Chemoenzymatic syntheses for the production of Rosuvastatin and other enantiomerically pure pharmaceutical building blocks

DISSERTATION

ZUR ERLANGUNG DES DOKTORGRADES

DER NATURWISSENSCHAFTEN

(DR. RER. NAT.)

VORGELEGT DER

FAKULTÄT FÜR CHEMIE

DER UNIVERSITÄT BIELEFELD

VON

DIPL.-CHEM.

RICHARD RONDANG BONAR METZNER

AUS WAIBLINGEN

BIELEFELD, MÄRZ 2014

ERSTGUTACHTER: Prof. Dr. Harald Gröger

ZWEITGUTACHTER: Prof. Dr. Norbert Sewald

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Die vorliegende Arbeit wurde am Institut für Organische Chemie des Departments Chemie und Pharmazie der Friedrich-Alexander Universität Erlangen-Nürnberg (02/2011 bis 04/2011), im Enzyme Laboratory des Biotechnology Research Center der Toyama Prefectural University, Toyama, Japan (01/2013 bis 07/2013) und in der Organischen Chemie I der Fakultät für Chemie an der Universität Bielefeld durchgeführt.

Die Ausarbeitung erfolgte unter der Betreuung von Prof. Dr. Harald Gröger in der Zeit von Februar 2011 bis März 2014.

Es wurden keine anderen als die in dieser Arbeit angegebenen Hilfen verwendet.

Die Dissertation wurde selbstständig verfasst und hat in der gegenwärtigen oder einer anderen Fassung noch nicht einer anderen Fakultät oder Hochschule vorgelegen.

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Bielefeld, den 21.03.2014 _____

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Teile dieser Arbeit sind bereits veröffentlicht oder zur Veröffentlichung eingereicht:

PUBLIKATIONEN

R. Metzner, S. Okazaki, Y. Asano, H. Gröger,
"Cyanide-free enantioselective synthesis of nitriles: Synthetic proof of a biocatalytic concept and mechanistic insights", **2014**, *submitted*.

PATENTE

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European Patent Application No. 13158655.4 – 1501, *unpublished* (filing date: March 11, 2013).

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Weitere im Rahmen dieser Promotion entstandene Veröffentlichungen:

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"Ett snyggt laboratorium innebär en lat kemist."

- A tidy laboratory means a lazy chemist. –

Jöns Jacob Berzelius, 1779 - 1848

1 PREFACE

Many active pharmaceutical ingredients and their syntheses are often more than 20 years old, thus based on a different era of synthesis and also a different sense of the purpose of chemistry. In view of more environmentally benign and economically attractive processes, their synthetic routes are being improved continuously and can still be optimized by the manufacturing companies. Within these enhancements, homogenous and heterogenous methodologies are investigated by working groups specialized in the field of organo-, metal- and biocatalysis, with increasing tendency to imitate nature. For example, organocatalytic processes are often based on proline derivatives, asymmetric synthesis are strengthened by using chiral auxiliars based on amino acids, functional-group interconversions (FGI) substitute expensive stoichiometric reagents by using cheap cosubstrates as donor or transfer reagents. By choosing a desired reaction from a complex cascade, it is often underestimated how the reaction system will behave and usually compromises have to be made. This can be seen in the general acceptance of certain mandatory structures where in some cases even the slightest alteration leads to incompatibility and a lack of conversion. The results are undesired enantiomers, side-products and maybe also unstable products. However, these drawbacks are also quite often readily circumvented by chemical methods.

Joining this aim for an interdisciplinary change, the overall concept of this work was to further increase the implementation of biocatalysis into organic syntheses. To elucidate this, the following projects were focused on the production of various pharmaceutical target structures like statins, dipeptidyl peptidase inhibitors, HIV-1 protease inhibitors, antibiotics and dopamine β-hydroxylase inhibitors. By using alcohol dehydrogenases, proteases, lipases, aldoxime dehydratases and ene reductases, alternative processes for either well established or novel compounds were developed. By adjusting of the preceding or subsequent chemical steps, the previously discussed possible restrictions of biocatalysis were approached to demonstrate the high value for chemoenzymatic processes.

2 CHEMOENZYMATIC SYNTHESIS OF ROSUVASTATIN

The following project was generously funded by Sandoz GmbH, Kundl, Austria. The aim of this collaboration was the establishment of an alternative synthetic route for the active pharmaceutical ingredient (API) of Crestor, Rosuvastatin. Rosuvastatin, as the name implies, belongs to the drug class of statins which are used for the treatment of dyslipidemia, *i.e.* for lowering the cholesterol levels, by inhibiting the enzyme 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG-CoA reductase). Several approaches for the synthesis of the generic[i] were investigated. However, since it is always reasonable to stay with certain structures already established in Good Manufacturing Practices (GMPs) from an industrial point of view, most of the upcoming experiments were focused on a specific route (see **Figure 1**). Some accompanying ideas were only investigated to a minor extend and will appear in the respective chapters without impact on the final route. Nevertheless they are included in this work for the purpose of encouraging academical research in a broadened fashion, away from traditional chemistry towards alternative syntheses.

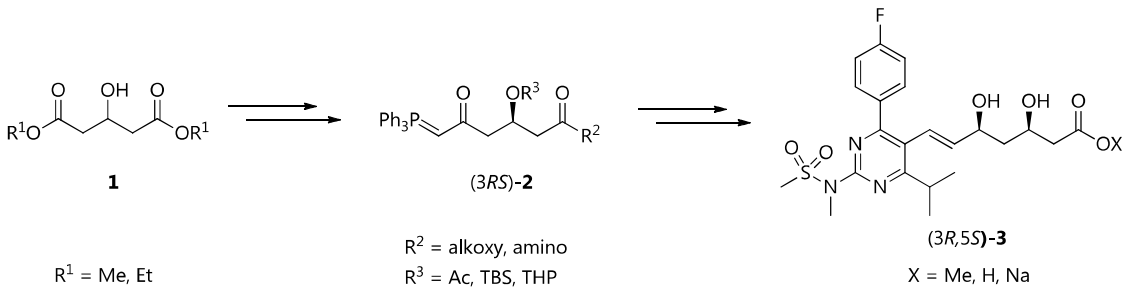


Figure 1. General project overview with target molecules throughout the synthetic route towards Rosuvastatin.

[i] A generic is defined as a copy of an established brand drug without the need for animal studies, clinical studies or tests regarding the bioavailability, because it is bioequivalent to its brand counterpart, *Center for Drug Evaluation and Research, U.S. Food and Drug Administration*

2.1 PATENT EXPIRATION OF DRUGS AND THE POTENTIAL OF NEW PROCESSES FOR GENERICS

Currently the pharmaceutical industry is facing numerous blockbuster drugs losing their patent protection between 2011 and 2016. With the expiration of Pfizer's Lipitor (generic name Atorvastatin), Eli Lilly's Zyprexa (Olanzapine) and a couple of other patents in 2011, no less than 32.2 billion USD in drug sales became accessible to low-cost generics in 2012 alone[1]. In the next three years, 27 more top-selling drugs will lose their patent protection (see **Figure 2**). This is already and will continue to be an unprecedented boost of process and drug development.

Year	Brand name	Manufacturer	Generic	Ann. Sales
2011	Lipitor	Pfizer	Atorvastatin	5.329
	Zyprexa	Eli Lilly	Olanzapin	2.496
	Levaquin	Johnson & Johnson	Levofloxazin	1.312
	Concerta	Johnson & Johnson	Methylphenidat	929
	Protonix	Pfizer	Pantoprazole sodium	690
2012	Plavix	Bristol-Myers Squibb	Clopidogrel	6.315
	Seroquel	AstraZeneca	Quetiapin	3.771
	Singulair	Merck & Co	Montelukast	3.356
	Actos	Takeda	Pioglitazon HCl	3.275
	Lexapro	Forest Laboratories	Escitalopram	2.122
	Diovan	Novartis	Valsartan	2.093
	Viagra	Pfizer	Sildenafil	1.007
	Provigil	Cephalon	Modafinil	916
	Geodon	Pfizer	Ziprasidon	907
2013	Oxycontin	Purdue Pharma LP	Oxycodone HCl	2.800
	AcipHex	Eisai / Janssen Pharma	Rabeprazole sodium	n.n.
	Zometa	Novartis	Zoledronate	1.500
	Xeloda	Genentech/Roche	Capecitabine	648
	Opana ER	Endo Pharma	Oxymorphone HCl	385
	Asacol	Warner Chilcott	Mesalazin	800
2014	Nexium	AstraZeneca	Esomeprazole	4.900
	Cymbalta	Eli Lilly	Duloxitine HCl	4.000
	Celebrex	Pfizer	Celecoxib	2.500
	Lunesta	Sunovion Pharma	Eszopiclone	631
	Evista	Eli Lilly	Raloxifene HCl	1.300
	Sandostatin LAR	Novartis	Octreotide acetate	1.300
	Actonel	Warner Chilcott	Risedronate	1.600
2015	Abilify	Bristol-Myers Squibb	Aripiprazole	4.600
	Copaxone	Teva Pharma	Glatiramer acetate	3.570
	Gleevec	Novartis	Imatinib mesylate	4.260
	Namenda	Forest Laboratories	Memantine HCl	n.n.
	Combivent	Boehringer Ingelheim	Albuterol / Ipratropium	965
	Zyvox	Pfizer	Linezolid	325
	Prezista	Janssen Therapeutics	Darunavir	888
	Avodart	GlaxoSmithKline	Dutasteride	973
2016	Crestor	AstraZeneca	Rosuvastatin	6.000
	Benicar	Daiichi Sankyo	Olmesartan medoxomil	2.500
	Cubicin	Cubist Pharma	Daptomycin	644

Figure 2. Expired and scheduled to be expiring patent protection of pharmaceutical blockbusters between 2011 and 2016.[1]

Racemic or undefined compounds (green), achiral compounds (red), natural products with static stereochemistry (blue) and enantiopure compounds (black)

A close collaboration between industry and academia lies at hand, with room for improvements in all fields of chemistry, including biocatalysis. This is already reflected in the rise of publications aimed for new methodologies and sustainable organic synthesis for the production of generics. For example, new methods for the synthesis of statin side-chains have been developed and patented by various companies, e.g. Lek Pharmaceuticals and DSM.[2,3] The extent of improvement varies from simple crystallization procedures to chemoenzymatic total syntheses. Codexis also developed a bienzymatic process for the synthesis of ethyl (*R*)-4-cyano-3-hydroxybutanoate, the chiral precursor for the side-chain of Atorvastatin.[4] Starting from commercially available ethyl 4-chloro-3-oxobutanoate **4**, they combined an enantioselective, biocatalytic reduction *via* ketoreductase (KRED) with a substitution process *via* halohydrin dehalogenase (HHDH) (see **Figure 3**). The HHDH initially catalyzes the elimination of hydrochloric acid under formation of the chiral epoxide (*S*)-**6**

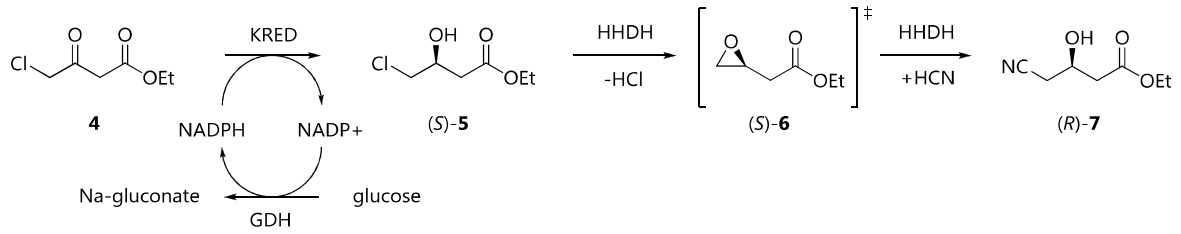


Figure 3. Two-step, three enzyme process for the precursor of the Atorvastatin side-chain by MA *et al.*.[4]

which is then hydrocyanated to the corresponding β-hydroxy nitrile (*R*)-**7**. After extensive molecular biological alterations, the recombinant enzymes overcame the drawbacks of their wild-type's poor stability and low catalyst loadings, leading to a space-time yield of 672 g/L product per day, which was isolated in 92% yield and had an excellent enantiomeric excess of >99.5% ee. Noteworthy, the highly toxic reagent hydrogen cyanide is produced *in situ* by an automatic acid-base titration with aqueous sodium cyanide as titer solution. Although sodium cyanide is also highly toxic, the described method allows an easier handling of this reagent and prevents the intoxication *via* inhalation or skin resorption. In this case the formation of hydrogen cyanide is coupled to the elimination of hydrogen chloride, which in the end reduces the concentration of hazardous free hydrogen cyanide to a minimum. Also from Codexis, a fascinating biocatalytic ketone reduction for the single stereogenic center of the leukotriene receptor antagonist Montelukast sodium (Singulair, Merck) is reported (see **Figure 4**, page 6).[5] Usually, oxidoreductases have a very high enantioselectivity when specific steric properties of ketone substituents are fulfilled. High selectivities are achieved when one of these substituents are methyl-, chloromethyl- and ethyl-groups.

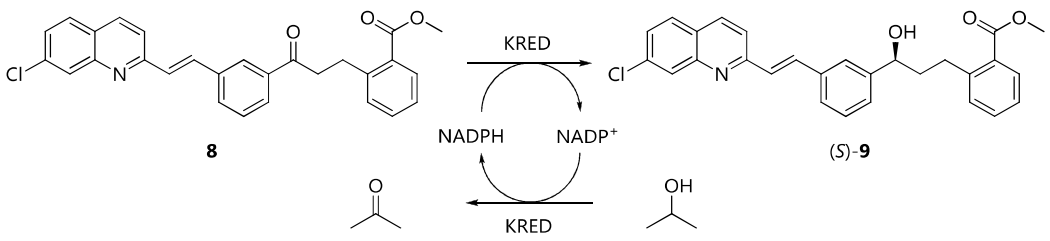
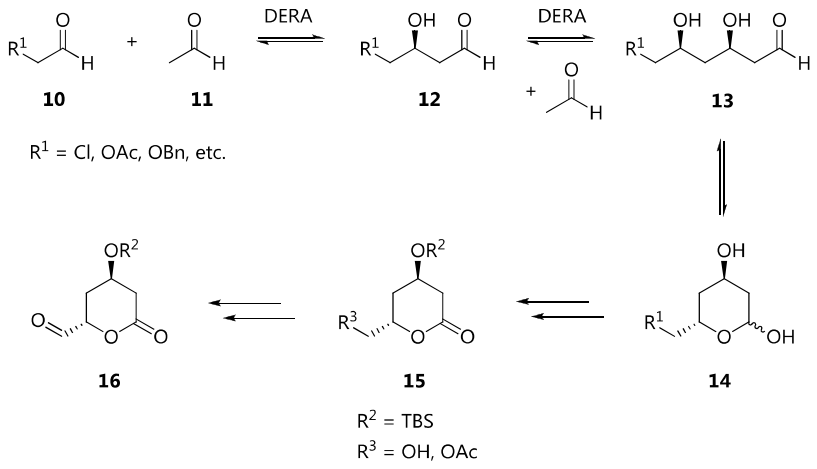


Figure 4. Enantioselective reduction of a bulky ketone for the production of the Montelukast precursor (*S*)-**9** by LIANG *et al.*.[5]

However, only a few examples in literature are highly selective for ketones bearing two bulky substituents.[6] In the case of Montelukast, the prochiral bulky ketone **8** was reduced at a substrate concentration of 100 g/L with quantitative conversions, leading to yields of 90-98% and an excellent enantiomeric excess of >99.9% ee. The expensive cofactor $NADP^+$ (1 mg/g substrate) is regenerated *in situ* with the cosubstrate 2-propanol in a toluene/alcohol/buffer mixture. The process currently runs on an impressive 230-kg scale. In the case of the HMG-CoA reductase inhibitor Rosuvastatin, another novel process was published very recently by Lek Pharmaceuticals.[7] This process uses whole-cell catalysis based on the enzyme 2-deoxyribose-5-phosphate aldolase (DERA) for the diastereoselective synthesis of a lactol precursor for the statin side-chain (see **Figure 5**).

Figure 5. Whole-cell DERA process for the enantio- and diastereoselective production of chiral lactols as precursors for statin side-chains based on aldehydes by Lek Pharmaceuticals.[7]



By this methodology the two stereogenic centers of the API[i] are inserted by only one enzyme in a highly enantio- and diastereoselective manner. After subsequent protection and oxidation of the lactol **14**, the resulting heterocyclic aldehyde **16** is then coupled with the phosphine-derivative of the

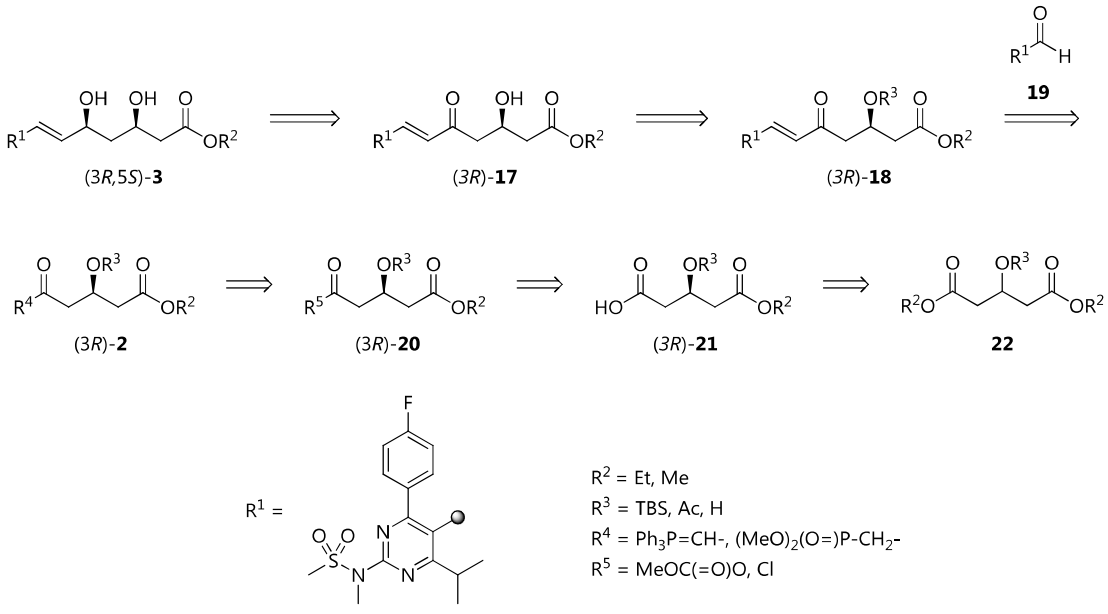
[i] Active pharmaceutical ingredient

heteroaromatic core of the respective drug. The final process had a productivity of 50 $g*L^{-1}*h^{-1}$ with >80% yield and excellent selectivities of >99% ee for aldol **12** and >99.8% de for 1,3-diol **13**. Nevertheless, there are still some difficulties for this lactol strategy. The product needs to be stabilized through oxidation of the lactol **14** to its corresponding lactone **15**. This oxidation process was also realized but shows only up to 84% yield for various oxidants. Furthermore, the reaction itself needs to be monitored and controlled since the reactants **10** and **11** can act as donor and acceptor, leading to product mixtures. This was largely achieved by fed-batch processing and sequential addition of reactants to the reaction mixture. Despite these drawbacks, this route also demonstrates the versatility and potential of enzymes for the industrial production of pharmaceutical compounds.

2.2 RETROSYNTHETIC STRATEGIES

Based on the preceding work of numerous groups and companies, different retrosynthetic routes for the production of enantiomerically pure intermediates were investigated in this work, starting in all cases from a prochiral diester of type **22** (see **Figure 6**), which is desymmetrized by a hydrolase. Since the heteroaromatic core of Rosuvastatin is achiral and commercially available as aldehyde **19**, no synthetic strategies for it were examined. The project was focused on the production of the enantiomerically pure phosphor reagent

Figure 6. Retrosynthetic approach by hydrolytic de-symmetrization of prochiral glutaric diesters.



(*3R*)-**2**. The corresponding ylide would be synthesized by a substitution reaction with a nucleophilic triphenylphosphoranylidene. In general, it would be possible to alkylate triphenylphosphine with a corresponding α-haloketone, but the synthesis of this reactant would be quite complex. Moreover, haloketones are often made from the respective carboxylic acid derivatives and would therefore not shorten the route but require significantly harsher conditions. The activated carboxylic acid derivative (3*R*)-**20** would be generated from the carboxylic acid (3*R*)-**21**, which itself would be obtained by desymmetrization of the prochiral diester **22**. The hydrolytic strategy would have the benefit of producing intermediates which are already established as GMP[i] compounds.

Furthermore, a second strategy based on the opposite enantioselectivity of lipases was investigated. In this way, the desymmetrization would be achieved through aminolysis, leading to amides of type (3*S*)-**26** (see **Figure 7**) which would still be reactive at the desired carbonyl moiety. This way the phosphoranylidene intermediate (3*R*)-**2** would also bear the necessary absolute configuration. The drawback for an industrial application of an aminolysis is of course the lack of information for all intermediates since most of them are unknown to literature. However, from an academic point of view, unknown compounds can be of even greater interest. As it can always be expected from retrosynthetic strategies, inevitable changes had to be made throughout the project regarding protective groups and process chemistry.

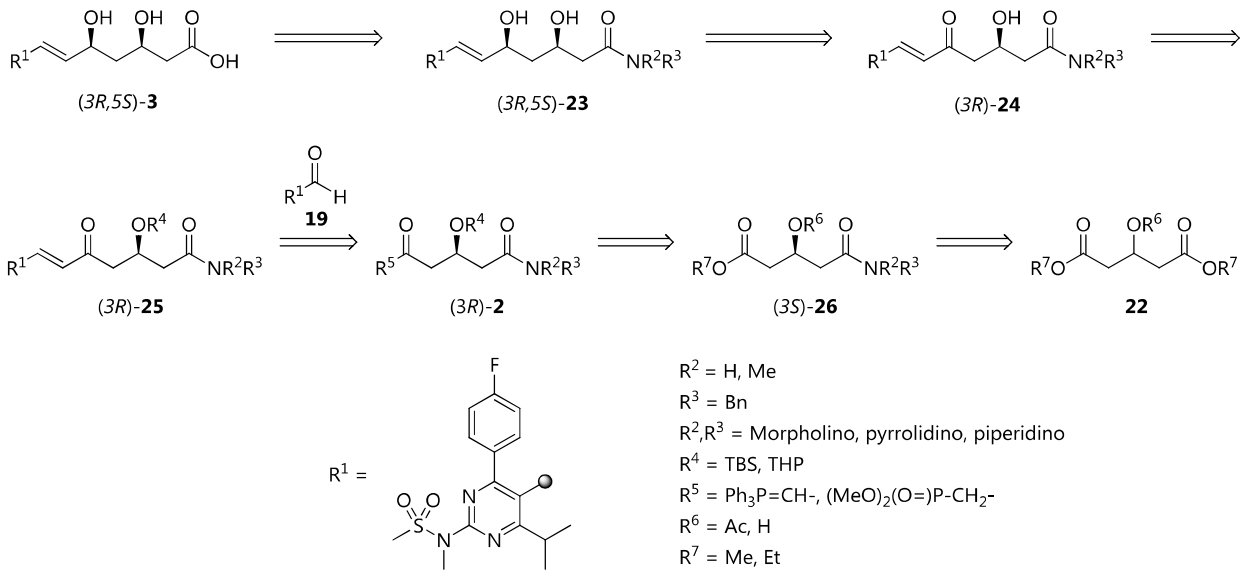


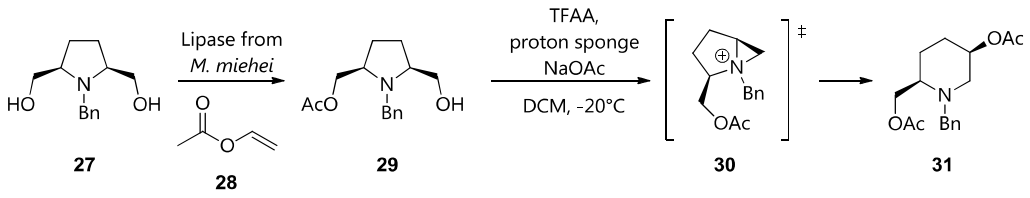
Figure 7. Retrosynthetic approach by aminolytic desymmetrization of prochiral glutaric diesters by the use of (*S*)-selective lipases.

[i] Good Manufacturing Process

2.3 BIOCATALYTIC DESYMMETRIZATIONS FOR ENANTIOPURE BUILDING BLOCKS

The examples given in chapter 2.1 (page 4) for new biocatalytic processes as part of chemical syntheses for the production of generics introduced the chirality by the addition of a nucleophile, e.g. an enol or a hydride, to a carbonyl compound, converting the prochiral substrate into a chiral product. The enantiospecificity was based on the binding orientation of the substrate and the direction of the nucleophilic attack on the *Re* or *Si* face. In the case of a desymmetrization process, the stereogenic centers are already present in the structure. The selectivity of the enzyme is then determined by the stronger binding constant for the two possibly orientations of the. For example, this methodology was used by THIEL and DESKA for the combination of a biocatalytic acetylation with a chemical ring expansion for the synthesis of bifunctionalized *N*-heterocycles in a highly enantio- and diastereoselective manner (see **Figure 8**).[8] The starting material, a symmetric *meso*-diol of a *bis*-hydroxymethyl-substituted heterocycle **27**, is first desymmetrized by

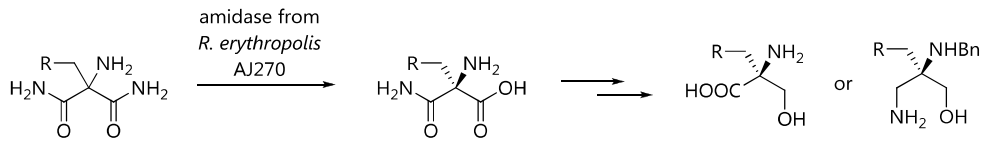
Figure 8. Biocatalytic desymmetrization and chemical ring-elongation for the synthesis of enantio- and diastereomerically pure bifuntionalized *N*-heterocycles by THIEL and DESKA.[8]



27 **28** **29** **30** **31**

acetylation with vinyl acetate (**28**) and a lipase from *Mucor miehei* as biocatalyst. The remaining carbinol moiety is then transformed *via* an aziridinium intermediate **30** to the thermodynamically preferred ring-elongated heterocycle **31**. While this reaction is based on a *meso*-compound, ZHANG *et al.* successfully desymmetrized disubstituted malonamides of type **32** (see **Figure 9**) to their chiral monoamides **33** by using an amidase from

Figure 9. Biocatalytic desymmetrization of disubstituted α-aminomalonamides by ZHANG *et al.*[9]



32 **33** **34** **35**

up to 98% yield
up to >99.5% ee

Rhodococcus erythropolis.[9] The products could be used for the production of α–substituted serine analogues **34** and β,β-disubstituted γ-amino alcohols **35**, respectively. Various alkyl-substituted substrates **32** were converted in high yields of up to 98% and with excellent enantioselectivities of up to >99.5% ee. The substrates are readily accessible by alkylation of dialkyl 2-aminomalonates and subsequent ammonolysis with aqueous or methanolic ammonia.

The desymmetrization of prochiral α,α-disubstituted dialkyl malonates **36** (see **Figure 10**) was also investigated by TENBRINK *et al.* as part of a sequential one-pot process for the combination with an olefin metathesis reaction in aqueous media.[10] The cyclic product **38** could be used for the production of the unusual, cyclic α-amino acid **39**. However, the enantiomeric excess with crude pig liver esterase as biocatalyst was poor, leading to the desired

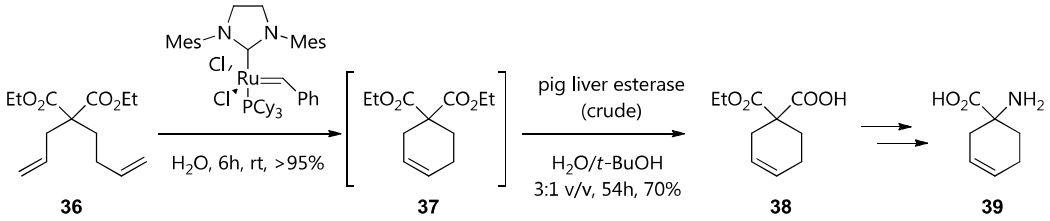


Figure 10. Biocatalytic desymmetrization of cyclic dialkymalonates after ring-closing metathesis reaction by TENBRINK *et al.*.[10]

monoester with only 7% ee. This is nevertheless reasonable as the only differentiation between the two substituents is a double bond and no large substituent as in most desymmetrization reactions.

For example, different 3,4-disubstituted *meso*-pyrrolidines, exemplified by pyrrolidine **40** (see **Figure 11**), were desymmetrized by the use of a monoamine oxidase (MAO) from *Aspergillus niger* by KÖHLER *et al.*.[11] The FAD[i]-dependent enzyme oxidizes the pyrrolidine **40** to the corresponding 1-pyrroline **41** in a highly enantioselective manner. This product can be used

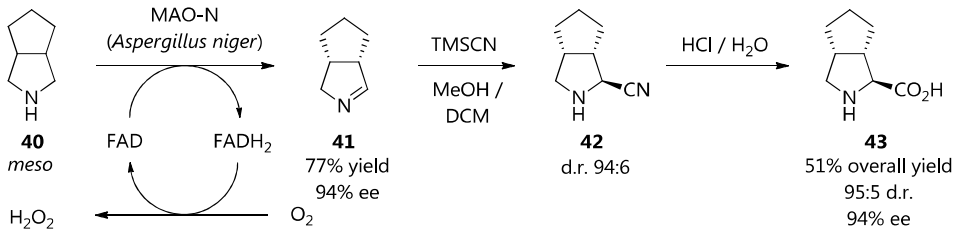


Figure 11. Biocatalytic desymmetrization of pyrrolidines with a monoamine oxidase (MAO-N) for the chemoenzymatic synthesis of proline derivatives by KÖHLER *et al.*.[11]

[i] Flavin-adenine dinucleotide (FAD)

for a subsequent *trans*-selective addition reaction, e.g. with trimethylsilyl cyanide, to obtain enantiopure aminonitrile **42** or the respective proline derivative **43**. The substituents at the stereogenic centers are large enough to obtain products in high yields of up to 79% with very high enantioselectivities of 94-99% ee.

2.4 BIOCATALYTIC HYDROLYSIS OF DIALKYL 3-OXYGLUTARATES

The hydrolytic desymmetrization of diethyl 3-hydroxyglutarate (**1a**) was already investigated by COHEN and KHEDOURI[12] using α-chymotrypsin (CHY) and BROOKS and PALMER[13] using both CHY and pig liver esterase (PLE) as biocatalysts. Their results were confirmed by SANTANIELLO *et al.* who compared the selectivities of the free dialkyl 3-hydroxyglutarates of type **1** with *O*-acylated derivatives of type **22**.[14] The latter reported a rather poor enantioselectivity for the hydroxyglutarate **44a** (see ***Figure 12***) of 55 ± 5% ee in contrast to good to excellent enantioselectivities of 84-95% ee for the acylated diesters **44c** and **44d**, depending on the alkyl ester, with the diethyl ester being superior to the dimethyl ester. When using diethyl 3-hydroxyglutarate and its derivatives with α-chymotrypsin, the same enhancement in enantioselectivity was observed in this work[i]

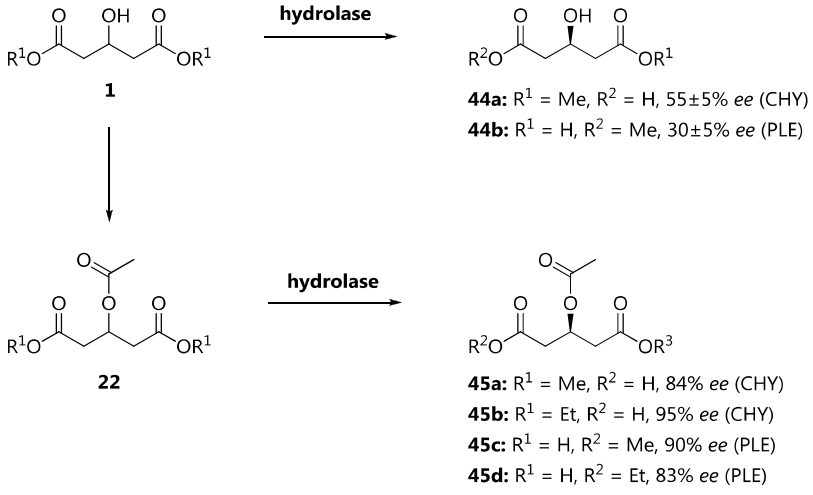


Figure 12. Desymmetrization of dialkyl 3-hydroxyglutarate and its *O*-acetyl derivative *via* α-chymotrypsin (CHY) and pig liver esterase (PLE) by SANTANIELLO *et al.*.14

[i] The enantiomeric excess was determined by coupling of the monoester with optically pure (*R*)- or (*S*)-1-phenylethylamine, respectively, and subsequent quantification of the diastereomers by ^{1}H-NMR spectroscopy and/or chiral normal-phase HPLC (see chapter 8.6).

The mechanism of α-chymotrypsin as serine protease is known in literature and can be described generally by the catalytic triad formed between Asp^{102}, His^{57} and Ser^{195}, with the latter serving as the nucleophile. The substrate, in this case the prochiral 3-hydroxy diester, is bound near Ser^{195} (see **Figure 13**). This serine is activated by the high pK_a of the adjacent His^{57} nitrogen atom. Its pK_a is elevated in the presence of Asp^{102} in its carboxylate form. The activated serine

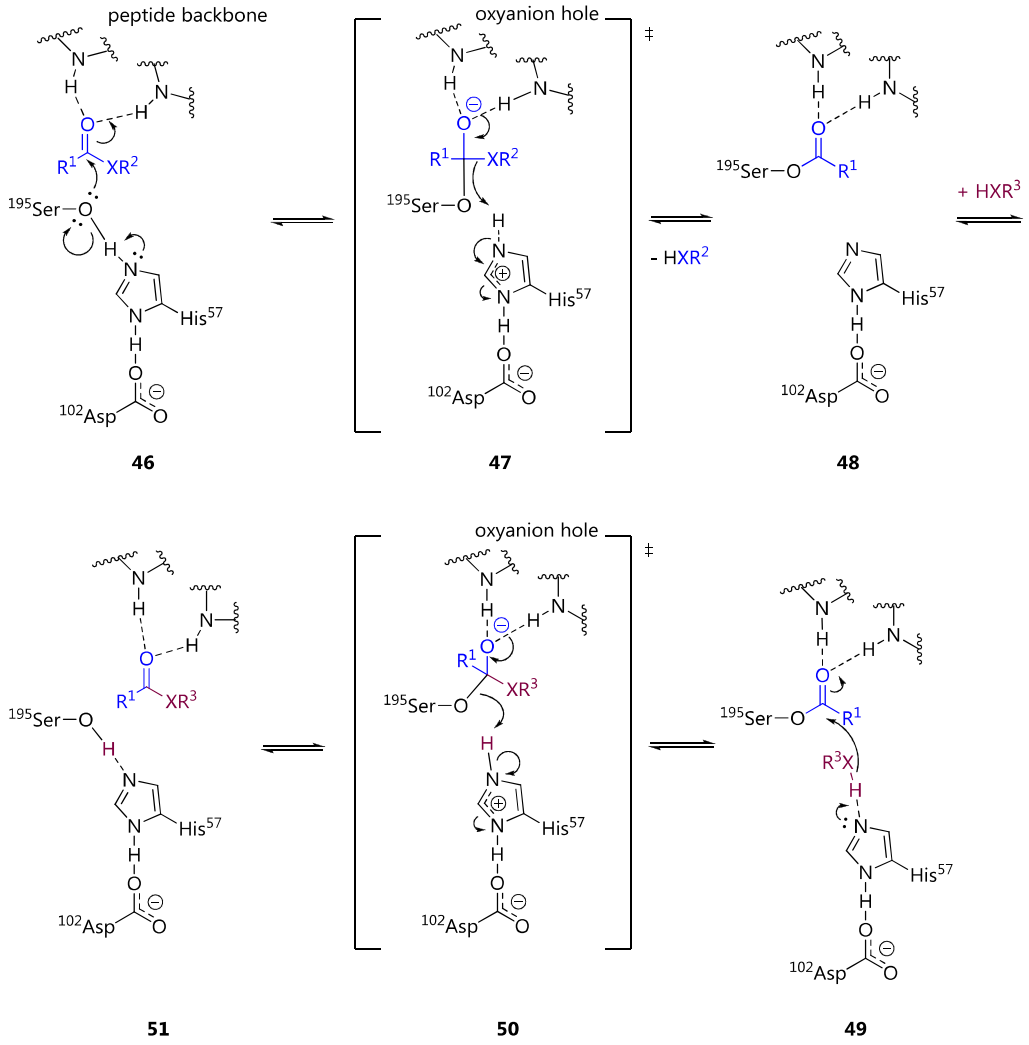
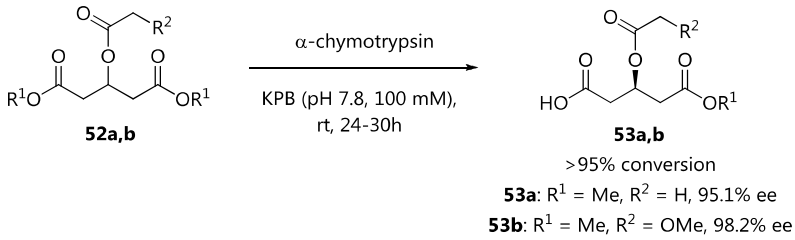


Figure 13. Addition-elimination mechanism of carboxylic acid derivatives *via* serine hydrolases.

then acts as a strong nucleophile, attacking the carbonyl of the substrate **46**. This leads to a tetrahedral intermediate **47**, which is stabilized by an oxyanion hole formed by the amide protons of the surrounding peptide backbone. The carboxylic acid derivative is cleaved under the formation of the acylated enzyme **48**. In a similar matter, the desired nucleophile is activated by His^{57} and attacks again the carbonyl of the covalently bound substrate **49**. It is therefore transferred from Ser^{195} to the nucleophile **51**. The catalytic mechanism is completed by diffusion of the product to the medium and diffusion of another

substrate molecule into the enzyme pocket. This is the crucial part of the entire reaction because the product could also be recognized as a substrate. Thus, the equilibrium of the overall reaction has to be directed towards the desired product. With the nucleophile being water, this is readily achieved with the resulting product being a carboxylic acid which is deprotonated and resonance-stabilized in its carboxylate form, thus inert towards nucleophilic attacks. By using alcohols, amines or thiols as nucleophiles, the reaction is usually controlled by an excess of the nucleophile or the removal of eliminated nucleophile, for example due to a higher vapor pressure or lower solubility in the reaction medium. In the present route, water served as nucleophile and the irreversibility of the saponification forced the equilibrium to the desired chiral monoester.



52a,b

53a,b
>95% conversion
53a: R^1 = Me, R^2 = H, 95.1% ee
53b: R^1 = Me, R^2 = OMe, 98.2% ee

Figure 14. Enhancement of enantioselectivity in the presence of an α-heteroatom reported by ÖHRLEIN and BAISCH.[15]

ÖHRLEIN and BAISCH reported an enhanced enantioselectivity of α-chymotrypsin by using a methoxyacetyl protecting group instead of acetyl.[15] It is commonly observed that α-heteroatoms are beneficial for the enantioselective binding of carboxylic esters and amides to hydrolases. The desymmetrization of diethyl 3-(methoxyacetyl)-oxyglutarate **52b** (see **Figure 14**) proceeded with an enantioselectivity of 98.2% ee. Based on these results, various *O*-acylated derivatives were synthesized in this study to reproduce the excellent enantioselectivity with α-chymotrypsin. In addition, varying the size and properties of the 3-acyloxy group could also facilitate the product isolation in later parts of the synthetic route, preferably by crystallization.

Using a desymmetrization strategy for the production of enantiomerically enriched *O*-TBS-protected 3-hydroxypentanoic monoesters is a challenging method in industry. In many cases, the enantioselectivity of a chemical desymmetrization is realized by diastereomeric coupling with another enantiomerically pure compound, preferably an alcohol or amine. In the case of amines, the resulting amides are cleaved by oxidation under harsh conditions. KONOIKE and ARAKI on the other hand used the lithium alkoxide **55** (see **Figure 15**, page 14) of enantiomerically pure benzyl (*R*)-mandelate for the

diastereoselective opening of TBS-protected glutaric anhydride **54** with a diastereomeric ratio of 9:1 for the (*3'R,2R*)-diastereomer **56**.[16]

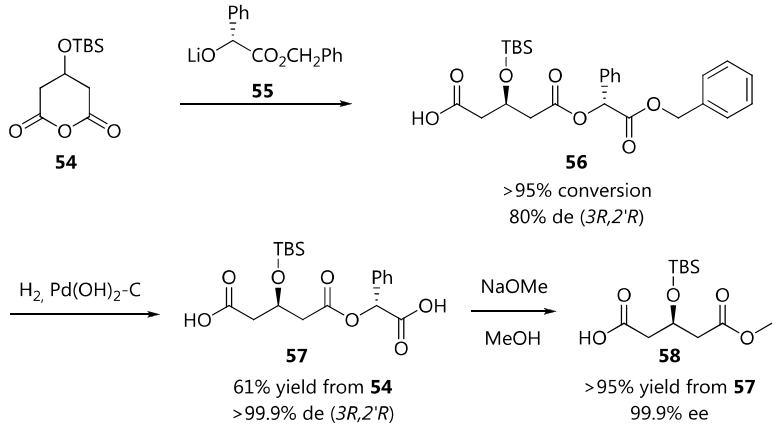


Figure 15. Chemical desymmetrization of prochiral TBS-protected glutaric anhydride by KONOIKE and ARAKI.[16]

Subsequently, a three-step purification procedure with hydrogenolysis of the monoester **56** was realized. After crystallization of the dicarboxylic acid **57** with 61% yield and with an excellent diastereoselectivity of 99.9% de, the transesterification with sodium methoxide in methanol yielded the desired (*3R*)-TBS-monomethyl ester **58** in enantiomerically pure form. With the enantioselective desymmetrization of the prochiral TBS-protected diester by the use of hydrolases in this work, the overall yield would probably be improved. In addition, the use of chiral auxiliaries and fractionized crystallization would be circumvented. Even if the desymmetrization requires a different *O*-derivative, this route would still have the potential to be superior, since all modifications would insert or remove parts of bulk chemicals which are both cheap and easily accessible. This biocatalytic approach was studied in a previous work from NOVÁK *et al.* in which the TBS-protected dimethyl ester **59** (see **Figure 16**) was hydrolyzed by using pig liver esterase.[17] Due to the lack of information about the reaction time, the activity cannot be discussed.

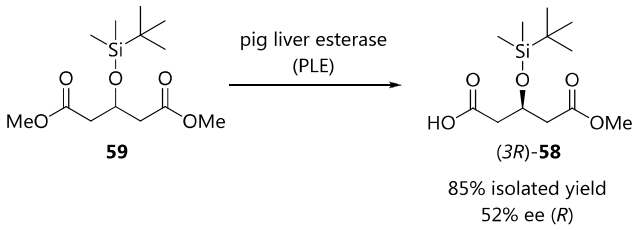
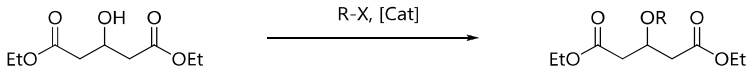


Figure 16. PLE-catalyzed desymmetrization of 3-TBS-DHG with low enantioselectivity by NOVAK *et al.*[17]

Nevertheless, the isolated product (3*R*)-**58** showed only a low enantiomeric excess of 52% ee after derivatization to its corresponding phenylethylamide and analysis of the diastereomeric excess.

2.4.1 SUBSTRATE SYNTHESIS

The literature-known derivatives were synthesized as benchmark substrates for the establishment of analytical methods or the determination of enantioselectivity and efficiency of the biocatalysts (see **Table 1**). Among the chosen substrates, diethyl 3-hydroxyglutarate (**1a**) was also used for the synthesis of reference mixtures of enantiomers with around 60% ee. The acetate **22a** and methoxyacetate **22e** were used for comparing the results



1a **22**

Entry	**22**	R[a]	X	Cat. (mol%)	Solvent	Time [h]	Temp. [°C]	Conv. [%][b]	Yield [g (%)]
1	**a**	Ac	OAc	$Zn(ClO_4)_2$ (0.1)	-	2	20	>95.0	0.84 (85.3)
2	**b**	Boc	OBoc	$Zn(OAc)_2$ (10.0)	-	6	60	>95.0	1.11 (91.2)
3	**c**	TFA	OTFA	$Zn(ClO_4)_2$ (1.0)	-	2	20	>95.0	1.07 (89.1)
4	**d**	PhAc	Cl	-	-	16	0-20	>95.0	5.30 (82.2)
5	**e**	MeOAc	Cl	Pyridine (110)	CH_2Cl_2	24	0-20	92.1	3.54 (64.0)
6	**f**	TBS	Cl	1*H*-imidazole (150)	THF	24	20	>95.0	0.58 (36.4)
7	**g**	THP	-	PPTS[c]	CH_2Cl_2	21	20	>95.0	1.68 (58.3)

Table 1. Protective groups for the secondary alcohol of diethyl 3-hydroxyglutarate

(a) Acetyl (Ac), *tert*-butyloxycarbonyl (Boc), trifluoroacetyl (TFA), phenylacetyl (PhAc), methoxyacetyl (MeOAc),*tert*-butyldimethylsilyl (TBS), tetrahydropyran-2-yl (THP) (b) Determined by integration of ^{1}H-NMR signals for **1a** and *O*-derivatives (c) Pyridinium *p*-toluenesulfonate

obtained in this work with the literature-reported enantioselectivities of >95% ee. The trifluoroacetate **22c** was chosen for analytic purposes, e.g. determination of product-related conversion and side-product formation by ^{19}F-NMR spectroscopy, and its smooth cleavage in the following steps. The phenylacetate derivative **22d** could improve the crystallization of intermediates. To circumvent the need for an exchange of the *O*-acyl moiety, substrate **22f**, bearing a *tert*-butyldimethylsilylether protective group, was also investigated. As a synthon with similar sterical influence, the *tert*-butyloxycarbonyl- (Boc-) protected compound **22b** was another promising candidate. And finally, since the silyl ether is later cleaved to silanol, which cannot be separated by liquid-liquid extraction, the tetrahydropyranyl

derivative **22g** was another alternative. Literature shows almost similar reaction properties for THP and TBS.[18] Notably, THP has the benefit of being hydrolyzed under mild conditions to 5-hydroxypentanal, an aldehyde which could be easily separated by liquid-liquid extraction with bisulfite solutions. As can be seen in **Table 1** (page 15, entries 1-5), the conversion and isolated yields of these substrate syntheses are good to excellent in almost all cases.

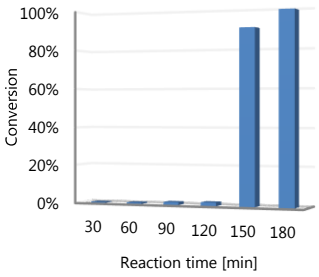


Figure 17. Time-course of the acetylation of **1a** with zinc perchlorate in acetic anhydride.

Remarkable is the synthesis of the acetate derivative **22a** (entry 1), which was achieved under solvent-free conditions with zinc perchlorate hexahydrate in acetic anhydride. However, after analyzing the time-dependent conversion rate of the reaction by direct ^{1}H-NMR integration without work-up[i] (see **Figure 17**), the mechanism is more likely autocatalytic. The exponential reaction rate after almost no conversion for two hours suggests an unidentified catalytic side-reaction, in this case probably by acetic acid formed during the reaction. The low catalyst loading of 0.1 mol% zinc perchlorate is presumably the chain reaction starter with acetic acid as BRÖNSTED acid catalyst (see **Figure 18**). Furthermore, the crude product could was used without purification. The only side-product acetic acid was neutralized with a base before adding the biocatalyst for the subsequent desymmetrization.

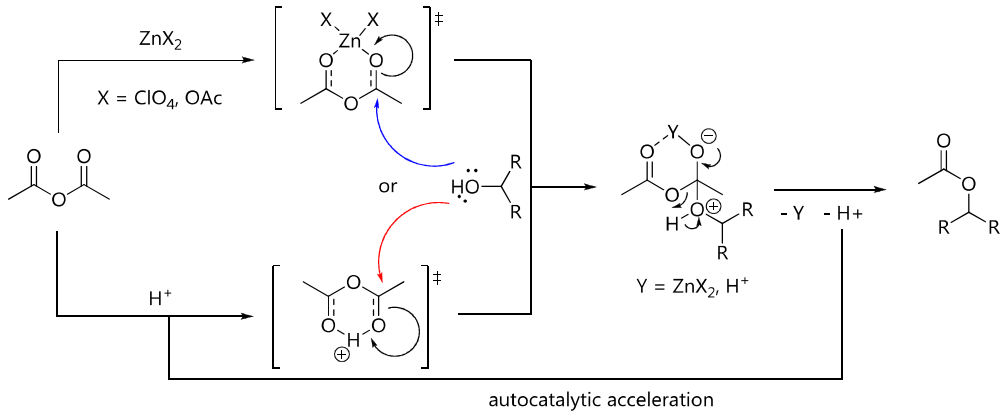


Figure 18. Proposed mechanism of acylation with acetic anhydride under solvent-free conditions with parallel LEWIS-acid (blue) and BRÖNSTED-acid catalysis (red).

[i] The absence of solvent, the non-paramagnetic metal catalyst and the nearly stoichiometric amount of reagent made this reaction convenient for monitoring by ^{1}H-NMR.

2.4.2 Analytical methods for the determination of the activity and enantioselectivity of hydrolases

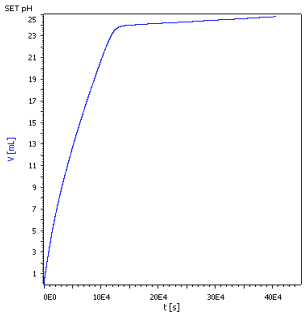


Figure 19. Exemplary titration graph for the hydrolytic desymmetrization in aqueous buffered solutions.

Hydrolytic reactions were conducted in a Titrino apparatus for maintaining the weak basic pH of 8.0 throughout the reaction. The formed carboxylic acid was neutralized by titration of aqueous sodium hydroxide solution (0.2 - 4.0 M). The consumption of titer was automatically recorded and direct proportional to the amount of product formed (see **Figure 19**). The conversion was additionally confirmed after extractive work-up by ^{1}H-NMR analysis[i] of the crude product (see **Figure 20**). The enantiomeric excess was determined according to literature protocols by coupling of the acetylated monoester **45b** (see **Figure 21**, page 18) with an enantiopure chiral amine, i.e. (*S*)- or (*R*)-1-phenylethylamine **61**, respectively. The formed diastereomers of type **62** were analyzed by ^{1}H-NMR spectroscopy and chiral normal-phase HPLC for their diastereomeric excess, which is equal to the enantiomeric excess of the monoester.

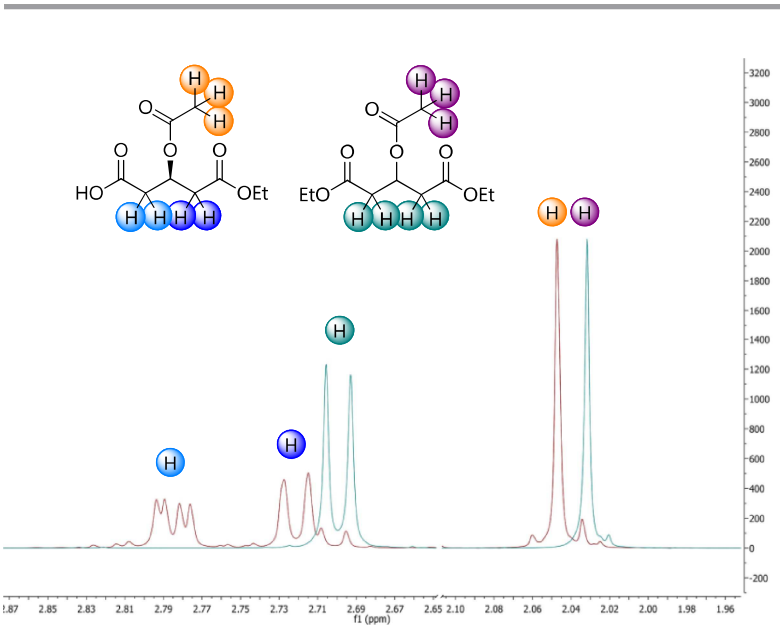


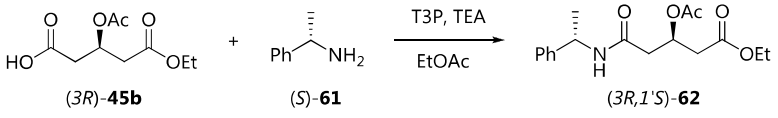
Figure 20. ^{1}H-NMR signals suitable for the determination of conversion for the hydrolytic desymmetrization.

Tetramethylsilane (TMS, δ = 0.0 ppm) was used as shift reference prior to stacking.

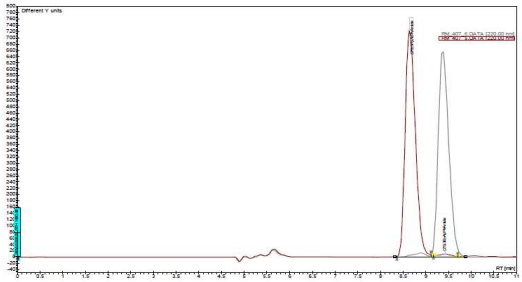
In general the enantiomeric excess of a compound should be measured without derivatization since most of the common coupling reagents have a slight tendency to isomerize chiral centers in the vicinity of the coupling bond. Experimental data for many reagents can be found in literature which suggest

[i] Most of the signals do not shift sufficiently for differentiation of product and starting material. Integration of ethyl signals gave poor reproducibility. Exceptions suitable for integration after Lorentz fitting are shown in **Figure 20**.

an expectable error of at least 1.0 % ee for the remaining enantiomeric excess.[19] Unfortunately, the investigated monoesters have only aliphatic hydrocarbons and carboxylate derivatives with only minimal absorbance of UV/Vis wavelengths. Thus, the previously mentioned error margins were taken as they were the monoester **45b** was derivatized to its diastereomeric phenylethylamide of type **62** by using the coupling reagent T3P (*tris*-*n*-propylphosphonic anhydride, see **Figure 21**, page 18). Due to its low tendency to epimerize the coupling compounds, the resulting diastereomeric excess should be close to the actual enantiomeric excess of the monoester.



(*3R*)-**45b** (*S*)-**61** (*3R,1'S*)-**62**



RM_407_5.DATA [220,00 nm]

Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	(2'S,3R)-AcPhAmide	8,64	98,42	723,7	189,3	98,424
1	(2'S,3S)-AcPhAmide	9,44	1,58	9,2	3,0	1,576
Total			100,00	732,8	192,3	100,000

Figure 21. Exemplary HPLC chromatogram of diastereomeric phenylethylamides by T3P coupling.

Diastereomerically enriched mixture of (2'*S*,3*R*)-phenylethylamide (red, 96.8% de) in comparison with its (2'*R*,3*R*)-isomer (grey) as reference.

The subsequent deacetylation of the *O*-protecting acetate was basically monitored accordingly by consumption of titer for maintaining the pH. Unfortunately there was evidence of base-catalyzed elimination of acetic acid due to the high molarity of the titer. Since this side-reaction would both eliminate the acetyl signal in ^{1}H-NMR spectra and disturb the methylene signals, the product-related conversion was determined by integration of the chiral carbinol **60** (4.47 ppm, see **Figure 22**, page 19). The absence of the acetic ester leads to a strong shift towards high-field. Since the free carbinol **60** is also reactive under the coupling reactions for the determination of the enantiomeric excess, the product was first acetylated again with excess acetic anhydride and then coupled with T3P and phenylethylamine to check for isomerization. The HPLC separation of isomers was conducted according to the method described for the desymmetrization.

Since the crude products were used without further purification, quantitative analysis of the ^{1}H-NMR spectra had to be made. Ideally, a convenient NMR standard, which is not compartmented in a glass capillary, should have a high purity and a distinctive signal in an isolated region of the spectrum. Such an isolated area would be between 4.60 and 5.30 ppm in both spectra (see **Figure 22**).

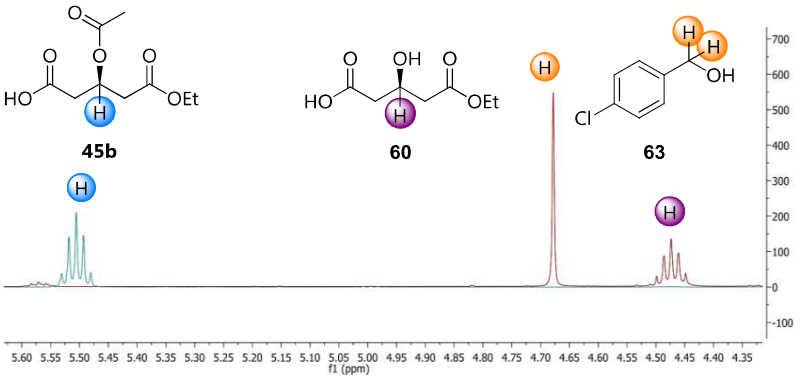


Figure 22. ^{1}H-NMR spectra with an internal standard for quantitative analysis of the product content.

Signals in this region can be seen for benzylic methylene compounds such as benzylamine and benzyl alcohol. For a more convenient handling and purification, *p*-chlorobenzyl alcohol was used. This compound is crystalline, commercially available in high purity of >99.9% and has a distinctive singlet at 4.68 ppm in $CDCl_3$. The product content and its purity were therefore calculated by the weigh-in of NMR standard and the mass ratio of the mixture according to the following equations:

$$\mathrm{PC} = \mathrm{m\ (pClBnOH)} * \frac{\mathrm{Int\ (pClBnOH)} * \mathrm{M(pClBnOH)}}{\mathrm{H\ (pClBnOH)}} \Big/ \frac{\mathrm{Int\ (Product)} * \mathrm{M\ (Product)}}{\mathrm{H\ (Product)}}$$

$$\mathrm{PP} = \frac{\mathrm{PC}}{\mathrm{m\ (Crude\ product)}}$$

PC = Product content [mg]

m (pClBnOH) = weigh-in NMR standard [mg]

Int (pClBnOH) = Integral for *p*-chlorobenzyl alcohol NMR signal (4.68 ppm)

M (pClBnOH) = Mol. weight (pClBnOH) = 142.58 g/mol

Int (Product) = Integral for product NMR signal

M (Product) = Mol. weight (Product) [g/mol]

PP = Product purity [%]

m (Crude product) = weigh-in crude product [mg]

2.4.3 BIOCATALYTIC DESYMMETRIZATION OF DIALKYL 3-HYDROXYGLUTARATES BY α-CHYMOTRYPSIN

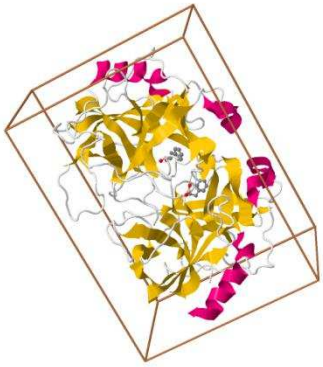


Figure 23. Crystal structure of the α-chymotrypsin dimer with the complexed inhibitor phenylethane boronic acid (BLEVINS and TULINSKY).[20]

The synthesized derivatives of diethyl 3-hydroxyglutarate were all subjected to the desymmetrization with α-chymotrypsin (**Figure 23**) as already proven active biocatalyst. The reactions were performed with a Titrino apparatus, which simultaneously measures the pH of the aqueous reaction mixture and titrates with a given solution of base to neutralize the product and maintain the pH, a prerequisite for maintaining a high enzyme activity. Unfortunately, the already TBS-protected substrate **22f**, did not show any conversion (see **Figure 24**). This is congruent with common observations throughout biocatalysis that bulky substituents in the vicinity of the reactive carbonyl structure can have a strong impact on both activity and selectivity of the enzyme, if not completely inhibiting the desired reaction. The absence of activity during the α-CHY-catalyzed desymmetrization was also observed for the bulky *O*-protected derivatives in this work, i.e. the phenylacetate **22d**, the *tert*-butyloxycarbonate **22b** and the tetrahydropyran-2-yl acetal **22g**, which showed no conversion. Aside from bulkiness, the trifluoroacetate **22c** revealed itself as highly labile towards hydrolysis in aqueous buffer solutions, which was the major reactivity observed for this compound.

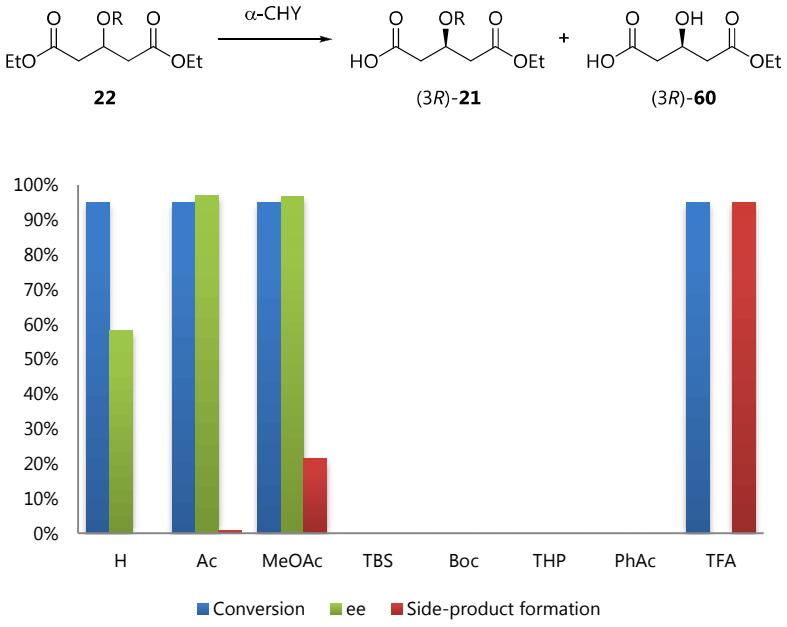


Figure 24. Biocatalytic desymmetrization of 3-DHG and *O*-protected derivatives by α-chymotrypsin (α-CHY).

O-protected derivatives: tert-butyldimethylsilyl (TBS), tert-butyloxycarbonyl (Boc), tetrahydropyran-2-yl (THP), tri-fluoroacetyl (TFA); side products: deprotected monoester (MeOAc), deprotected diester (TFA).

The product of this hydrolysis is in fact the O-unsubstituted diethyl 3-hydroxyglutarate **1a** and is further converted by α-chymotrypsin with poor enantioselectivity (see **Figure 12**, page 11). As it was expected, the acetate **22a** and methoxyacetate **22e** showed high activity in combination with excellent enantioselectivities of >95% ee. Interestingly, the methoxyacetate **22e** also showed significant hydrolysis to the 3-hydroxy monoester of type **60** up to 21%. This was also considered beneficial for the exchange to the TBS protective group. Surprisingly, the second hydrolysis could not be enhanced by prolonged reaction times or higher basicity of the reaction medium. The chemical saponification of the methoxyacetate **21e** led to significant loss of yields due to parallel saponification of the remaining ethyl ester. The sole substrate exhibiting the desired reactivity was indeed the acetate **22a**, which did not show any side-reactions. Occasional appearance of an α,β-unsaturated diester by base-induced elimination of acetic acid was observed for highly concentrated titer (4.0M sodium hydroxide) and was prevented by slow titration or higher dilution of titer.

2.4.4 DEACYLATION OF THE 3-ACYLOXY MOIETY

The deprotection of *O*-acylated carbinols in the presence of other esters was quite challenging. Standard wet-chemical approaches by acid- or base-catalyzed hydrolysis were futile since the hydrolysis of the second alkyl ester led to the loss of chirality and thus decreased the yield of the desired monoester. Although α-chymotrypsin showed the desired activity for the methoxyacetyl substrate with around 20% conversion (see **Figure 24**, page 20), the hydrolysis could not be enhanced by either prolonged reaction time or higher enzyme amount. Therefore, a selection of hydrolases was screened for activity but none of them showed any conversion (see **Table 2**, entries 3-5, page 22). A lipase-catalyzed transesterification with various alcohols was also investigated (see **Table 2**, entries 1-2, 6-8, page 22). *Candida antarctica* lipase B showed moderate activity up to 30% product-related conversion. Nevertheless, the conversion was never quantitative, leading to a mixture of acylated and deacylated monoester which would have to be separated by column chromatography. The most promising solution was found with an industrially applied hydrolase for the specific cleavage of an acetyl group from cephalosporin C, thus named cephalosporin C acetylesterase (CAE, see **Figure *25***, page 21).[21] The enzyme was originally isolated from *Bacillus subtilis* and overexpressed in recombinant *E. coli*. It was further immobilized on a commercial carrier for the large-scale production of deacetyl-7-amino-

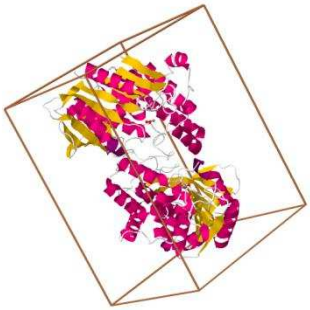
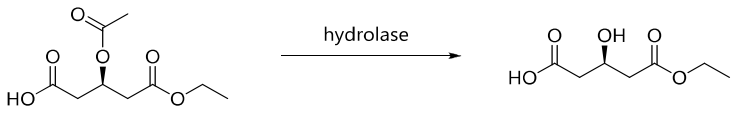


Figure 25. Crystal structure of cephalosporin C deacetylase from *Bacillus subtilis* complexed with acetate (VINCENT et al.).[21]

cephalosporanic acid (HACA) by combination with a D-amino acid oxidase from *Trigonopsis variabilis*.[22]

Table 2. Removal of the *O*-acetyl protective group by the use of hydrolases.

(a) The pH was not maintained in this first experiment and decreased to 3-4 which might have led to inactivation of the enzyme (b) Due to high solubility of the product in water the extractive work-up led only to moderate yields.



(*3R*)-**45b** (*3R*)-**60**

Entry	Substrate [mmol]	Biocatalyst (g)	Additive (eq.)	Substrate [M]	Conversion [% (h)]	Yield [g (%)]
1	10.0	CAL-B (1.00)	EtOH (2.00)	1.43	22.0 (15.5)	n.d.
2	10.0	CAL-B (1.00)	EtOH (10.0)	1.43	30.1 (76.5)	n.d.
3	1.25	Amano PS SD (0.05)	-	0.05	4.76 (24.0)	n.d.
4	1.25	Amano AK (0.05)	-	0.05	7.41 (24.0)	n.d.
5	1.25	Amano AYS (0.05)	-	0.05	4.76 (24.0)	n.d.
6	5.00	CAL-B (0.50)	EtOH (17.1)	0.50	5.66 (24.0)	n.d.
7	5.00	CAL-B (0.50)	EtOH (34.2)	0.50	<1.00 (24.0)	n.d.
8	4.58	CAL-B (0.20)	MeOH (5.38)	0.76	<1.00 (24.0)	n.d.
9	5.00	CAE (0.05)	-	1.00	12.3[a] (18.5)	n.d.
10	10.0	CAE (0.05)	-	1.00	89.5 (5.5)	1.47 (74.8)[b]
11	40.0	CAE (0.50)	-	0.80	>95.0 (4.5)	5.43 (77.0)[b]
12	48.5	CAE (0.50)	-	4.00	>95.0 (8.0)	4.28 (50.1)[b]

In the case of the acetylated monoester (*3R*)-**45b**, CAE showed complete conversion in aqueous buffer, even in the presence of stoichiometric amounts of ethanol, which corresponds to the product mixture of the desymmetrization (see **Table 2**, entries 11-12, page 22). Remarkably, only the acetate is cleaved while the ethyl ester remains untouched. Control experiments with **1a** and the acetate-protected diester **22a** showed no conversion and full conversion to 3-DHG, respectively (see **Figure 26**, page 23). In any case, desymmetrization did not occur with CAE. The fact that Cephalosporin C acetylesterase accepts carboxylates while most hydrolases are only active for esters and amides makes this enzyme extremely valuable.

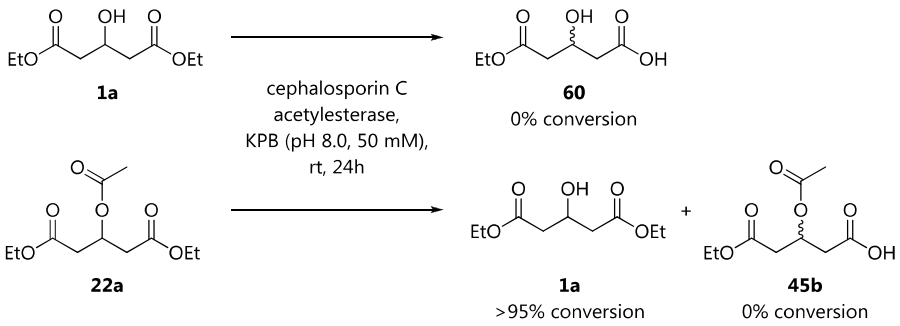


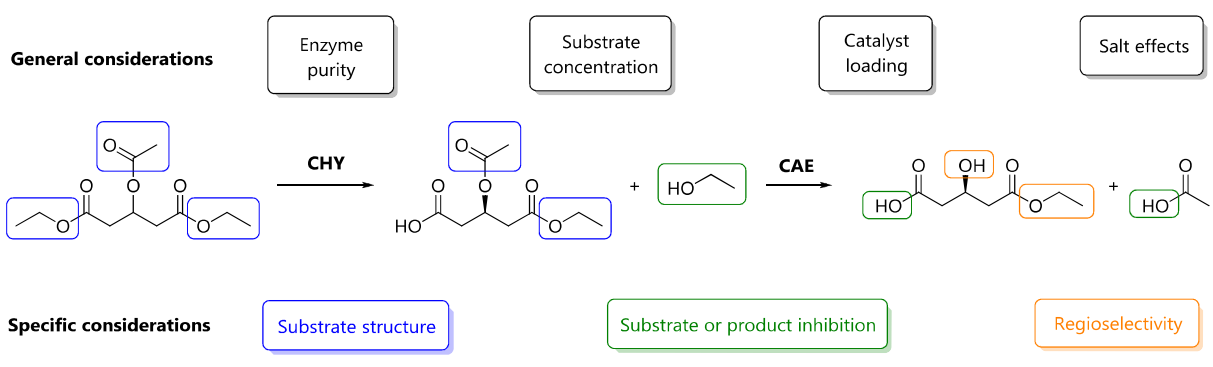
Figure 26. Control experiments for a possible desymmetrization of dialkyl 3-oxyglutarates by cephalosporin C acetylesterase (CAE).

Table 2 (page 22) shows the examined hydrolases for hydrolysis and alcoholysis of the *O*-acetyl group. After the experiments with CAE demonstrated quantitative conversion and excellent regioselectivity, no further hydrolases were screened.

2.4.5 Investigation of optimum parameters for the biocatalytic desymmetrization and deacetylation

The establishment of a bienzymatic synthesis for the enantioselective production of ethyl (*3R*)-hydroxyglutarate from the prochiral *O*-acetylated diethyl 3-hydroxyglutarate was already a great. Nevertheless, the productivity of the process needed to be improved by modifying various parameters (see **Figure 27**). Since the experiments were always terminated after 100% consumption of theoretical titer amount, comparing the reactions by conversion alone would be difficult. The reaction time itself would also be

Figure 27. Optimization of reaction parameters for cost-effective production of enantiomerically enriched monoesters by hydrolytic biotransformations



ambiguous since it depends on the absolute amount of substrate in the given experiment, which was altered in the range of 50 mM to 4.0 M. Thus, the following results will be given in calculated data for the volumetric productivity of a reactor under the respective reaction conditions (g/(L*d), grams of product per liter of reaction medium per day), which would be identical to the space-time-yield under continuous operation. Since the biocatalyst has the highest cost factor, additional values of both catalyst concentration (g/L, grams of enzyme formulation per liter of reaction medium) and catalyst loading (g(Sub)/g(CHY), grams of substrate per gram of enzyme formulation of α-chymotrypsin or its mixture with trypsin) will be presented. The cost-related amount of enzyme formulation necessary for the production of one kilogram of product per day can be directly estimated from the ratio of the volumetric productivity (blue) to the catalyst concentration (red) and is therefore not presented separately.

2.4.5.1 SUBSTRATE STRUCTURE

The effect of the 3-hydroxy protective group was already discussed in chapter 2.4.3 (page 20). It accelerated the hydrolytic reaction and enhanced the enantiomeric differentiation of α-chymotrypsin from moderate 60% ee to excellent >98% ee. Also of importance was the kind of alkyl ester used for the reaction. The readily available diesters of 3-hydroxyglutarate are the dimethyl **1b** and diethyl ester **1a**, respectively, with the diethyl ester being the more economical one. However, the commercially available building block of the Rosuvastatin side-chain, the chiral phosphor ylide, consists of the corresponding methyl ester. Thus both dialkyl esters were compared for their productivity in the biocatalytic desymmetrization (see **Figure 28**).

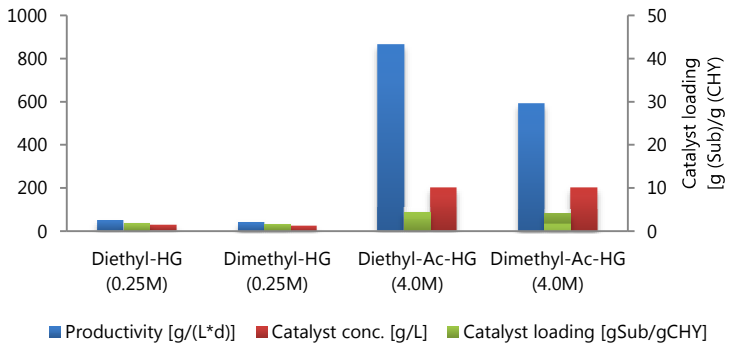


Figure 28. Biocatalytic desymmetrization of different dialkyl esters of 3-hydroxyglutarate and its *O*-acetyl derivative.

The free hydroxyesters were converted in nearly the same reaction rates at 0.25 mol/L substrate concentration, whereas the corresponding *O*-acetyl esters showed a strong dependency on the chain length. The acetylated diethyl ester was desymmetrized with more than 40% higher activity at 4.0 mol/L substrate concentration. Since this substrate was converted The influence of the alcoholic side-product on the enzyme activity was not investigated.

2.4.5.2 Substrate Concentration

Biocatalytic reactions are often limited by the necessity of a low substrate concentration. This is due to the fact that many enzymes tend to be inhibited by a high excess of substrate, especially if the product has also high binding constants in the active center. In the case of hydrolases, this inhibition is often prevented by the strong difference in polarity of substrate and product. This fact also explains the significant higher reaction rates of hydrolases in relation to oxidoreductases, for example. The enzyme with the highest known reaction rate is the acetylcholine esterase with a turn-over frequency (TOF) of >10,000 s^{-1}.[23] The substrate concentration of the biocatalytic desymmetrization of diethyl 3-acetoxyglutarate **22a** was successfully improved from 50 mM to 4.0 M, which corresponds to a theoretical product concentration of >800 g/L. at 100% conversion (see **Figure 29**).

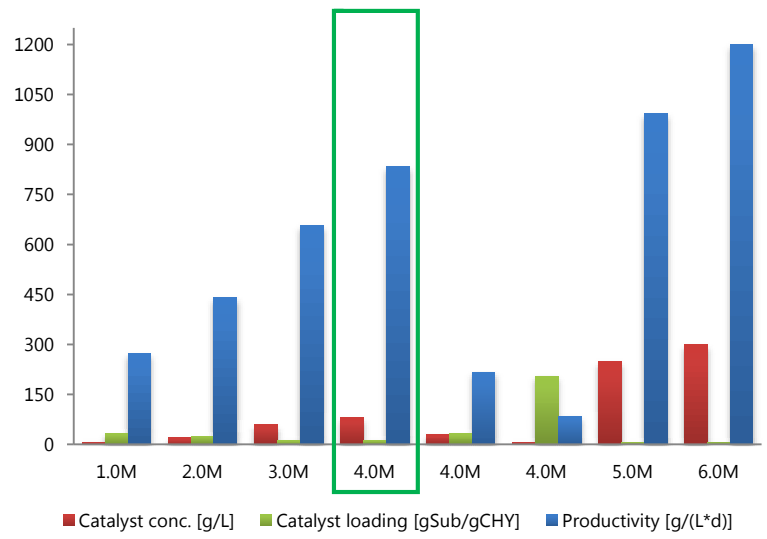


Figure 29. Biocatalytic desymmetrization with different substrate concentrations of diethyl 3-acetoxyglutarate **22a** and improved process for 4.0 mol/L substrate concentration (green square).

Notably, the solubility of the substrate in the aqueous reaction mixture is rather low, leading to a biphasic system in almost 1:1 volumetric ratio (buffer/substrate). Further upscaling to 5.0 M and even 6.0 M showed a dramatic decrease in activity (see **Figure 29**, page 25, ratio of volumetric productivity (blue) to catalyst concentration (red)). However, this could also be due to the increased viscosity of the reaction mixture and the aggravated titration under pH control. Nevertheless, the concentration of 4.0 M was sufficient for industrial applicability. Furthermore, the productivity was tremendously improved by combining a moderate catalyst concentration of 80 g/L with a low catalyst loading of 12.3 g diethyl 3-acetoxyglutarate per g of α-chymotrypsin (see **Figure 29**, page 25, green square).

2.4.5.3 Enzyme Formulations

There are numerous suppliers of α-chymotrypsin, comprising a variance of purified or crude enzymes as well as mixtures of α-chymotrypsin and trypsin. To evaluate the efficiency of these formulations for the desymmetrization route and the need of a recycling procedure for the homogenously dissolved biocatalyst, different enzyme formulations were examined. Of great interest was the usability of 1:1 α-chymotrypsin/trypsin mixtures, which are considerably cheaper than the isolated enzyme. Additionally, three proteins which were isolated from the purified α-chymotrypsin formulation by coworkers of the Hummel group were also used.

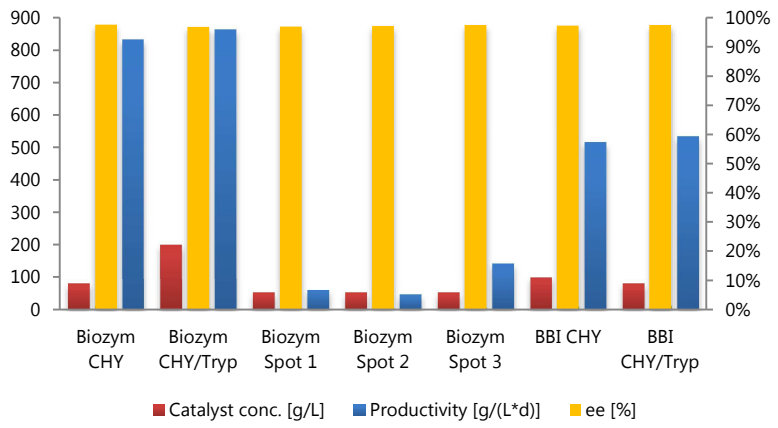


Figure 30. Various commercial formulations of α-chymotrypsin and isolated proteins (prepared by coworkers of the Hummel group) for the de-symmetrization of diethyl 3-acetoxyglutarate.

The major concern of these experiments was the enantiomeric excess of the product, since protein impurities could lead to side-reactions and a lower enantioselectivity. Fortunately, neither the presence of trypsin nor the isolation of one of the three proteins in the purified α-CHY altered the enantioselectivity to a notable extent. All formulations showed an excellent enantioselectivity of around 97% ee (see **Figure 30**, page 26). Even more, the economically more attractive α-chymotrypsin/trypsin mixtures also showed the desired high productivities of >800 g/L (Biozym) and >500 g/L (BBI). The need for 2.0 - 2.5 equivalents of enzyme amount for the mixture of Biozym in relation to the purified enzyme is readily amortized by its substantially lower prizing.

2.4.5.4 Effects of salts and additives on the volumetric productivity

Apart from the substrate and the enzyme itself, the reaction could also be affected by other solutes. When conducting the reaction in pure water instead of buffer, the reaction rate decreased slightly, suggesting a dependency on the ionic strength of the reaction medium, or the fluctuation of pH in the absence of a buffered medium (see **Figure 31**). The choice of additives was imposed by the combination with the preceding acetylation as well as the use of isolated proteins of a commercial α-chymotrypsin formulation.

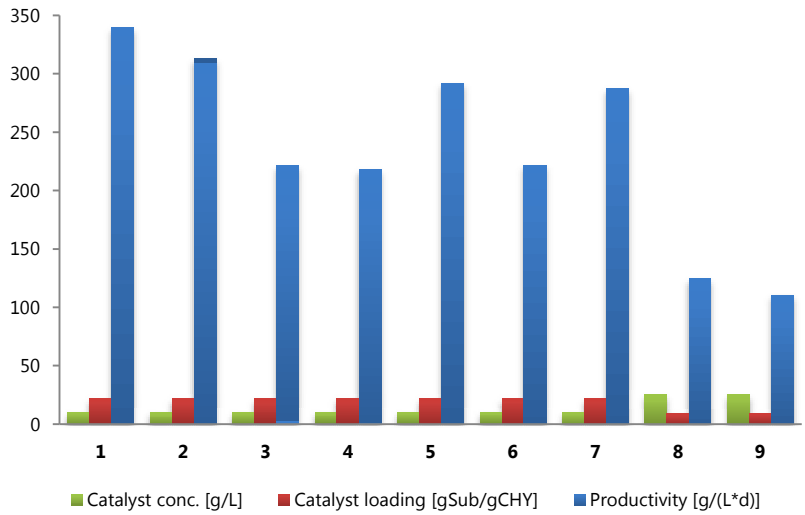


Figure 31. Effect of additives on the productivity of the biocatalytic desymmetrization of **22a** with α –chymotrypsin.

*Additive: none (**1**) 1.2 M sodium chloride (**2**) 1.2 M sodium acetate (**3**) 2.4 M sodium acetate (**4**) 1.0 mM zinc perchlorate (**5**) 1.0 M ammonium sulfate (**6**) reaction in dist. water (**7**) α-Cyclodextrin (**8**) 15 vol-% 2-propanol (**9**).*

The solvent-free acetylation led to a crude product comprising the acetylated diester and stoichiometric amounts of acetic acid, a small excess of acetic

anhydride (0.1 equivalents) and the catalytic amount of zinc perchlorate (0.001 equivalents). After dissolving this crude product in aqueous buffer and neutralizing the side-products with an aqueous sodium base (carbonate, bicarbonate or hydroxide, 1.2 equivalents), the reaction medium with 1.0 mol/L substrate would consequently have an additional ionic strength of dissolved 1.2 mol/L sodium acetate prior to the addition of the biocatalyst (see **Figure 31**, page 27, entry 3). The effect of catalytic amounts of zinc perchlorate was also studied separately (entry 5). Coworkers of the HUMMEL group isolated three proteins from the purified α-chymotrypsin batch (Biozym) which was used for the majority of the given experiments. The final separation step was done with an eluent of aqueous ammonium sulfate (1.0 mol/L). Since ammonium sulfate does not only increase the ionic strength but also lower the solubility of proteins, its effect on the reaction was also examined (entry 6). All additives were tested under the same reaction conditions with 1.0 mol/L substrate concentration and 100 mg of α-chymotrypsin. The effect on the volumetric productivity of the reaction, defined as the amount of product (in grams) per liter of reaction medium and per day, is illustrated. The highest reaction rate is observed under the standard reaction conditions in aqueous buffer. Catalytic amounts of zinc perchlorate show nearly the same decrease as the reaction in pure water (entries 5 and 7). In contrast to that, the presence of sodium acetate and ammonium sulfate did have a strong influence on the productivity, however arbitrarily related to their respective concentrations (entries 3, 4 and 6). Interestingly, sodium chloride had nearly no effect on the reaction, even at relatively high concentration of 1.2 mol/L (entry 2).

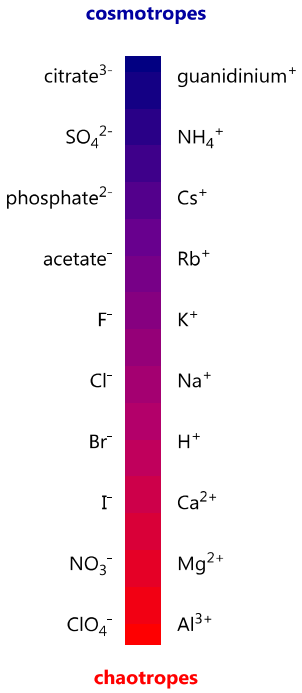
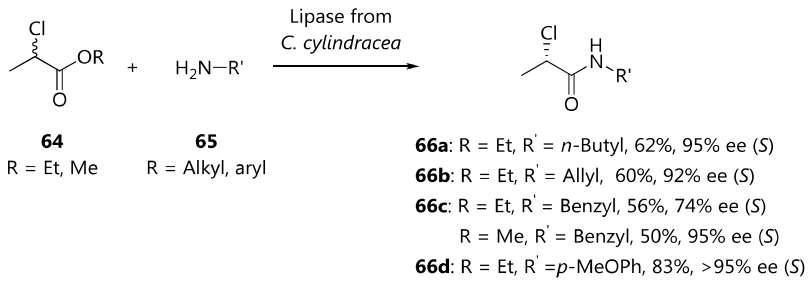


Figure 32. Hofmeister series of cations and anions altering the properties of dissolved proteins and the solvent in aqueous solutions from high stability (cosmotropes) to high solubility (chaotropes) accompanied by a decreesing surface tension.

This discrepancy could suggest ionic effects related to the HOFMEISTER series, with the acetate, ammonium and sulfate ions leading to a lower solubility of the biocatalyst and/or an increase in surface tension and therefore weaker mixing of the substrate phase with the aqueous phase (see **Figure 32**).[24] However, doubling of the acetate concentration did not show any effect on the reaction, contradicting the proposed ionic effects. Summing up these results, the effects of ionic additives on the volumetric productivity did not show a clear tendency. In general, the highest productivity was achieved in aqueous phosphate buffer (50 mM, pH 8.0, entry 1) without other solutes but was almost unchanged in the presence of sodium chloride (see **Figure 31**, page 27, entry **2**).

2.5 ENANTIO- AND DIASTEREOSELECTIVE AMINOLYSIS OF CHIRAL CARBON ACID ESTERS

Apart from resolving carbonacid esters by enantioselective hydrolysis in aqueous media, some hydrolases are also capable of resolution processes in organic media. This way the corresponding donor can be changed from water to all kinds of nucleophiles, e.g. alcohols, amines or thiols. Furthermore, most of these lipases are fairly stable in organic media and at elevated temperatures. Comprehensive work in the field of lipase-catalyzed aminolysis of chiral carbonacid esters has been done by GOTOR *et al.*, mostly by using commercially available lipases from *Candida* sp..[25,26]



64
R = Et, Me

65
R = Alkyl, aryl

66a: R = Et, R' = *n*-Butyl, 62%, 95% ee (*S*)
66b: R = Et, R' = Allyl, 60%, 92% ee (*S*)
66c: R = Et, R' = Benzyl, 56%, 74% ee (*S*)
R = Me, R' = Benzyl, 50%, 95% ee (*S*)
66d: R = Et, R' =*p*-MeOPh, 83%, >95% ee (*S*)

Figure 33. Lipase-catalyzed enantioselective aminolysis of α-halocarboxylic acid esters by GOTOR *et al.*[26]

For instance, the resolution of α-halo esters of type **64** (see **Figure 33**) was achieved with a lipase from *Candida cylindracea* (CCL) in *n*-hexane as reaction medium at low or elevated temperatures.[26] This process is of particular importance since halogenated esters would also alkylate the amine under common amide synthesis conditions. With CCL, primary amines of type **65** react with excellent enantioselectivities under formation of the corresponding amides. Notably, the enantiomeric excess is strongly dependent on the alkyl ester of the substrate **64** and the structure of incorporated amine. Even aromatic amines can be used, e.g. *p*-methoxyaniline, with high yields and excellent enantioselectivites of >95% ee (**66d**). In addition, GARCIA-URDIALES *et al.* reported the enantioselective ammonolysis of ethyl 4-chloro-3-hydroxy-butanoate **67** (see **Figure 34**, page 30) in various ammonia-saturated organic solvents in the presence of lipase B from *Candida antarctica*.[27] These reports indicate clearly that lipases show enantioselectivity for α- and β-stereogenic centers, with a strong dependency on the structure of the nucleophile. The enantiomeric excess improves with increasing polarity of the nucleophile, with ammonia and primary achiral amines giving the highest selectivities.

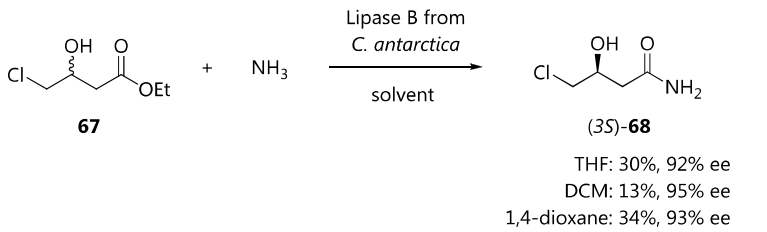


Figure 34. Lipase-catalyzed enantioselective ammonolysis of β-hydroxycarboxylic acid esters by GARCIA-URDIALES *et al.*[27]

Remarkably, α-chiral amines are also applicable, giving diastereomers in enriched mixtures, as shown by VÖRDE *et al.* for the diastereoselective aminolysis of racemic ethyl 2-methyloctanoate with enantiomerically pure (*R*)- or (*S*)-1-phenyl-ethylamine.[i28] Unfortunately, the diastereomeric excess was only low to moderate, with the best result being 54% de at 3% conversion in dioxane. Due to the necessity for enantiomerically pure amine, this diastereomeric approach was not investigated in the present work.

2.5.1 LIPASE-CATALYZED DESYMMETRIZATION BY AMINOLYSIS WITH VARIOUS PRIMARY AND SECONDARY AMINES

RIISE MOEN *et al.* successfully desymmetrized diethyl 3-hydroxyglutarate with lipase B from *Candida antarctica* (CAL-B) through hydrolysis in aqueous buffer.[29] Remarkably, CAL-B shows enhanced activity and enantioselectivity in comparison to α-chymotrypsin for **1a** as substrate. Furthermore, the immobilized catalyst can readily be recycled by filtration and resuspension in buffer. However, the enantiopreference of CAL-B is inverted in comparison to α-chymotrypsin, leading to the corresponding (*S*)-enantiomer of the monoester **60** by hydrolytic desymmetrization. In contrast to that, LÓPEZ-GARCÍA *et al.* successfully used primary amines in the desymmetrization, leading to their corresponding amines with high conversions and good to excellent enantioselectivities of 88-99% ee (see **Error! Not a valid bookmark self-reference.**). [30] Notably, also the O-unprotected diester of 3-hydroxyglutarate was converted in 98% and with >99% ee. This is important as it opens the possibility of introducing the tert-butyldimethylsilyl protective group after the desymmetrization. Based on these preceding reports, the aminolytic desymmetrization was further investigated in this work. The inverted selectivity of the lipase for producing the (3S)-amide was still a drawback.

[i] According to their publication the reaction is enantioselective. However, they obtain different conversions and selectivities for (*R*)- and (*S*)-amine. Therefore the reaction with CAL-B should be defined as diastereoselective.

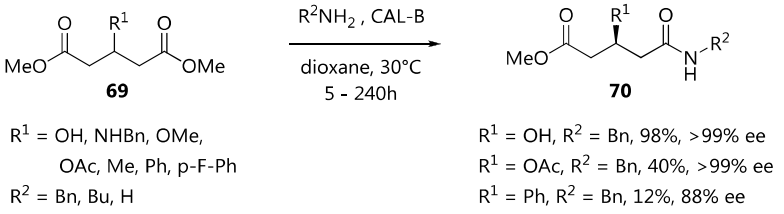


Figure 35. Enantioselective desymmetrization of 3-substituted dimethyl glutarates with benzylamine in dioxane by LÓPEZ-GARCÍA *et al.*[30]

By using sterically demanding nucleophiles, the lipase-catalyzed desymmetrization would initially generate the undesired (*S*)-enantiomer (*3S*)-**26**, but with increased reactivity for the remaining ethyl ester. Consequently, the substitution reaction with the phosphoranylidene would then again yield the desired (*R*)-enantiomer of the stabilized ylide (*3R*)- **2** (see **Figure 36**).

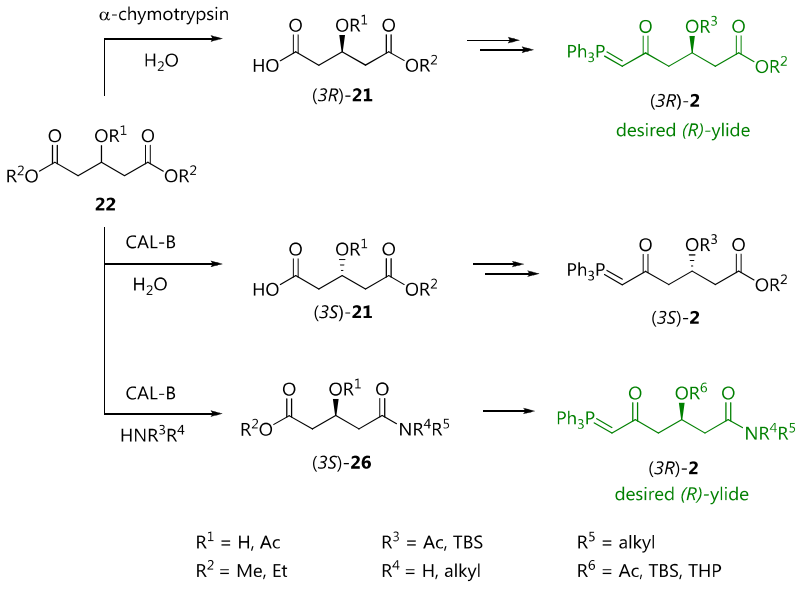
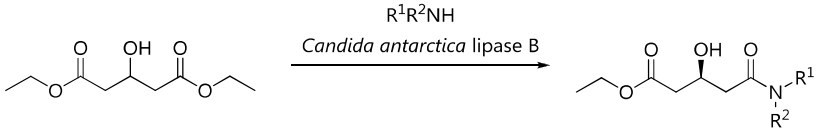


Figure 36. Enantiopreference of biocatalytic desymmetrizations *via* α-chymotrypsin and *Candida antarctica* lipase B with water and amines, leading to the stabilized phosphor ylides with the desired absolute configuration.

Benzyl amine was regarded as a promising and sterically demanding amine for this methodology. Additionally, the benzyl group would certainly facilitate UV/Vis analysis of most intermediates. The desymmetrization of **1a** with benzylamine was successfully realized in this study, with quantitative conversion and an excellent enantioselectivity of >93% ee (see **Table 3**, page 32, entries 1-5). Besides, the corresponding amide is readily purified by recrystallization in MTBE.



22 (*3S*)-**26**

Entry	R^1	R^2	**22** [mmol]	CAL-B [mg]	Temp. [°C]	Solvent	Conv. [%]	Yield [g(%)]
1	H	Bn	2.00	180	20	dioxane	>95	0.52 (98.9)
2	H	Bn	2.00	180	20	THF	>95	0.47 (89.3)
3	H	Bn	2.00	180	20	MTBE	>95	0.49 (93.1)
4	H	Bn	2.00	180	20	toluene	84.0	n.d.
5	H	Bn	10.0	907	20	MTBE	>95	2.20 (83.1)
6	Me	Bn	10.0	900	reflux	MTBE	7.2	0.16 (5.6)
7	Et	Et	2.00	180	reflux	MTBE	0	-
8	morpholine		10.0	900	reflux	MTBE	n.d.	0.86 (35.0)
9	pyrrolidine		5.00	110	20	MTBE	55.9	n.d.
10	pyrrolidine		5.00	0	20	MTBE	22.0	n.d.
11	pyrrolidine		5.00	110	60	pyrrolidine	>95.0[b]	n.d.
12	piperidine		5.00	113	reflux	MTBE	32.0	n.d.

Table 3. Lipase-catalyzed aminolysis[a] of 3-DHG with various primary and secondary amines in organic solvents.

(a) General reaction conditions: 1.0 eq. amine (except entry 10), 0.5 M substrate concentration (b) Double aminolysis to the corresponding prochiral bis-amide.

What remained questionable was the reactivity of the benzylamide during the following synthetic steps. For example, ASENSIO *et al.* tosylated *N*-benzyl acetamide in 60% conversion by using the lithium amidate.[31] This lithium amidate of type **71** could also be formed in the present synthesis by an acid-base equilibrium during the ylide substitution (see **Figure 37**, page 33). This species could then also cyclize intramolecularly and form a number of undesired side-products of type **72** and **73**. To circumvent this problem, a couple of secondary amines were also examined for the biocatalytic desymmetrization (see **Table 3**, entries 6-12). Most of them were indeed active, nevertheless generally to only minor extent. This is in accordance with some results by DHAKE *et al*. for piperidine.[32] Its low activity for aminolytic ester substitution was also reproduced in the present work (entry 12). Interestingly, pyrrolidine and morpholine showed higher conversion of 56% and 35%, respectively (see ***Table 3***, page 32, entries 8-9). The benefit of morpholine was a crystalline product which was readily purified by recrystallization in light petroleum ether. The reaction with pyrrolidine demonstrated the limits of lipase-catalyzed desymmetrization. At a reaction temperature of 60°C with the donor as solvent in more than 10-fold molar excess, the reaction went straight through to the bisamide (see **Table 3**, page 32, entry 11).

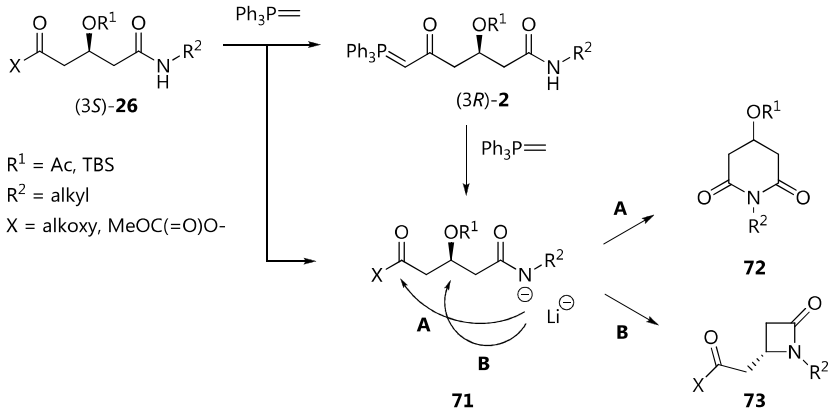


Figure 37. Possible intramolecular side-reactions by introducing primary amides in the synthetic route.

Judging from the absence of this second aminolysis for primary amines, the reactivity of secondary amines for uncatalyzed aminolysis does in fact play an important role for the optical purity of the product. For this reason the reaction between diethyl 3-hydroxyglutarate and pyrrolidine was reproduced without biocatalyst (entry 10). After the same reaction time as the lipase-catalyzed reaction and the respective work-up procedure, the crude product was analyzed by NMR spectroscopy. The conversion of the uncatalyzed reaction in MTBE at room temperature was determined to be 22%. By taking this uncatalyzed conversion to the racemic monoamide into consideration, the moderate enantiomeric excess of 59.1% ee (see **Figure 40**, page 35) at 55.9% conversion (see **Table 3**, page 32, entry 9) could actually be significantly higher, more precisely up to 97.6% ee. This would be in good agreement for the excellent enantioselectivity observed when using benzyl amine as donor reagent (>93% ee, see **Figure 39**, page 34).

2.5.2 Analytical methods for the determination of Conversion and enantioselectivity

The conversion of the prochiral diester to its chiral monoamide was determined according to the method described for hydrolytic products (see **chapter 2.4.2**, page 17) by integration of significant signals in the ^{1}H-NMR spectrum. *In situ*-monitoring of the reaction progress by titration, which was applicable for hydrolytic reactions, was not possible for these reactions. The reaction products, *i.e.* the chiral monoamide, remaining prochiral diester as well as the substituted alcohol, cannot be detected by such methods. A possibility would

be an alcohol dehydrogenase-coupled oxidation of the carbinol to the corresponding aldehyde and quantification *via* photometric assays with carbonyl-detecting reagents. Since the reaction proceeds in MTBE this approach would need an alcohol dehydrogenase tolerant to organic solvents. Since this methodology was not available in this work, the conversion of all aminolytic reactions were solely analyzed by isolated product quantification and NMR integration (see **Figure 38**).

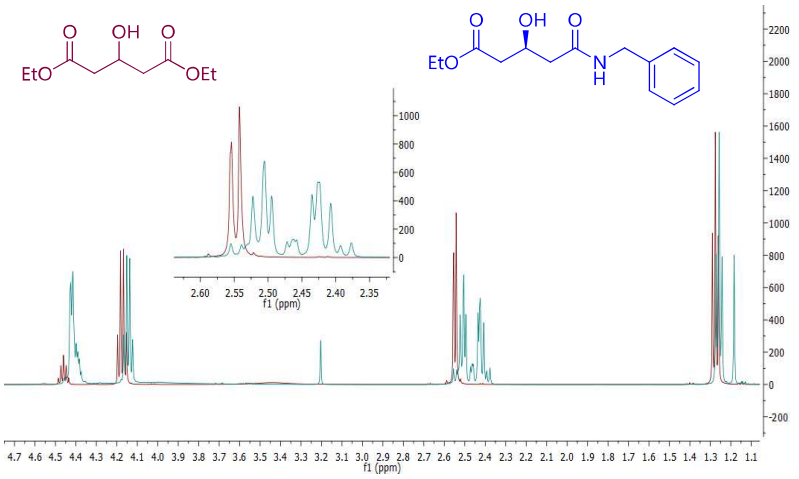
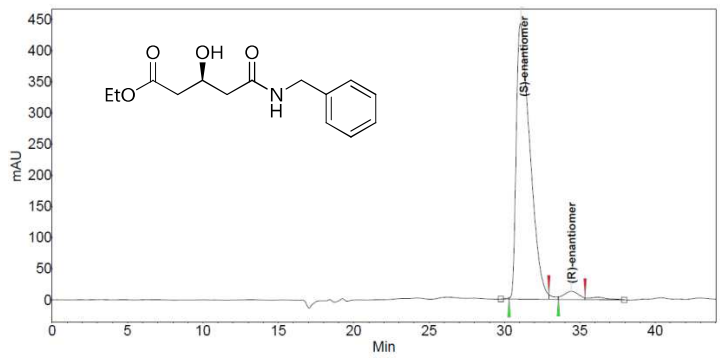


Figure 38. NMR spectra of the prochiral substrate and the enantiomerically enriched N-benzylamino monoester after purification.

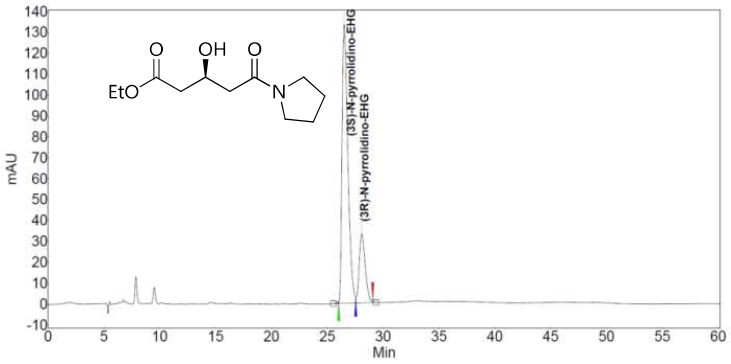
Despite literature-known methods for HPLC analysis of the chiral monoamides, only the *N*-benzyl amide (see **Figure 39**) and the *N*-pyrrolidino amide (see **Figure 40**, page 35) were successfully separated on chiral HPLC for the determination of the enantiomeric excess.



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(S)-enantiomer	31,06	96,85	442,1	464,4	96,854
2	(R)-enantiomer	34,42	3,15	13,2	15,1	3,146
Total			100,00	455,3	479,5	100,000

Figure 39. HPLC chromatogram of the enantiomerically enriched *N*-benzylamino monoester

Both separations were sufficient for integration, however should be improved for further studies on aminolytic desymmetrizations of 3-DHG, including separation of substrate and diamide, which was observed at higher reaction temperatures and excess amine (see ***Table 3***, page 32, entry 10). Analysis by quantitative HPLC measurement should be possible by simple filtration of the immobilized biocatalyst and direct measurement of the MTBE reaction solution. This methodology was not further investigated in the present work.



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
2	(3S)-N-pyrrolidino-EHG	26.50	79.57	133.6	80.8	79.571
1	(3R)-N-pyrrolidino-EHG	28.08	20.43	33.1	20.7	20.429
Total			100.00	166.7	101.5	100.000

Figure 40. HPLC chromatogram of the enantiomerically enriched *N*-pyrrolidino monoester.

2.6 SUBSEQUENT CHEMICAL ROUTE TO AN ENANTIOPURE KEY INTERMEDIATE

Up to now the absolute configuration was introduced by a biocatalyst in excellent selectivity. The task for the following chemical reactions (see **Figure 41**, page 36) was to achieve high conversions and yields under retention of the absolute configuration and without any racemization of the stereogenic center. The biocatalytic deacetylation proceeded without epimerization and yielded the desired deprotected (*3R*)-monoester **60** in quantitative conversion and excellent yields of up to 95%. The optical purity was determined to be >97% ee according to the method described in **chapter 2.4.3** on page 18. Since the monoesters with the free hydroxy group and the acetyl-protecting group were obtained with excellent enantiomeric excess, the following steps were conducted for both of them in parallel.

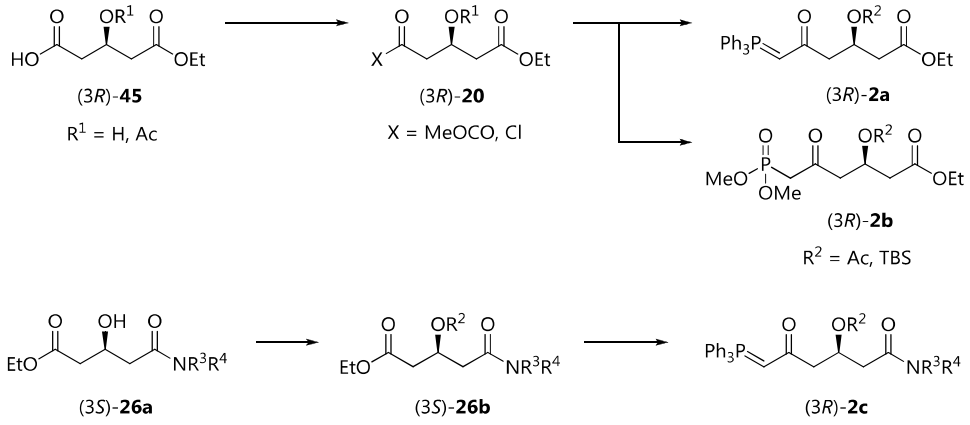


Figure 41. Chemical derivatization of enantiomerically enriched monoesters to their corresponding phosphor reagents

2.6.1 PROTECTIVE GROUP CHEMISTRY FOR THE 3-HYDROXY GROUP

The subsequent activation of the monoester was going to be done by nucleophilic substitution, thus the potentially nucleophilic secondary alcohol had to be masked. A common protecting group in this case is the *tert*-butyldimethylsilyl ether, which is highly stable towards strongly basic reaction conditions and nucleophilic substitution. This protective group is also used in the literature-known synthesis of Rosuvastatin.[16] Usually, *tert*-butyldimethylchlorosilane **74** is activated *in situ* in the presence of 1*H*-imidazole **75** (see **Figure 42**). The intermediate **76** is then used to silylate a nucleophile, which in this case is the secondary alcohol of (3*R*)-**60**. However, after extraction of the crude product, the appearance of a by-product was observed. By

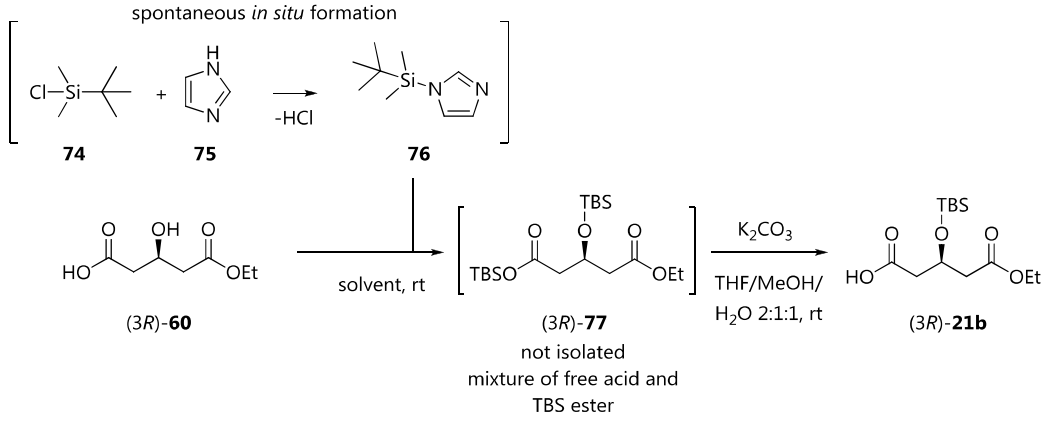
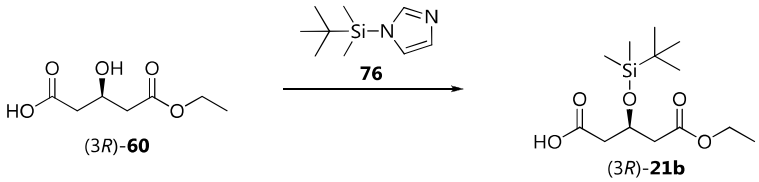


Figure 42. Reaction scheme for the TBS protection of ethyl (*3R*)-hydroxyglutarate

analysis of the ^{1}H-NMR spectra and the reaction mechanism, the impurity was identified as the corresponding silyl ester (3*R*)-**77**.

The procedure was therefore changed to an excess of chlorosilane **74** and a subsequent hydrolysis in aqueous basic medium as previously described by SCHOENHERR *et al.*. [33] The literature protocol was partially changed since the reported 6-fold excess of inorganic base seemed to be exaggerated. The hydrolysis was successfully achieved with 0.5 equivalents of inorganic base to maintain the basic pH in the solution. In addition, the aqueous work-up after silylation, which was done in the literature procedure, was eliminated since it was redundant. Under the optimized reaction conditions (see **Table 4**), the desired TBS-protected monoester (3*R*)-**21b** was isolated as a mixture with residual silanol. Although the protection group is labile to acidic pH, the aqueous phase needed at least pH 5 to obtain yields above 60% after three extractions. Saturation with sodium chloride led to an increased yield of both product and silanol. This impurity could only be partly removed by distillation, preferably in its hemihydrate form.[34] Even at high vacuum and elevated temperatures the viscosity of the product made it hard to completely distillate the remaining silanol. Since it was not clear how this impurity would affect the following reactions, the crude product was initially used without further purification.



(3*R*)-**60** (3*R*)-**21b**

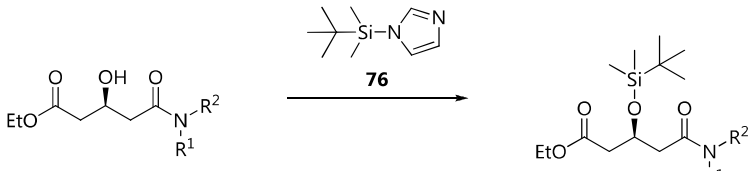
Entry	**60** [mmol]	TBS-Cl (eq.)	1*H*-imidazole (eq.)	Solvent	T [°C]	Time [h]	Conversion [a] [% (h)]	Yield [g (%)]
1	5.00	2.20	6.00	THF	20	17	>95	2.26 (75)
2	10.0	1.10	3.00	MTBE	20	26	43	n.d.
3	5.00	1.50	2.50	MTBE	reflux	12	59	n.d.
4	5.00	2.00	3.00	MTBE	reflux	20	89	1.82 (52)
5	30.8	2.20	2.20	MTBE	reflux	6	87	10.5 (51)
6	40.0	2.20	2.20	2-MeTH	80	18	32	n.d.
7	100	2.00	2.00	THF	20	21	>95	9.80[b] (24)
8	117	2.00	2.00	EtOAc	20	19	78	n.d.
9	100	2.00	2.00	THF	20	17	>95	16.8[b] (58)

Table 4. TBS-protection of ethyl 3-hydroxyglutarate in various organic solvents

(a) The conversion is determined after basic hydrolysis of the silyl ester and subsequent extraction from the acidified aqueous phase (b) Calculated from the crude product by quantitative NMR analysis with p-chlorobenzyl alcohol as internal standard.

The TBS-protection of desymmetrized primary and secondary amides was realized according to the above mentioned method. Since amides have only low solubility in water, the extraction could be done at a basic pH of around 8. This way, the acid-labile protective group did not hydrolyse during the work-up. Unfortunately, the product was again mixed with silanol. All amides were purified *via* column chromatography on silica to remove the remaining side-product (see **Table 5**). Notably, the primary amide positions were not silylated under the reaction conditions.

Table 5. TBS-protection of various enantiomerically enriched β-hydroxyamides



(3*S*)-**26a** (3*S*)-**26b**

Entry	R^1	R^2	TBS-Cl (eq.)	1*H*-Imidazole (eq.)	Conv. [%]	Isol. yield [g(%)]
1	H	Bn	1.1	3.0	>95.0	0.49 (64.5)
2	Me	Bn	2.0	3.0	>95.0	0.20 (66.0)
3	Morpholine		2.0	3.0	>95.0	0.12 (83.4)

2.6.2 ACTIVATION OF THE CARBOXYLIC ACID

The phosphor ylide was going to be generated *via* nucleophilic substitution, so the *O*-protected carboxylic acids had to be activated. Some common activation methods for carboxylic acids were investigated for this purpose. The monoesters were transformed into the corresponding acid chlorides or mixed anhydrides of type **78** (see **Figure 43**, page 39) as suggested by some literature protocols.[35] Whereas the formation of the acid chloride seemed to be the more convenient method, the purification of the crude product was not trivial. The analysis of the crude product also revealed the presence of various impurities which could not be identified within this work. In contrast to that the

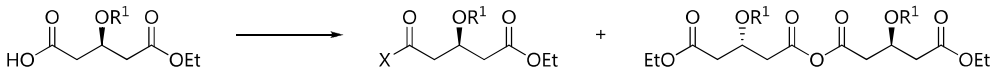


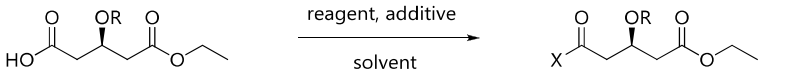
Figure 43. Reaction scheme for the synthesis of activated carboxylic acid derivatives

reaction with methyl chloroformate for the synthesis of the mixed anhydride with a carbonate leaving group proved itself to be straightforward. The quantitative conversion was determined by ^{13}C-NMR analysis for the acid chloride since the only clear observable difference in NMR spectroscopy is the low-field shift of the carboxyl-carbon.

In the case of the methyl carbonate derivative, the conversion was also quantitative. Even after purification on silica to remove residual silanol from the preceding reaction, the pure compound showed a methyl integral not corresponding to the theoretical number of protons. This could be explained by a mixed anhydride of type **78** (see **Figure 43**) of two monoesters.

Since the two carboxylates share a C_2-axis, diastereomeric shifts are not observable. The integrals determined by manual integration suggested the lack

Table 6. Synthesis of activated carbonacid derivatives by literature-known protocols

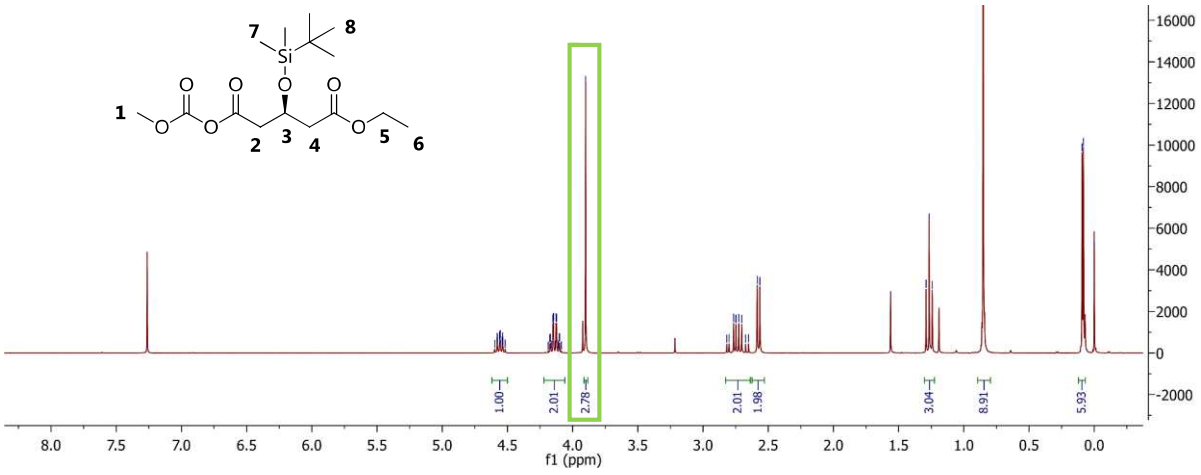


21a: R = Ac
21b: R = TBS

20a: R = Ac, X = MeOC(=O)O-
20b: R = TBS, X = MeOC(=O)O-
20c: R = Ac, X = Cl

Entry	R (mmol)	Reagent (eq.)	Additive (eq.)	Solvent (°C)	Conv. [% (h)]	Yield [g (%)]
1	Ac (1.60)	ClCOOMe (1.25)	TEA (1.50)	Toluene (-40)	86.3 (0.5)	0.29 (60.6)
2	Ac (5.00)	ClCOOMe (1.25)	TEA (1.50)	MTBE (-40)	>95.0 (0.5)	0.94 (68.1)
3	Ac (4.00)	$C_2O_2Cl_2$ (1.30)	DMF (0.01)	DCM (0-20)	>95.0 (4.0)	n.d.
4	TBS (3.77)	$(H_3C)_3CCOCl$ (1.03)	TEA (1.03)	THF (0)	0.0 (3.0)	-
5	TBS (3.44)	TosCl (1.10)	TEA (1.10)	MTBE (20)	0.0 (24.0)	-
6	TBS (2.00)	ClCOOMe (1.65)	TEA (1.25)	MTBE (-60)	>95.0 (1.0)	0.28 (33.8)
7	TBS (15.0)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	78.7 (2.0)	5.64 (n.d.)
8	TBS (12.6)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	91.7 (2.0)	3.17 (66.1)
9	TBS (5.00)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	91.0 (16.5)	1.16 (66.8)

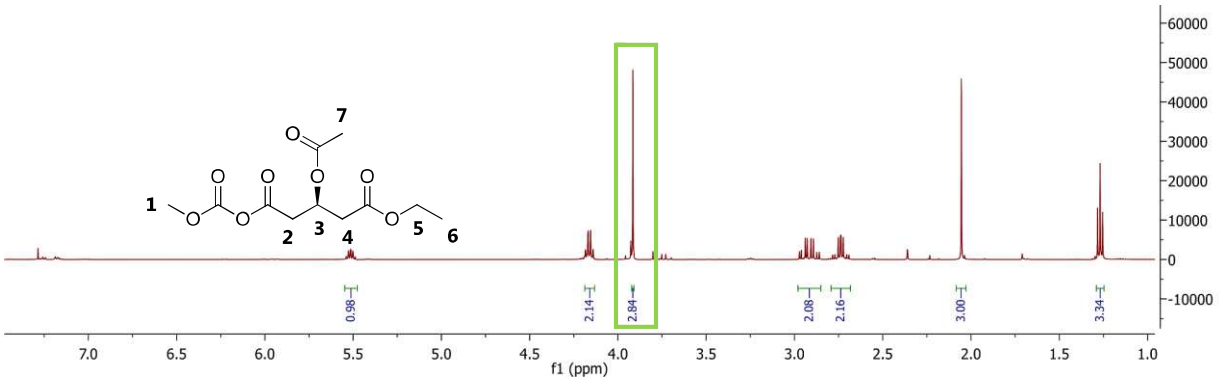
of protons for the methylcarbonate signal, but the analysis of the Lorentz-fitted spectrum[36] suggested quite the opposite (see **Table 7**, page 40). The number of protons for each position was determined by normalizing each signal separately to its theoretical value and calculating an average of all other signals as well as the standard deviation (STDEV). The latter was determined to be 6.1 - 6.5% for every integral. This leads to the conclusion that the integral of the methylcarbonate (see **Table 7**, 3.90 ppm) cannot account for quantification of the proposed impurity **78** (see **Figure 43**, page 39). The corresponding spectrum of the acetate-protected mixed anhydride showed an enhanced standard deviation of 4.3% (see **Table 8**, page 41) for every signal. Despite the fact that the manual integration suggested a lack of desired product, the validation by Lorentz-fitting stood again in contrast.



Signal shift [ppm]	**4.56**	**4.13**	**3.90**	**2.74**	**2.57**	**1.27**	**0.85**	**0.09**
Number of protons	1	2	3	2	2	3	9	6
Position	**3**	**5**	**1**	**2**	**4**	**6**	**8**	**7**
Calculated protons	1.02	1.98	3.38	1.90	1.92	3.18	8.26	5.96
STDEV	0.06	0.13	0.20	0.11	0.12	0.19	0.50	0.36
STDEV [%]	6.1	6.5	6.1	6.1	6.1	6.1	6.1	6.1

Table 7. Analysis of the ^{1}H-NMR spectrum of the mixed TBS-anhydride

These findings indicate that integration of ^{1}H-NMR spectra for the quantification of impurities is only reliable to a moderate extend. In general, the activation with methyl chloroformate was superior to the formation of the acid chloride, in terms of conversion and purity of the intermediate. There was also no concrete evidence of a formation of the proposed anhydride **78** from two monoester molecules. Pivaloyl and *p*-toluenesulfonyl chloride were also



Signal shift [ppm]	5.51	4.16	3.92	2.91	2.74	2.05	1.27
Number of protons	1	2	3	2	2	3	3
Position	**3**	**5**	**1**	**2**	**4**	**6**	**7**
Calculated protons	1.03	1.98	3.00	1.97	1.95	2.83	3.28
STDEV	0.04	0.09	0.13	0.08	0.08	0.12	0.14
STDEV [%]	4.3	4.3	4.3	4.3	4.3	4.3	4.3

Table 8. Analysis of the ^{1}H-NMR spectrum of the mixed O-acetyl anhydride

tested for the activation of the monoesters but exhibited no product-related conversion in the crude product. Whether this resulted from low reactivity or due to hydrolysis during the extractive work-up was not determined. The literature procedure was done in toluene, with triethylamine as assisting base for the neutralization of the byproduct hydrochloric acid. Interestingly, triethylamine hydrochloride precipitated out of the reaction mixture when *tert*-butyl methyl ether (MTBE) was used as reaction solvent. The precipitation was not quantified but could be useful for further reaction optimization and combination with preceding or following reaction steps.

2.6.3 PREPARATION OF THE STABILIZED PHOSPHOR YLIDE

The C=C bond formation of an aldehyde with phosphor ylides based on phosphoranylidenes or phosphonates are known as WITTIG and HORNER-WADSWORTH-EMMONS (HWE) reaction, respectively. These two reactions have similar reaction mechanisms. In general, both reactions produce predominantly *E*-alkenes. The main difference between WITTIG and HWE reagents lies in their

basicity and nucleophilicity. The β-carbonyl moiety leads to an increased stability of the reagents. The benefit of the WITTIG reagent is its reactivity even in the absence of additional base. Most notably is a recent publication of BENCH *et al.* on the synthesis of phosporanylidenes of type **81** (see **Figure 44**, page 42) from acetone and phosphines of type **79** by using a biconcave cobalt perfluorophthalocyanine (**80**) and oxygen, leading to the desired ylide with water as the only by-product.[37] Despite it being an initial study with no sophisticated data for lab scale procedures, this surely opens new possibilities for stabilized WITTIG reagents without the need for α-halogenated ketones.

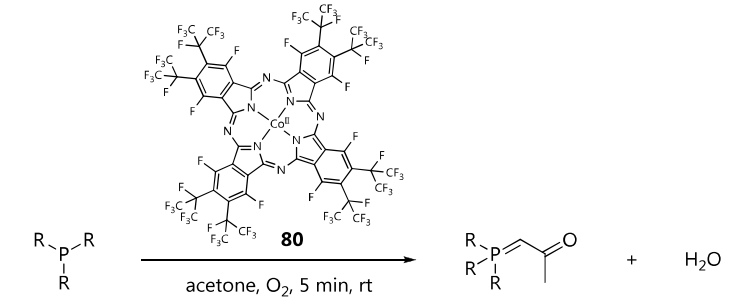


Figure 44. Synthesis of stabilized WITTIG reagents by cobalt (II)- perfluorophthalocyanine-catalyzed oxidative coupling of acetone with phosphines in the presence of oxygen (BENCH *et al.*).[37]

Commonly, WITTIG reagents can be prepared by two general strategies. The alkylation of trisubstituted phosphines of type **82** (see **Figure 45**) is by far the most convenient way and has been established for various C=C coupling reactions. In the case at hand the formation of such an α-haloketone **83** starting from a carboxylic acid derivative would require certain harsh conditions which are unfavorable for an industrial application. Therefore, the method of choice was the substitution of a carboxylic ester derivative **88** with a trisubstituted phosphoranylidene **87**. This reagent is formed *in situ* by deprotonation of trisubstituted phosphonium halides **86** in the presence of strong bases, e.g. *n*-butyllithium or lithium *N,N*-diisopropylamide.

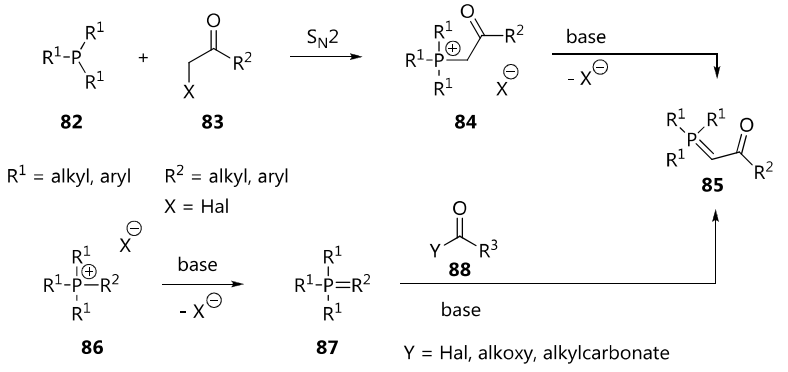


Figure 45. Common strategies for the synthesis of stabilized WITTIG reagents

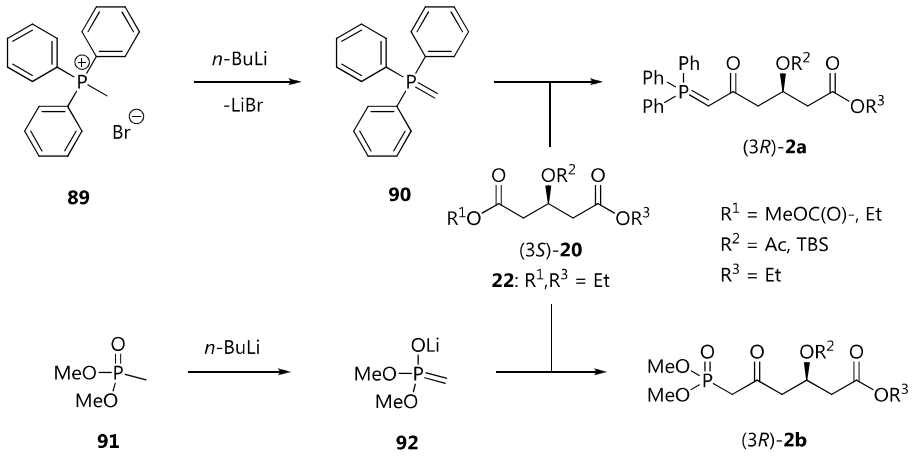
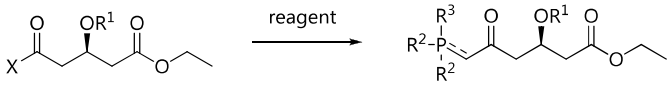


Figure 46. Synthesis of stabilized WITTIG and HWE reagents by nucleophilic substitution of carboxylic acid derivatives

Since the latter is mostly used for its coordinating counter ion, *n*-butyllithium was used for complete deprotonation and its irreversibility caused by the formation and evaporation of *n*-butane and therefore shifting of the acid-base equilibrium to the desired product. The progress of the reaction can even be monitored by plain sight. The colorless phosphonium halide **89** (see **Figure 46**) is suspended in THF while the corresponding ylide **90** is highly soluble. Moreover, due to delocalization with the aromatic rings and the phosphorous d-orbital, the reagent solution has an intense orange color. The complete conversion is monitored by the change of a suspension to a deep orange solution. This is also a convenient indicator for remaining base, which in the case of *n*-butyllithium would lead to undesired side-reactions. To circumvent this, the reaction solution should always maintain a small amount of dispersed phosphonium salt. After addition of the activated carboxylate of type **20** or **22**, the substitution reaction leads to decoloration of the solution. Unfortunately, the substitution product has acidic α-protons which neutralize one equivalent of reagent. For this reason the reaction had to be done with an at least 2-fold amount of reagent. It was not tested if the precipitating starting material **89** could be isolated by filtration and reused for another batch, however it seems reasonable. Apart from the reaction with the activated carboxylic acid derivatives (see **Table 9**, page 44, entries **4**, **7-9**), the ylide was also formed from the corresponding prochiral TBS-protected diethyl ester to obtain the racemic compound (entry **5**). As expected, the ethyl ester had a low reactivity with **90** and only 27% product-related conversion was observed. Interestingly, the same reaction with the HWE phosphonate **92** (entry **6**) showed complete conversion but also side-product formation and loss of chirality by silanol elimination, suggesting lower selectivity for the desired product.

Table 9. Synthesis of phosphor ylides as WITTIG and HWE reagents



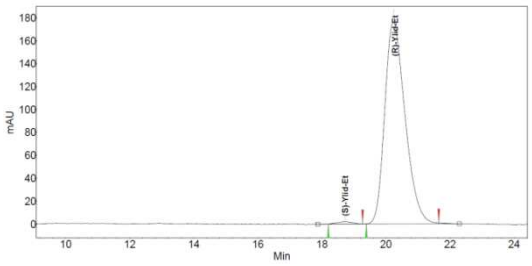
(3*S*)-**20** or **22**

(3*R*)-**2a**: R^1 = Ac, TBS, R^2 = Ph, R^3 = Ph
(3*R*)-**2b**: R^1 = TBS, R^2 = OMe, R^3 = (=O)

Entry	Reagent (eq)	**20** (R^1) (mmol)	X	Solvent (°C)	Conv. [% (h)]
1	Ph_3PCH_3Br (2.0)	H (3.60)	BnNH[a]	THF/toluene (-55)	n.d.[b]
2	Ph_3PCH_3Br (2.0)	2-THP (2.30)	BnNH[a]	THF (-55)	n.d.[b]
3	Ph_3PCH_3Br (2.0)	Ac (2.50)	Cl	THF (-55)	n.d.[c]
4	Ph_3PCH_3Br (2.0)	Ac (2.30)	MeOCO	THF (-55)	n.d.[c]
5	Ph_3PCH_3Br (2.0)	TBS (**22**) (1.00)	EtO	THF (-15)	27.0 (3.0)
6	$(MeO)_2POCH_3$ (4.0)	TBS (**22**) (2.00)	EtO	THF (-78)	74.0 (2.5)[d]
7	Ph_3PCH_3Br (2.0)	TBS (0.80)	MeOCO	THF/toluene (-70)	61-69[e] (3.0)
8	Ph_3PCH_3Br (2.5)	TBS (3.67)	MeOCO	THF (-78)	n.d.[f]
9	Ph_3PCH_3Br (2.5)	TBS (9.00)	MeOCO	THF (-78)	n.d.[f]

(a) (S)-enantiomer through lipase-catalyzed aminolysis (b) not determined; the lower pKa of protic moieties (NH < OH < methylene) led to various side-products which could neither be isolated nor identified (c) not determined; the strongly basic methylene compound led to major elimination of acetic acid with the loss of chirality (d) quantitative conversion of the starting material, mixture of at least four products incl. elimination products with loss of chirality, 26% product-related conversion (e) conversion was determined by the conversion of aldehyde in the subsequent WITTIG reaction, incl. correction of 1.1 eq. aldehyde and 97% conversion of the reference reaction (f) not determined, isolation methods were tested but unsuccessful

The enantiomerically enriched phosphor ylide (3*R*)-**2a** is an important intermediate since the established industrial route to Rosuvastatin is based on the following WITTIG reaction. It is also the commercially available intermediate (methyl ester) and thus the benchmark for the desired novel process. The remaining impurities led to no satisfying crystallization, regardless of the investigated solvents.



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(S)-Ylid-Et	18,72	0,91	2,0	1,2	0,906
2	(R)-Ylid-Et	20,24	99,09	176,0	129,7	99,094
Total			100,00	178,0	130,9	100,000

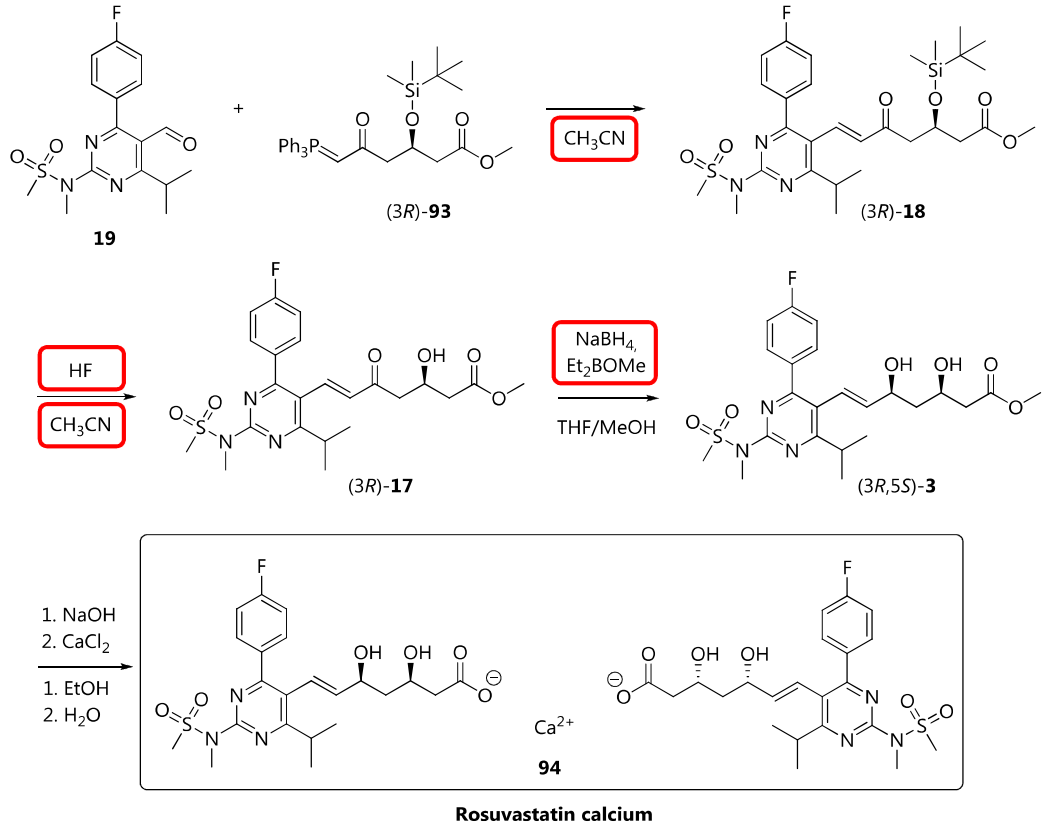
Figure 47. HPLC chromatogram of enantiomerically enriched TBS-protected phosphoranylidene ethyl ester after preparative TLC purification and calculation of the enantiomeric excess

Therefore, the compound was used without further purification in the following WITTIG reaction. The product-related conversion was determined by calculating the conversion of heteroaromatic aldehyde consumed in the subsequent reaction. The small excess of ylide (1.1 equivalents) in the reaction procedure was also taken into account. Nevertheless, since the optical purity of this ylide is crucial for the route, the crude product (see **Table 9**, page 44, entry 9) was partially purified by preparative thin-layer chromatography on silica and analyzed by chiral HPLC to reveal an excellent enantiomeric excess of 98.2% ee (see **Figure 47**, page 44), which was considered sufficient for the following steps.

2.7 SYNTHESIS OF THE ACTIVE PHARMACEUTICAL INGREDIENT ROSUVASTATIN

The heteroaromatic core of Rosuvastatin is commercially available as the corresponding aldehyde **19** (see **Figure 48**), which can be used directly for the

Figure 48. Patent procedure for the production of Rosuvastatin calcium from heteroaromatic aldehyde.[38]



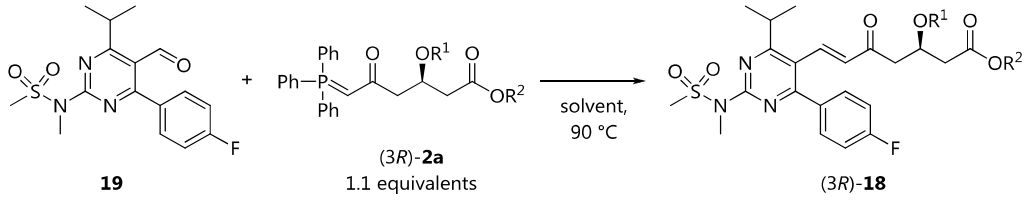
WITTIG reaction. The product (3*R*)-**18** is then deprotected for a diastereoselective reduction of the remaining carbonyl group. Subsequently, the alkyl ester of Rosuvastatin ((3*R*,5*S*)-**3**) is saponified to obtain the sodium salt of the API. The patented procedures by WATANABE *et al.* had some hazardous conditions which were objectable, like the deprotection of the silyl ether by hydrogen fluoride or the use of acetonitrile as solvent.[38] Notably, a recently published solvent guide from Sanofi Aventis defines acetonitrile as the preferable aprotic polar solvent for industrial processes.[39] Furthermore, the stoichiometric use of reducing agent and LEWIS base for the diastereoselective reduction of the β-hydroxyketone (3*R*)-**17** were investigated for an alternative route *via* alcohol dehydrogenases.

2.7.1 WITTIG REACTION WITH THE HETEROAROMATIC CORE OF ROSUVASTATIN

Table 10. WITTIG reaction with phosphoranylidene and heteroaromatic aldehyde

(a) acetonitrile (MeCN), potassium phosphate buffer (KPB), 2-propanol (iPA), tert-butyl methyl ether (MTBE) (b) Determined by ^{1}H-NMR integration from the crude product (c) Purified by column chromatography on silica.

The WITTIG reaction for the C=C coupling of the heteroaromatic aldehyde **19** and the phosphor ylide of type (3*R*)-**2a** was first reproduced with commercially available methyl ester (see ***Table 10***, entries **1-4**, **6**). Since the stabilized WITTIG reagent does not need additional base, the reaction proceeded smoothly in a mixture of both compounds, with the ylide in slight excess of 1.1 equivalents. In contrast to the literature procedure[38], the present study also used protic solvents (entries **3-5**) and even water (entry **2**) which were proven to be



19 (3*R*)-**2a** 1.1 equivalents (3*R*)-**18**

Entry	R^1	R^2	**19** [mmol]	Solvent[a]	Conv.[b] (time) [% (h)]	Isolated yield[c] [g (%)]
1	TBS	Me	0.90	MeCN	69.2 (12)	n.d.
2	TBS	Me	0.90	H_2O	68.5 (12)	n.d.
3	TBS	Me	0.90	KPB/iPA	97.2 (12)	0.43 (78.6)
4	TBS	Me	2.85	iPA	97.4 (8.5)	1.43 (82.6)
5	TBS	Et	0.49	iPA	78.8 (6.0)	n.d.
6	TBS	Me	28.9	MTBE	98.0 (18.0)	n.d.

convenient solvents.

Remarkably, the reaction seems to be accelerated in protic solvents, possibly due to the stabilization of the betain intermediate. In view of a possible biocatalytic reduction with alcohol dehydrogenases, a mixture of buffer and 2-propanol (3:1 v/v) was also successfully used for the reaction. The presence of 2-propanol is a convenient way of substrate-coupled regeneration of the expensive cofactor nicotinamide adenine dinucleotide (NADH). The only side-product of the WITTIG reaction and therefore the sole impurity, triphenylphosphine oxide, was separated by column chromatography for analytical purposes. Its effect on biocatalytic reductions was not investigated. To eliminate a possible inhibitory activity, the phosphine oxide was always removed in the initial experiments. The moderate conversion for the synthesized TBS-protected phosphoranylidene (3*R*)-**2a** is due to the conversion of the preceding ylide formation. Most likely the ethyl ester reacted to the same extent with the aldehyde **19** as the commercial methyl ester. Thus, the lower conversion was reasoned with the lower content of ylide reagent of only about 70-80 mol-%. The presence of a fluorine substituent in the aldehyde is helpful when analyzing the following reaction steps by NMR spectroscopy. In the proton-decoupled ^{19}F-NMR spectra, the fluorine signal shifts sufficiently to determine product formation, but only qualitatively due to the necessary proton-decoupling (see **Figure 49**). The recorded spectra after purification *via* column chromatography show a high-field shift for the product relative to the aldehyde. An unidentified side-product is also visible between 108.2-108.4

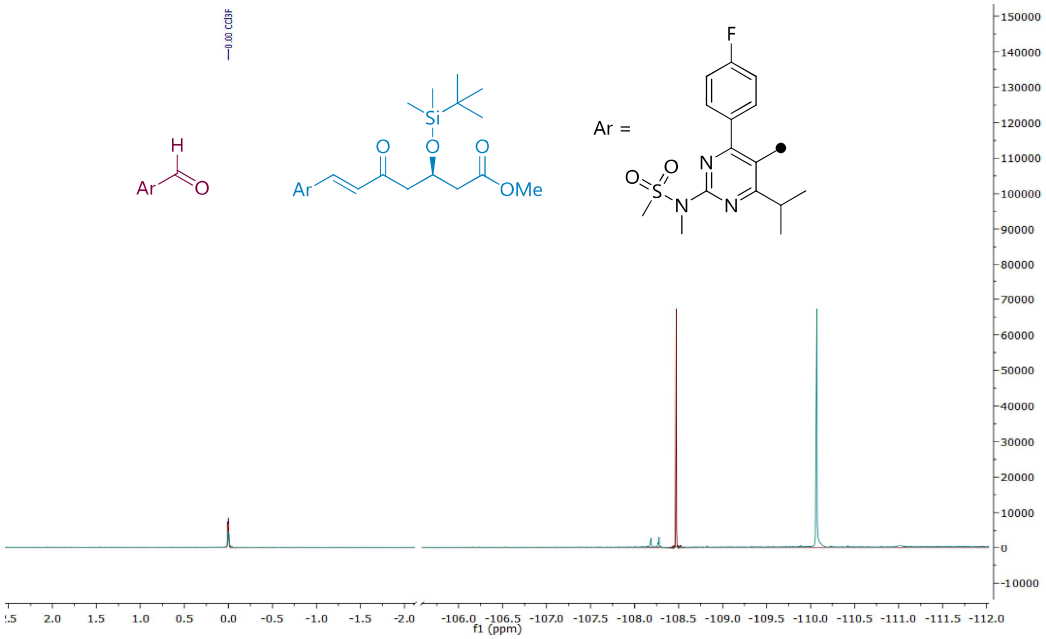


Figure 49. Proton-decoupled ^{19}F-NMR spectra of the WITTIG reaction and the hetero-aromatic aldehyde as reference.

ppm, which could not be separated from the product, but was separated after the following step.

2.7.2 DEPROTECTION OF THE 3-HYDROXY GROUP

The O-protective group chemistry is necessary for ylide formation and possibly also prevents the product from reacting with excess ylide. However, after the WITTIG reaction, the protecting group became obsolete and even a hindrance for the following reduction. It was therefore removed to obtain the chiral β-hydroxyketone as starting point for the following diastereoselective reduction. Although most of the literature-known procedures for the removal of *tert*-butyldimethylsilyl ethers use the extremly toxic hydrogen fluoride or easier to handle fluoride salts, silyl ethers also tend to hydrolyse in acidic aqueous media. This hydrolytic approach is of course more desirable for an industrial process in regard of process safety. Hence, the TBS group was successfully hydrolyzed by the use of both tetra-*n*-butylammonium fluoride or a mixture of hydrochloric acid and ethanol, with quantitative conversions and excellent yields (see **Table 11**, page 49).

Apart from the toxicity of fluoride, the two discussed methodologies differ also in their course of action (see **Figure 50**). The fluoride acts as a nucleophile and attacks directly at the silicon due to its ability to incorporate d-orbitals. The

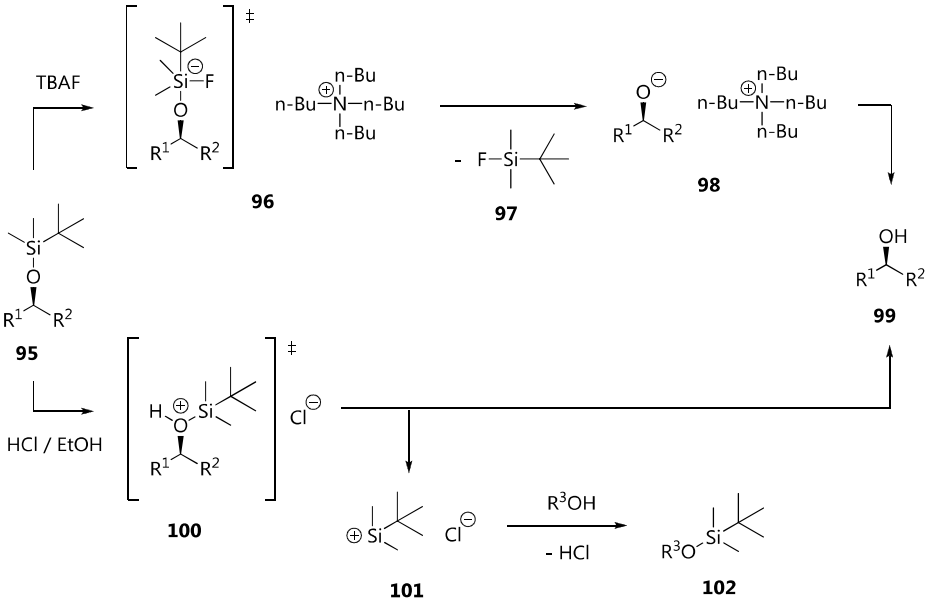
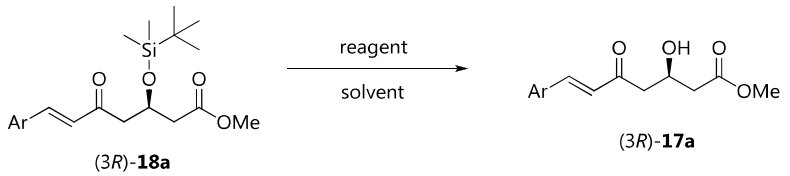


Figure 50. TBS-deprotection mechanisms of fluoride reagents and aqueous acid solutions

formed trigonal bipyramidal intermediate **96** eliminates the highly stable *tert*-butyldimethylfluorosilane (**97**). This reaction is driven by the Si-X bond strengths, which can be seen in their respective dissociation energies. For trialkylsilylethers, the Si-O bond has a dissociation energy of around 122 kcal/mol, while the trialkylfluorosilanes are in the region of 158 kcal/mol.[40] The free carbinol **99** is then formed by aqueous work-up of the TBA salt **98**. The hydrolytic approach starts with the electrophilic attack of a proton to the oxygen of the silylether **95**, leading to the intermediate **100**. The *tert*-butyldimethylsilyl cation **101** is stabilized by its high electron density and the +I effects of the alkyl substituents. The carbinol **99** is eliminated and the silyl cation reacts with nucleophiles in the solution, e.g. ethanol or water. Both methods will produce the desired free carbinol **99** under retention of the stereogenic information.



(3*R*)-**18a** (3*R*)-**17a**

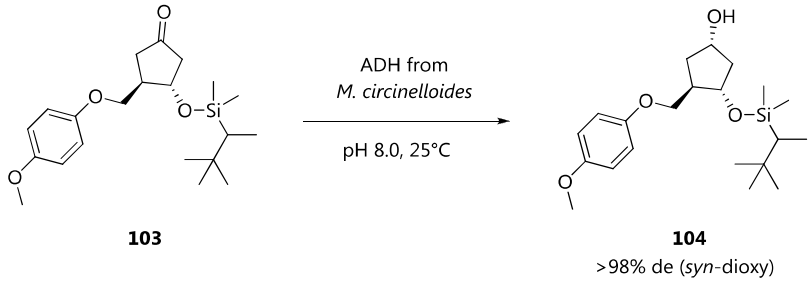
Table 11. TBS-deprotection of WITTIG reaction products by fluoride and acidic treatment

Entry	(3*R*)-**18a** [mmol]	Reagent	Solvent	Conversion [%]	Yield [g(%)]
1	0.69	TBAF	THF	>95.0	n.d.
2	2.30	HCl	EtOH	>95.0	1.00 (88.1)

The product of the hydrolytic deprotection was purified by column chromatography since all protic components, including *tert*-butyldimethylsilanol and its hemihydrate, had to be removed prior to the following reduction with sodium borohydride. This could become unnecessary if a biocatalytic reduction can be realized since the biocatalyst would naturally be highly chemoselective towards ketone reduction. Hence, it would be possible to use crude products and eliminate another necessary purification step.

2.7.3 DIASTEREOSELECTIVE REDUCTION *VIA* ALCOHOL DEHYDROGENASES

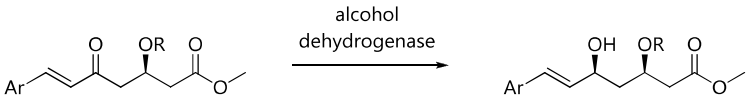
The biocatalytic reduction by the use of an alcohol dehydrogenase (ADH) was bilaterally investigated together with researchers from the HUMMEL group who screened alcohol dehydrogenases of various organisms for the enantioselective reduction of both *O*-protected and free β'-hydroxy enones of the Rosuvastatin methyl ester. Preliminary experiments were focused on a general activity for the corresponding substrate. To the best of our knowledge, a similar biotransformation was only achieved once for the cyclic β-silyloxy ketone **103** (see **Figure 51**) by BORTHWICK *et al.*.[41] They screened alcohol dehydrogenases from various organisms for the diastereoselective reduction of **103**. Active biocatalysts from *Mucor racemosus* and *Mucor circellinoides* showed the desired selectivity for the *syn*-product **104**, with an ADH from *M. circellinoides* as the superior one. After optimization of the reaction conditions, they were able to reduce the cyclic ketone **103** at 5 g/L substrate concentration with 62% yield and an excellent diastereoselectivity of >98% de.



103 **104**
>98% de (*syn*-dioxy)

Figure 51. Diastereoselective reduction of a bulky β-silyloxy ketone using an alcohol dehydrogenase (BORTHWICK *et al.*).[41]

The presence of the *p*-fluorophenyl moiety in Rosuvastatin and its specific shift in ^{19}F-NMR spectra made it possible to analyze the screening samples for product-related conversion. Usually, ADH screening assays are photometric measurements of the conversion-dependent decrease of NAD(P)H absorbance. However, side product formations or depletion can also lead to a decrease of absorption and falsify the results. Therefore, samples with positive photometric activity were extracted with MTBE and additionally analyzed by ^{19}F-NMR spectroscopy. Unfortunately, as depicted in **Table 12** (page 51), none of the tested alcohol dehydrogenases were able to reduce the *O*-TBS protected β'-hydroxy enone. This is much likely due to the increased bulkiness of the *tert*-butyldimethylsilyl ether.



(3*R*)-**18a**: R = TBS
(3*R*)-**17a**: R = H

(3*R*,5*RS*)-**3**: R = H
(3*R*,5*RS*)-**105**: R = TBS

Table 12. ADH-Screening[a,b] for the reduction of TBS-protected or free β'-hydroxy enones of Rosuvastatin methyl ester.

(a) General reaction conditions: 5 mM substrate (solution in tert-butyl methyl ether), 0.5 M triethylamine, 1 mM $MgCl_2$, 1 mM NAD^+, 1 mM $NADP^+$, D-Glucose, GDH, 30 °C, overnight (b) Determined by analysis of the ^{19}F-NMR spectrum. "+" corresponds to a new peak occurring after the enzymatic reaction. (c) For the definition of Ar see **Figure 49**, page 47.

Entry [a]	Organism	Activity [b] (+ / -) for (3*R*)-**18a**[c]	(3*R*)-**17a**[c]
1	*Kocuria rosea*	-	-
2	*Rhizobium rhizogenes*	-	-
3	*Lactobacillus paracasei* ssp. *tolerans*	-	-
4	*Streptococcus thermophilus*	-	-
5	*Brochotrix thermospecta*	-	-
6	*Lactobacillus kefir*	-	-
7	*Dactylosporangium fulvum*	-	-
8	*Dactylosporangium roseum*	-	-
9	*Streptococcus ferus*	-	-
10	*Dactylosporangium thailandense*	-	-
11	*Rhizopus oryzae*	-	-
12	*Aspergillus oryzae*	-	-
13	*Weissella confusa* (*Lactobacillus confusus*)	-	-
14	*Bacillus subtilis* ssp. *spizizenii*	-	-
15	*Streptomyces antibioticus*	-	-

The protective group was removed and the free β-hydroxyketone was used in another screening with the same organisms. Even for this more accessible substrate, no active biocatalyst was identified.

2.7.4 DIASTEREOSELECTIVE REDUCTION UNDER LEWIS-ACID COORDINATION

The chemical pathway to Rosuvastatin methyl ester was achieved by the NARASAKA-PRASAD reduction.[42,43] This reaction is specifically used for the diastereoselective reduction of β-hydroxyketones to their corresponding *syn*-diols by the use of sodium borohydride. The enantioselectivity is achieved by incorporation of a LEWIS acid like diethyl methoxyborane **106** in stoichiometric amounts (see **Figure** , page 52).

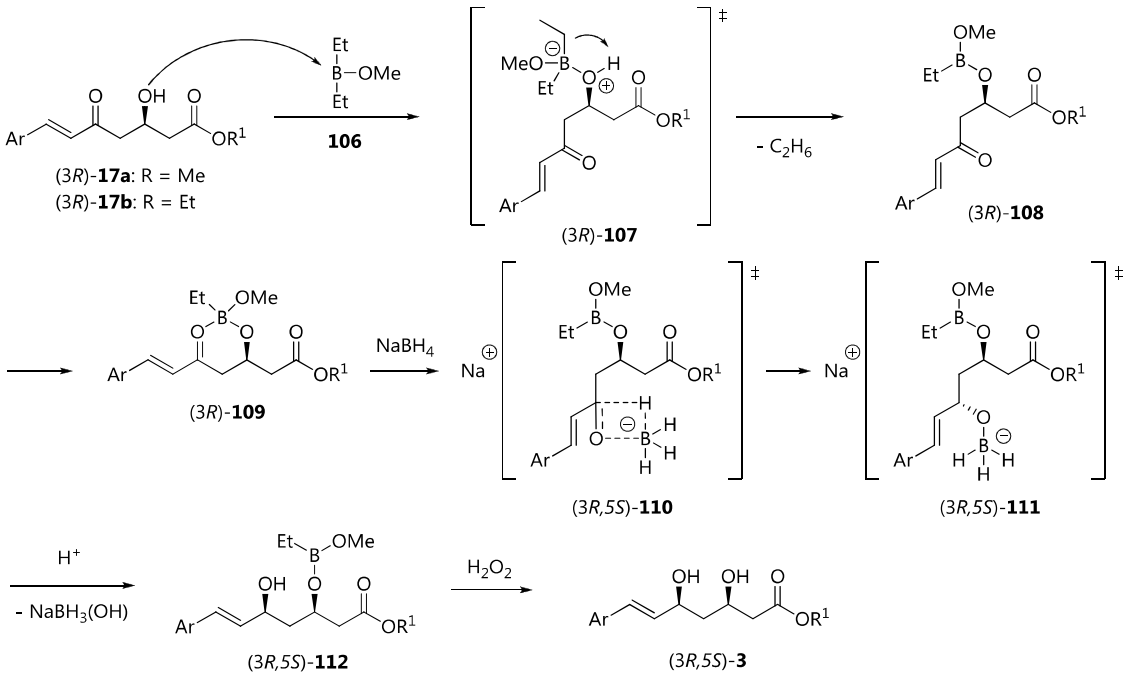


Figure 52. Diastereoselective NARASAKA-PRASAD reduction and oxidative cleavage for the synthesis of Rosuvastatin methyl ester.

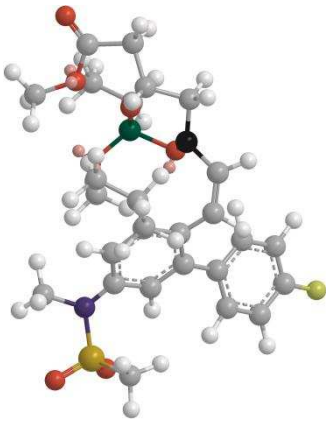
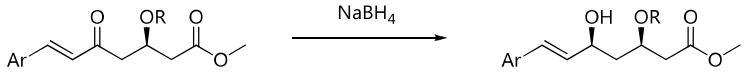


Figure 53. Reaction intermediate **109** (**Figure**) with polarized carbonyl (black) and open *Re* face (minimized energy model)

An ethyl substituent is eliminated as ethane during the coordination of the chiral carbinol **17**. The boronate **108** then acts as sterical hindrance and directs the *Re*-face attack of the usually unselective borohydride. The resulting borate **111** is then oxidatively cleaved by hydrogen peroxide for retention of the absolute configuration. Although the literature mechanism suggests the substitution of both ethyl groups by two β-hydroxy ketone molecules, each of them acting as bulky steric hindrance for the *Si* face of another, the highest diastereomeric excess was achieved with a stoichiometric amount of lewis acid at a low temperature of -78°C. Nevertheless, since the heteroaromatic core of Rosuvastatin is very bulky, the coordination of the second molecule could still have been incomplete prior to the addition of sodium borohydride, thus leading to a lower selectivity of the hydride transfer.

Figure illustrates a minimized energy model of the 1:1 substitution intermediate for stoichiometric amounts of borane. The coordination of the carbonyl oxygen to the boron atom leads to a boat configuration with blocked *Si* face. The hydride transfer is restricted to the *Re* face, thus producing the 1,3-*syn*-diol. At higher temperatures, the vibrational energy seems to be high enough to promote the corresponding chair conformation with opening of the *Si* face for hydride transfer. In general, the diastereomeric excess was enhanced by an increased amount of coordinating borane **106** as well as minimizing the enthalpy of the system by operating at low temperatures. It is reasonable that

the diastereoselectivity can be maximized by prolonged period of reagent addition between the LEWIS acid and the borohydride.



(3*R*)-**18a**: R = TBS
(3*R*)-**17a**: R = H

(3*R*,5*RS*)-**3**: R = H
(3*R*,5*RS*)-**105**: R = TBS

Table 13. Diastereoselective reduction of β-chiral enone with $NaBH_4$ and with or without LEWIS-acid coordination.

Entry	**17**/**18** (R) [mmol]	Additive (eq.)	Solvent (°C)	Conv. (time) [%(h)]	d.r.	Yield [g (%)]
1	TBS (0.06)	-	THF (0)	>95 (1.0)	80:20	n.d.
2	H (2.30)	Et_2BOMe (0.5)	THF (-15)	>95 (1.0)	66:34	1.20 (n.d.)
3	H (2.00)	Et_2BOMe (0.5)	THF (-70)	>95 (2.0)	65:35	0.92 (n.d.)
4	H (1.00)	Et_2BOMe (1.0)	THF (-15)	>95 (1.0)	80:20	n.d. (-)
5	H (1.00)	Et_2BOMe (1.0)	THF (-70)	>95 (1.0)	>98:2	n.d. (-)

Remarkably, the unselective reduction of the TBS-protected enone showed a diastereomeric excess of 60% de at 0°C, whereas the diastereoselectivity for the NARASAKA-PRASAD reduction was also 60% de at -15 °C and stoichiometric amounts of LEWIS acid. The bulky silyl ether seems to have a similar directing effect as the boronic ester.

2.8 DEVELOPMENT OF THE PROCESS CHEMISTRY

After the validation of all separate steps for the synthesis of Rosuvastatin alkyl ester, the experiments were focused towards a higher efficiency. The obvious drawback of the previous chapters was the purification of certain intermediates by column chromatography. This method is not feasible for large-scale production of APIs. Therefore, multiple strategies for the elimination of purification steps and the reduction of waste were examined.

2.8.1 RECYCLING OF BIOCATALYSTS

A common method for the reduction of costs and waste in biocatalysis is the recycling of the catalysts. This is reasonable for low catalyst loadings (mol substrate / mol catalyst), when the costs for the biocatalyst surpass the costs for the product itself. The necessity of purified biocatalysts for a high chemo- or enantioselectivity could also favor a recycling procedure, due to their sometimes cost- and work-intensive purification. Moreover, high tolerance to organic media would in general facilitate recycling of the biocatalyst by extraction or filtration. For example, lipases are often immobilized on heterogeneous carriers like acrylic resins (*Candida antarctica* lipase B) or diatomite (Amano PS SD, *Pseudomonas cepacia* or *Burkholderia cepacia*). This way the catalysts can be compartmented in a packed-bed reactor or continuously stirred tank reactor (CSTR), while the substrates are dissolved in an organic solvent and pumped through the immobilisate.

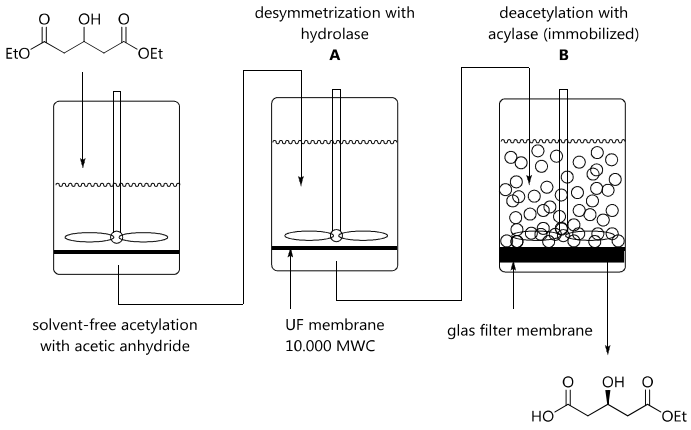


Table 14. Recycling[a] of homogenous and heterogeneous biocatalysts in the production of (*3R*)-EHG.

Entry	Spec. Activity **A** [U/g][b,c]	Rel. activity[d] **A** [%]	Spec. Activity **B** [U/g][b,c]	Rel. activity[d] **B** [%]
1	101 (100)	100	88.9 (100)	100
2	99.1 (98.1)	98.1	88.3 (99.3)	99.3
3	83.2 (82.4)	82.4	79.9 (89.9)	89.9
4	46.6 (46.1)	46.1	79.4 (89.3)	89.3
5	25.6 (25.3)	25.3	79.0 (88.9)	88.9

(*a*) *Each cycle was terminated after reaching >95% conversion judging by the titration curve of aqueous base; α-Chymotrypsin (CHY,* ***A****) was recycled by filtration over an ultrafiltration (UF) membrane and diluted with new reaction medium; Cephalosporin C acetylesterase* (*CAE,* ***B***) *was used in immobilized form, separated by filtration over a glass frit and resuspended in the next UF membrane filtrate. (b) One unit is herein defined as one µmol of substrate converted per minute related to full conversions instead of initial reaction rates (c) Grams of enzyme formulation (purified (****A****) / immobilized (****B****) (d) Remaining activity relative to preceding cycle.*

However, continuous processes with hydrolytic reactions are difficult to realize since the maintaining of basic pH is mandatory. By pumping the

substrate/product mixture through an enzyme column, the pH is permanently lowered and the biocatalysts in the rear part of the column are inactivated, if not denaturized. Hence, the single batch reactor with an automatic titration with aqueous base is combined with a filtration step to remove the biocatalyst from the product solution. After resuspension in buffer solution, the biocatalyst is reused. For homogenous biocatalysts, this filtration can be realized by an ultrafiltration membrane, usually with a molecular weight cut-off of around 25% of the catalyst's molecular weight. Since α-chymotrypsin was used in homogenous solution, an ultrafiltration was implemented to recycle the biocatalyst. After 24 hours of desymmetrization reaction, the solution is pumped through an ultrafiltration membrane in a stirring cell (0.6 bar argon gas, 5.6 bar internal pressure). **Table 14** (page 54) shows the remaining activities of α-chymotrypsin (**A**, Biozym, purified) within each desymmetrization cycle. A continuous decrease of activity was observed over five cycles, which implies the need for at least partial supplementation of biocatalyt after each cycle. The filtrate was directly used for the deacetylation reaction with cephalosporin C acetylesterase (CAE) while the filter residue was taken up in aqueous buffer and directly used for the next reaction batch. The deacetylation catalyst in its immobilized form was recycled by filtration over a glass frit (Por4, 16 um) and resuspension in the filtrate of the next desymmetrization reaction. This procedure was repeated until five reaction cycles were completed. The results show almost complete retention of starting activity for the first two cycles of the immobilized cephalosporin C acetylesterase (**B**, see **Table 14**, page 54, entries 1-2). After storage at 4-8°C for two weeks, the next cycle showed a decrease of activity (Entry 3), while the following cycles were again in the range of the third cycle (entries 4-5). This could be explained by the absence of buffer solution between cycles 2 and 3, whereas its presence seems advantageous for the stability of the immobilized enzyme.

Figure 54. Stirring cell with ultrafiltration membrane for the removal of dissolved α-chymotrypsin after the desymmetrization reaction.

2.8.2 WORK-UP PROCEDURES CIRCUMVENTING CHROMATOGRAPHY

The main reasons for column chromatography were the elimination of silanol from the silyl ether protection step and the deprotection of this silyl ether after the WITTIG reaction. In addition, triphenylphosphine oxide had to be removed after the WITTIG reaction.

SILANOL REMOVAL FROM CRUDE PRODUCTS

For moderate volatile impurities like *tert*-butyldimethylsilanol, a distillation could be reasonable as purification method. With a boiling point of 109 °C under atmospheric pressure, this seems also facile to achieve. However, the high viscosity of the TBS-protected monoester as well as the β-hydroxy ketone of Rosuvastatin made it very difficult. Remarkably, the silanol could be partly removed *via* distillation and condensed as colorless oil and colorless needles. The reason for crystallization is the formation of a silanol hemihydrate with residual water. This hydrate was serendipitously discovered by BARRY *et al.*[34] after the precipitation of colorless needles from recrystallization liquor.

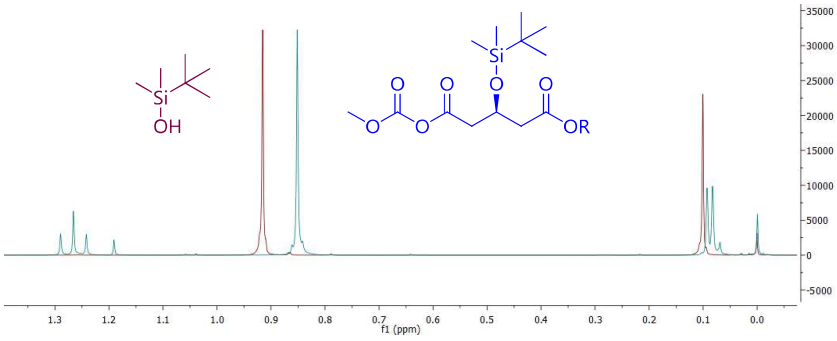


Figure 55. ^{1}H-NMR segment of TBS-protected mixed anhydride and *tert*-butyldimethylsilanol as reference for purity control

They determined the crystal structure by X-ray diffraction and calculated a rather low density of 0.996 g/cm^3 which explains its volatility. This hemihydrate was removed by either precipitation at low temperatures and subsequent filtration (residual content <20%) or by high vacuum distillation from crude products[i] (residual content <10%). Moreover, xylene was successfully utilized in an azeotrope distillation (b.p. 110°C, atm.) to remove the silanol and its hemihydrate to a residual content of <5%. The determination of purity was achieved by quantitative ^{1}H-NMR analysis of the silylated product and *tert*-butyldimethylsilanol as reference (see ***Figure 55***, page 56).

TRIPHENYLPHOSPHINE OXIDE REMOVAL FROM CRUDE PRODUCTS

Since triphenylphosphine oxide (TPPO) is not volatile, distillation was not an option. However, TPPO shows poor solubility in certain organic solvents, e.g. MTBE. At low temperatures, precipitation and filtration sufficed for the removal of >90% TPPO impurities in the first cycle. Additional impurities prevented

[i] A separate cooling trap for the hemihydrate is strongly recommended. Membrane pumps and the nearest lab technician responsible for replacing the membranes will most likely agree.

further precipitation of TPPO. It has a very distinctive ^{31}P-NMR shift at 29.8 ppm (relative to Me_3PO_4 in acetone-d_6, 4.00 ppm) which was used to qualitatively determine its absence or presence, respectively.

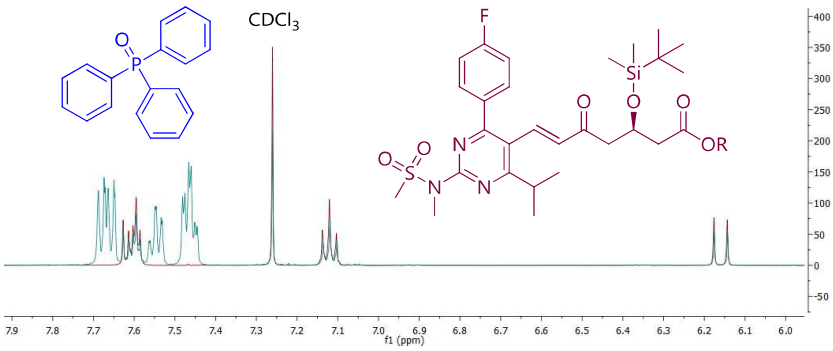


Figure 56. ^{1}H-NMR segment of the WITTIG product and triphenylphosphine oxide as reference for purity control

Since the phosphorous Nuclear-Overhauser-Effect (NOE) is inconsistent, integration of ^{31}P-NMR spectra is inconclusive. Fortunately for the case of Rosuvastatin, the few aromatic signals of the *p*-fluorophenyl substituent do not overlay with the TPPO signals in the ^{1}H-NMR (see ***Figure 56***). Therefore, quantitative analysis of remaining TPPO was done *via* integration of the latter in relation to the internal standard *p*-chlorobenzyl alcohol which was already described in chapter **2.4.2** for the quantification of the desymmetrized monoesters.

2.8.3 MULTI-STEP REACTIONS WITHOUT ISOLATION OF INTERMEDIATES

Most of the patented processes for the production of APIs were initially designed for a high yield of the target molecule. To reach this goal, solvents for each reaction were often selected for solubility reasons or beneficial effects on the reaction progress. For example, the use of diethyl ether for Grignard reactions, dispite its hazardousness, is still common sense. In many cases, the reaction proceeds only with symmetrical, aliphatic and unbranched ethers. Di-*n*-butylether was also successfully used in such reactions. However, its high boiling point of 110°C is a drawback for volatile products. In regard of more sustainable process chemistry, generic processes now use solvents with low environmental impact, like ethyl acetate, alcohols or ideally water. In addition, separate steps with the same compatibility towards a certain solvent can be combined in a sequential one-pot or even tandem synthesis. In the recent past, some major breakthroughs were achieved by this solvent engineering

approach, reducing the amount of total solvent used in the downstream processing.

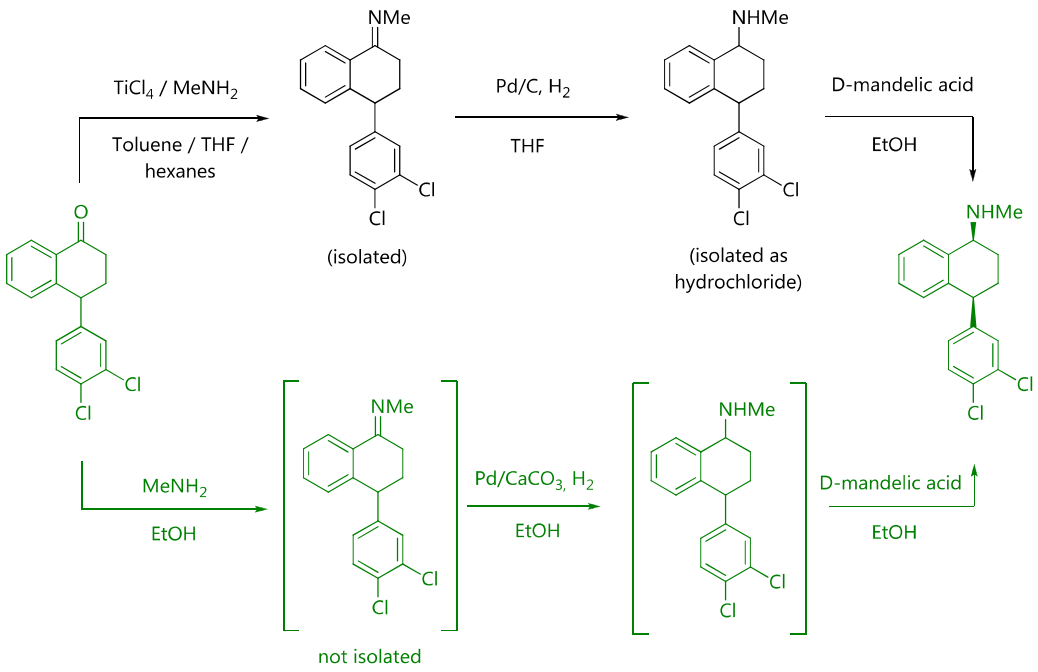


Figure 57. Solvent engineering for an improved production of Sertraline mandelate by TABER *et al.*[44]

For example, researchers from Pfizer improved their synthesis of Sertraline hydrochloride by using ethanol as the sole solvent (see **Figure 57**).[44] By elimination of all intermediary isolation steps, the process was converted from a three-step synthesis in THF, toluene, hexanes and ethanol to a three-step one-pot process in only ethanol with isolation of the API as mandelate salt.

Starting from this aim, the reactions for the synthesis of Rosuvastatin were tested in various solvents for the assessment of compatible reactions. During this analysis, some advantageous properties were also discovered. For example, the biocatalytic processes for the desymmetrization and deacetylation of diethyl 3-acetoxyglutarate both used an aqueous buffer system at pH 8.0 and were therefore compatible without an extractive work-up (see **Figure 57**, page 59). Furthermore, the acetylation of **1a** proceeded without any solvent. Thus, the first reaction was also combined with the biocatalytic reaction by simple addition of aqueous reaction medium and neutralization of the remaining byproduct, acetic acid, by neat sodium carbonate. The additional salt loading and increased ionic strength did not exhibit any inhibitory effects for neither of both biocatalytic reactions (see chapter **2.4.5.4**, page 25).

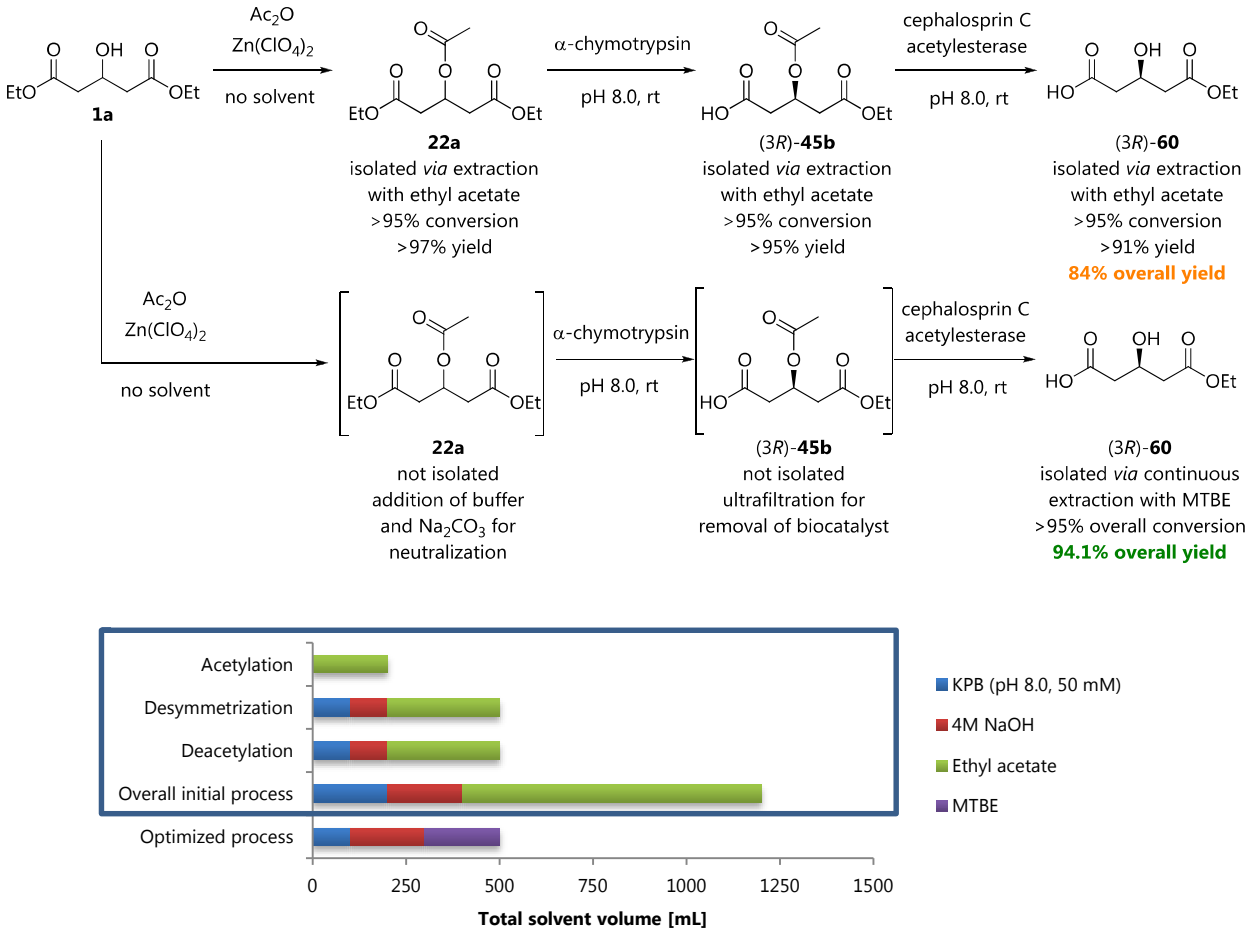


Figure 57. Multi-step synthesis for the production of enantiomerically pure (3*R*)-**60** in comparison to the separate steps (square).

DERIVATIZATION FOR THE SYNTHESIS OF THE TBS-PROTECTED MIXED ANHYDRIDE

The resulting free β-hydroxyacid is then extracted and *tert*-butyldimethyl-silyl-(TBS-) protected (see **Figure 58**, page 60). This formation of silyl ether is compatible with ethyl acetate, MTBE and other water-immiscible solvents, which could be used for extraction of the β-hydroxy carboxylic acid. The silylation in MTBE did not only afford the desired mixture of silyl ether and silyl ester, it also showed low solubility for imidazole salt byproducts and the silanol, making it possible to purify the crude product by filtration of precipitating impurities at low temperatures. Addition of the THF/methanol/water mixture and potassium carbonate led to a biphasic system, which was sufficient, however slower for the hydrolysis of the silyl ester. Again, the extractive work-up could be combined with MTBE. Since methanol had to be removed prior to the addition of methyl chloroformate because of its own reactivity, this was the first step which needed to be completely dried. The

previously discussed silanol hemihydrate was also reactive under the following conditions, reducing the excess of chloroformate to below stoichiometric

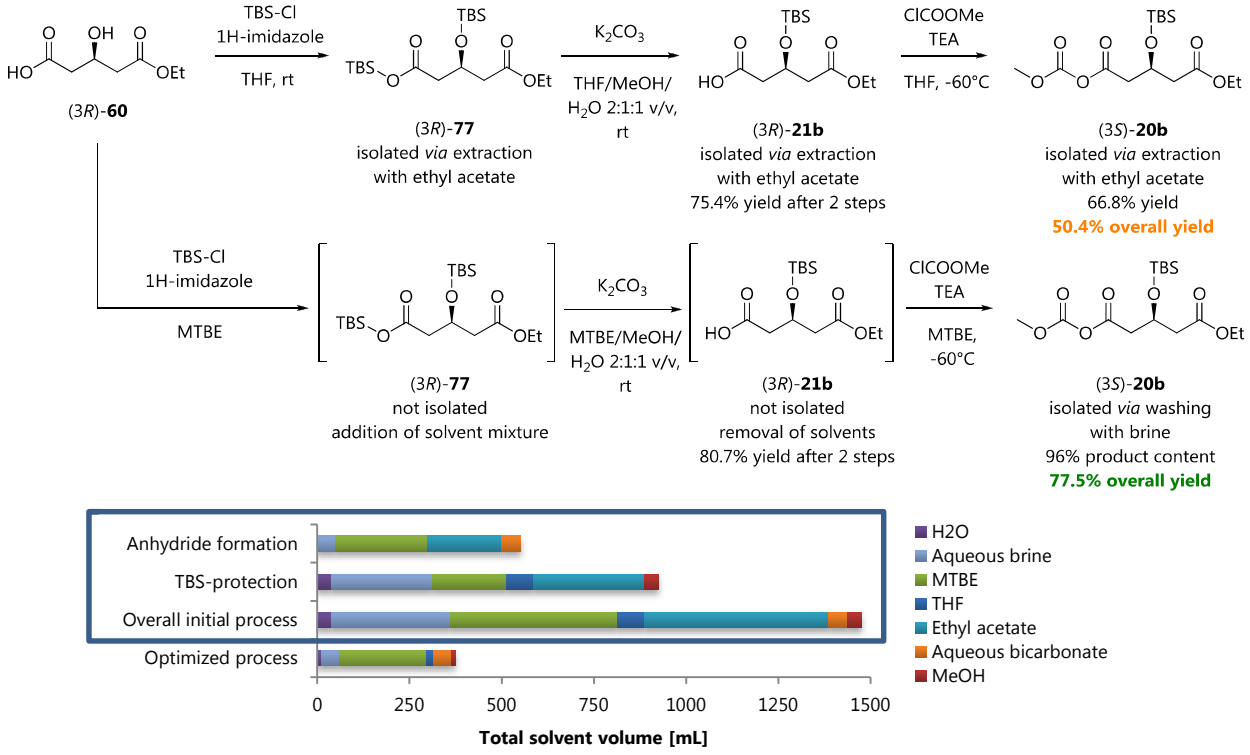


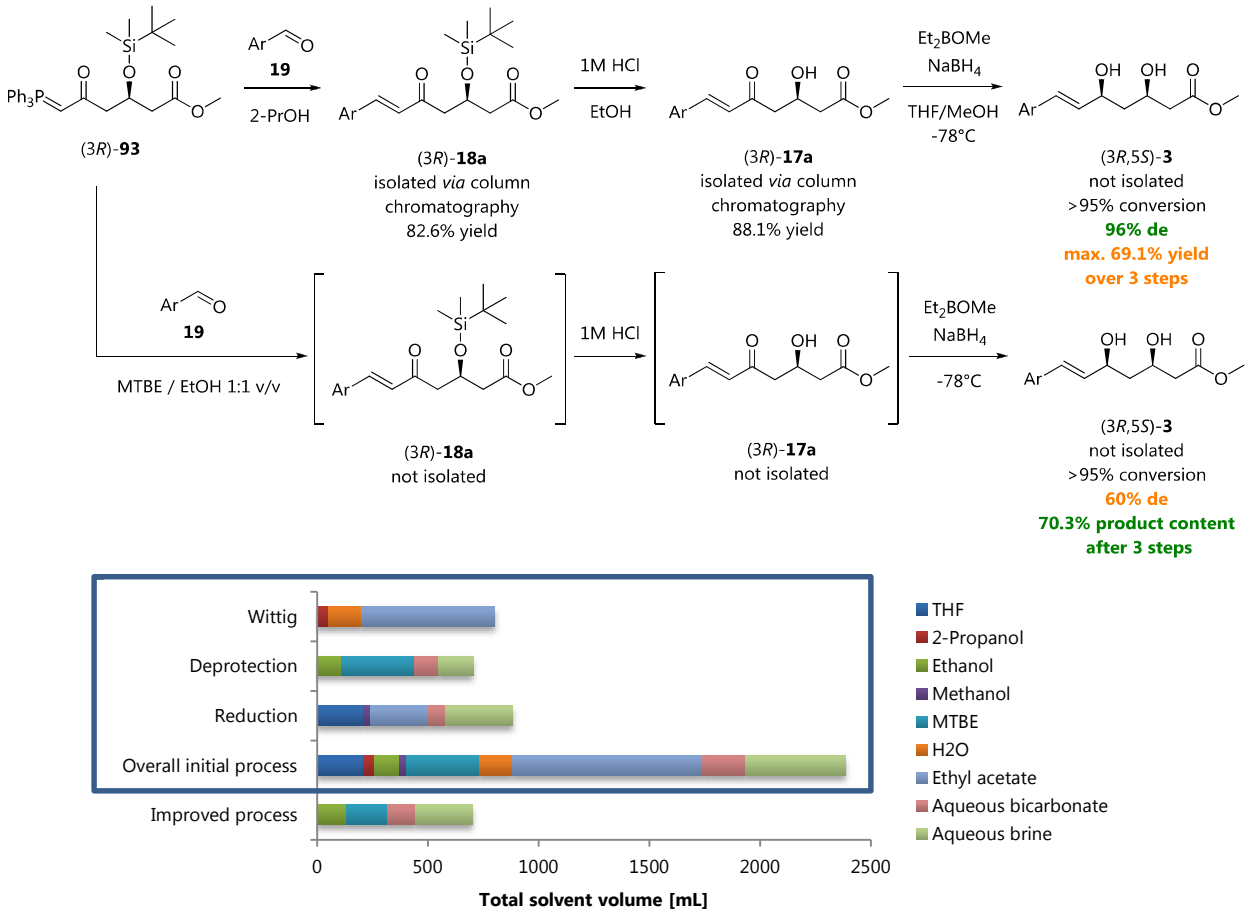
Figure 58. Solvent engineering for the subsequent derivati-zation to the TBS-protected mixed anhydride. Comparison of the multi-step synthesis and the isolated reaction steps (square).

amounts. This purification seemed to be mandatory and was also tolerable after four consecutive steps without necessary purification. The activation of the TBS-protected monoester was also realized in MTBE, with the additional benefit of triethylamine hydrochloride as the sole impurity being hardly soluble. Again, this reaction solution was readily purified by precipitation and filtration at low temperatures. Excess of methyl chloroformate was still present and needed to be quenched and removed *via* addition of aqueous sodium bicarbonate solution and phase separation. The compatibility of the ylide formation in MTBE was not tested.

Synthesis of Rosuvastatin Methylester from the enantiopure phosphor ylide

The synthesis of Rosuvastatin methyl ester starting from the commercial ylide was also investigated for possible enhancement regarding solvents (see **Figure 59**, page 61). Since MTBE had proven itself to be highly versatile as reaction and extractive solvent, it was also chosen for this examination. The Wittig reaction already turned out to be proceeding in any examined organic

solvent, with higher reaction rates observed in protic solvents or mixtures with water. The deprotection of the silyl ether **18a** in aqueous media produced the corresponding silanol as persistent impurity. Thus, an alcoholic medium seemed favorable to transetherate the protecting group into a more convenient impurity, which could possibly be removed *via* evaporation. The



following diastereoselective reduction of **17a** was usually conducted in a methanolic THF medium. Therefore, the use of MTBE and ethanol as the only solvents was reasonable.

While the WITTIG reaction proceeded as expected, the deprotection of the silyl ether did not. The conversion was not quantitative and led to a mixture of β-silyloxyketone and β-hydroxyketone. The coordination of LEWIS-acid was therefore restricted to the deprotected hydroxy group and the diastereoselective reduction produced the desired (5*S*)-carbinol **3** in only 60% de. After oxidative cleavage with hydrogen peroxide and additional acidic workup to remove remaining silylether, the content of Rosuvastatin methyl ester was quantified *via* ^{1}H-NMR spectroscopy. The low conversion for the

Figure 59. Solvent engineering for the production of Rosuvastatin methyl ester starting from the phosphoranylidene. Comparison of the multi-step synthesis and the isolated reaction steps (square).

deprotection step could probably be circumvented by a prolonged reaction time, hence leading to quantitative elimination of silanol. The diastereoselectivity of the subsequent reduction would thus be enhanced compared to the already determined 96% de under these reaction conditions.

2.8.4 CRYSTALLIZATION OF INTERMEDIATES

The desymmetrization with α-chymotrypsin showed an excellent enantioselectivity of >97% ee for the desired monoester. To further increase the optical purity of the desired phosphorous ylide, a fractionized crystallization was also examined (see **Figure 60**).

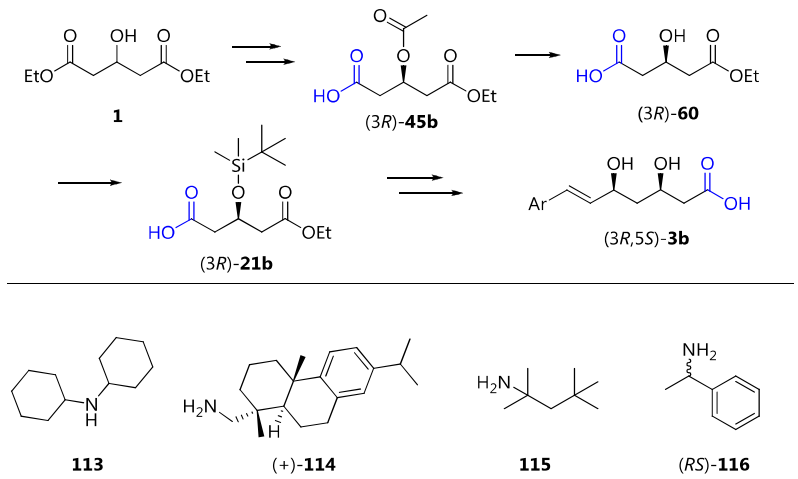
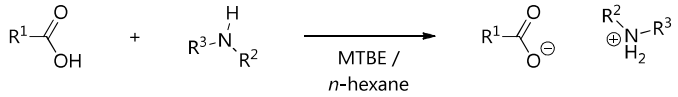


Figure 60. Intermediates bearing carboxylic acid moieties and amines examined in crystallization of enantiomeri-cally enriched and purified ammonium salts.

Since four intermediates are carboxylic acids, the crystallization with different amines was reasonable. Among commonly used amines for the enrichment of chiral carboxylic acids are dicyclohexylamine **113**, *tert*-octylamine **115** and dehydroabietylamine **114**, which were used in this study (see **Table 15**). Moreover, chiral amines such as 1-phenylethylamine **116** can also be applied for diastereopreferencial crystallization. The intermediates **45b**, **60** and **21b** were successfully crystallized with dicyclohexylamine from a mixture of MTBE and *n*-hexane, unfortunately, due to fast crystallization, with an unsatisfying rise of optical purity to a maximum of 98% ee. Notably, the dicyclohexylamine salt of the TBS-protected monoester **21b** was also used directly for the chloroformate activation reaction (see **Figure 61**, page 63).



Entry	R^1-COOH[a]	R^2R^3NH	Cryst.	Isol. Yield [g (%)]	ee[b] [%]
1	(*3R*)-**45b**	**113**	+	0.70 (38.1)	98.0
2	(*3R*)-**45b**	(*RS*)-**116**	-	-	-
3	(*3R*)-**60**	**113**	+	0.95 (57.9)	97.2
4	(*3R*)-**60**	(+)-**114**	+	1.89 (71.3)	98.4
5	(*3R*)-**60**	(*RS*)-**116**	-	-	-
6	(*3R*)-**21b**	**113**	+	1.15 (53.0)	n.d.
7	(*3R*)-**21b**	(+)-**114**	-	-	-
8	(*3R*)-**21b**	(*RS*)-**116**	-	-	-
9	Rosuvastatin	**115**	-	-	-

Table 15. Purification of carboxylic acid intermediates by crystallization of their ammonium salts (see **Figure 60**, page 62).

(a) 4.6 mmol, except for entries 4 and 7 (5.8 mmol) (b) Determined after acidic work-up and phenylethylamine derivatization.

The byproduct dicyclohexylamine **113** precipitated as hydrochloride from the MTBE reaction mixture. Due to its cationic species, it was also inert towards carbamate formation. However, the reaction showed only 84% conversion, possibly due to the sterically demanding cyclohexyl groups. This reaction was not further investigated, but gives rise to an improved purification method for the silylation in combination with the carboxylic acid activation.

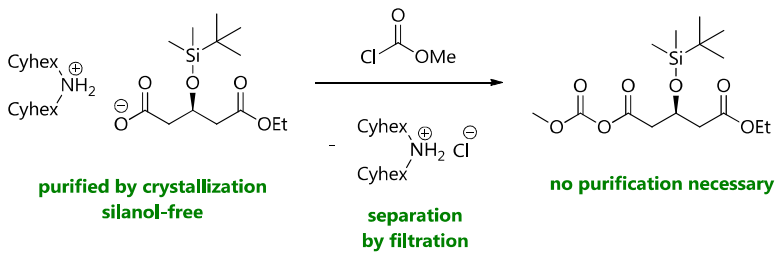


Figure 61. Advantageous use of a crystallization procedure for (*3R*)-TBS-EHG (**21b**) in view of purity control for the mixed anhydride

The present study on a biocatalytic desymmetrization route to the blockbuster drug Rosuvastatin, which had an annual world market sale of 6.253 billion US dollars in 2012, demonstrated the difference between an ideal process on paper and the often more complex chemistry of the realized synthesis. The initial route was adjusted several times to circumvent chemical as well as biocatalyst weaknesses. Nevertheless, the final route to the enantiomerically enriched key intermediate, the chiral phosphoranylidene **93**, was successfully realized with high yields, excellent enantioselectivities and numerous improvements which made this process sophisticated enough to be of high industrial value. The most notable fact is that the bienzymatic process to ethyl (3*R*)-hydroxyglutarate (**60**), which was necessary to increase the enantioselectivity of the hydrolase α-chymotrypsin from moderate 60% ee to excellent >97% ee, is also the most stable part of the route (see **Figure 62**). The substrate concentration of 4.0 mol/L of **22a** was even more fascinating, as it is in fact a binary mixture of aqueous reaction buffer and an organic substrate

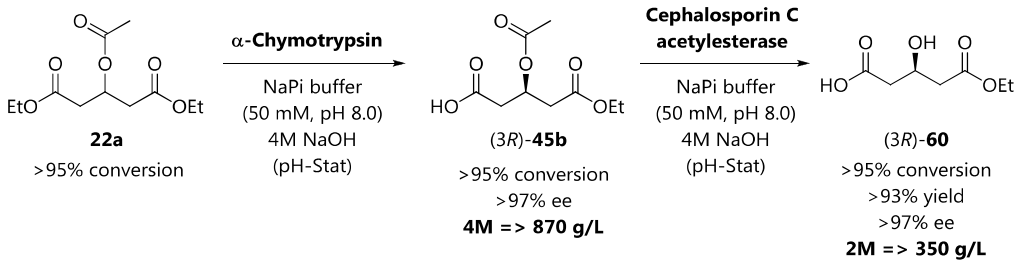


Figure 62. Bienzymatic process for the production of the enantiomerically enriched monoester (3*R*)-60 at high substrate concentrations.

phase of around 1:1 volumetric ratio. By combining the highly enantioselective desymmetrization and a subsequent chemoselective deacetylation with cephalosporin C acetylesterase (CAE), the enantiomerically enriched ethyl (3*R*)-hydroxyglutarate (**60**) was produced in more than 350 g/L, which is remarkable for a biocatalytic process.

The subsequent derivatization to the enantiomerically enriched ylide (*3R*)-**93b** (see **Figure 63**, page 65), which was used as a benchmark intermediate for the industrial feasibility of the process, was established using literature-known protecting groups and activated carboxylic acid derivatives. The desired ylide could not be isolated in pure crystalline form. However, the content of ylide was determined by the conversion of aldehyde in the following WITTIG reaction (see, page 66). With respect to the already established WITTIG reaction with the

commercial ylide and the need for 1.1 equivalents of reagent, the conversion of aldehyde revealed an ylide content of 78-87%. The ylide was partially purified by preparative TLC and analyzed *via* chiral HPLC. The enantiomeric excess was calculated to be 98.2% ee, an optical purity which was considered to be sufficient for an industrial application.

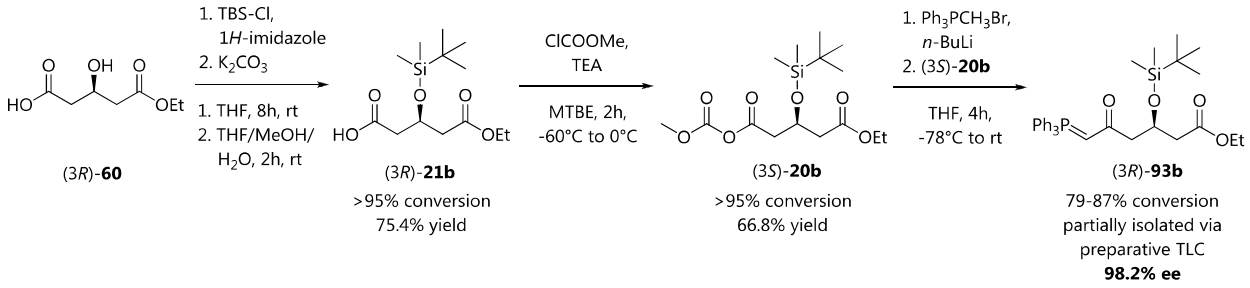


Figure 63. Final route for the production of chiral phosphoranylidene (3*R*)-**93b**

Another major achievement of this work was the combination of compatible reactions by solvent engineering and the elimination of purification steps. By doing so, the overall synthetic route was basically reduced to a single necessary purification of intermediate (3*S*)-**20** in 9 consecutive steps (see **Figure 64**, page 66). Nevertheless, this purification is not trivial, with the *tert*-butyldimethylsilanol as the major impurity. Within this work, a possible solution for this problem was found in the crystallization of the preceding product by the use of dicyclohexylamine. The corresponding ammonium salt can be purified by recrystallization and used directly for the following anhydride formation. This is only one of numerous future improvements which revealed themselves during this project. From an enzymatic point of view the top priority for upcoming improvements would without a doubt be an alcohol dehydrogenase which reduces the β-hydroxyketone **113** in high diastereoselectivity, eliminating stoichiometric amounts of diethylmethoxyborane and sodium borohydride. Since the preceding WITTIG reaction is enhanced by aqueous or protic solvents, the reaction medium would most likely be compatible. Furthermore, the chemoselectivity of a biocatalyst instead of hydride reagents would be advantageous for present impurities.

Moreover, the route to the enantiomerically enriched O-protected monoester could tremendously be shortened by a hydrolase accepting the TBS protecting group on the prochiral starting material **1a** without losing activity or enantioselectivity. The TBS group is also the weakest point of this route from a chemical point of view, since its insertion and removal is bound to silanol

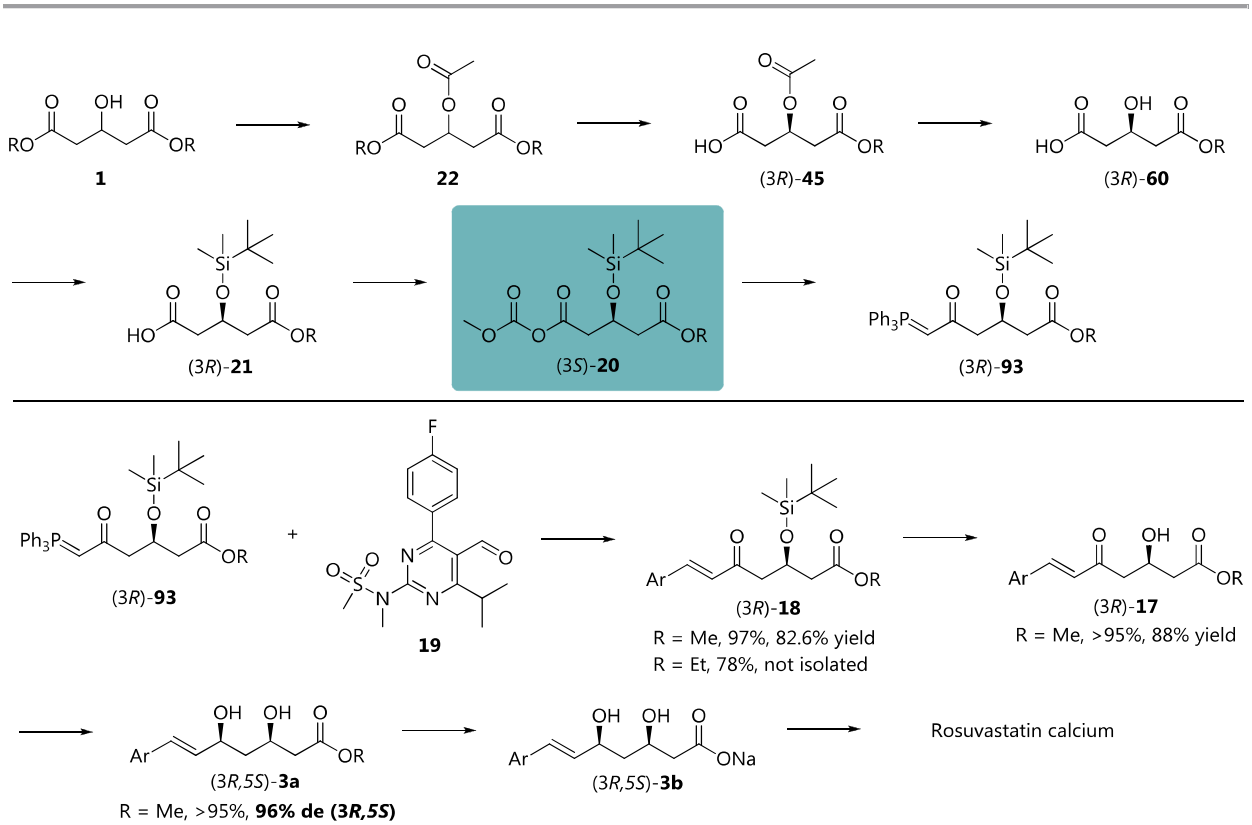
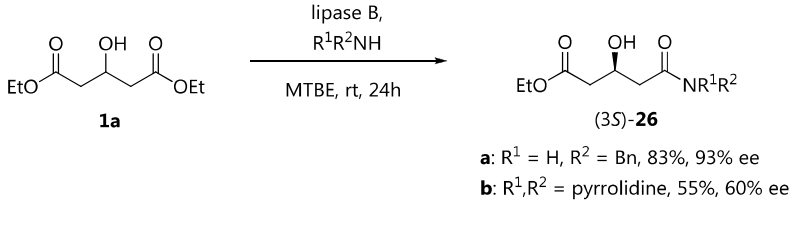


Figure 64. Overall synthetic route of Rosuvastatin calcium with only one mandatory purification step (blue).

impurities which are not easy to eliminate and also not inert towards some of the reaction conditions. Since the tetrahydropyran-2-yl group has the same stability properties but can be converted into an extractable aldehyde, it would certainly be the more favorable choice.

Finally, a desymmetrization process by a lipase-catalyzed aminolysis with primary and secondary amines was investigated (see **Figure 65**). Lipase B from *Candida antarctica* was successfully used to desymmetrize **1a** with benzylamine, in good yields of 83% and very good enantioselectivity of 93% ee. However, the presence of a relative acidic amide proton led to significant side-product formation during the ylide synthesis.

Figure 65. Lipase-catalyzed desymmetrization of **1a** with primary and secondary amines.



Changing the enzymatic desymmetrization to secondary amines was only moderately successful, accompanied by an uncatalyzed chemical aminolysis at even room temperature and therefore a low enantiomeric excess of the chiral monoester. By using pyrrolidine as nucleophile, a moderate optical purity of 60% ee was achieved in parallel to an uncatalyzed aminolysis yielding a racemate. The reaction was reproduced in an uncatalyzed experiment and the result was used to recalculate the enantioselectivity of the isolated enzymatic process. Under the premise of equal reaction conditions, the enantioselectivity was determined to be 97.6% ee. These are most promising results for future work in the chemoenzymatic synthesis of pharmaceutical building blocks and generics.

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3 OVEREXPRESSION AND APPLICATION OF ALDOXIME DEHYDRATASES FOR THE SYNTHESIS OF ENANTIOPURE NITRILES AS PHARMACEUTICAL BUILDING BLOCKS

This project was a collaboration project between the organic synthetic working group of Prof. Harald Gröger (Organic Chemistry I, Bielefeld University, Bielefeld, Germany) and the molecular biology working group of Prof. Yasuhisa Asano (Enzyme Laboratory, Toyama Prefectural University, Kosugi, Toyama, Japan). It was generously funded with a Pre-Doctoral Fellowship by the Japan Society for the Promotion of Science (JSPS) on recommendation of the German Academic Exchange Service (DAAD). The following experiments were conducted during a six-months research stay at the japanese host institution from January 2013 – July 2013. The main interest focused on a possible application of aldoxime dehydratases, a recently discovered enzyme belonging to the class of lyases, for the enantioselective synthesis of nitriles (see **Figure 66**).

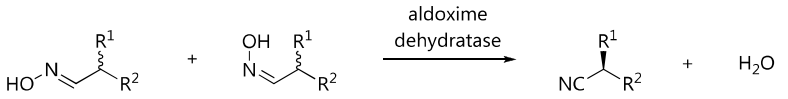


Figure 66. General project overview for the synthesis of enantiopure nitriles by biocatalytic aldoxime dehydration.

3.1 ENANTIOSELECTIVE SYNTHESIS OF α-CHIRAL NITRILES AS BUILDING BLOCKS IN THE PHARMACEUTICAL INDUSTRY

Nitriles as carbonyl synthons can be widely applied in the manufacture of carboxylic acids, amides, amines and ketones (see **Figure 67**). This is based on the straightforward introduction of the nitrile group by nucleophilic substitution of alkyl halides with the pseudohalide cyanide or the addition of cyanide to aldehydes, e.g. for the STRECKER synthesis of α-amino acids.[45] The nucleophilic substitution of alkyl halides follows a second order mechanism under complete inversion of the preceding absolute configuration, enabling the synthesis of enantiomerically pure nitriles.

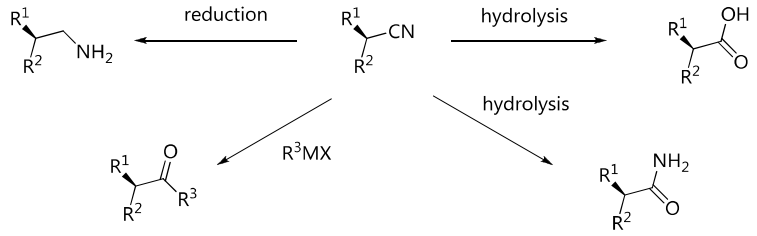


Figure 67. Derivatization of chiral nitriles towards pharmaceutically relevant building blocks.

Nevertheless, enantiomerically pure alkyl halides are generated from their corresponding enantiomerically pure carbinols. This need for optically pure starting materials is a crucial drawback. Furthermore, the convenience of this nucleophilic carbonyl derivative is overshadowed by the reagents toxicity. Methodologies to circumvent the use of cyanide have been published in recent years, but to the best of the author's knowledge, none of the following reactions is currently conducted on industrial scale. CZEKELIUS and CARREIRA successfully converted enantiomerically enriched β-chiral nitroalkanes of type **117** to their corresponding nitriles of type **119** by a chemical two-step elimination strategy (see **Figure 68**, page 71).[46]

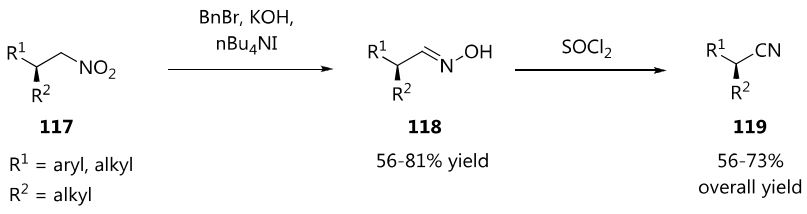
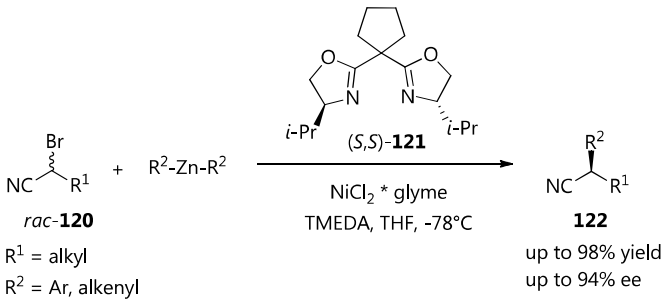


Figure 68. Two-step chemical derivatization of β-chiral nitroalkanes to α-chiral nitriles by CZEKELIUS and CARREIRA.[46]

No optical purities are given for the overall process, which would be the most interesting information since the intermediate of type **118** and the product **119** should more or less be epimerizable under the reaction conditions. For the reason that enantiopure β-chiral nitroalkanes are not trivial to synthesize, this methodology is certainly restricted. Very recently, CHOI and FU reported an enantioselective, nickel-catalyzed NEGISHI cross-coupling reaction with chiral bidentate ligands of type (*S*,*S*)-**121** (see **Figure 69**).[47]

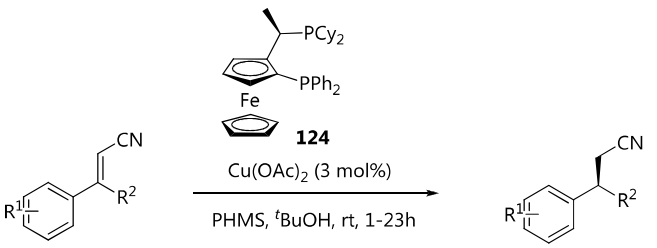


rac-**120**
R^1 = alkyl
R^2 = Ar, alkenyl

122
up to 98% yield
up to 94% ee

Figure 69. Enantioselective C-C coupling reaction of racemic α-bromonitriles (*rac*-**120**) with zinc organyles in the presence of enantio- and diastereomerically pure bidentate ligands by CHOI and FU.[47]

Another metal-catalyzed process for the production of chiral nitriles was realized by a copper hydride reduction of α,β-unsaturated nitriles of type **123** with the use of a JOSIPHOS ligand (**124**) and stoichiometric amounts of hydrosilanes (see **Figure 70**).[48]



E- or *Z*-**123**
R^1 = H, 4-Cl, 4-Me, 2-Me
R^2 = Me, Et, ^{i}Pr

125
81-95% isolated yield
94-99% ee (*R*) based on *E*-**123**
99% ee (*S*) based on *Z*-**123**

Figure 70. Enantioselective reduction of β-substituted cinnamonitriles **123** by a copper hydride with JOSIPHOS (**124**) as chiral ligand and stoichiometric amounts of hydrosilanes as hydride donor by LEE *et al*..[48]

Very recently, an organocatalytic enantioselective synthesis of chiral α-aryl nitriles has been developed by coworkers of the LIST group (see **Figure 71**, page 73).[49] Starting from racemic nitriles, they synthesized prochiral silyl ketene imines of type **126** which are subsequently protonated by a chiral organic phosphate **127**. The stoichiometric proton donor is methanol which is itself silylated in return.

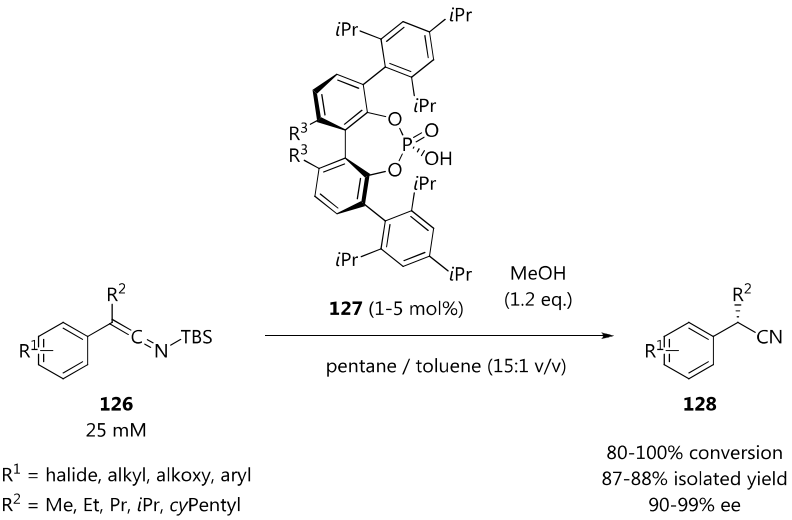


Figure 71. Enantioselective protonation of silyl ketene imines with the use of organocatalysts by GUIN *et al.*[49]

A biocatalytic alternative to these metal- and organocatalytic approaches has also been reported by KOSJEK and her coworkers from Merck, reducing α,β-unsaturated nitriles of type **129** with an enoate reductase.[50] The stoichiometric hydride donor in this case is sodium phosphite, which recycles the oxidized nicotinamide cofactor (NAD(P)$^+$) to its reduced form by a phosphite dehydrogenase (PTDH)[51] present in the reaction mixture (see **Figure 72**).

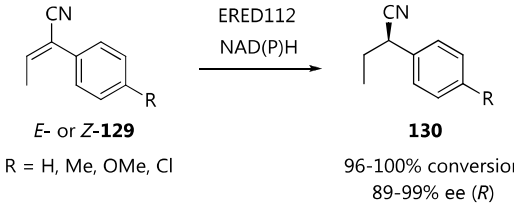


Figure 72. Enantioselective reduction of α,β-disubstituted α-aryl acrylonitriles of type **126** with an enoate reductase by KOSJEK *et al.*[50]

POSSIBLE IMPLEMENTATION OF AN ALDOXIME DEHYDRATION IN THE PRODUCTION OF ACTIVE PHARMACEUTICAL INGREDIENTS

Beside the use as a precursor for previously discussed chiral structures, nitriles are also implemented directly as pharmacologically active structures in drugs such as Vildagliptin, Saxagliptin and Sapacitabine (see **Figure 73**). While the first two compounds are used as dipeptidyl-peptidase 4 inhibitors for the

treatment of diabetes, the latter is currently in the clinical phase III for the treatment of leukemia. The inhibitory effect towards peptidases is based on the structure of the nitrile as a carbonyl synthon. The peptidase acts on the nitrile, transforming it into an unreactive enzyme-aldimine complex.

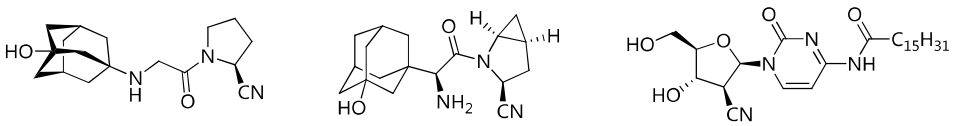


Figure 73. Active pharmaceutical ingredients possessing α-chiral nitrile moieties.

In the case of Vildagliptin, the chiral nitrile is derived from L-proline amide by trifluoroacetic anhydride dehydration. This is advantageous as L-proline is commercially available in enantiomerically pure form. For more complex structures like Saxagliptin or Sapacitabine, the proline-type or furanose-type structure syntheses become sophisticated and replaceable. As aldoximes can be produced by the condensation of aldehydes with hydroxylamine, the relevant structures could be generated from their corresponding aldehyde derivatives, which will be discussed in chapter 3.3 (page X).

3.2 ENANTIOSELECTIVE BIOCATALYTIC DEHYDRATION OF ALDOXIMES

All of the methods above have certain drawbacks of either using enantiomerically pure precursors or enantiomerically pure metal-catalysts. Additionally, the substrate structures are sometimes a prerequisite, e.g. the α,β-substitution pattern for enoate reductases. Therefore, finding a catalyst which is able to accept a wide range of molecular structures and combining it with a feasible substrate synthesis is of high interest. By using an aldoxime dehydratase, the synthetic pathway based on aldehydes could have additional advantages, such as a nearly perfect atom economy (see **Figure 74**) and the absence of stoichiometric reagents or expensive cofactors.

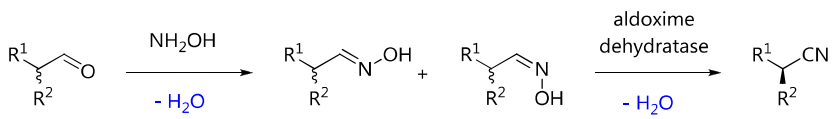


Figure 74. General synthetic route for the synthesis of enantiomerically pure nitriles starting from racemic aldehydes.

3.2.1 STATE OF THE SCIENTIFIC KNOWLEDGE OF THE ALDOXIME DEHYDRATASE MECHANISM

Aldoxime dehydratases were first characterized by ASANO and KATO during the investigation of the aldoxime-nitrile pathway in plants and microorganisms.[52,53] This pathway describes a part of the degradation of α-amino acids to their corresponding lower homologues of nitriles, which are further degraded to carboxylic amides and acids. The understanding of these biological pathways is of importance for the synthesis of bio-based structures or the development of new catalytic systems. The formal dehydration of aldoximes to their nitriles is catalyzed by a ferrous (Fe^{2+}) heme unit, with protic amino acid side-chains like histidine and serine in the vicinity.[54,55] The oxidation state of the iron heme was investigated by various groups and their results suggest the necessity of ferrous (Fe^{2+}) heme rather than ferric (Fe^{3+}) heme for successful catalysis. Moreover, flavin mononucleotide (FMN) or reducing agents like sodium sulfide are necessary for high activity. This effect implies an oxidative inactivation of the active center to the ferric (Fe^{3+}) state, either by the reaction itself or in a side-reaction with molecular oxygen.

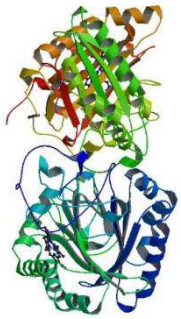


Figure 75. Crystal structure of the biological assembly of aldoxime dehydratase from *Pseudomonas chlororaphis* (*OxdA*) in complex with butyraldoxime (NOMURA *et al.*).[55]

In addition, the ligand binding was determined by several crystal structures of aldoxime dehydratases from *Pseudomonas chlororaphis* (*OxdA*, see **Figure 75**, page 75)[55] or *Rhodococcus erythropolis* (*OxdRE*, see **Figure 76**)[54], with the aldoxime directly bound to the ferrous heme unit with its nitrogen atom. It has been reported that the reaction only proceeds with this binding conformation and does not occur for the binding of the aldoxime oxygen. This inactive binding motif is mainly observed for the ferric oxidation state, which was artificially induced by oxidation. Furthermore, NOMURA *et al.* elucidated the mechanism by crystal structure and spectroscopic analysis and postulated an iron-(IV) carbimide intermediate.[55] They also examined mutants obtained by alanine exchange for increase or decrease of enzymatic activity. As it is predictable, histidine (His^{320}) and serine (Ser^{219}) were obligatory for activity (see **Figure 77**) and their substitution to alanine led to complete loss of activity. Also, an adjacent arginine residue (Arg^{178}) enhanced the reaction by a 5-fold rate, completing a catalytic triad responsible for the elimination of the aldoxime hydroxy group. Other residues in the vicinity of the active center were also exchanged by alanine but showed only minor effects on the residual activity. Notably, the exchange of a tyrosine led to a 1.7-fold increase of activity related to the wild-type. This could be explained by expansion of the active center and a correlating facilitated substrate binding.

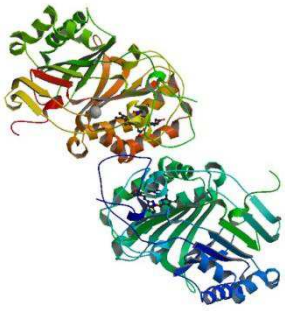


Figure 76. Crystal structure of the biological assembly 1 of aldoxime dehydratase from *Rhodococcus erythropolis* (*OxdRE*) in complex with propionaldoxime (SAWAI *et al.*).[54]

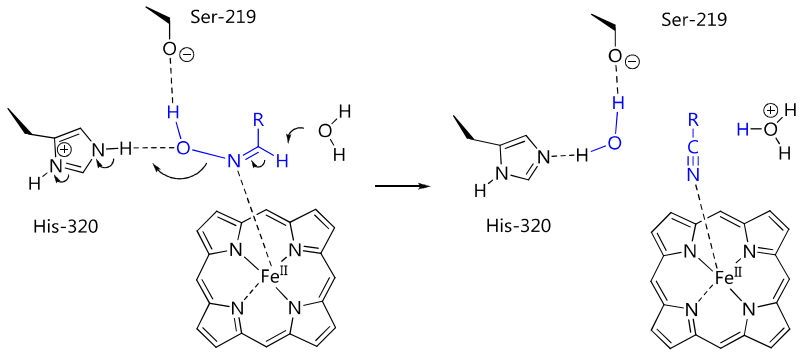


Figure 77. Proposed overall dehydration process based on the crystal structure of *OxdA* and necessary protein residues from alanine exchange studies by NOMURA *et al.*.[55]

Looking at the proposed reaction pathway starting from aldehydes and hydroxylamine it seems favorable to generate the aldoxime *in situ* by spontaneous condensation in aqueous medium. Unfortunately, XIE *et al.* demonstrated in a scaled-up process (0.75 M substrate concentration) for the synthesis of 3-phenylpropionitrile that the aldoxime dehydratase is inhibited by both aldehyde and hydroxylamine, even as residual impurities from a separate aldoxime formation.[56] Remarkably, a simple washing of the crude product with water was sufficient to remove excess hydroxylamine.

3.2.2 OVEREXPRESSION AND PURIFICATION OF RECOMBINANT ALDOXIME DEHYDRATASES

From an academic point of view it was important to have a reference reaction to gain insights into the reaction, preferably an already established substrate. The dehydration of phenylacetaldoxime was chosen as the higher chiral homologue 2-phenylpropionaldoxime, more specifically its corresponding aldehyde, is commercially available. The achiral reference substrate was converted with a remarkably high activity of up to 14,000 units per liter of culture (U/L_{cult})[57] by an aldoxime dehydratase from *Bacillus* sp. OxB-1, overexpressed in recombinant *E. coli*, which was therefore used as a reference catalyst system. Notably, the overexpression in the heterologous host amplified the wild-type activity by more than thousand-fold. In preceding studies by KATO *et al*. it was already reported that this aldoxime dehydratase from *Bacillus* sp. (*OxdB*) showed no activity towards 2-phenylpropionaldoxime.[58] Nevertheless, this substrate was also investigated. Other chiral aldoximes were also to be tested in the present work, none of them being screened before. The

biocatalyst was overexpressed according to the literature procedures and used, unless stated otherwise, as resting cell suspension in phosphate buffer (KPB).

Figure 78. SDS-PAGE of the purification procedure of *OxdB* from rec. *E. coli* HB101 /pOxD-9OF; 8-30 µg protein were placed on each lane.

MP = Marker proteins (Phosphor-ylase b (97.4 kDa), Serum albumin (66.2 kDa), Ovalbumin (45.0 kDa), Carbonic anhydrase (31.0 kDa), Trypsin inhibitor (21.5 kDa), Lysozyme (14.4 kDa)), Cell-free extract (1), 40-70% $(NH_4)_2SO_4$ precipitate (2), DEAE-Toyopearl (3), Butyl-Toyopearl (4), Superdex 200 16/600 (5).

The heterologous host system *E. coli* HB101, already transfected with the plasmid pOxD-9OF, was used in the present study.[57] The aldoxime dehydratase was additionally purified for comparison with other formulations, e.g. resting cells, acetone-dried cells and the cell-free extract after sonication. The purification procedure was first described by KATO and ASANO[57] and was reproduced to yield purified *OxdB* with the reported literature activity (8.35 U/mg, 8.55 U/mg WT[i]), however with a relatively low overall yield of 5.7 mg. The cell-free extract was purified by ammonium sulfate precipitation (40-70% saturation), DEAE- and Butyl-Toyopearl ion-exchange chromatography as well as a preparative gel-filtration chromatography on a Superdex 200 (16/600)

Table 16. Purification steps for *OxdB* from recombinant *E. coli* HB101 / pOxD-9OF.

Entry	Method	Total Protein (mg)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification (fold)
1	Cell-free extract	1357	0.21	279.2	100	1.0
2	40-70% $(NH_4)_2SO_4$	958.8	0.23	220.5	79.0	1.1
3	DEAE-Toyopearl	244.0	0.34	82.96	29.7	1.6
4	Butyl-Toyopearl	42.97	1.80	77.35	27.7	8.6
5	Superdex 200	5.690	8.47	48.19	17.3	40

column connected to an ÄKTA purifier unit. After the final purification step an SDS-PAGE analysis (see **Figure 78**, page 77) showed a single band at approximately 40 kDa molecular weight, corresponding to the literature value of *OxdB* from *Bacillus* sp. (40,000 Da)[58]. The first ion-exchange chromatography on a *N,N*-(diethylamino)ethyl-Toyopearl (DEAE-Toyopearl) removed more than 70% of total extractable protein of the cell-free extract (see **Table 16**, entry 3). Although the pooled fractions 10 - 17 (244.0 mg) contained all of the active protein (see **Figure 79**), almost 63% of the total activity was lost during the procedure. This shows the rather weak stability of *OxdB* after separation from its recombinant host cell.

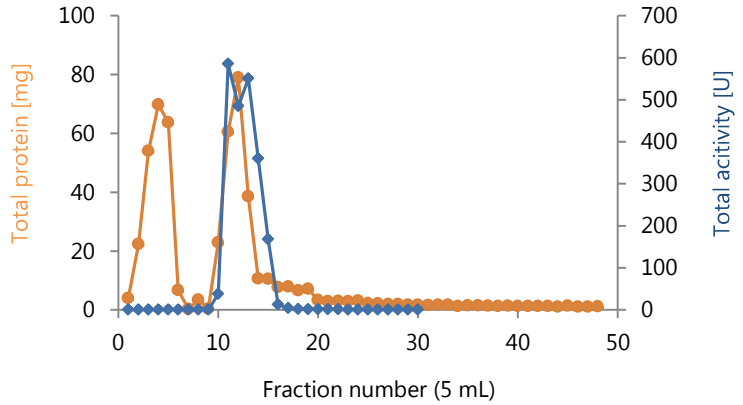


Figure 79. DEAE-Toyopearl purification of *OxdB*. Each fraction was analyzed by Bradford assay and subsequent biotransformation for protein content and specific activity towards *Z*-phenylacetaldoxime.

The subsequent purification step on a Butyl-Toyopearl ion-exchange column could further remove inactive proteins, with the pooled fractions 16 - 20 (see **Figure 80**) maintaining more than 93% of total active protein. However, the resolution of this procedure seems quite poor, hence the need for small volume fractions.

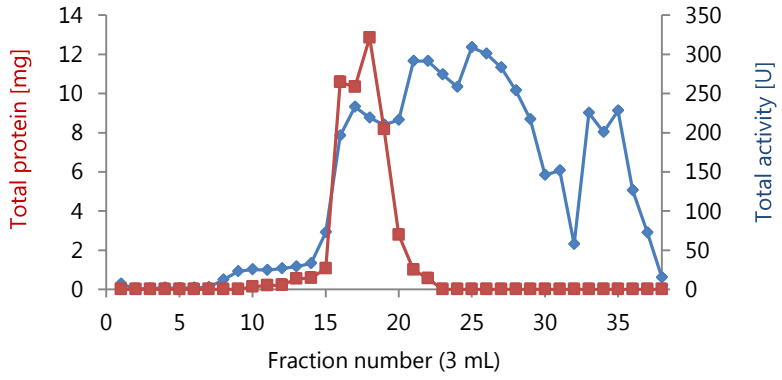


Figure 80. Butyl-Toyopearl purification of *OxdB*. Each fraction was analyzed by Bradford assay and subsequent biotransformation for protein content and specific activity towards *Z*-phenylacetaldoxime.

The final gel-filtration chromatography successfully removed the remaining inactive protein contaminants. Due to its poor resolution (see **Figure 81**,

page 79) and the instability of the protein, nearly 38% of total active protein from Butyl-Toyopearl was lost in this process. The purified *OxdB* solution (fraction 17) showed a specific activity of 8.47 U/mg for *Z*-phenylacetaldoxime under the literature-assay conditions. The purified *OxdB* remained unstable, losing its entire remaining activity over the following 120 hours.

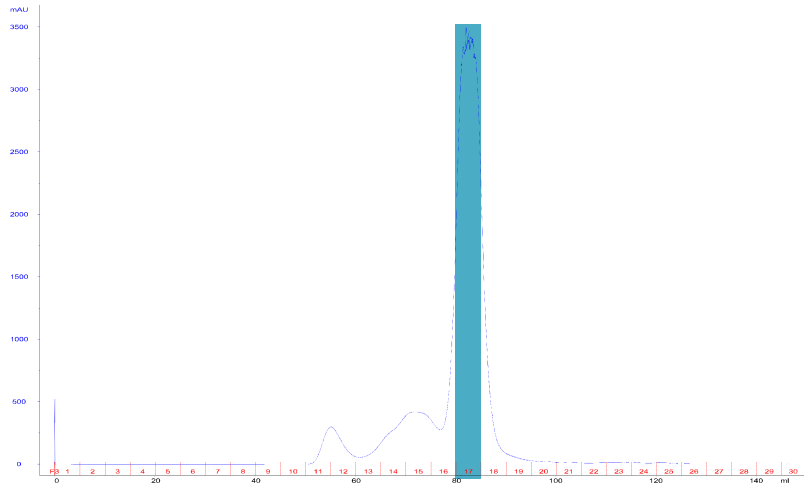


Figure 81. Superdex 200 (16/600) purification of *OxdB*. Fraction 17 was analyzed by Bradford assay and subsequent biotrans-formation for protein content and specific activity towards *Z*-phenylacetald-oxime.

Therefore, whole-cell catalysis seems to be the preferable method for using aldoxime dehydratases, at least up to date. In contrast to the purified enzyme, whole-cells are also superior in regard of their separation from biotransformations, their reusability, if staying intact, and their heightened stability in solution. In addition, acetone-dried cells of *E. coli* HB101 / pOxD-9OF were prepared according to a literature protocol from ASANO for the preparation of acetone-dried whole-cells of a phenylalanine dehydrogenase.[59]

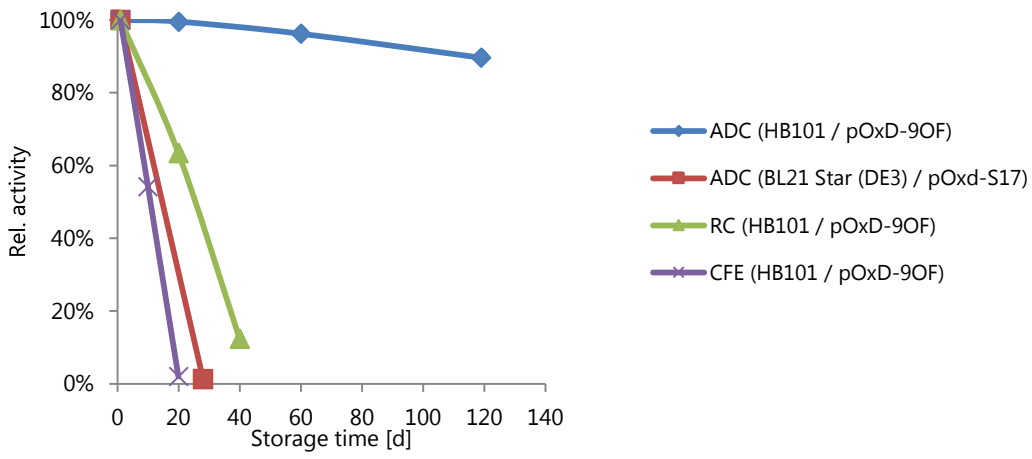


Figure 82. Storage stability of different enzyme formulations of recombinant *E. coli* HB101/pOxD-9OF.

Abbreviations: acetone-dried cells (ADC), resting cell suspension (RC), cell-free extract (CFE)

Impressively, these acetone-dried cells maintained about 90% of the initial specific activity for more than four months while being stored at -20 °C (see **Figure 82**, page 79). This is a promising factor on possible future applications on industrial level, since the preparation of the whole-cells can be separated from the manufacturing of chiral nitrile compounds.

3.2.3 SUBSTRATE DIVERSITY AND STEREOPREFERENCES FOR ACHIRAL ALDOXIMES

A crucial point for many enzymatic reactions is the substrate spectrum. For example, lipases react significantly faster with apolar substrates like carboxylic esters or alcohols than amides or carboxylates. Alcohol dehydrogenases have often a higher selectivity for ketones bearing small substituents. Since the aldoxime dehydration is part of the microbial degradation of α-amino acids, it is reasonable to presume that all amino acid side-chains, whether aliphatic or aromatic, are accepted as substrate structures. In fact, this seems to be the case for aldoxime dehydratases, as they were already proven to be highly versatile in this manner. Many aliphatic, aromatic, even heteroaromatic aldoximes were successfully screened with aldoxime dehydratases from *Bacillus* sp.[56,58], *Rhodococcus* sp.[60,61,62,63], *Pseudomonas* sp.[64] and *Fusarium* sp.[65] (see **Table 17**). While the synthesis of *Z*-phenylacetaldoxime, the standard substrate for the

Table 17. Exemplary substrate spectrum for aldoxime dehydratases based on literature reports.[56,58,60-63]

Entry	Substrate	*Oxd*	K_m [mM]	Rel. activity [%]
1	*Z*-phenylacetaldoxime	*Bacillus* sp. OxB-1	0.872	100
2	*Z*-3-phenylpropionaldoxime	*Bacillus* sp. OxB-1	1.36	62.9
3	*E/Z-n*-capronaldoxime	*Bacillus* sp. OxB-1	6.12	57.1
4	*E/Z*-indoleacetaldoxime	*Bacillus* sp. OxB-1	2.42	39.3
5	*Z*-napthacetaldoxime	*Bacillus* sp. OxB-1	0.846	4.48
6	*E/Z*-2-phenylpropionaldoxime	*Rhodococcus* sp. N-771	n.d.	232
7	*E/Z*-cyclohexanecarbaldoxime	*Rhodococcus* sp. N-771	n.d.	610
8	*E/Z*-Isobutyraldoxime	*Rhodococcus* sp. N-771	n.d.	67.1
9	*Z*-3-phenylpropionaldoxime	*R. globerulus* A-4	2.31	253
10	*E/Z*-2-phenylpropionaldoxime	*R. globerulus* A-4	11.9	19.7

aldoxime dehydratase from *Bacillus* sp. OxB-1, is significantly affected by the preferential crystallization of the Z-isomer over the *E*-isomer, most of the other aliphatic aldoximes can only be generated as *E/Z*-mixtures, with thermodynamic preference for the *E*-aldoxime. Aromatic aldoximes are an exception since the overlap with the aromatic system is impeding the *E/Z*-isomerism. The stereopreference of *E*- and *Z*-aldoximes was first described by KATO *et al.*, showing that *OxdB* has a higher activity for *Z*-aldoxime dehydration at 30 °C.[58] What was surprising during this study is the fact that a handful of chiral aldoximes was initially screened for activity and also enantioselectivity. These chiral aldoximes, *i.e.* *E/Z*-2-phenyl-propionaldoxime and mandelic aldoxime, were determined to be either not active or leading to a racemic mixture of the corresponding nitriles.[58,63] Nevertheless, since these results were only based on two chiral substrates and a couple of already identified aldoxime dehydratases, the present study was conducted for a number of additional chiral aldoximes and was initially focused on the screening for more active microorganisms.

3.3 CHIRAL SUBSTRATE SYNTHESIS AND REFERENCE COMPOUNDS

The previously discussed pharmaceutical target structures (**chapter 3.1**, page 79), more precisely their necessary aldehyde precursors, could be synthesized through a hydroformulation approach (see **Figure 83**). This hydroformulation, also known as Oxo synthesis or ROELEN reaction, adds carbon monoxide to an olefin under reductive conditions with molecular hydrogen to form aldehydes.[66]

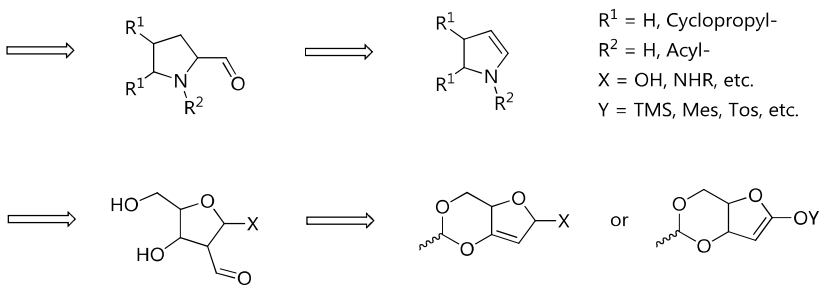


Figure 83. Retrosynthetic approach based on a hydroformulation process for the synthesis of obligatory aldehyde precursors.

It is widely applied in industry and efforts were also made to transfer this valuable method to aqueous or biphasic reaction mixtures, like the RHÔNE-POULENC process for the production of *n*-butanal from propene.[67]

Interestingly, not only alkenes but protected enamines and enol ethers can be hydroformulated. For example, LEIGHTON and O'NEIL successfully hydroformulated cyclic enol acetals of type **131** for the synthesis of polyol aldehyde structures **132** of natural compounds (see **Figure 84**).[68]

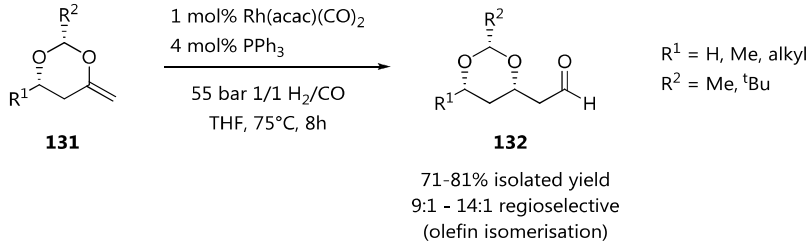


Figure 84. Hydroformylation of exocyclic enol ethers for the synthesis of aliphatic polyol aldehydes by LEIGHTON and O'NEIL.[68]

However, in this work, the biotransformation of racemic aldoximes was first investigated by using commercially available aldehydes (see **Figure 85**). The synthesis of aldoximes from their corresponding aldehydes can be realized under numerous conditions more or less optimized for the corresponding aldehydes. The methods range from simple mixing of aldehyde and hydroxylamine, or one of its readily available and easier to handle salts, to heterogenous catalysis with a solid inorganic base under solvent-free conditions, or just with stirring over molecular sieves. There are more than enough methodologies to generate aldoximes in excellent yields, even in aqueous media facilitating combination with an enzymatic reaction in a one-pot process.

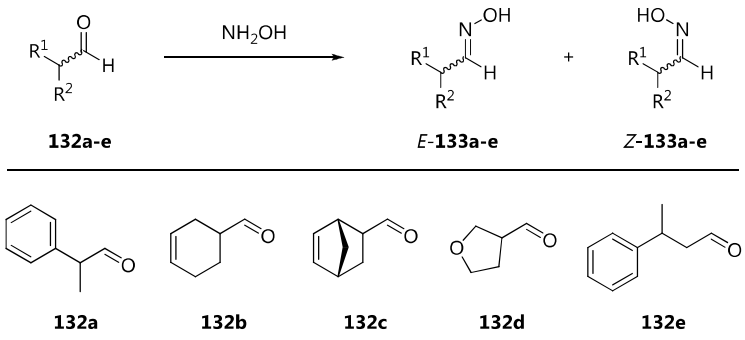


Figure 85. Substrate synthesis of α- and β-chiral aldoximes for the screening of enzymatic activity of *OxdB* and a possible enantioselectivity.

However, since it was already intensively investigated by KATO *et al.*58 that the aldoxime dehydratase from *Bacillus* sp. OxB-1 shows different activities for *E*- and *Z*-aldoximes of the same aldehyde, it seemed reasonable to explore

synthetic conditions to generate either one isomer or the other, separately. In some cases, predominantly aromatic aldehydes but also for phenylacetaldehyde, the isomers differ not only in their enzymatic activity but also in their physicochemical properties, e.g. melting points or solubility. For phenylacetaldehyde, the *Z*-aldoxime was isolated from a mixture through fractionized crystallization. The literature also offers a number of methodologies specifically designed to synthesize aromatic *Z*- or *E*-aldoximes, which were also tested for applicability in this work.

The broad substrate spectrum of aldoxime dehydratases made it possible to vary the structures of chiral aldoximes to get a wide, however rational view on stereopreferences. For this matter, five small and structurally different aldehydes were used as starting materials (see **Figure 85**, page 82). The closest α-chiral aldehyde related to the standard substrate phenylacetaldehyde is 2-phenylpropionaldehyde (**132a**) with an additional methyl substituent. This substrate was tested by KATO *et al*. and showed no activity for the aldoxime dehydratase from *Bacillus* sp. (*OxdB*) [58] and no selectivity for *OxdRE* (*Rhodococcus erythropolis*)[56]. Regardlessly, it was still chosen for screening purposes in the present study. High activities for aromatic aldoximes in preceding studies suggested a general acceptance of cyclic structures directly bound to the carbaldoxime. Thus, 3-cyclohexene-1-carbaldehyde (**132b**) was examined as a chiral cyclic substrate. The flexibility of cycloalkanes can sometimes complicate the analysis. Therefore, the rigid 5-norbornene-2-carb-aldehyde (**132c**) was used as an analog to **132b**, without the ability to change the ring conformation and coincidentally enhance structure complexicity. Furthermore, the impact of a heteroatom was tested in view of biologically active chiral nitriles based on proline derivatives. However, unprotected pyrrolidine derivatives could not be synthesized in the present study. Hence, tetrahydrofuran-2-carbaldehyde (**132d**) was chosen as a substitute. Remarkably, addition of the synthesized compounds to the growing medium of *OxdB* resulted in complete inhibition in all but one cases. As can be seen in **Figure 86**, the cells of *E. coli* HB101 / pOxD-9OF grew also on a medium containing tetrahydrofuran-2-carbaldoxime **133d** (5 mM). The reason for this specific tolerance was not determined. Last but not least, identifying mid-range selectivities for aldoxime dehydratases were also of interest so that 3-phenylbutyraldehyde (**132e**) was chosen as well. Its stereogenic center is an additional methylene group away from the aldoxime. With this set of structure- and electronically diverted compounds, standard protocols for the formation of aldoximes and their corresponding racemic nitriles were conducted.

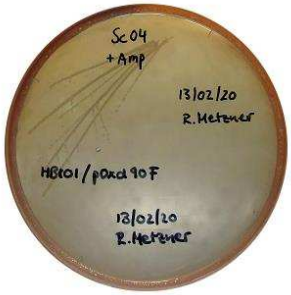
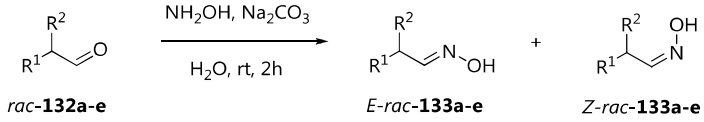


Figure 86. Agar plate with grown recombinant E. coli HB101 / pOxD-9OF on LB medium containing 5 mM tetrahydrofuran-2-carbald-oxime.

3.3.1 RACEMIC ALDOXIMES THROUGH CONDENSATION OF RACEMIC ALDEHYDES WITH HYDROXYL AMINE

It is commonly known in literature that aldehydes and hydroxylamine spontaneously condensate to the corresponding aldoxime under the elimination of water. This process is nearly solvent-independent. However, the solubility of the reagent could play an important role in terms of conversion and isolation. Hydroxylamine is highly hazardous in its free form, with reports stating explosions at around 70 °C. Even more, aqueous solutions were reported to explode at 100 °C. Since hydroxylamine salts are readily available and easier to handle, the reagent was formed *in situ* with an adequate base. In view of an environmentally benign process the reaction was first tested in pure water with hydroxylamine salts like chloride and phosphate. Inorganic bases like sodium acetate, sodium hydroxide or sodium carbonate were used due to their high solubility in water. However, sodium hydroxide was ruled out for the possibility of a CANNIZZARO-type side-reaction and the disproportionation of starting material. Sodium acetate reacted as desired, but the byproduct acetic acid needed to be removed *via* extensive washing or in high vacuum. The most convenient base was in fact sodium carbonate, leading to the evolution of gaseous carbon dioxide and thus facilitating the isolation process. In this case, the corresponding aldoxime was extracted from an aqueous sodium salt solution of the hydroxylamine salt counter ion.



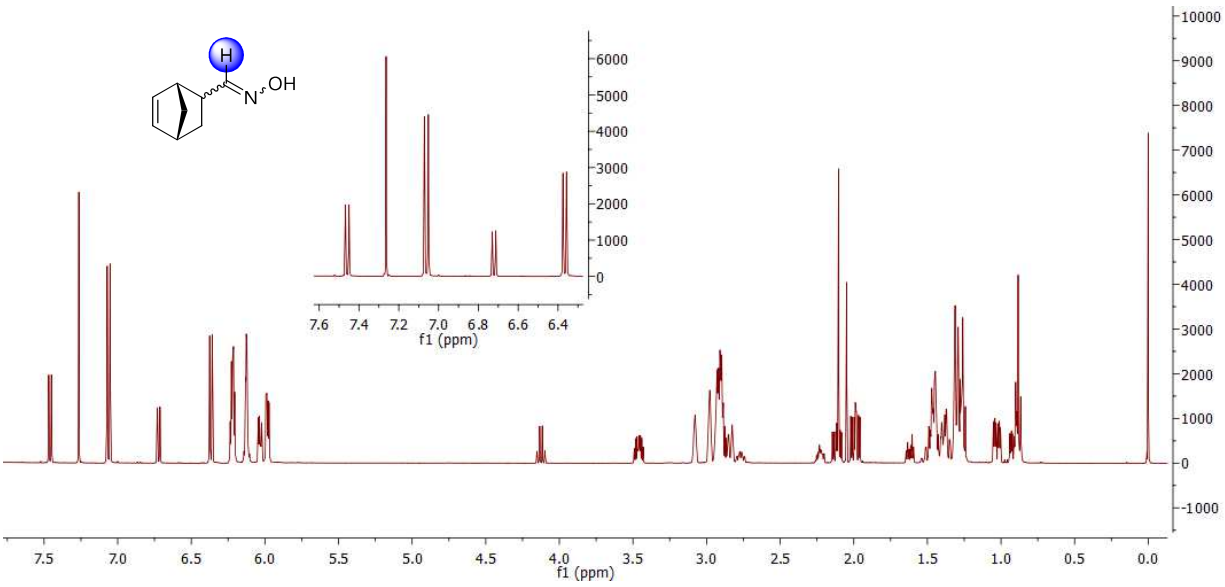
rac-**132a-e** *E-rac*-**133a-e** *Z-rac*-**133a-e**

Table 18. Substrate synthesis of racemic aldoximes by condensation of racemic aldehydes with hydroxyl-amine.

(a) Determined by integration of ^{1}H-NMR spectra (b) Purification *via* column chromatography or Kugelrohr distillation (c) NMR analysis showed a diastereomeric excess of 40% de for the α-stereogenic center.

Entry	**132**	R^1	R^2	Conversion [%]	E/Z[a]	Isol. Yield[b] [g(%)]
1	**a**	Ph	Me	>95	72:28	8.46 (94.5)
2	**b**	1-Cyclohex-3-ene		>95	65:35	3.63 (96.8)
3	**c**	2-Norborn-5-ene		>95	55:45[c]	2.40 (44.1)
4	**d**	3-Tetrahydrofuran		>95	50:50	3.93 (75.9)
5	**e**	2-Ph-Pr	H	>95	50:50	3.03 (82.5)

Interestingly, all aldehydes showed nearly quantitative conversion despite their overall poor solubility in the reaction medium water. In addition, the low solubility of the aldoximes was useful for an extractive isolation of the crude product. Except for substrate *E/Z*-**133d**, all aldoximes were purified by column chromatography on silica with different eluent mixtures of *n*-hexane[i] and ethyl acetate. *E/Z*-Tetrahydrofuran-2-carbaldoxime (*E/Z*-**133d**) was purified by Kugelrohr distillation. This methodology was also applicable for other substrates, but the heating of the compounds led to almost 1:1 mixtures of *E*- and *Z*-aldoxime, aggravating the establishment of analytical methods later on. Notably, the bicyclic substrate **133c** showed a diastereomeric excess of 40% de



for the α-stereogenic center. This can be seen in its proton NMR spectrum since the additional two stereogenic centers of the bridged rings lead to four distinctive diastereomers (**Figure 87**).

This diastereomeric excess is based on the production of the starting material **132c**. These norbornene derivatives are prepared *via* DIELS-ALDER reaction (see **Figure 88**) between cyclopentadiene (**132**) and a MICHAEL acceptor of type **134**. DIELS-ALDER reactions are *endo*-selective by definition, which would give the corresponding (*2R*)-diastereomer of **135** in high excess. Detailed analysis of ^{1}H-NMR and two-dimensional correlation spectra (^{1}H-COSY) were used for the determination of the chemical shifts of all diastereomers (see **Figure 89**, page 86). Unfortunately, with two different bridgeheads of the bicyclus,

[i] The use of toxic *n*-hexane was due to the solvent stock of the host institution. The separation should be reproducible with less hazardous cyclohexane/ethyl acetate mixtures.

regioisomers of the 2-carbonyl moiety cannot be discriminated in NMR spectroscopy but will most likely be discriminated by the biocatalyst.

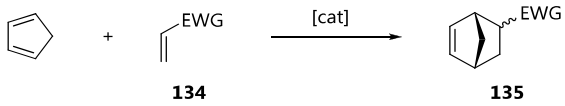


Figure 88. DIELS-ALDER reaction of cyclopentadiene and α,β-unsaturated carbonyl compounds leading to diastereomerically enriched *endo*-products.

Hence, the separation of eight isomers would have been necessary to analyze the biotransformation. In the present study, this separation could not be realized. Therefore, the following results obtained during the biotransformation of 5-norbornene-2-carbaldoxime (**133c**) could not be interpreted conclusively.

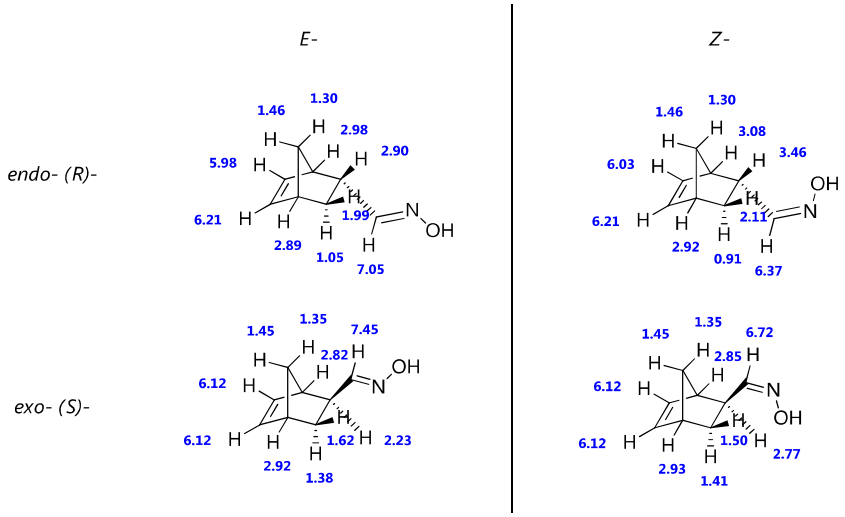


Figure 89. Identification of all ^{1}H-NMR shifts for the separated diastereomers by ^{1}H-COSY spectra. Enantiomers of the aldoxime moiety, thus additional four isomers, cannot be discriminated by proton NMR spectroscopy.

3.3.2 RACEMIC NITRILES BY PHOSPHINE OXIDE-CATALYZED DEHYDRATION

The reference compounds, the corresponding racemic nitriles, were synthesized according to a literature protocol from DENTON *et al.* for the triphenylphosphine oxide (**135**)-catalyzed dehydration of aldoximes (see **Figure 90**, page 87).[69] There are actually a couple of methods known in literature for the catalytic dehydration of aldoximes; however most of them are unselective. They are based on the substitution of the aldoxime hydroxy group by chloride and subsequent elimination of hydrochloric acid. This process also shows the limitation for these reactions, since basically all alcohols in the

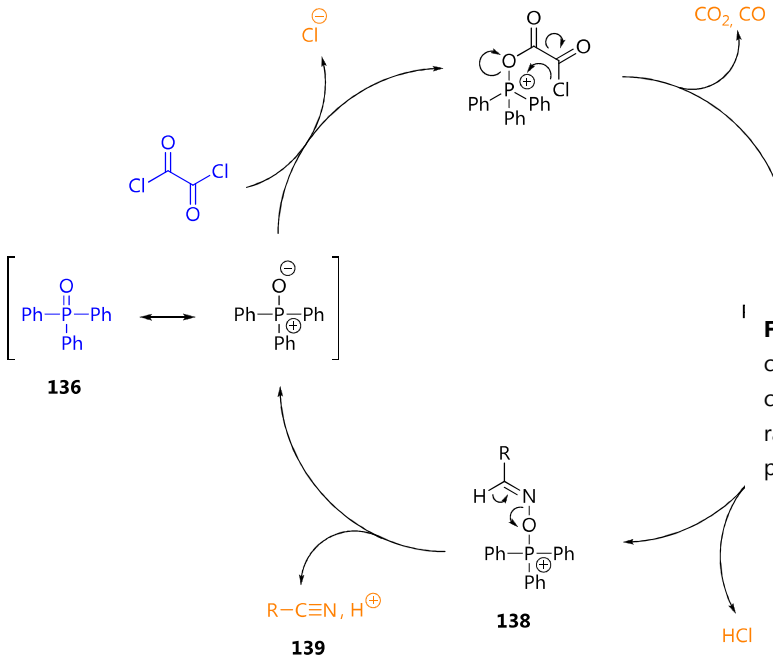
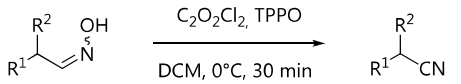


Figure 90. Phosphine oxide-catalyzed dehydration process for the synthesis of racemic reference compounds.

starting material will be converted as well. Additionally, nucleophilic positions have to be masked by protecting groups. There is not one enantioselective dehydration process known in literature, which could generate enantiomerically pure nitriles from racemic aldoximes.



E/Z-rac-**133a-e** *rac*-**139a-e**

Entry	**133**	R^1	R^2	Conversion[a] [%]	Isol. Yield[b] [g(%)]
1	**a**	Ph	Me	>95.0	0.20 (30.5)
2	**b**	1-Cyclohex-3-ene		>95.0	0.14 (58.3)
3	**c**	2-Norborn-5-ene		>95.0	0.32 (73.6)
4	**d**	3-Tetrahydrofuran		>95.0	0.36 (74.1)
5	**e**	2-Ph-Pr	H	>95.0	0.42 (94.5)

Table 19. Reference compounds by phosphine-oxide catalyzed dehydration of aldoximes.

(a) Determined by integration of ^{1}H-NMR spectra (b) Purification *via* column chromatography or Kugelrohr distillation.

The reactive species in the given protocol is most likely an intermediary chlorotriphenylphosphonium ion **137**, which is substituted by the aldoxime **133** to generate the unstable aldoximophosphonium ion **138**, which produces the desired nitrile of type **139** under elimination of triphenylphosphine oxide (**135**) and a proton. The overall reaction is impressively sophisticated, with three gaseous byproducts (CO, CO_2, HCl) as the driving force. All aldoximes were converted quantitatively and purified *via* column chromatography on silica or Kugelrohr distillation, respectively. As they were only used as analytical reference compounds, the isolated yield was not considered an issue and cannot be used for comparison with the yield of the biocatalytic transformation. **Table 16** (page 87) shows the results of the unselective dehydration of the investigated chiral aldoximes.

3.4 Analytical methods for conversion and selectivity

The establishment of analytical methods for the determination of both enzyme activity and enantioselectivity is always the initial key for investigating biocatalysts. Depending on the respective substrate or product structures, this can be achieved either *in situ* or after extractive work-up. As usual, the main advantage of *in situ* analysis is the higher sample throughput and the unchanged mixture content. The literature protocol for the standard substrate is based on quantitative product determination in the aqueous reaction solution by reversed-phase HPLC (acetonitrile/water mixtures as eluent). The enantiomeric excess was subsequently determined by extraction of the products into an organic solvent, i.e. methyl *tert*-butylether (MTBE), and analysis thereof on a normal-phase chiral HPLC column (*n*-hexane/2-propanol mixtures as eluent).

3.4.1 E/Z-2-Phenylpropionaldoxime / 2-Phenylpropionitrile

The closest phenylacetaldoxime (PAOx) – related substrate also had the benefit of bearing an aromatic ring, which facilitated the HPLC analysis by UV absorption. The product-related conversion could therefore be determined *in situ* by separating the reaction mixture on a reversed-phase column with an eluent of 30% acetonitrile in water (see **Figure 91**). Furthermore, after verification with reference mixtures, by-products were identified, *i.e.* hydrolysis

to the corresponding aldehyde and formation of its hydrate. The equilibrium between those impurities impeded the quantitative analysis of the hydrolytic side-reaction.

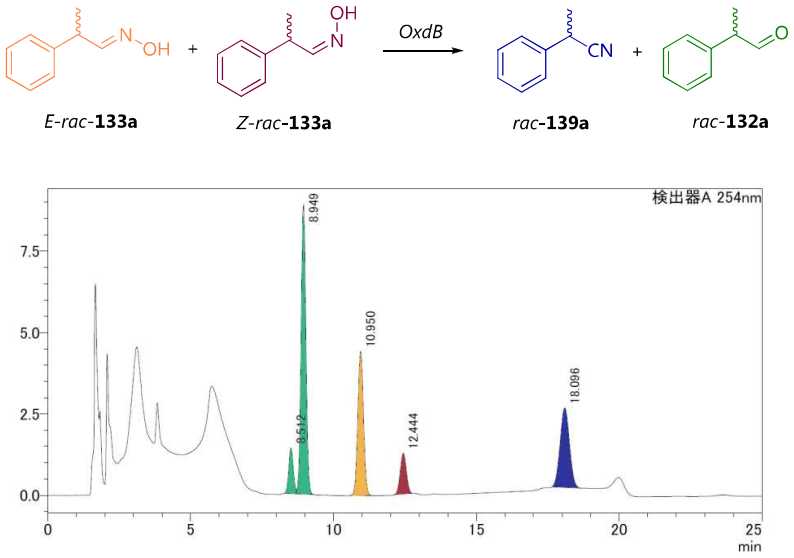


Figure 91. Reversed-phase HPLC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of 2-phenylpropionitrile (**139a**).

Peaks: 2-phenylpropionaldehyde and hydrate (green), *E*-2-phenylpropionaldoxime (orange), *Z*-2-phenylpropionaldoxime (red), 2-phenylpropionitrile (blue).

The separation of all isomers of substrate and product on a Chiralcel OJ-H column was beneficial for the kinetic analysis within *E/Z*-mixtures. In addition, after separating the enriched product from non-quantitative biotransformations, the absolute configurations of all isomers were successfully determined by comparison of the optical rotary power with a literature reference for (*R*)-**139a** (see **Table 20**).[70]

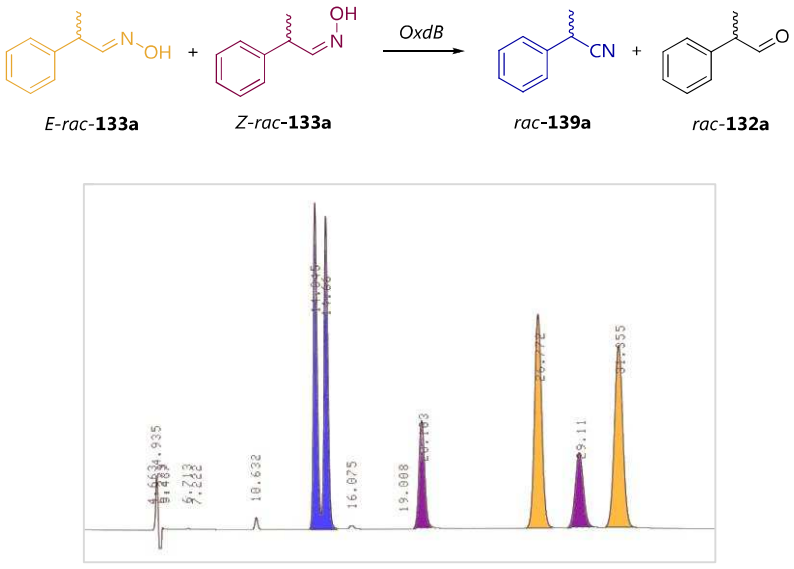
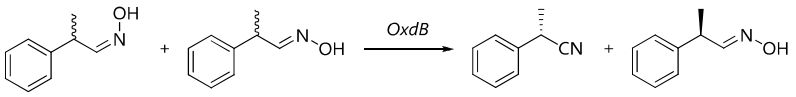


Figure 92. HPLC analysis of the extracted product mixture of the aldoxime dehydratase-catalyzed synthesis of 2-phenylpropionitrile (**139a**).

Peaks: 2-phenylpropionitrile (blue), E-2-phenylpropionaldoxime (orange), Z-2-phenylpropionaldoxime (purple).

Table 20. Identification of isolated products[a] and remaining substrates by measurement of the optical rotary power.

(a) Lit. value for *(R)*-2-phenyl-propionitrile $[a]^{D}_{20}$ = +9.5 (c 2.4, $CHCl_3$) (b) Determined after column chromatography on silica (c) Determined from ^{1}H-NMR samples.



Z-rac-**133a** *E-rac*-**133a** *(S)*-**139a** *E-(R)*-**132a**

5 mM substrate concentration
1 mmol *E/Z*-**133**

Entry	Isolated compound	Isolated yield[b] [mg (%)]	*ee* [%]	$[\alpha]^{D}_{23}$ (c, $CDCl_3$)[c]
1	(*S*)-**139a**	68.4 (52.1)	65.2	- 4.40 (2.25)
2	*E*-(*R*)-**133a**	61.6 (41.2)	>99.8	- 7.57 (1.32)
3	(*R*)-**139a**	34.2 (13.0)	67.3	+ 5.57 (0.85)

Since the distribution coefficients for the extraction of the product mixture were not equal for all compounds, the conversion was not determined on chiral normal-phase HPLC. The enantiomeric excess for each component could be determined since enantiomers do not differ in their biphasic distribution.

3.4.2 E/Z-Cyclohex-3-ene-1-carbaldoxime / Cyclohex-3-ene-1-carbonitrile

Stereogenic centers in cyclic substrates can be quite interesting when it comes to ring inversion processes depending on the substitution. For cyclohexene derivatives, different substituent effects were studied by Lambert *et al.*.[71] As a rule of thumb, electron-withdrawing groups (EWG) enhance ring inversion by stabilizing the planar intermediate. For the present study on enantioselective aldoxime dehydration this can be rather challenging since substrate and product both have an EWG. In the case of cyclohex-3-ene-1-carbonyl derivatives, this could lead to epimerization of the product, independent of an enantiopreference of the aldoxime dehydratase. The conversion was successfully quantified by reversed-phase HPLC analysis of the reaction mixture (see **Figure 93**, page 91).

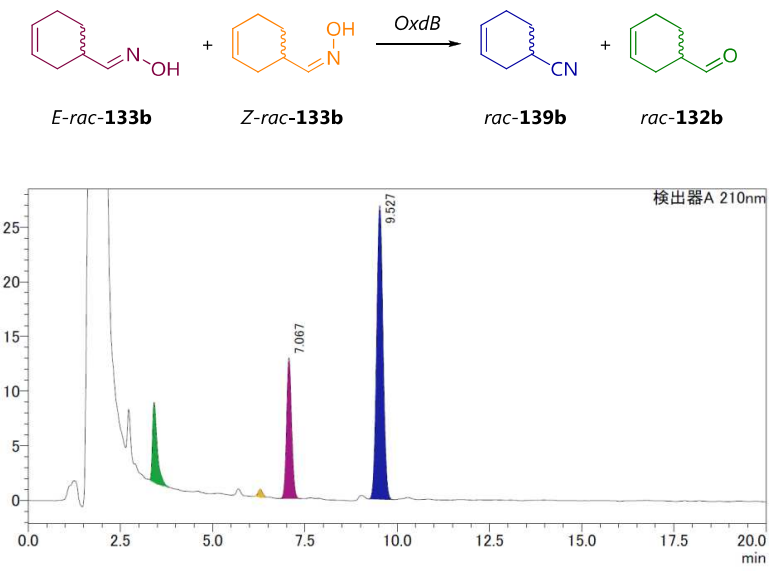


Figure 93. HPLC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of 3-cyclohexene-1-carbonitrile (**139b**).

Peaks: 3-cyclohexene-1-carbaldehyde (green), *Z*-3-cyclohexene-1-carbaldoxime (orange), *E*-3-cyclohexene-1-carbaldoxime (purple), 3-cyclohexene-1-carbonitrile (blue).

Unfortunately, the enantiomeric excess could not be determined by UV detection on a chiral HPLC. This missing link was closed by a chiral GC column and flame-ionization detection (FID). While the product could be readily separated into both enantiomers, the chiral aldoximes could not be separated (see **Figure 94**). In addition, due to the lack of literature values for the optical rotary power of the components, the major enantiomer of the product could not be identified yet.

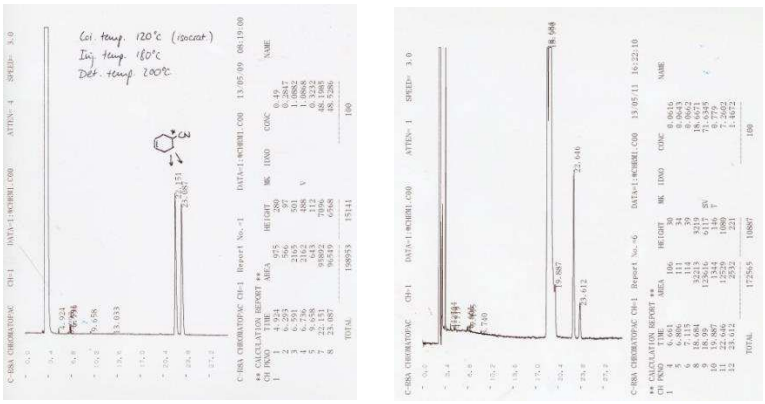


Figure 94. GC analysis of the product mixture of the aldoxime dehydratase-catalyzed synthesis of 3-cyclohexene-1-carbonitrile.

Spectra: Reference mixture of rac-3-cyclohexene-1-carbonitrile (left) and biotransformation with *OxdB* (right).

3.4.3 *E/Z*-5-NORBORNENE-2-CARBALDOXIME / 5-NORBORNENE-2-CARBONITRILE

The previously discussed ring inversion can be suppressed by further substituents. In addition, bridging of the aliphatic ring system can also be useful. The restriction of conformational interchange and increase of structure complexity was initially chosen for a better understanding of the enzyme kinetics.

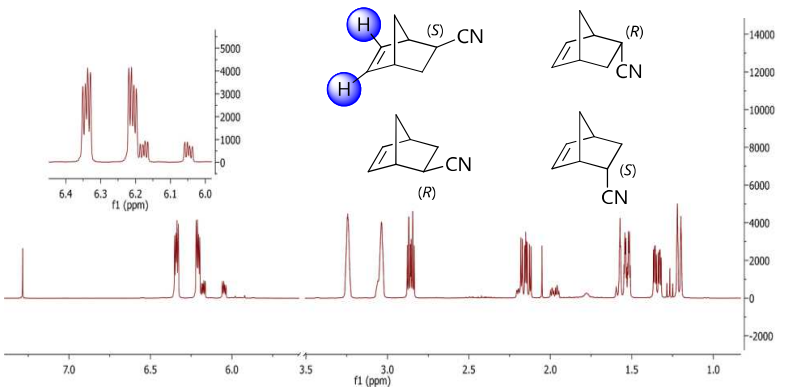


Figure 95. NMR analysis of the racemic mixture of 5-norbornene-2-carbonitrile (**139c**) with two distinguishable diastereomers and two regioisomers, presumably in 1:1 ratio.

Nevertheless, the different bridgeheads of this bicyclic substrate led to the formation of eight substrate isomers, a complex mixture that could not be fully separated within this project.

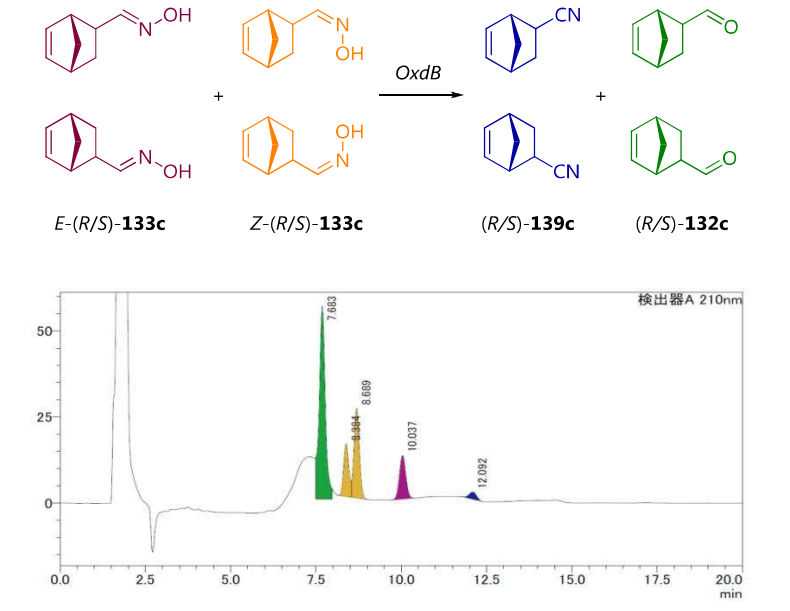


Figure 96. HPLC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of 5-norbornene-2-carbonitrile (**139c**).

Peaks: 5-norbornene-2-carbaldehyde (green), *Z*-5-norbornene-2-carbaldoxime and diastereomer (orange), *E*-5-norbornene-2-carbaldoxime and diastereomer (purple), 5-norbornene-2-carbonitrile (blue).

The NMR spectrum of the TPPO-catalyzed dehydration reaction confirms the enrichment of *endo*-components in about 5:1 ratio (see **Figure 95**, page 92). Unfortunately, the regioisomers of these compounds, cannot be regarded as equal for *OxdB* binding. The four stereoisomers could not be separated by any available method during this study. HPLC analysis of the reaction mixture on a reversed-phase HPLC column demonstrated the need for a different assay to determine conversion and enantiopreference of aldoxime dehydratases for these bicyclic structures (see **Figure 96**, page 92).

3.4.4 *E/Z*-Tetrahydrofuran-3-carbaldoxime / Tetrahydrofuran-3-carbonitrile

In view of the chemoenzymatic synthesis of pyrrolidine derivatives for the production of dipeptidyl-peptidase inhibitors like Vildagliptin, a heterocyclic substrate was also investigated.[72] In this work, tetrahydrofuran-3-carboxaldehyde **132** was used as a commercially available substitute. This substrate showed an enhanced solubility in aqueous reaction media and was also the sole compound which did not inhibit growth of the recombinant *E. coli* cells on an agar plate. The purification of substrate and reference compound was established by Kugelrohr distillation, thus leading to nearly 1:1 mixtures of *E/Z*-aldoxime **133d**, aggravating the differentiation by UV absorption analysis.

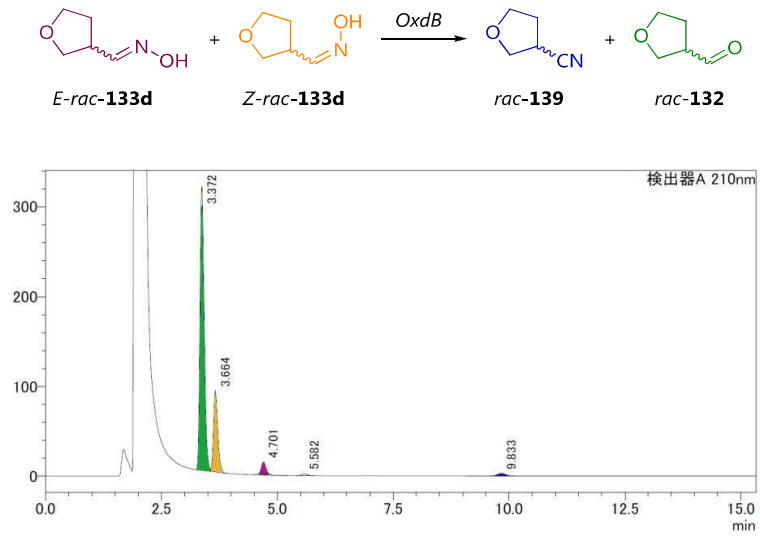


Figure 97. HPLC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of tetrahydrofuran-3-carbonitrile (**139d**).

Peaks: Tetrahydrofuran-3-carbaldehyde (green), *Z*-tetrahydrofuran-3-carbaldoxime (orange), *E*-tetrahydrofuran-3-carbaldoxime (purple), tetrahydrofuran-3-carbonitrile (blue).

However, based on the fact that *Z*-aldoximes have a higher UV extinction coefficient than *E*-aldoximes, the HPLC analysis of a 1:1 mixture could be used for the assignment of *E*- and *Z*-conformation (see **Figure 97**, page 93).

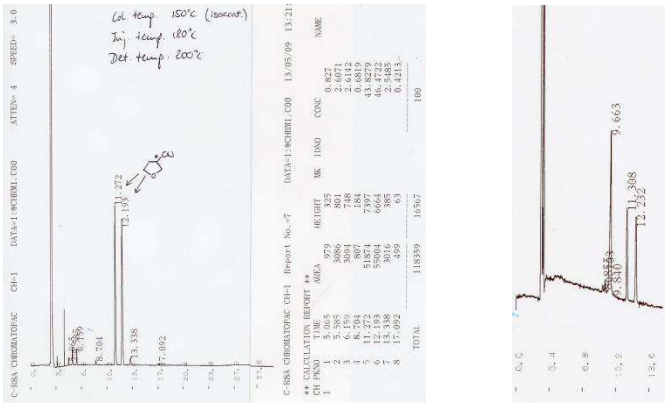


Figure 98. GC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of tetrahydrofuran-3-carbonitrile (**139d**).

Spectra: Reference mixture of *rac*-tetrahydrofuran-3-carbonitrile (left) and biotransformation with *OxdB* (right).

Also in this case, HPLC analysis by UV detection was troublesome since nitriles have nearly no UV absorption. Again the solution of this analytical problem was found in chiral GC analysis and FID spectra (see **Figure 98**).

3.4.5 *E/Z*-3-PHENYLBUTYRALDOXIME / 3-PHENYLBUTYRONITRILE

This β-chiral substrate had again an aromatic moiety and was therefore readily observed by UV absorption on both reversed-phase and normal-phase HPLC.

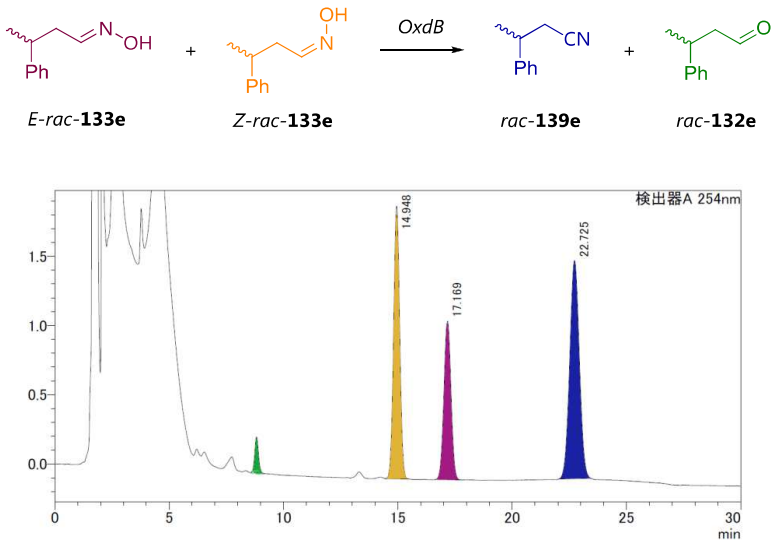
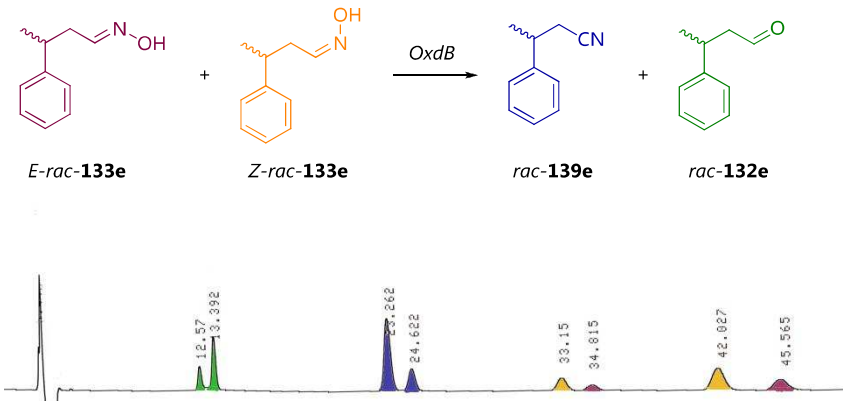


Figure 99. Reversed-phase HPLC analysis of the reaction mixture of the aldoxime dehydratase-catalyzed synthesis of 3-phenylbutyronitrile (**139e**).

Peaks: 3-Phenybutyraldehyde (green), *Z*-3-phenylbutyraldoxime (orange), *E*-3-phenylbutyraldoxime (purple), 3-phenylbutyronitrile (blue).

Interestingly, the aldoxime formation led in all cases to 1:1 mixtures of *E*- and *Z*-isomers. In combination with the higher molar extinction of the *Z*-isomer, this was again beneficial for the assignment of isomers by UV absorption (see **Figure 99**).



E-*rac*-**133e** *Z*-*rac*-**133e** *rac*-**139e** *rac*-**132e**

Figure 100. Normal-phase HPLC analysis of the extracted product for the determination of the enantiomeric excess of 3-phenylbutyronitrile (**139e**).

Peaks: 3-Phenylbutyraldehyde (green) 3-phenylbutyronitrile (blue), *Z*-3-phenylbutyraldoxime (orange), *E*-3-phenylbutyraldoxime (purple).

The separation of enantiomers was also successfully achieved in this study (see **Figure 100**). Unfortunately, no literature data for the identification of the preferred enantiomers was available. The enantiopreference will therefore be given as the *major*-enantiomer. This substrate is also of interest from another point of view. Since the stereogenic center is not enolizable, a possible epimerization of the product for α-chiral nitriles can be ruled out and the enantiomeric excess should be directly related to the biocatalyst.

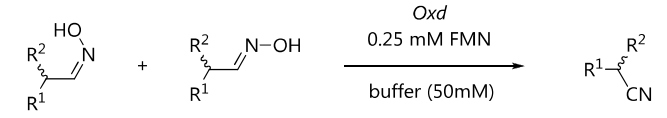
3.5 CASE STUDIES REVEALING A CONFORMATION-DEPENDENT SELECTIVITY

After the establishment of analytical methods for the product-related conversion of the aldoxime dehydratase-catalyzed synthesis of chiral nitriles, the compounds were tested for their specific activity in relation to the achiral standard substrate *Z*-phenylacetaldoxime. For these initial preparative screening experiments, *E/Z*-mixtures were examined by the literature protocol of KATO *et al.*[58] Surprisingly, the allegedly inert substrate *E/Z*-2-phenylpropionaldoxime (**133a**) was infact highly active for the aldoxime dehydratase from *Bacillus* sp. OxB-1 (see **Table 21**, page 96, entry 1). With around 3,200 U/L of culture, the relative activity was about 23 % at 30 °C, with full conversion determined by quantitative HPLC analysis. The substrate was also tested for another aldoxime dehydratase from *Rhodococcus* sp., which was

reported to be active yet unselective for this compound.[63] The specific activity was also high in this case, but even lower than for the *OxdB* biotransformation (entry 2). To reduce the enzymatic activity and investigate a possible enantiopreference of the enzyme, the screening was also done at a lower temperature of 8 °C. The activity decreased to about 1/18 of its initial activity at 30 °C (entry 4).

Table 21. Microscale screening for active biocatalysts at elevated and lower temperatures

(a) *OxdB* = HB101 / pOxD-9OF, *OxdRG* = BL21 Star DE3 / pOxdS17 (b) Determined by quantitative HPLC measurement after 60 seconds reaction time (c) Units per mg of acetone-dried cells, not purified protein.



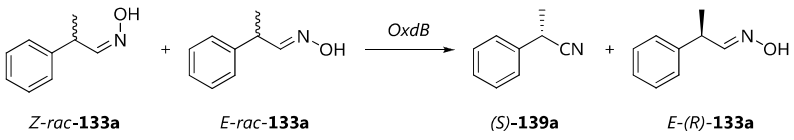
E-rac-**133a-e** *Z-rac*-**133a-e** (*R/S*)-**139a-e**

Entry	133	*Oxd*[a]	Temp. [°C]	Total activity[b] [U/$L_{culture}$]	Whole-cell activity[c] [U/mg]
1	**a**	*OxdB*	30	3,190	-
2	**a**	*OxdRG*	30	2,010	-
3	**a**	*OxdB*	30	-	0.13
4	**a**	*OxdB*	8	170	-
5	**b**	*OxdB*	8	30	-
6	**c**	*OxdB*	8	81	-
7	**d**	*OxdB*	8	n.d.	-
8	**e**	*OxdB*	8	205	-

In addition, the other chiral substrates were also tested at this reaction temperature and showed significantly lower activites, nevertheless sufficient for preparative experiments. The only compound which could not be analyzed for conversion was tetrahydrofuran-3-carbaldoxime (**133d**), which was therefore not investigated in more detail. Notably, the more complex structure of 5-norbornene-2-carbaldoxime (**133c**) showed a higher activity than its unbridged counterpart **133b** (entries 5 and 6). This indicates conformational changes during the binding of 3-cyclohexene-1-carbaldoxime (**133b**), slowing down the dehydration process.

3.5.1 BIOCATALYTIC SYNTHESIS OF ENANTIOMERICALLY ENRICHED NITRILES BY THE USE OF ALDOXIME DEHYDRATASES IN RECOMBINANT *E. COLI*

The screening experiments were promising for the preparative synthesis of the chiral nitriles. To get a more detailed insight into the time-course of the reaction and to clarify important parameters like the K_M value or a product inhibition, the biotransformations were scaled up from 0.5 mL to 5.0 mL., in some cases even to 50 mL. This allowed the analysis of more than one aliquot of the reaction mixture after certain timeframes. More importantly, by observing the enantiomeric excess of the formed nitrile over time, a fascinating r appeared. The enantiomeric excess of the product was shifting over the course of the reaction. This was first observed during a biotransformation of *E/Z*-2-phenylpropionaldoxime with a diastereomeric ratio of 4:1 (*E/Z*, see **Figure 101**). At a substrate concentration of 5 mM, which was the standard concentration in the screening experiments, an initially high ee value decreased during the first minutes of the biotransformation. Nevertheless, the ee value increased again after reaching about 20% conversion.



Z-rac-**133a** *E-rac*-**133a** *(S)*-**139a** *E-(R)*-**133a**

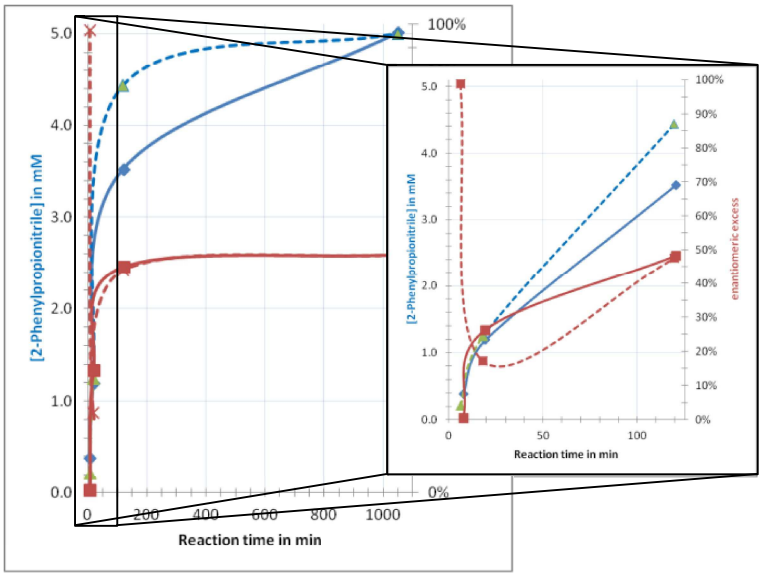
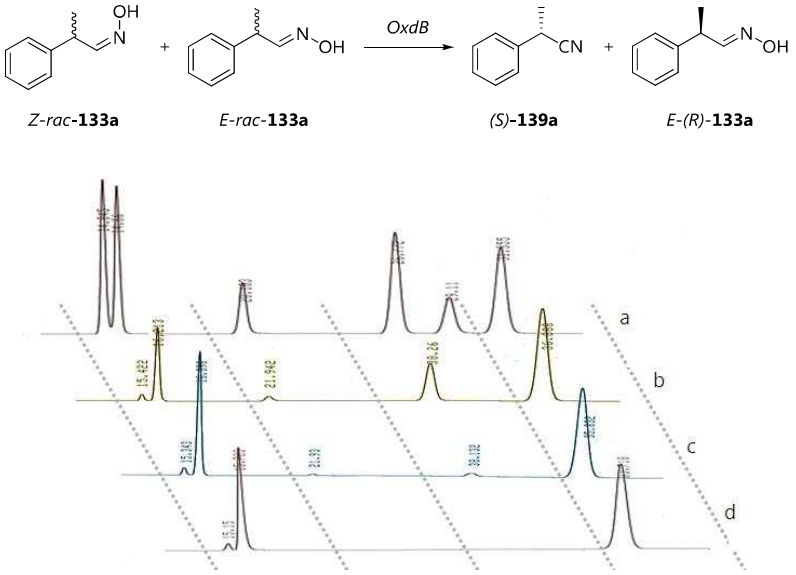


Figure 101. Time-course of the aldoxime dehydratase catalyzed synthesis of 2-phenylpropionitrile (**139a**).

By taking a closer look at the chiral HPLC spectra during the reaction progress, it was observed that both *Z*-enantiomers are converted, with kinetic differentiation, but in general, leading to a racemate at full conversion. In contrast to that, the *E*-aldoxime was only converted with a certain absolute configuration, which was later determined according to **Table 20** (page 90). The remaining enantiomer of the *E*-aldoxime was not converted, even after 24 hours in the presence of biocatalyst (see **Figure 102**). This was somehow surprising as the same experiment at an elevated temperature of 30 °C led to full conversion and a perfect racemate.

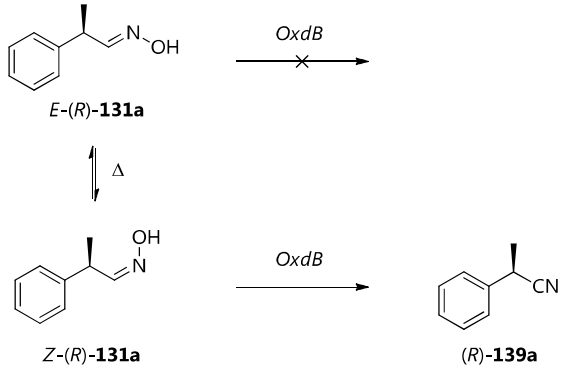


Z-*rac*-**133a** *E*-*rac*-**133a** *(S)*-**139a** *E*-*(R)*-**133a**

Figure 102. Chiral HPLC chromatograms from the time-course of the aldoxime dehydratase-catalyzed synthesis of 2-phenylpropionitrile (**139a**).

(a) Reference mixture of *rac*-2-phenylpropionitrile and *E/Z*-2-phenylpropionaldoxime (9:1) (b) Reaction mixture after 30 minutes (c) Reaction mixture after 2 hours (d) Reaction mixture after 24 hours

Under the given circumstances this could be explained by an isomerization process between *E*- and *Z*-aldoximes in a thermodynamically controlled equilibrium (see **Figure 103**). The corresponding *Z*-isomer would then be active for *Oxd* dehydration and thus lead to full conversion and a racemic product.



E-(*R*)-**131a**

Z-(*R*)-**131a** (*R*)-**139a**

Figure 103. Thermodynamically controlled isomerization of inert *E*-(R)-aldoxime to active *Z*-(R)-aldoxime and subsequent dehydration by *OxdB*.

Besides the enantioselectivity of the aldoxime dehydration, it was also investigated if the substrate concentration could be enhanced. Running the biotransformation at 5 mM aldoxime concentration, which corresponds to less than 1 g/L, would certainly be irrelevant for an industrial application. Therefore, the reaction was scaled up to 10 mM, 25 mM, 50 mM and 100 mM. The conversion rate decreased with increasing substrate concentration, but not proportional to the product concentration, suggesting a substrate inhibition above around 20 mM (see **Figure 104**).

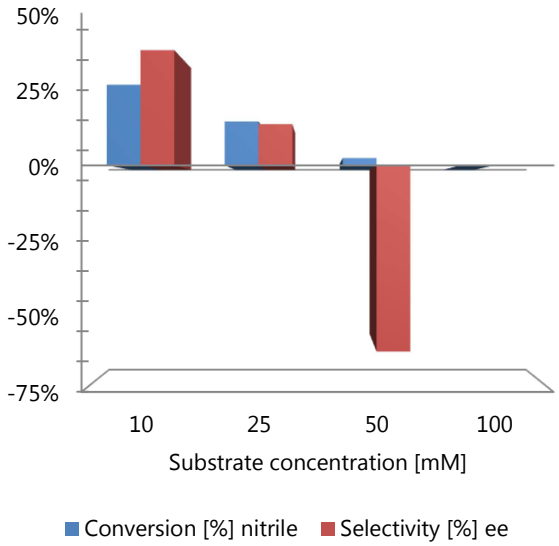


Figure 104. Scale-up of the biotransformation for the synthesis of 2-phenyl-propionitrile (**139a**).

Even more intriguing was the enantiomeric excess of the product obtained during the scale-up experiments. At low conversions, the absolute configuration of 2-phenylpropionitrile was inverted. The product was isolated by column chromatography and the opposite configuration was confirmed by measuring the optical rotary power (see Table 20). The inverted enantiopreference can be explained by the high substrate concentration of 50 mM. At this concentration, presumably all components of the mixture are above their Km or even double Km value, hence being converted in the highest possible reaction rates. Judging from the kinetic differentiation on the chiral HPLC spectra (see **Figure 102**, page 98), the *Z*-aldoxime is converted at first, with the *Z*-(*R*)-enantiomer being faster converted than its enantiomer. Due to the inhibition at around 2.4 % (see **Figure 104**, 50 mM) this led to an enhanced conversion of *Z*-(*R*)-aldoxime and thus the preferred synthesis of (*R*)-2-phenylpropionitrile.

3.5.2 ENANTIOSELECTIVITY CHANGE ACCORDING TO *E/Z*-CONFORMATION

Based on the preceding findings it was necessary to separate the aldoxime diastereomers and reproduce the experiments with isomerically pure compounds. The separation of *E*- and *Z*-aldoximes by column chromatography seemed manageable and was successfully achieved with the crude product of the aldoxime condensation. Two fractions were obtained with sufficient purity of *E*-*rac*-2-phenylpropionaldoxime (98% de) and *Z*-*rac*-2-phenyl-propionaldoxime (84% de). Better separation should be achievable by using a UV detector, which was not available during this study. The biotransformations with *OxdB* were reproduced and showed the same behavior as with the mixtures (see **Figure 105**). While *Z*-*rac*-**133a** was at first converted with a high enantioselectivity for the (*R*)-enantiomer, the (*S*)-enantiomer was subsequently transformed to obtain a nearly perfect racemate of **139a** at full conversion. Since 4% of inert *E*-(*R*)-aldoxime was present in the substrate, the reaction stopped at 96% conversion and with an enantiomeric excess of 4% ee for the (*S*)-nitrile. On the other hand, the enzymatic dehydration of *E*-*rac*-**133a** at 8 °C led to the predicted full conversion of *E*-(*S*)-**133a** to its corresponding (*S*)-nitrile **139a** and no conversion of *E*-(*R*)-**133a**. Due to the residual 1% of *Z*-aldoxime in the substrate, the product showed a lower, however still excellent enantiomeric excess of 98% ee for the (*S*)-nitrile.

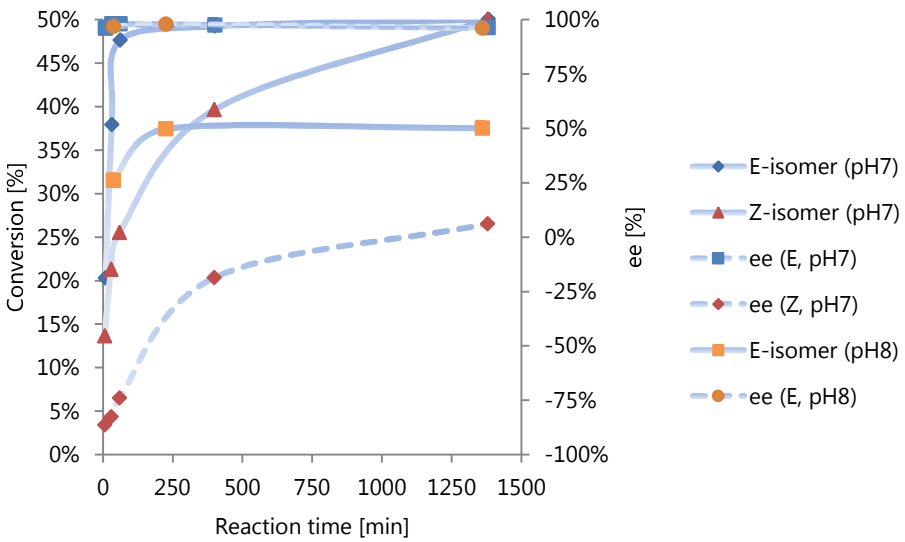
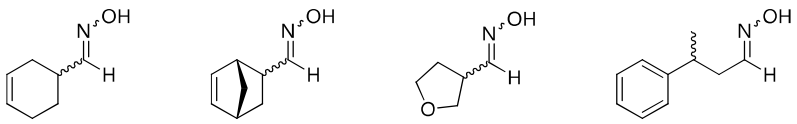


Figure 105. Biotransformation of isomerically enriched *E*- or *Z*-**131a** by *OxdB* at low temperature (8°C).

3.5.3 QUALITATIVE KINETICS FOR THE CONVERSION OF RACEMIC *E*- AND *Z*-ALDOXIMES

With the literature-unknown and now identified enantioselectivity of the aldoxime dehydratase from *Bacillus* sp. OxB-1 in recombinant *E. coli* for the conversion of *E*-2-phenylpropionaldoxime, the other chiral substrates **133b-e** were also investigated (see **Figure 106**). Since their enzymatic activity was already proven in the microscale experiments, the following-up biotransformations were focused on a change in enantioselectivity during the time-course of the reaction. This phenomenon can be used as a direct implication of diastereomeric differentiation. Due to the schedule of the research stay at the host institution, only qualitative results were obtained.



133b **133c** **133d** **133e**

Figure 106. Additional chiral substrates for the investigation of a non-linear behaviour of the enantiomeric excess.

E/Z-3-CYCLOHEXENE-1-CARBALDOXIME (**133B**)

As it was already mentioned for the initial experiments, the ring inversion of 3-cyclohexene derivatives could alter the enantiomeric excess of the remaining substrate and the corresponding nitrile. Apart from this, the non-linear behaviour of the enantiomeric excess during conversion observed for 2-phenylpropionaldoxime was also present in the biotransformations with *E/Z*-3-cyclohexene-1-carbaldoxime. Unfortunately, the *E/Z*-mixture could not easily be separated and the highest purity of *E*-**133b** achieved was an *E/Z* ratio of 89:11 after column chromatography. Nevertheless, even with using a substrate mixture of **133b** with an *E/Z* ratio of 2.2:1, non-linear behaviour was indeed observable (see **Figure 107**, page 102), with an initial high selectivity for one enantiomer. Even more intriguing were the final conversion of 76% and the final enantiomeric excess of 17% ee.

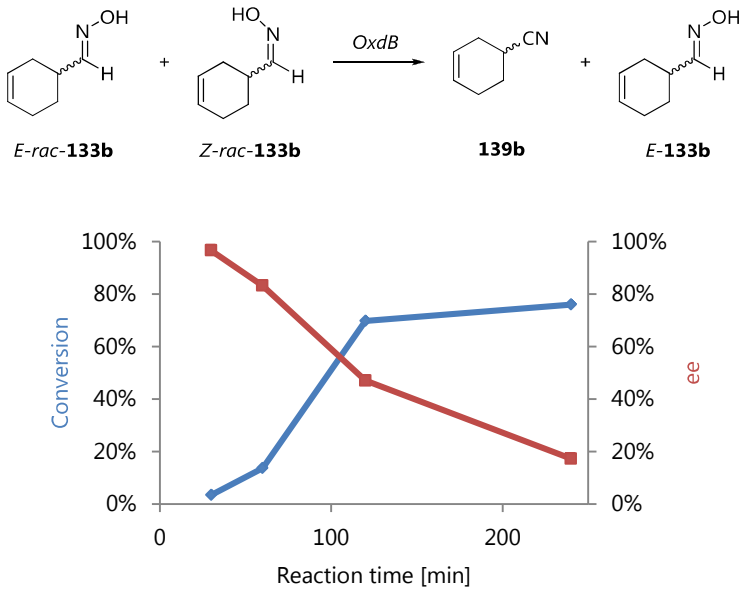


Figure 107. Biotransformation of isomerically enriched *E*-**133b** by *OxdB* at low temperature (8°C).

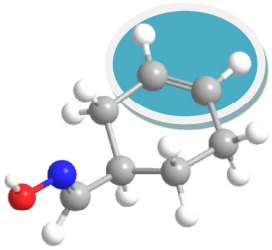


Figure 108. Chiral discrimination of *E/Z*-3-cyclohexene-1-carbaldoxime (**131b**) by only one unsaturated bond (emphasized).

The reaction did not proceed any further, which again suggests the existence of an inert aldoxime enantiomer. Due to the lack of literature references, the absolute configuration of the major enantiomer of **139b** could not be identified yet. This result is very surprising since the only differentiation of the biocatalyst is the unsaturated bond in the cyclohexene ring (see **Figure 108**). More studies on this substrate will be done in the future, as it would certainly have an impact on the synthesis of enantiomerically pure cyclic nitriles.

E/Z-5-NORBORNENE-2-CARBALDOXIME (**133c**)

Implementing a bridged 3-cyclohexene as substrate seemed favorable to prevent the decrease of the enantiomeric excess by ring inversion. However, during the analysis of the starting material it became clear that the mixture of a total of eight isomers would be extremely difficult. Since the isomers could not be separated on chiral HPLC, it was not distinguishable which isomers are preferably converted by the enzyme. What could be determined, however, was again a non-quantitative conversion of the substrate and a final enantiomeric excess of 66% ee, which was in the range of the enantiomeric excess of the starting material (see **Figure 109**, page 103). This leads to the conclusion that there are at least some isomers which are not converted by the biocatalyst, but their identities remain unclear up to now.

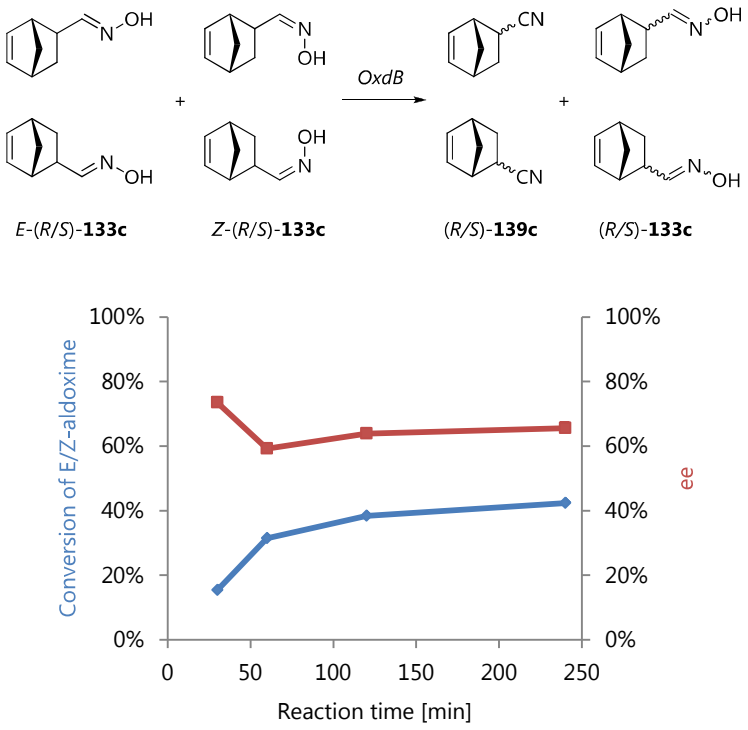


Figure 109. Biotransformation of isomerically enriched *E/Z*-**133c** by *OxdB* at low temperature (8°C).

E/Z-TETRAHYDROFURAN-3-CARBALDOXIME (**131D**)

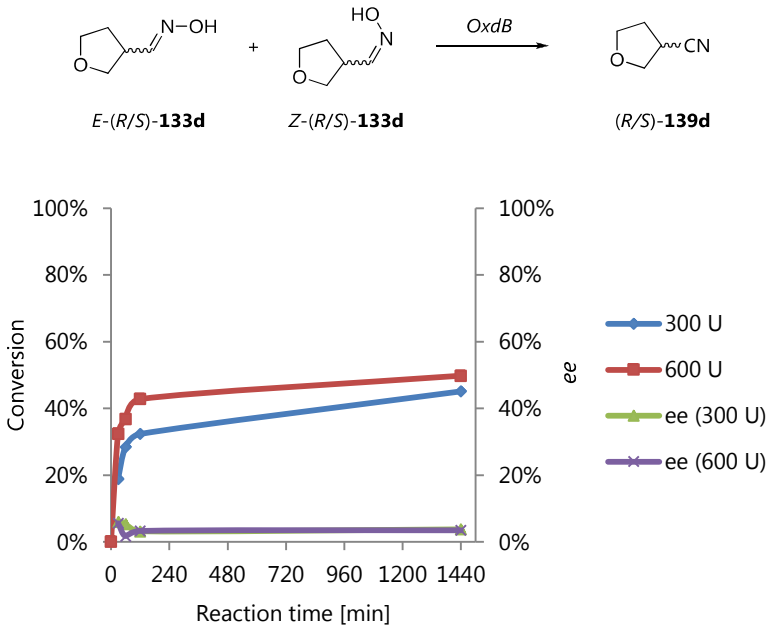
Since the aim of this project was also the synthesis of pharmaceutically relevant heterocyclic nitriles of type **140** (see **Figure 110**), substrate **133d** was of great interest to get insights into the biocatalytic transformation of heteroaromatic cyclopentane structures.

140 **133d**

Figure 110. Structural analogy of investigated substrate **133d** with pyrrolidine-type heterocyclic aldoximes.

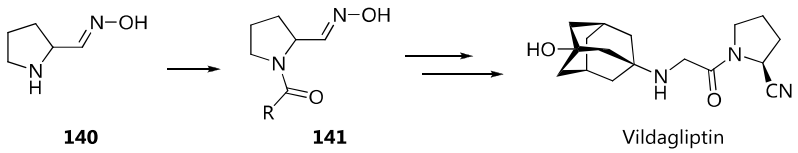
Unfortunately, the specific activity could only be determined by reversed-phase HPLC after reaching about 20% conversion at 5 mM due to the low molar extinction of the product **139d**. Nevertheless, the reaction was monitored for a change in enantioselectivity non-proportional to the conversion of **133d**. The aldoxime dehydratase from *Bacillus* sp. OxB-1 showed no enantioselectivity by GC analysis of the product mixtures. At any stage of the reaction, a racemate was obtained (see **Figure 111**, page 104).

Figure 111. Biotransformation of racemic *E/Z*-**133d** by *OxdB* at low temperature (8°C).



Since also this reaction stops at about 50 % conversion, it remains unclear if there is an inhibition by one of the stereoisomers or if one or more stereoisomers are again inert substrates. This issue will certainly be adressed in future experiments but could not be clarified in the present study. Since tetrahydrofuran is known to undergo conformational changes even at room temperature (signals with higher order in NMR spectra) this could also indicate that the product racemizes by ring-inversion after a possible enantioselective dehydration.

Figure 112. Alteration of pyrrolidine-type substrates in view of facilitated derivatization and enhanced discrimination by *Oxd*s.



The target molecules, bearing a pyrrolidine ring system, would of course have similar properties. An alternative methodology would be the use of acylated pyrrolidine derivatives of type **141** (see **Figure 112**, page 104) to increase the barrier for conformational changes and also enhance the enantiospecific discrimination by the aldoxime dehydratase.

E/Z-3-PHENYLBUTYRALDOXIME (**133E**)

The only β-chiral substrate investigated in the present study, namely 3-phenylbutyraldoxime (**133e**), should elucidate two more informations on the applicability of aldoxime dehydratases. On the one hand, β-chiral nitriles are not prone to epimerization and could therefore give indications on a potential enzyme-induced epimerization of the products after the dehydration. On the other hand, the selectivity of the dehydration could be enhanced by a chain-elongation, with the substituents being closer orientated to the protein backbone and therefore presumably bound with stronger stereospecific discrimination.

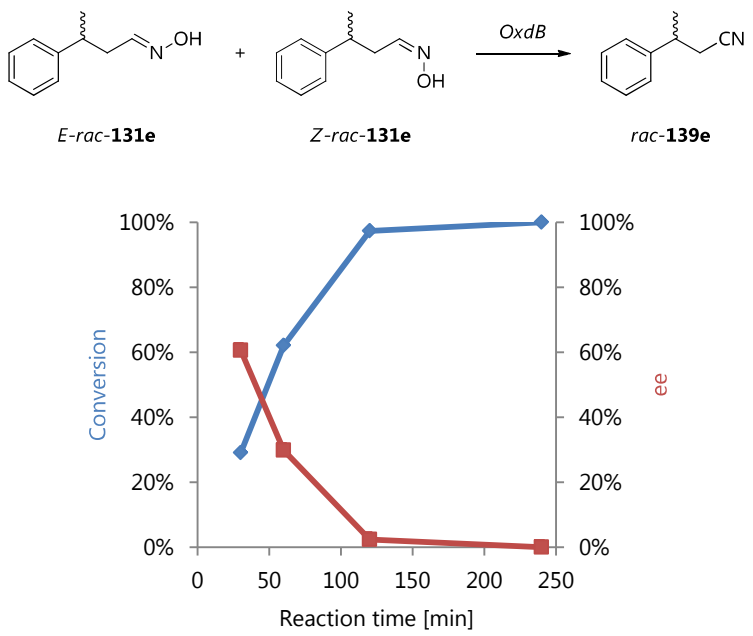
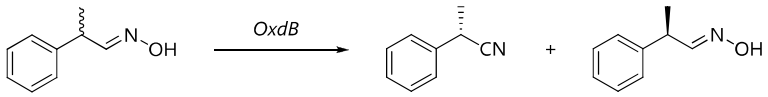


Figure 113. Biotransformation of racemic *E/Z*-**131e** by *OxdB* at low temperature (8°C).

Also with this substrate, the aldoxime dehydratase first produces the corresponding nitrile with a high enantiomeric excess (60 % ee at 30 % conversion, see **Figure 113**) which decreases steadily and in relation with the conversion until yielding a racemic product at full conversion. This indicates an enantioselectivity for one enantiomer but also the lack of an unreactive isomer, thus leading to a racemate in the end. It is yet promising that there is also a kinetically preferred enantiomer of this β-chiral compound and it should be worthy to manipulate the biocatalyst to further reduce or completely eliminate the activity for the enantiomer with the lower reaction rate. Notably, the β-chiral aldoxime was the only substrate which did not include an inert isomer.

The successful synthesis of enantiomerically pure (*S*)-2-phenylpropionitrile by the use of an aldoxime dehydratase was an encouraging result. This process is somehow limited by the need for isomerically pure *E*-aldoxime and reaching only 50 % conversion for the desired nitrile. While the specific synthesis of *E*-aldoximes remained unresolved during the present study, the racemization of the remaining *E*-(*R*)-aldoxime was examined. The most common way to epimerize α-chiral carbonyl compounds in aqueous media is by changing the pH towards an either more acidic or more basic value and thus initiating an enolization process. With regard of maintaining enzymatic activity throughout the epimerization, the reaction was reproduced at elevated pH values of 8, 9 and 10 (see **Table 22**). The reaction rates slowed down with increasing pH. Nevertheless, the reaction yielded around 50% of unreacted *E*-(*R*)-**133a**.



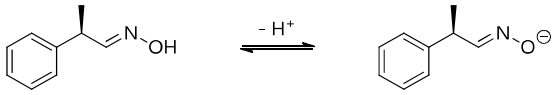
E-rac-**133a** *(S)*-**139a** *E-(R)*-**133a**

Table 22. pH-Dependence of the biocatalytic dehydration of *E-rac*-**133a** in aqueous reaction media.

(a) Significant hydrolysis of the substrate (b) No hydrolysis, only product-related conversion (c) HPLC analysis of the remaining aldoxime shows no racemization (up to 24 hours).

Entry	Buffer (50mM)	pH	Conversion [%]	ee [%]
1	NaOAc	5.0	<0.5[a]	n.d.
2	KPB	7.0	27.7[b]	98.1[c]
3	KPB	8.0	37.2[b]	97.9[c]
4	Ethanolamine	9.0	8.9	n.d.
5	Ethanolamine	10.0	7.3[a]	n.d.

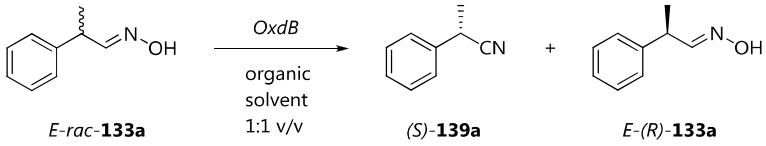
This inability to epimerize can be rationalized by the lower pK_a of the aldoxime proton in contrast to the α-proton. The deprotonated species **142** seems to be stable towards epimerization and also towards biotransformation, therefore slowing down the reaction rate (see **Figure 114**).



E-(R)-**131a** *E-(R)*-**142**

Figure 114. pH-Dependent deprotonation of the free aldoxime, counteracting a pH-induced epimerization of the stereogenic center.

From these results it seemed favorable to extend the methodologies towards biphasic reaction media, containing an aqueous solution for the biocatalytic dehydration and an organic solution for the *in situ* racemization of inert *E*-(*R*)-**133a** to *E*-(*S*)-**133a**. To get a general view on required reagents for the epimerization it was first determined which organic solvent can be combined with the enzymatic reaction. A number of typical water-immiscible solvents were examined in a biphasic biotransformation for their effect on the whole-cell catalyst.



E-rac-**133a** *(S)*-**139a** *E-(R)*-**133a**

Table 23. Compatibility of organic solvents for the biocatalytic dehydration of *E-rac*-**133a** in binary reaction media.

(a) Strong denaturation visible, no further conversion detected

Entry	Solvent	Conversion [%]	*ee* (**139a**) [%]	*ee* (*Z*-**131a**) [%]	*ee* (*E*-**131a**) [%]
1	MTBE	33.8	29.3 (*S*)	85.8 (*S*)	27.7 (*R*)
2	EtOAc	20.0	17.2 (*R*)	79.3 (*S*)	6.8 (*R*)
3	*n*-Hexane	62.5	60.5 (*S*)	-	>99.8 (*R*)
4	DCM[a]	21.6	50.4 (*R*)	97.0 (*S*)	6.70 (*R*)
5	*n*-BuOAc	20.4	44.8 (*S*)	30.6 (*R*)	29.4 (*R*)
6	$CHCl_3$[a]	7.40	56.3 (*R*)	5.50 (*S*)	0.50 (*R*)
7	THF	22.8	13.0 (*S*)	38.3 (*S*)	13.7 (*R*)
8	2-PrOH	33.3	39.6 (*S*)	0.20 (*R*)	37.2 (*R*)

The experiments clearly show an unchanged biotransformation for the binary mixture with *n*-hexane (**Table 23**, page 107, entry 3) with quantitative conversion of all active isomers. Other common solvents like MTBE, ethyl acetate and butyl acetate showed a decrease in conversion, with the product mixture showing enantiomerically enriched compounds (entries 1-2 and 5). Halogenated solvents like dichloromethane and chloroform led to a visible denaturation of the cells during the reaction (entries 4 and 6). Nevertheless, the biotransformation in the binary medium with dichloromethane showed almost equal conversion to ethyl acetate, suggesting denaturation of the whole-cells but not necessarily of the aldoxime dehydratase. THF and 2-propanol were also investigated, leading to a more or less homogenous reaction mixture (entries 7 and 8). Of all tested solvents, *n*-hexane was chosen as the most promising for

further development of a racemization process without influencing the whole-cell catalysis.

SOLUBILITY AND DISTRIBUTION IN BINARY REACTION MEDIA WITH N-HEXANE

By introducing an organic and immiscible solvent, the substrate concentration in the aqueous phase is subjected to change. Therefore, the solubility of **133a** and the corresponding nitrile **139a** had to be determined (see **Figure 115**).

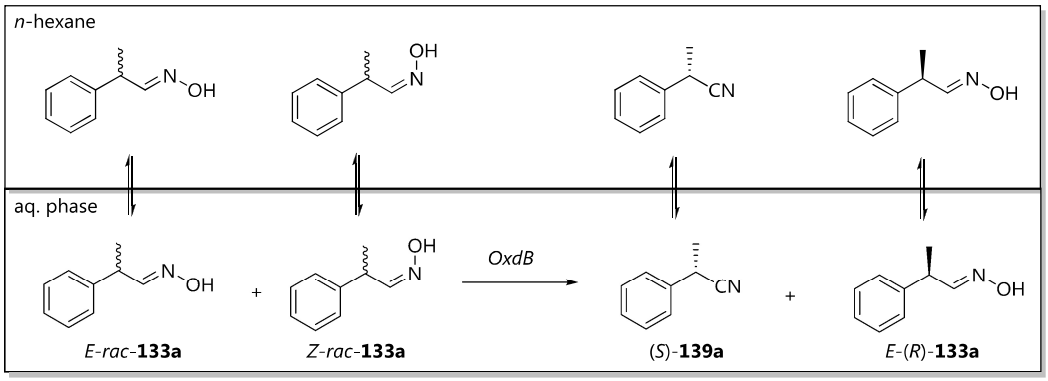
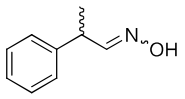


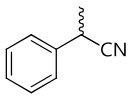
Figure 115. General scheme for the distribution of reagents and products in a binary reaction mixture.

Judging from the preceding experiments with increased substrate concentration it was favorable to reach around 5 – 10 mM for an unchanged biotransformation. Interestingly, by mixing an excess of substrate with the aqueous buffer medium of the reaction, a saturated substrate solution with a concentration of 20.8 ± 0.2 mM was obtained (see **Table 24**, entries 1-3). With this high solubility, the binary mixture had to be engineered not to exceed the limitation of 5 – 10 mM.

Table 24. Solubility of substrate of product in the buffered reaction medium without organic solvent.



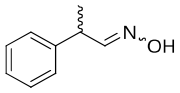
E/Z-rac-**133a**



rac-**139a**

Entry	Compound	Concentration [mM]	Solubility [g/L]
1	E/Z-**133a**	20.8 ± 0.1	
2	E/Z-**133a**	20.8 ± 0.3	3.1 ± 0.0
3	E/Z-**133a**	20.8 ± 0.2	
4	**139a**	13.7 ± 1.8	1.8 ± 0.2

Different concentrations of the substrate were prepared in pure *n*-hexane and mixed with an equal volume of the aqueous buffer.



E/Z-rac-**133a**

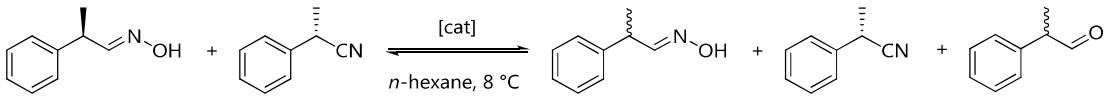
Table 25. Distribution of the substrate in 1:1 v/v binary mixtures with *n*-hexane.

Entry	Organic phase concentration *E/Z*-**133a** [mM]	Aqueous phase concentration *E/Z*-**133a** [mM]
1	10	1.45
2	20	2.49
3	30	3.23
4	40	3.81
5	50	4.13

After reaching the distribution equilibrium, the substrate concentration in the aqueous phase was quantified by reversed-phase HPLC. Due to its low polarity the substrate was significantly enriched in the organic phase and did not exceed 5 mM concentration even when using a 50 mM solution of *E/Z*-**133a** in *n*-hexane (see **Table 25**, entry 5). This was also very promising for increasing the product concentration of the process from 1 g/L (aqueous mixture) to around 7.5 g/L (binary mixture). These results were the basis for the following trials of an *in situ* racemization for a dynamic kinetic resolution of (*S*)-2-phenylpropionitrile ((*S*)-**139a**).

Table 26. Racemization tests of the product mixture of the biotransformation under Bronsted acid or Lewis base catalysis.

(a) 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]-octane (DABCO), N,N-dimethyl-4-aminopyridine (DMAP) (b) Hydrolysis to the corresponding aldehyde.



E-(*R*)-**133a** >99% ee | (*S*)-**139a** 78% ee | *E-rac*-**133a** | (*S*)-**139a** | *rac*-**132a**

Entry	Catalyst[a] (eq.)	Mode of action	E/Z	*E-(R)*-**133** [% ee]	(S)-**139** [% ee]	**132**[b] [%]
1	none	-	99:1	>99	78	-
2	PhCOOH (1.0)	Brønsted acid	71:29	>99	78	63
3	DBU (1.0)	Lewis base	99:1	>99	64	0.0
4	DABCO (1.0)	Lewis base	99:1	>99	n. d.	0.0
5	DMAP (0.3)	Lewis base	99:1	>99	n. d.	0.0

The adjustment of pH in organic media was achieved by the addition of organic acids and bases and their effect on enantiomerically pure substrate and product was examined by chiral HPLC analysis (see **Table 26**, page 109). Addition of a Brønsted acid, e.g. benzoic acid, the remaining enantiomerically pure *E*-(*R*)-aldoxime isomerized to its *Z*-diastereomer and also hydrolyzed to the corresponding aldehyde in about 63% conversion, presumably with residual water from either the crude product of the biotransformation or the reagent itself (entry 2). Notably, neither the enantiomeric excess of the remaining aldoxime nor of the present nitrile product changed in this acidic medium. In contrast to that, commonly used nitrogen-containing Lewis base catalysts like 1,4-diazabicyclo[2.2.2]-octane (DABCO) and N,N-dimethylaminopyridine (DMAP) did not exhibit any effect on the product mixture (entries 4 and 5). As opposed to this, 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) did affect the enantiomeric excess of the mixture, unfortunately, only regarding the enantiomeric excess of the nitrile (entry 3). While the remaining aldoxime remained untouched, the nitrile continuously epimerized towards its racemate. The reason for this behavior was not further investigated, but was supposedly related to the pK_a of the respective structures, as described for the pH-dependent racemization (see **Figure 114**, page 106). Since the problem seemed to be based on the free aldoxime, an additional step was considered for masking the free aldoxime *in situ*, racemizing this intermediate and unmask the free aldoxime again for the biotransformation.

3.7 EXPLORATION OF A LIPASE-COUPLED DYNAMIC KINETIC RESOLUTION PROCESS

GOTOR and MENENDEZ first achieved the oximolysis of different acetates by using lipases from *Pseudomonas* sp., *Candida cylindracea*, *Aspergillus niger*, *Humicola lanuginosa* and a porcine pancreatic lipase, showing different activities sufficient for preparative application (see **Figure 116**).[73]

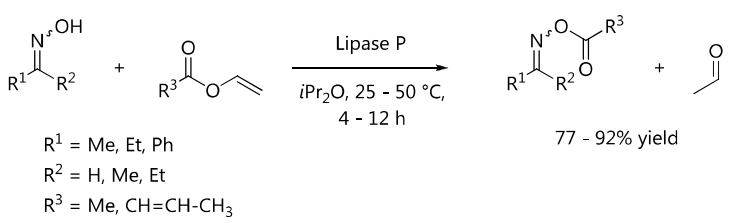


Figure 116. Lipase-catalyzed acylation of various ketoximes and benzaldoxime with vinyl carboxylates by GOTOR and MENENDEZ.[73]

Additionally, SALUNKHE and NAIR successfully acetylated different ketoximes of type **144** and also benzaldoxime with a lipase from *Pseudomonas cepacia*, immobilized on diatomite (Amano PS-D), and vinyl acetate (**145**) as highly active and irreversible acetyl donor (see **Figure 117**).[74]

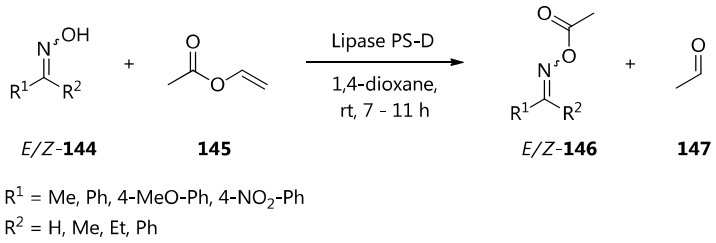


Figure 117. Lipase-catalyzed acetylation of various ketoximes and benzaldoxime with vinyl acetate by SALUNKHE and NAIR.[74]

These acetyl oximes of type **146** would mask the more acidic proton of the aldoxime, allowing the epimerization of the chiral aldoxime under base-catalysis. Masking of functional groups *in situ* is not trivial as the addition and the removal of the masking group should be coupled in an equilibrium to ensure reversibility (see **Figure 118**). In addition, the presence of an aqueous phase is complicating the procedure by the possibility of hydrolytic side-reactions.

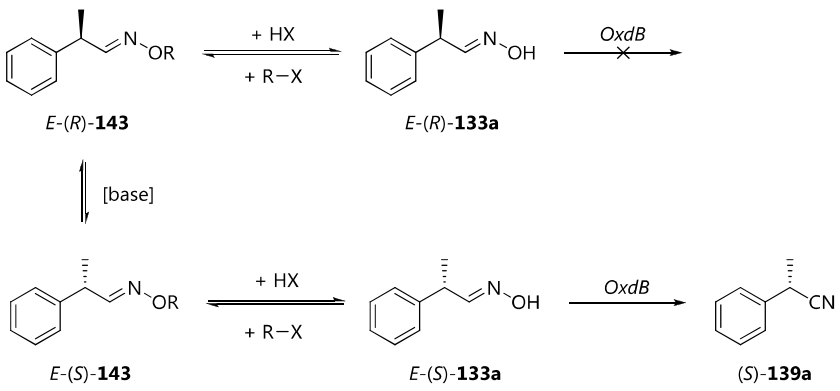


Figure 118. Proposed scheme of a reversible masking of free aldoximes and epimerization of their derivatives for a dynamic kinetic resolution.

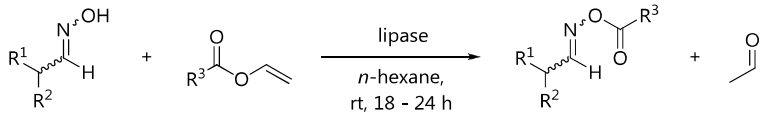
First of all, the right masking group had to be found. Since it should be able to withstand a binary mixture with water, chemical reactions were excluded. A second enzymatic reaction should be capable of working in a binary mixture and also have the necessary chemoselectivity to specifically react with free aldoxime. For this reason, the lipase-coupled acylation of the remaining aldoxime was investigated.

3.7.1 LIPASE-CATALYZED SYNTHESIS OF ALDOXIME ESTERS

In the present study, two of the chiral substrates were examined for this reaction, using the same lipase from *Pseudomonas cepacia* (Amano PS-D, immobilized on diatomite) or lipase B from *Candida antarctica* (CAL-B, immobilized on acrylic resin) and vinyl acylates (see **Table 27**).

Table 27. Lipase-catalyzed **a**cylation of chiral aldoximes with vinyl carboxylates in organic medium.

(a) Hydrolysis to the corresponding aldehyde (b) not detected.



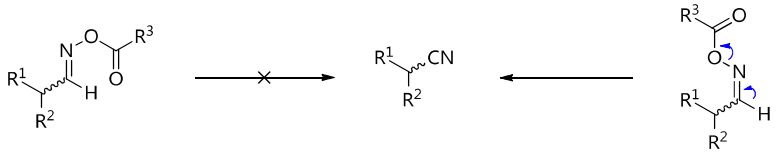
E/Z-**133a**: R^1 = Ph, R^2 = Me, R^3 = Me
E/Z-**133b**: R^1,R^2 = 3-cyclohexene, R^3 = Me, Ph

E/Z-**148a-c**

Entry	**133**	R^3	Lipase	Time [h]	**148a-c** [%]	**139a,b** [%]	E/Z **148a-c** [%]
1	**a**	Me	PS-D	12	85.1	14.3	81:19
2	**b**	Me	PS-D	18	10.6	34.4	88:12
3	**b**	Me	PS-D	72	26.7	31.1	92:8
4	**b**	Me	CAL-B	72	55.2	43.2	88:12
5	**b**	Ph	PS-D	72	n.d.[b]	n.d.[b]	n.d.[b]
6	**b**	Ph	CAL-B	72	n.d.[b]	n.d.[b]	n.d.[b]

The preliminary results were quite positive, with the aldoximes **133a** and **133b** being acetylated with high conversions in *n*-hexane, the benchmark solvent for the intended racemization process. All substrates and biocatalysts showed the necessary high activity for this oximolysis reaction.

Figure 119. E_2-elimination leading to a mixture of *E*-aldoxime esters and corresponding nitriles of the substrates **133a** and **133b**.



E-rac-**148**
syn

rac-**139**

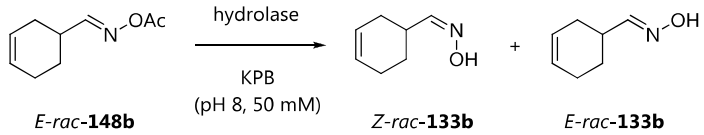
Z-rac-**148**
anti

In regard of an enantioselective reaction, which would facilitate the aldoxime dehydration by acylating only the inert substrate enantiomer, the products

were also analyzed by chiral HPLC for an enantiomeric excess of the acyl aldoximes. Racemic reference compounds were synthesized by standard procedures in form of a SCHOTTEN-BAUMANN reaction with the corresponding acyl chlorides and triethylamine (data not shown). It was during this reference synthesis that an isomeric enrichment was observed. By analyzing the NMR spectra of the crude product after removing the solvent, not only acyl aldoxime of type **148** was present but also the corresponding nitrile **139** obtained by carboxylic acid elimination. Noteworthy, this predominantly occurred with the *Z*-aldoxime esters of type *Z-rac*-**148** following an E_2-elimination mechanism (see **Figure 119**, page 112).

3.7.2 HYDROLASE-CATALYZED DEACYLATION OF ISOMERICALLY ENRICHED ALDOXIME ESTERS

With the prospect of synthesizing *E*-aldoxime esters from *E/Z*-aldoxime mixtures, it was further examined, if the cleavage of said acyl groups would then produce free *E*-aldoximes in high isomeric purity. While the experiments with the substrate *E-rac*-**148b** have to be regarded as preliminary results and will be subjected to optimzation in future studies, it was not possible to produce isomerically pure *E*-aldoximes from pure *E*-aldoxime esters by means of a biocatalytic hydrolysis at room temperature. This could be related to enzymatic isomerization or thermodynamically-induced isomerization at room temperature.



E-rac-**148b** *Z-rac*-**133b** *E-rac*-**133b**

Table 28. Hydrolase-catalyzed deacetylation of racemic aldoxime acetates **148b** in aqueous medium.

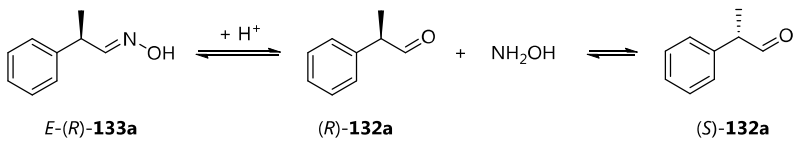
(a) Cephalosporin acetylesterase (CAE), Lipase from *Pseudomonas cepacia*, immobilized on diatomite (PS-D).

Entry	Hydrolase[a] (amount)	Time [h]	Conversion [%]	*E/Z*[a]
1	CAE (1.5 mL)	7.5	>95.0	72:28
2	Amano PS-D (50 mg)	19.5	>95.0	82:18

Nevertheless, the results indicated a controllable isomerization by either temperature or biocatalyst amount. This would also be adressed during the establishment of an *in situ* racemization process, since the racemic *Z*-aldoxime would also decrease the enantiomeric excess of the desired nitrile.

3.8 PURIFICATION OF ENANTIOMERICALLY PURE NITRILES FROM BIOTRANSFORMATIONS IN AQUEOUS MEDIUM

With a selective *E*-aldoxime synthesis and a dynamic kinetic resolution, the product mixture would certainly be of high purity for the desired nitrile. Nevertheless, the current process yielded a mixture of around 50% enantiomerically enriched nitrile and 50% remaining aldoxime. These compounds could easily be separated by column chromatography. However, chromatography should always be the last choice for purification, especially if the by-product could be reused. The enantiomerically pure remaining *E*-aldoxime does not epimerize, but is prone to isomerization and hydrolysis. So in general, it should be feasible to hydrolyze the aldoxime to its corresponding aldehyde, which is then again epimerized (see **Figure 120**). This racemate could then be used again as starting material or eliminated from the product mixture.



E-(*R*)-**133a** (*R*)-**132a** (*S*)-**132a**

Figure 120. pH-Induced hydrolysis of the remaining aldoxime to the epimerizable aldehyde.

3.8.1 HYDROLYSIS OF REMAINING ALDOXIME FROM PRODUCT MIXTURES AFTER BIOTRANSFORMATION

The effect of acidic media on the enantiomerically pure nitrile was already determined during the initial racemization tests in chapter **3.6** (see **Table 26**, page 109). A product mixture of the biotransformation containing enantiomerically enriched nitrile (*S*)-**139a** (78% ee) and enantiomerically pure *E*-(*R*)-**131a** (>99% ee) was subjected to pH change by addition of concentrated hydrochloric acid. The respective acid concentration was calculated and the mixture was stirred for 60 minutes at room temperature or 80 °C. The hydrolysis was determined for up to 0.2 M acid concentration and showed significant conversion of aldoxime to the corresponding aldehyde (see **Figure 121**, page 115).

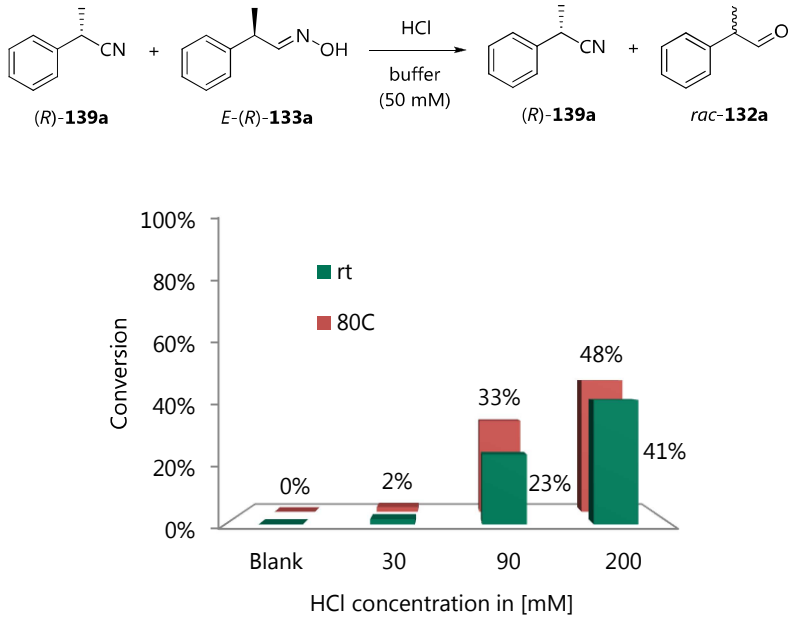


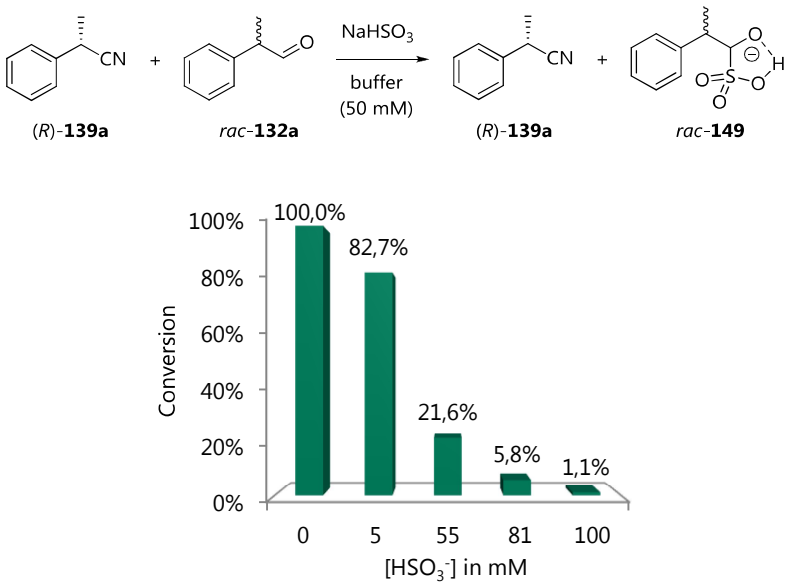
Figure 121. pH-Induced hydrolysis of the remaining aldoxime at different HCl concentrations and temperatures.

During the hydrolysis, the enantiomeric excess of the product remained stable, while the formed (*R*)-aldehyde immediately racemized in acidic aqueous solution. Notably, the hydrolysis was only negligibly accelerated at 80 °C. By raising the acid concentration to about 1.0 M, the reaction should be complete within 60 minutes, which is promising for this first step of the intended extractive purification method.

3.8.2 Extractive separation of racemic aldehyde from enantiomerically pure nitrile

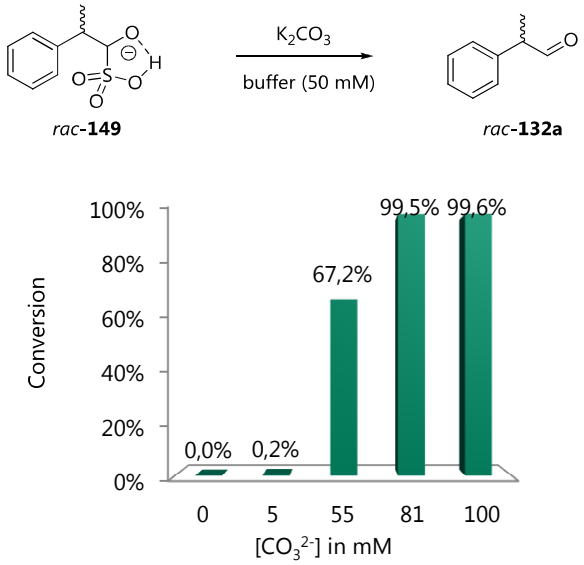
The extractive separation of racemic aldehyde was subsequently tested by bisulfite addition. Since bisulfite adducts of aldehydes are hardly soluble, sometimes even in aqueous media, this methodology would only allow the nitrile to be extracted. The relative content of aldehyde was analyzed by chiral HPLC from an extraction with MTBE. The solvent was chosen for its compatibility with the chiral stationary phase. The applicability for product mixtures was tested by adding enantiomerically pure nitrile as an internal reference. The mixture was subjected to aqueous solutions of sodium bisulfite with varying concentrations (see **Figure 122**, page 116).

Figure 122. Bisulfite addition and associated elimination of aldehyde from nitrile extraction with MTBE.



Each solution was stirred for 30 minutes at room temperature to reach an addition equilibrium prior to extraction. After complete extraction of the enantiomerically enriched nitrile **139a** (78% ee), the aqueous phase now consists of the pure bisulfite adduct. By raising the pH value to basicity, the remaining hydroxylamine from the hydrolytic reaction again condensed with the free aldehyde, yielding racemic *E/Z*-mixtures of aldoxime **133a**.

Figure 123. Cleavage of bisulfite adducts by basification with neat potassium carbonate and extraction of pure aldehyde with MTBE.



To investigate the cleavage of bisulfite adducts, a solution of bisulfite adduct was subjected to potassium carbonate addition. The current concentration was calculated related to the aqueous volume and the relative content of aldehyde

was again determined *via* extraction with MTBE and chiral HPLC analysis (see **Figure 123**, page 116). The mixtures were again stirred for 30 minutes at room temperature prior to extraction. The aldehyde was quantitatively recovered at carbonate concentrations above 80 mM. By these few extractive steps, the chiral nitrile was obtained in high purity and the remaining inert substrate was hydrolyzed and also extracted as highly pure starting material.

3.9 CONCLUSION AND OUTLOOK

The present work started from basically no literature regarding an enantioselectivity of aldoxime dehydratases (*Oxd*s). Even more, the only comments about chiral aldoximes which were actually converted by *Oxd*s stated the racemic composition of the products. By reducing the activity of the biocatalyst, i.e. an aldoxime dehydratase from *Bacillus* sp. OxB-1 overexpressed in recombinant *E. coli* HB101 / pOxD-9OF, and the free energy of the system with lowering the reaction temperature, an unidentified enantioselectivity for the chiral *E*-aldoxime of 2-phenylpropionaldehyde (*E*-**133a**) was observed for the first time. The kinetic progress of the enzymatic reaction was analyzed from a mixture of two diastereomers of *E*- and *Z*-aldoxime consisting of a racemate. By identifying an inert substrate, namely *E*-(*R*)-**133a**, as well as the absolute configuration of the enantiomerically enriched nitrile (*S*)-**139a**, the enantioselectivity for this substrate could be clarified.

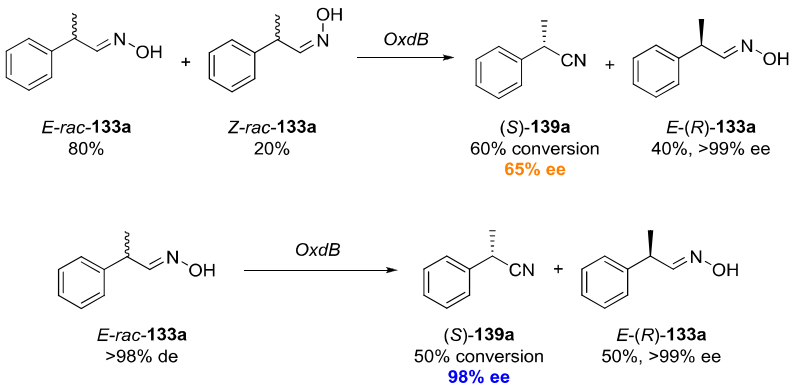


Figure 124. Synthesis of enantiomerically pure (*S*)-2-phenylpropionitrile by an enzymatic dehydration of a racemic aldoxime by means of a kinetic resolution.

Furthermore, by separating *E*- and *Z*-aldoximes from their mixture, essentially pure (*S*)-**139a** was synthesized in a kinetic resolution starting from racemic *E*-**133a** (>99% isomeric purity), with 50% conversion and an excellent

enantioselectivity of 98% ee (see **Figure 124**, page 117). This unusual enantiopreference for *E*-aldoximes was also observed for four other chiral substrates which were not investigated in more detail. The inert substrate and only by-product *E*-(*R*)-**133a** was also investigated for an *in situ* racemization process in view of a dynamic kinetic resolution. The inability to epimerize free aldoximes was to be circumvented by a lipase-coupled esterification in a binary mixture with *n*-hexane. This binary mixture revealed itself to be of even greater advance than initially planned. The biotransformation with a resting cell suspension of the aldoxime dehydratase was not altered by the presence of an equal volume of *n*-hexane. In addition, the low substrate concentration of 5 mM for the purely aqueous reaction mixture could be increased to at least 50 mM in a binary reaction mixture on the basis of a determined distribution coefficient for the chiral substrate.

Finally, a purification procedure for non-quantitative conversion to the enantiomerically pure nitrile was established which was solely done by pH adjustment and salt addition to obtain highly pure nitrile and also racemic aldehyde from the remaining impurities, which could be recycled for a following reaction batch.

Further studies on the enantiopreference of aldoxime dehydratases for other chiral aldoximes are in progress. Currently, efforts are made to crystallize the investigated aldoxime dehydratase and clarify the binding of substrates in the active center, with *in silico* studies confirming the experimental results and predict the necessary alterations to enhance the enantiopreference for the *Z*-aldoxime diastereomer. Pharmaceutically relevant target molecules will also be subject to experiments in the near future.

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4 BIOCATALYTIC REDUCTION OF BETA-OXO CARBONYL COMPOUNDS IN THE CHEMOENZYMATIC SYNTHESIS OF ENANTIO- AND DIASTEREOMERICALLY PURE PHARMACEUTICAL BUILDING BLOCKS

The following project consisted of two different target molecules, *i.e.* β-amino alcohols **150** and β-lactones **152**, with α,β-disubstitution patterns for the synthesis of diastereomers (see **Figure 125**). The β-amino alcohol project was first investigated within the own diploma thesis. While the general synthetic pathway was successfully established with remarkable observations, the search for active enzymes was challenging. The following chapters will investigate different approaches to circumvent biocatalytic bottlenecks of the used alcohol dehydrogenases.

The second target molecule, i.e. the β-lactone of type **152**, was part of a lab course project based on the literature-known and already observed activity and selectivity for the enantio- and diastereoselective reduction of α,β-disubstituted β-oxo esters. The respective chapters will give a brief overview of identified selectivities and the use of the corresponding β-hydroxy esters for the chemoenzymatic synthesis of β-lactones.

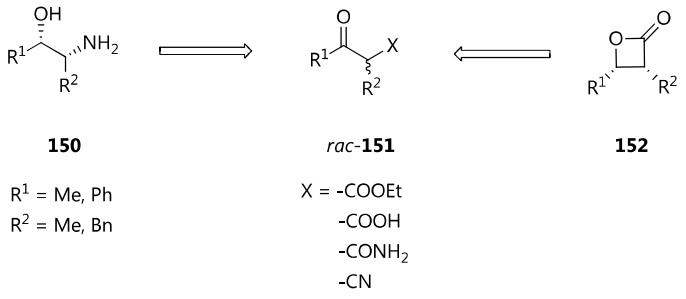


Figure 125. General project overview for the retro-synthetic approach to β-amino alcohols and β-lactones from racemic β-oxo carbonyl compounds.

4.1 COMBINATION OF ENZYMES IN THE SYNTHESIS OF ENANTIOMERICALLY PURE BUILDING BLOCKS

There are countless examples of successful biotransformations for simple substrate to product conversion in industrial syntheses.[75] They are also readily integrated in multi-step synthesis of high value.[76] Nevertheless, a real challenge still remains to combine multiple enzymatic steps, either with different enzymes or even one enzyme capable of multiple reaction types. The complexity of finding optimal conditions for more than one enzymatic step in the same system or having specific chemoselectivities is a demanding task. Most of the literature-known processes combining different enzymes in one pot are related to cofactor regeneration. The most common examples employ oxidoreductase enzymes, e.g. alcohol dehydrogenases or keto reductases, with nicotinamide adenine dinucleotide phosphate ($NAD(P)^+$) as hydride transfer reagent. For example, the use of a glucose dehydrogenase and the cheap stoichiometric "hydride donor" D-glucose is implemented in many NAD(P)H-dependent redox reactions.[77] This methodology of generating or regenerating reagents was also achieved by utilizing a glucose oxidase (GOX) for the *in situ* preparation of hydrogen peroxide (H_2O_2) from again cheap glucose (**153**) and molecular oxygen by KUMAR *et al.* (see **Figure 126**).[78] The combination of this process with a chloroperoxidase (CPO)-catalyzed oxidation was successfully used in the synthesis of benzoxazones and benzothiazones of type **159** starting from aldehydes of type **157**. This is also a good example of controlling the amount of highly reactive reagents *in situ*. Hydrogen peroxide would denaturate the biocatalyst in higher concentrations, thus limiting the direct addition of

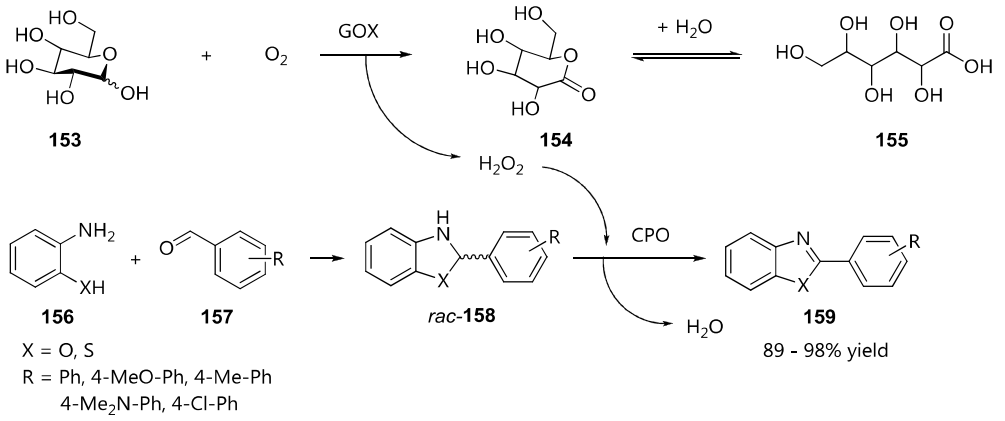
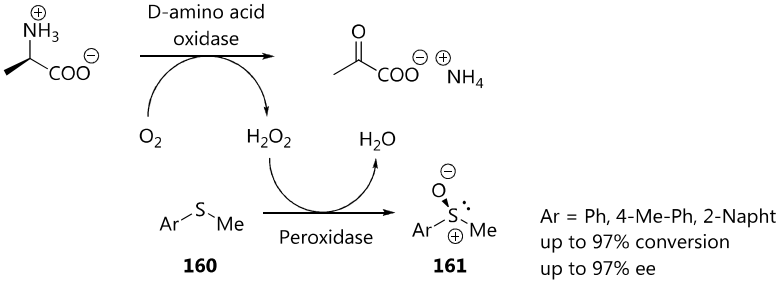


Figure 126. Bienzymatic synthesis of benzoxazones and benzothiazones by oxidative coupling of aromatic aldehydes with o-aminophenol and o-aminothiophenol by KUMAR *et al.*.[78]

commercially available H_2O_2 solutions.[79] By using glucose as a stoichiometric reagent, the total amount of reagent can easily be controlled, since both the

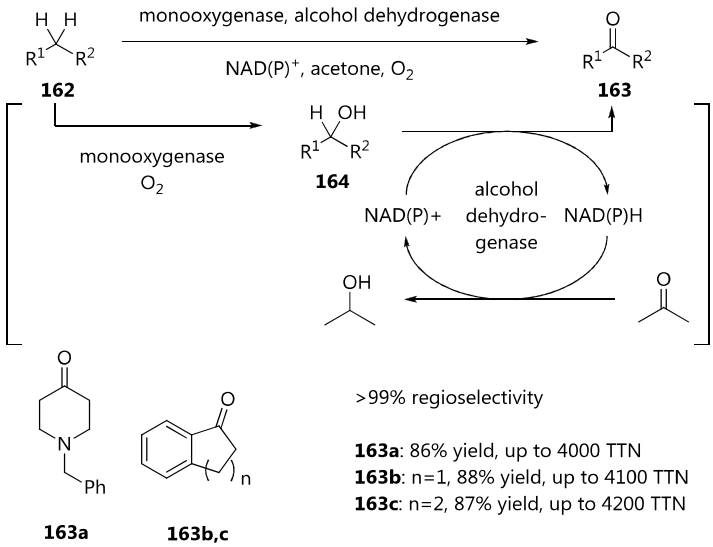
oxidative process by molecular oxygen and the subsequent hydrolysis of gluconolactone (**154**) to gluconate (**155**) are irreversible. This method was also established with a D-amino acid oxidase (DAO) for an enantioselective sulfoxidation by OKRASA *et al.*.[80] They prepared chiral aryl-methylsulfoxides of type **161** with good to excellent enantioselectivities of up to 97% ee (see **Figure 127**).



160 **161**

Figure 127. Bienzymatic enantioselective synthesis of chiral aryl methylsulfoxides with molecular oxygen as environmentally benign oxidation reagent by OKRASA *ET AL.*.[80]

This concept was later extended to heteroaromatic sulfoxides by the same research group.[81] Although *in situ* generation of reagents is of high interest in this field, also intermediary compounds from within a multi-step synthesis can be formed by the combination of enzymes.



162 **163** **164** **163a** **163b,c**

Figure 128. Bienzymatic tandem process for the synthesis of cyclic ketones from cyclic methylene groups by ZHANG *ET AL.*.[82]

ZHANG *et al.* successfully established a monooxygenase-catalyzed hydroxylation of cyclic methylene compounds **162** with an alcohol dehydrogenase-catalyzed oxidation to the corresponding ketones of type **163** (see **Figure 128**, page 122).[82] The low oxidative activity of an alcohol dehydrogenase from *Lactobacillus kefir* for the formation of the cyclic ketone was enhanced by the

addition of acetone as more active substrate towards reduction, thus pushing the equilibrium in the desired direction. By this tandem reaction they were able to oxidize the compounds **162a-c** to their corresponding ketones **163a-c** with yields of around 88% and with perfect regioselectivity. A bienzymatic process, consisting of a combination of a monooxygenase and an alcohol dehydrogenase as isolated enzymes, was recently also successfully established for the oxidation of cycloalkanes of type **164** (see **Figure 129**). STAUDT *et al.* prepared the corresponding cycloalkanones **165** with a P450 monooxygenase from *Bacillus megaterium*.[83] These cyclic ketones are used for the production of lactams, e.g. ε-caprolactam, which is one of the starting materials for nylon 6-6.

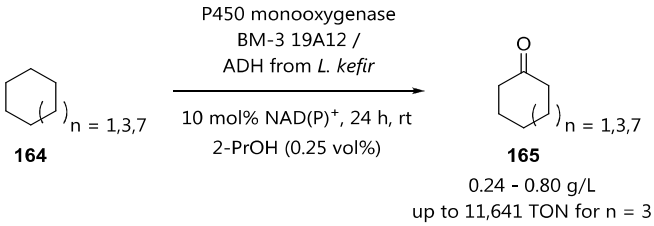


Figure 129. Bienzymatic tandem process for the synthesis of cycloalkanones by STAUDT *ET AL.*.[83]

Apart from the previously discussed functional group conversions, there are also some cases of multienzymatic processes for the kinetic resolution of racemates. For example, WU *et al.* prepared enantiomerically pure *(S)*-β-phenylalanine and related β-amino acids of type *(S)*-**166** by combining a phenylalanine aminomutase and a phenylalanine ammonia lyase (see **Figure 130**).[84] At first, the aminomutase converts the undesired *(R)*-β-phenylalanine enantiomer *(R)*-**166** into its *(S)*-*α*-phenylalanine analogue of type *(S)*-**167**. Subsequently, a phenylalanine ammonia lyase from *Rhodosporidium toruloides* converts the intermediate to its cinnamic acid deriva-tive **168**, therefore driving the equilibrium of the aminomutase reaction to completion. The enantiomerically pure *(S)*-β-phenylalanine was obtained after ion exchange chromatography.

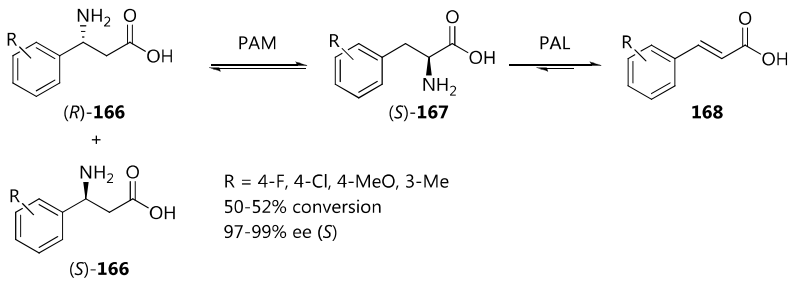


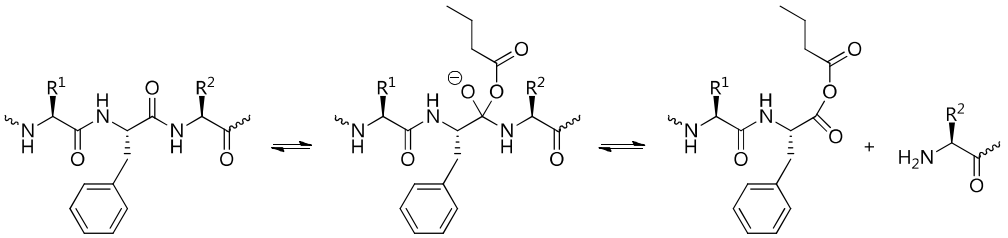
Figure 130. Bienzymatic kinetic resolution of *(S)*-β-phenylalanine derivatives by WU *ET AL.*.[84]

Abbreviations: phenylalanine aminomutase (PAM), phenylalanine ammonia lyase (PAL).

Also, some meta- and para-substituted β-phenylalanine derivatives **166** were obtained in about 50% conversion and with excellent enantioselectivities of 97-99% ee. This process could certainly be even more valuable with an equilibrium preferably producing the racemic β-amino acid **166** from the cinnamic acid **168**, turning it into a deracemization process.

4.2 SYNTHESIS OF BETA-AMINO ALCOHOL DERIVATIVES FOR THE PRODUCTION OF HIV-I PROTEASE INHIBITORS

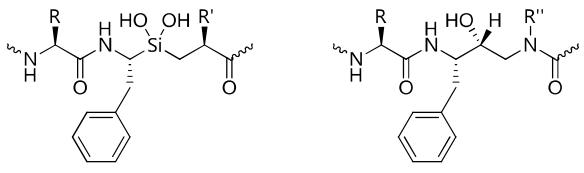
The fight against the human immunodeficiency virus (HIV) is still ongoing. In the past decades, numerous drugs were developed to inhibit its activitiy through different strategies. The inhibition of the HIV-I protease, an aspartate protease responsible for cleaving viral polypeptides into pharmacologically active proteins, is one target. The protease has a specific substrate binding pattern to recognize the crucial peptide bonds. This peptide bond is for example the carboxylate of L-phenylalanine (see **Figure 131**). Potent inhibitors



169 **170** **171a** **171b**

Figure 131. HIV-I protease-catalyzed cleavage of phenylalanine residues in viral peptide synthesis.

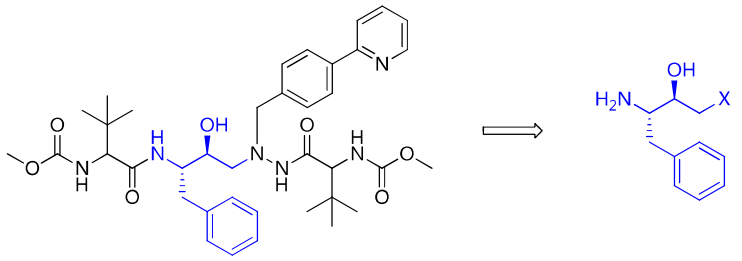
mimic the tetrahedral intermediate **170** prior to bond cleavage. The inhibitors contain usually tetrahedral structures not prone to bond breaking, such as geminal silandiols **172** or quarterny carbinols **173** (see **Figure 132**). This structure is also embedded into peptide-like structures to enhance substrate binding and decrease substitution by the original polypeptide substrate.



172 **173**

Figure 132. Phenylalanine derivatives mimicking the tetrahedral intermediate acting as active-site inhibitors.

A prominent example is Atazanavir (**174**), the active pharmaceutical ingredient of Reyataz®, manufactured by Bristol-Myers Squibb (see **Figure 133**).[85]



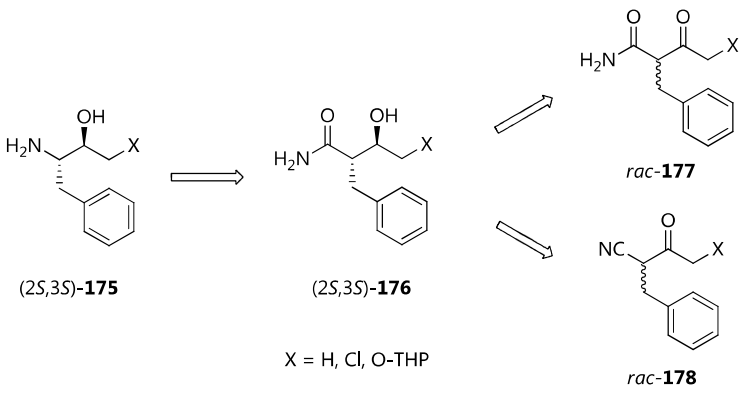
(2*S*,3*S*)-**174** (2*S*,3*S*)-**175**

Figure 133. Main pharmacologically active structure in HIV-1 protease inhibitor Atazanavir and identification of the necessary building block.

The enzyme-substrate complex is imitated by the chiral secondary carbinol **175** related to the phenylalanine carboxylate after aspartate addition. Since this carbinol will not eliminate under the given conditions, the substrate is blocking the active site of the protease and the formation of viral proteins is prevented.[86]

4.2.1 Retrosynthetic strategies

The main building block, the β-amino alcohol of type **175**, could be synthesized according to **Figure 134**. The functional-group interconversion of an aliphatic, primary amide of type **176** to its corresponding amine **175** can be accomplished *via* Curtius or Hofmann degradation. The latter was already investigated during the own diploma thesis and was proven to be straightforward, with retention of the absolute configuration.



(2*S*,3*S*)-**175** (2*S*,3*S*)-**176** *rac*-**177** *rac*-**178**

X = H, Cl, O-THP

Figure 134. Retrosynthetic approach to enantio- and diastereomerically pure β-amino alcohols starting from racemic β-oxocarbonyl compounds.

This carboxylic amide **176** can either be synthesized through ammonolysis of the corresponding carboxylic ester or by hydration of the respective carbonitrile. Due to the ultimate goal of implementing biocatalysis in as many reaction steps as possible, these conversions were also partially examined for an enzymatic alternative. The enantio- and diastereoselectivity of the chiral secondary carbinol of type **176** is introduced by a stereoselective ketone reduction *via* alcohol dehydrogenases. The benefit of using 1,3-dicarbonyl compounds like **177** or **178** lies in their tendency to racemize in aqueous media without additional catalyst, thus enabling a dynamic kinetic resolution process for the biocatalytic reduction.

4.2.2 PRELIMINARY RESULTS FOR THE REDUCTION OF α-SUBSTITUTED β-OXO AMIDES

The enantio- and diastereoselective reduction of β-oxo amides of type **177** for the synthesis of β-hydroxy amides like **176** as precursors for β-amino alcohols is an already known concept. However, most of the enzymatic literature examples have a very limited substrate scope. For instance, QUIRÓS *et al.* successfully reduced β-substituted β-oxo amides **179** with an alcohol dehydrogenase from *Mortierella isabellina* (see **Figure 135**).[87]

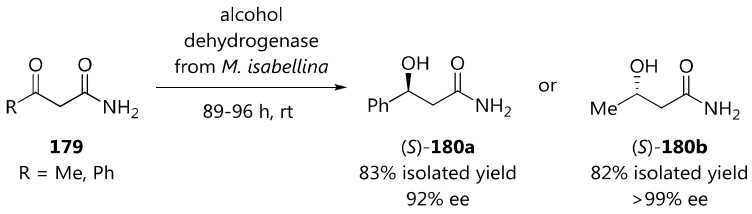


Figure 135. Enantioselective enzymatic reduction of β-substituted β-oxo amides to their β-hydroxy amides by QUIRÓS *et al.*[87]

The respective β-hydroxy amides **180a** and **180b** were obtained in high yields of 82-83% and with excellent enantioselectivities of 92% and >99% ee, respectively. While these biotransformations were restricted to α-unsubstituted β-oxo amides, ROZZELL and coworkers from BioCatalytics Inc. patented a closely related enzymatic reduction, however, with α,β-disubstituted short-chain aliphatic or β-aryl β-oxo amides.[88] Remarkably, the used alcohol dehydrogenase from *Geotrichum candidum* could also reduce cyclic β-oxo amides in a fermentation process and obtain the products in excellent enantioselectivities of >99% ee. They also applied commercially available baker's yeast from Sigma-Aldrich for the enantio- and diastereoselective

production of short-chain aliphatic β-hydroxy amides. Noteworthy, also β-oxo hydrazides, obtained from reacting the corresponding β-oxo esters with hydrazine, could be reduced to one diastereomer of the β-hydroxy hydrazides by fermentation with *Colletotrichum gloeosporioides*. However, no optical purities or even conversions are disclosed. QUIRÓS *et al.* also published the enantio- and diastereoselective reduction of cyclic β-oxo amides of type **181** (see **Figure 136**).[89]

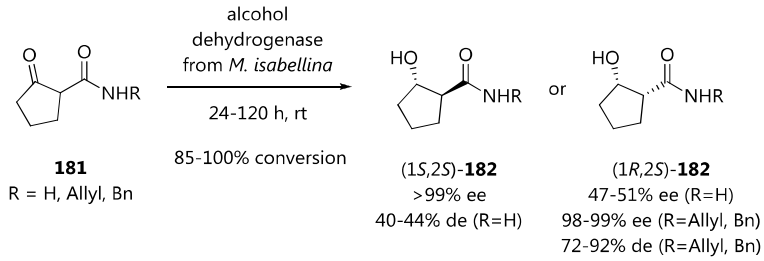


Figure 136. Enantio- and diastereoselective reduction of cyclic β-oxo amides to their β-hydroxy amides by QUIRÓS *et al.*.[89]

Surprisingly, the diastereoselectivity for this reduction changed completely by using either unsubstituted amides (*anti*-selective for **182**) or *n*-allyl and *n*-benzyl amides (*syn*-selective for **182**). In addition, the enantiomeric excess for the α-chiral center tremendously improved from around 50% ee to almost perfect 99% ee by using *n*-monosubstituted β-oxo amides.

In the preceding diploma study, β-oxo amides related to the phenylalanine-type β-amino alcohol **185a** and also a norephedrine-type structure **185b** were prepared according to literature-known procedures from commercially available ethyl acetoacetate (**183a**) and ethyl benzoylacetate (**183b**) (see **Figure 137**).[90] After ammonolysis of the racemic β-oxo esters **184** in aqueous ammonia solution, the crystalline β-oxo amides **185** were preparatively screened with a number of alcohol dehydrogenases provided by collaboration partners.

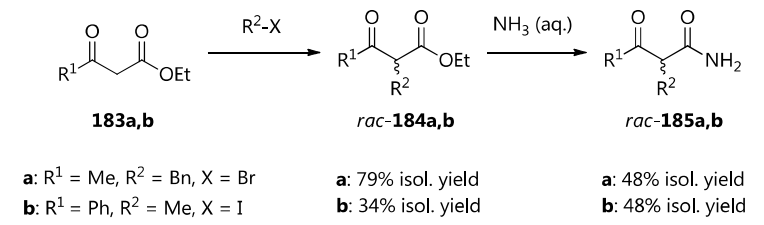
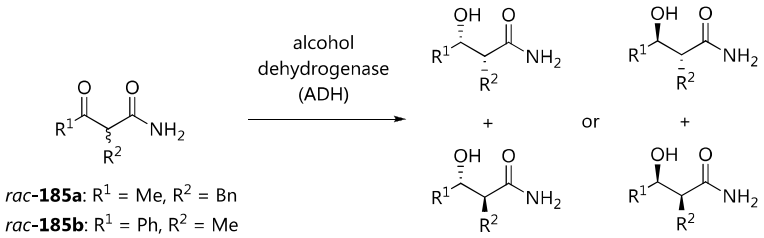


Figure 137. Substrate synthesis of racemic β-oxo amides during the diploma thesis.[90]

A preliminary photometric screening suggested sufficient activities of the investigated alcohol dehydrogenases for the reduction of the prepared β-oxo amides. Unfortunately, these photometric activities, related to the decrease in absorption of the cofactor NAD(P)H at 340 nm, could not be reproduced in preparative scale. In fact, none of the tested alcohol dehydrogenases showed activity for the reduction of **185a** or **185b** (see **Table 29**).

Table 29. Preparative screening of various alcohol dehydrogenases for the enantio- and diastereoselective reduction of α,β-disubstituted β-oxo amides.

(a) n.d. = not detected



rac-**185a**: R^1 = Me, R^2 = Bn
rac-**185b**: R^1 = Ph, R^2 = Me

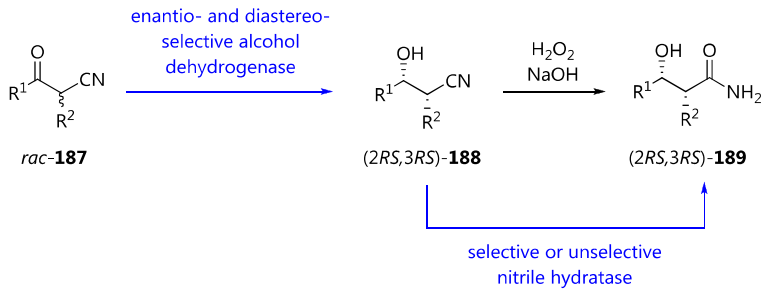
(2*RS*,3*RS*)-**186a,b**

Entry	**185**	ADH (selectivity)	Conversion[a] [%]	ee [%]	de [%]
1	**a**	*Rhodococcus sp. (S)*	n. d.	-	-
2	**a**	*evocatal 1.1.200 (R)*	n. d.	-	-
3	**a**	*Lactobacillus* sp. (*R*)	n. d.	-	-
4	**a**	*Thermoanaerobacter* sp. (*S*)	n. d.	-	-
5	**b**	*Rhodococcus sp. (S)*	n. d.	-	-
6	**b**	*evocatal 1.1.200 (R)*	n. d.	-	-
7	**b**	*Lactobacillus* sp. (*R*)	n. d.	-	-

These were somehow surprising results, since the corresponding racemic β-oxo esters of type **184** (see **Figure 137**, page 127) were readily converted by the same biocatalysts. Therefore, an explanation for the missing activity was focused on the difference between an ethyl ester and a primary amide. An initial idea of lower solubilities for the β-oxo amides was investigated by a quantitative ^{1}H-NMR analysis of saturated substrate solutions in D_2O and showed, on the contrary, higher aqueous concentrations. Another possible reason was assumed in the polarity of the active center, more specifically the amino acid side chains in the proximity of the carboxyl moiety.

4.2.3 REDUCTION OF α-CYANOKETONES

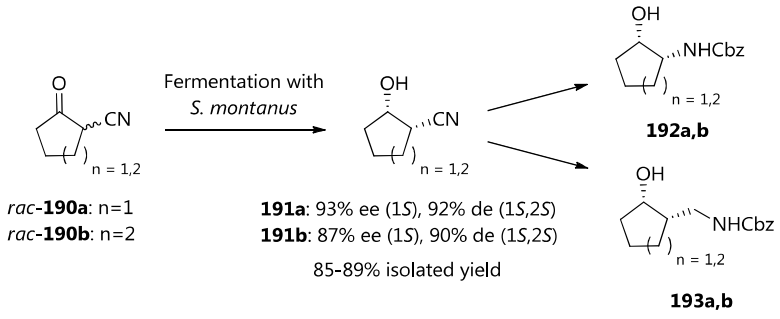
The disappointing results from the biocatalytic reduction of β-oxo amides for the syntheses of pharmaceutically relevant β-amino alcohols were the reason for reconsidering the synthetic route in this work. With the target compounds still being the β-hydroxy amides, the amide moeity was to be introduced after the enantio- and diastereoselective reduction with alcohol dehydrogenases. Thus, a secondary strategy was tested, with a "masked" carboxylic amide in form of its corresponding nitrile (see **Figure 138**). The use of nitriles instead of other amide derivatives like *N*-alkyl or even *N,N*-dialkyl amides is beneficial for the subsequent preparation of the unsubstituted primary amide.



rac-**187** (2*RS*,3*RS*)-**188** (2*RS*,3*RS*)-**189**

Figure 138. General concept for the biocatalytic reduction of β-oxo nitriles and subsequent formation of β-hydroxy amides.

This synthetic route starting from racemic β-oxo nitriles is in parts already literature-known for cyclic α-cyanoketones. DEHLI and GOTOR successfully reduced 2-cyanocycloalkanones of type **190** with alcohol dehydrogenases from different microorganisms in a highly enantio- and diastereoselective manner, with the best results obtained after fermentation with *Saccharomyces montanus* (see **Figure 139**).[91]



rac-**190a**: n=1
rac-**190b**: n=2

191a: 93% ee (1*S*), 92% de (1*S*,2*S*)
191b: 87% ee (1*S*), 90% de (1*S*,2*S*)
85-89% isolated yield

192a,b

193a,b

Figure 139. Dynamic kinetic resolution of α-cyano cycloalkanones and chemical derivatization to cyclic β-amino alcohols by DEHLI and GOTOR.[91]

The obtained 2-cyanocycloalkanols **191** were further derivatized to their corresponding β- and γ-amino alcohol derivatives **192** and **193** by chemical methods.

This approach to cyclic amino alcohols has some important advantages in contrast to, for example, epoxidation of cycloalkenes and diastereoselective ammonolysis. First of all, this biocatalytic reduction can be tuned to yield, in principle, all four stereoisomers of **191** separately. In addition, the substrate synthesis is straightforward. Aliphatic medium-chain dinitriles, which are commercially available, undergo an intramolecular CLAISEN-type condensation by addition of strong bases like sodium hydride or potassium *tert*-butoxide. The intermediary α-cyano ketimine is stabilized by tautomerization to the enamine and has to be hydrolyzed in acidic aqueous media at elevated temperatures. While this reaction benefits from the intramolecular preference and high chemo- and regioselectivity of using aliphatic dinitriles without additional functional groups, the adaptation for this work needed a more sophisticated approach.

4.2.3.1 SUBSTRATE SYNTHESIS OF Β-OXO NITRILES RELATED TO THE TARGET BUILDING BLOCK OF ATAZANAVIR

Retrosynthetic analysis of the β-amino alcohol structure **194** revealed two lead structures **197** for a biocatalytic reduction with alcohol dehydrogenases (see **Figure 140**). The main difference, the halogenated methylene group, is not trivial to introduce. The preference of halogenation with bromine or *N*-halosuccinimide (NXS) was in all cases the α-position, since it is activated by two electron-withdrawing groups (see **Figure 141**, page 131). Under weak

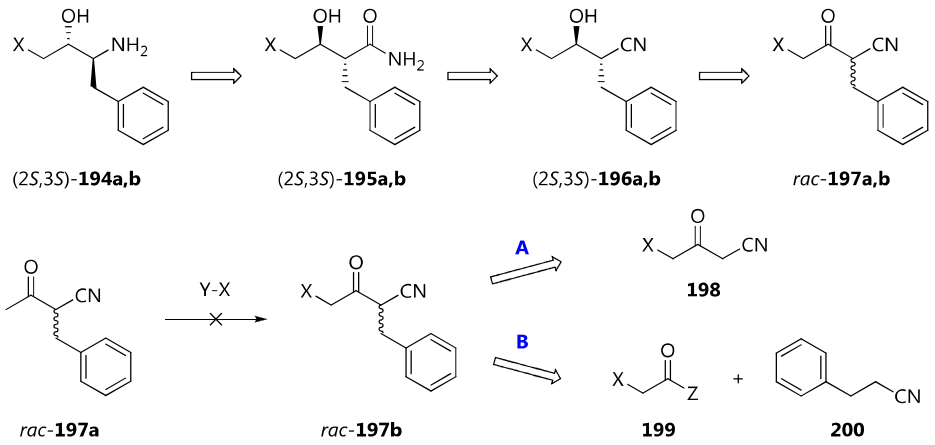


Figure 140. Retrosynthetic approach to the main Atazanavir building block starting from racemic β-oxo nitriles.

basic reaction conditions, this chloride **201** was then also eliminated as hydrochloric acid under the formation of the cinnamonitrile derivative **202**.

Nevertheless, the difference between a methyl and chloromethyl moiety should be low for alcohol dehydrogenases. For this reason, the unhalogenated β-oxonitrile **197a** was synthesized, also as a direct reference compound for the β-oxo amide **185a**, which was not reduced in preceding experiments (see **Table** , page 128).

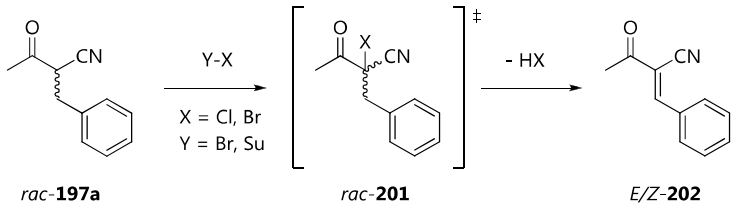


Figure 141. Unsuccessful halogenation due to wrong regioselectivity of the reaction.

While the acetyl-substrate **197a** was synthesized rather facile by a crossed CLAISEN-type reaction (see **Figure 140,** page 130, pathway **B**) between 3-phenylpropionitrile and ethyl acetate, the corresponding halogenated reagents degraded under the basic reaction conditions, leading to a crude product consisting mainly of starting material and unidentified impurities. In addition, the more chemo- and regioselective approach by an α-unsubstituted α-cyano-α'-haloacetone **198** could not be investigated due to the unavailability of the starting material (pathway **A**). Due to these reasons, pathway B was modulated to gain excess to the α'-substituted compound **197b** (see **Figure 142**).

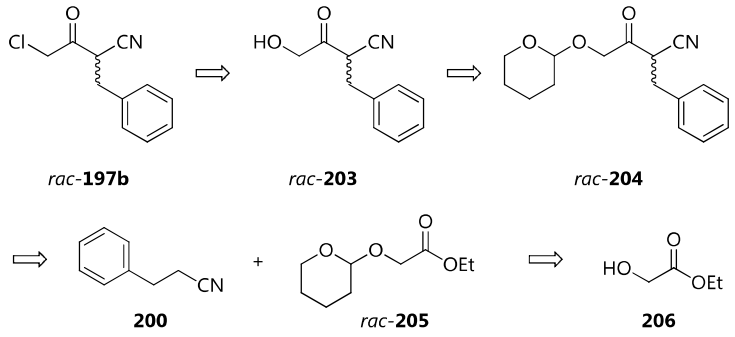
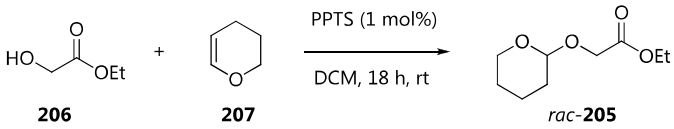


Figure 142. Modulation of the substrate synthesis for an enhanced chemo- and regioselectivity.

Ethyl glycolate (**206**) was chosen as commercially available precursor. For the chemoselectivity of the alkoxide-catalyzed condensation reaction, the α-hydroxy group was protected as tetrahydropyranyl (THP) acetal **205**. THP was considered as the optimal protective group due to its base-stability and its facile introduction and removal under neutral conditions, with pyridinium *p*-toluenesulfonate (PPTS) as catalyst (see **Figure 143**, page 132).



206 **207** *rac*-**205**

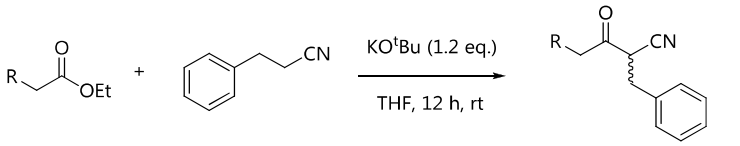
1.5 eq.

>95% conversion
90% isolated yield (8.5 g)

Figure 143. THP protection of ethyl glycolate under PPTS-catalysis.

Abbreviations: dichlormethane (DCM), pyridinium *p*-toluene-sulfonate (PPTS).

The protection of **206** was achieved on a 10-gram-scale. Excess of starting material 3,4-dihydro-2*H*-pyran (**207**) was removed at 60 °C in high vacuum. The catalyst was removed by filtration over a short silica column. In addition, the subsequent crossed CLAISEN condensation was also successful to a brief extend, reaching a product-related conversion of 10% (see **Figure 144**).



208 R = H
205 R = O-THP

200

rac-**197**: R = H
86% isol. yield
rac-**204**: R = O-THP
10% conversion

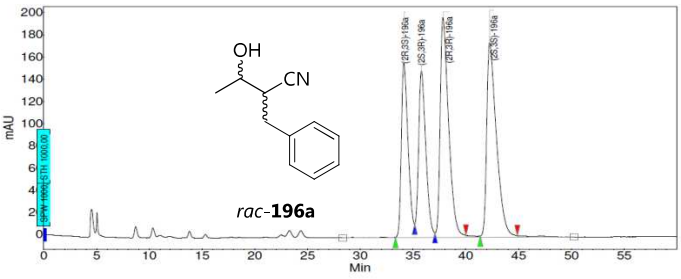
Figure 144. Crossed CLAISEN condensation for the synthesis of α-benzyl-β-oxo nitriles.

Abbreviations: potassium *tert*-butoxide (KO^{t}Bu), tetrahydrofuran (THF).

The low conversion in contrast to the reaction with ethyl acetate (**208**) could be related to the bulkiness of the THP group. The crude product *rac*-**204** was partially purified *via* preparative HPLC to increase the product content from about 15% to 56%, with 3-phenylpropionitrile (**200**) as the main impurity. The crude product could nevertheless be used as substrate for the biocatalytic reduction since none of the impurities has a ketone moiety and under the premise of them not acting as inhibitors.

4.2.3.2 ANALYTICAL METHODS FOR THE DETERMINATION OF THE CONVERSION AND ENANTIOSELECTIVITY

The β-hydroxy nitriles were analyzed by ^{1}H-NMR spectroscopy and chiral normal-phase HPLC (see **Figure 145**). The main interest was of course the identification of the respective enantio- and diastereomers of **196a** produced by different alcohol dehydrogenases.



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(2R,3S)-196a	34,13	19,22	157,1	116,6	19,216
2	(2S,3R)-196a	35,78	19,77	150,0	120,0	19,774
3	(2R,3R)-196a	37,84	30,26	198,0	183,7	30,265
4	(2S,3S)-196a	42,24	30,75	175,5	186,6	30,745
Total			100,00	680,6	607,0	100,000

Figure 145. HPLC analysis of the racemic reference compound for the bio-catalytic reduction of β-oxo nitrile **197a**.

Since the β-hydroxy nitrile **196a** is unknown to literature, the isomers had to be assigned by different assumptions. In general, the enantiospecificity of alcohol dehydrogenases is usually defined, especially with alkyl methylketones. Thus, an alcohol dehydrogenase from *Rhodococcus* sp., which is (*S*)-selective, was used to assign two of the diastereomers in the HPLC chromatogram at 34.1 min and 42.2 min according to their carbinol configuration (see **Figure 145**, page 133). The respective diastereomer was determined using the HPLC peak ratio of 3:2 and comparison with the ^{1}H-NMR spectra (see **Figure 146**, page 133). In these spectra, the diastereomers had also the ratio of 3:2, with the signals of the carbinol being sufficiently separated for integration. Nevertheless, the assignment of *syn*- and *anti*-diastereomers for acyclic organic molecules is not trivial. The preference of hydride addition during the reference synthesis with sodium borohydride produces *anti*-diastereomers. In addition,

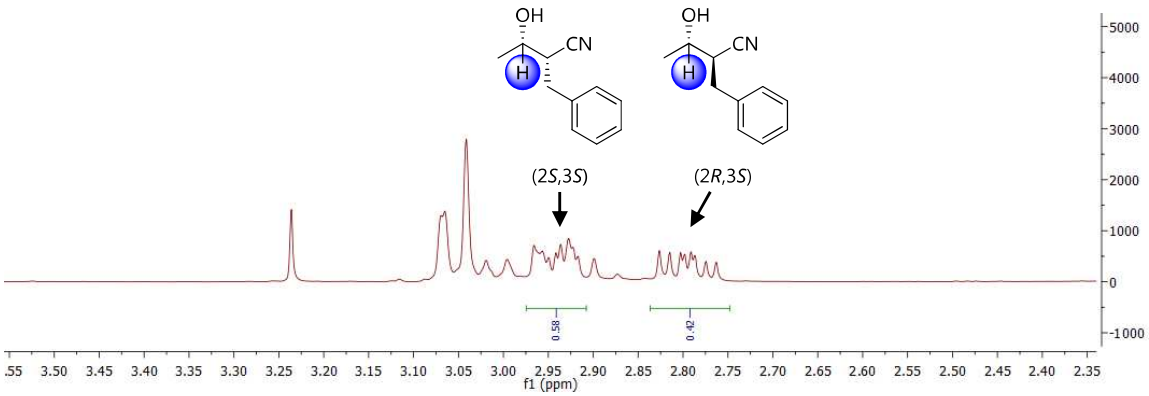


Figure 146. ^{1}H-NMR of diastereomers in accordance with the HPLC ratio of 3:2 and assignment to the *syn*- and *anti*-isomers.

the corresponding β-hydroxy esters and amides were intensively studied during the own diploma thesis.[90] By comparing the respective low-field shifts

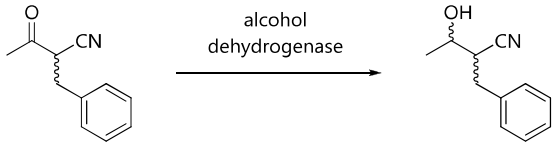
of the *syn*-diastereomers in relation to the *anti*-diastereomers, the HPLC isomers could be further assigned. However, it should be pointed out that only the derivatives of these β-hydroxy nitriles will give final proof of their absolute configuration.

4.3.3.3 BIOCATALYTIC REDUCTION OF β-OXO NITRILES RELATED TO THE BUILDING BLOCK OF ATAZANAVIR

The dehalogenated substrate **197a** was preparatively screened for active and stereoselective alcohol dehydrogenases. As anticipated, the biocatalysts showed high activities for the conversion of the racemic β-oxo nitrile (see **Table 30**, page 134). The corresponding β-hydroxy nitrile **196a** was synthesized with quantitative conversion and with excellent enantioselectivities for both *(S)*- and *(R)*-carbinols, corresponding to the enantioselectivity of the biocatalysts. In these initial preparative screenings with high enzyme amounts, the diastereomeric excess was also increased, confirming a dynamic kinetic resolution process. The absolute configuration of the products was determined in analogy to extensive NMR studies on the β-hydroxy amide shifts during the diploma thesis.[90] The diastereomers obtained in enantiomerically pure form are expected to be *syn*-configured for the alcohol dehydrogenases from *Rhodococcus* sp. and the bacterial ADH evocatal 1.1.200 (entries 1-2) and *anti*-configured for the alcohol dehydrogenase from *Lactobacillus kefir* (entry 3).

Table 30. Preparative screening of various alcohol dehydrogenases for the enantio- and diastereo-selective reduction of β-oxo nitriles.

(a) Determined by chiral HPLC analysis (b) Determined by ^{1}H-NMR and chiral HPLC analysis.



rac-**197a** (2*RS*,3*RS*)-**196a**

Entry	ADH (selectivity)	Conversion [%]	ee[a] [%]	de[b] [%]
1	*Rhodococcus* sp. *(S)*	>95	>99 (3*S*)	82.4 (*syn*)
2	evocatal 1.1.200 *(R)*	>95	>99 (3*R*)	65.7 (*syn*)
3	*Lactobacillus kefir* (*R*)	>95	>99 (3*R*)	40.2 (*anti*)

Thus, three of the four possible stereoisomers of **196a** were successfully generated by these biocatalysts, at least in highly enantio- and diastereomerically enriched form.

4.4 HOFMANN DEGRADATION OF β-HYDROXY AMIDES

In parallel to the enzymatic reductions, the subsequent chemical derivatization of β-hydroxy amides of type **195** to the corresponding β-amino alcohols **194** was examined (see **Figure 140**, page 130). Due to the lack of active biocatalysts for the β-oxo amides (see **Table 29**, page 128), the following experiments were conducted with a diastereomerically enriched racemic mixture of β-hydroxy amides of type **195**. The enrichment of diastereomers can be explained by the

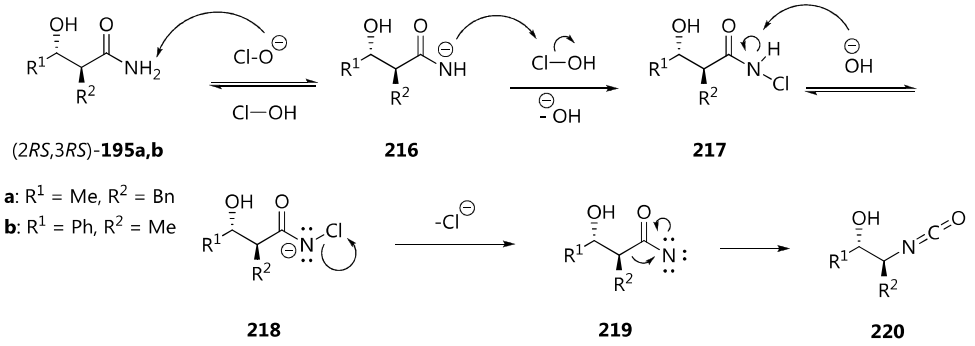


Figure 147. Proposed mechanism of the hypochlorite-induced HOFMANN rearrangement of diastereomeric β-hydroxy amides.

steric hindrance of the α-chiral substituent, thus preferring the hydride addition to form the *anti*-hydroxy amide. The HOFMANN rearrangement can be used for the functional-group interconversion (FGI) of these carboxylic, primary amides to their corresponding chain-shortened amines. This reaction occurs with halonium ions in basic media, usually by combining the haline with aqueous sodium hydroxide. In the present work, the β-hydroxy amide **195a** was degraded with a commercially available sodium hypochlorite solution (15 w-%, see **Figure 149**, page 136). The basic reagent first deprotonates the primary amide **195** in an equilibrium with its corresponding base **216**, which is in return chlorinated to **217** (see **Figure 147**). This additionally activated chloroamide is deprotonated to its anion **218** and undergoes spontaneous elimination of chloride under formation of a six-electron nitrene intermediate **219**. This nitrene rearranges in a highly concerted manner to the isocyanate **220**, concluding this HOFMANN degradation. For this reason, α-chiral amides can be converted under complete retention of the absolute configuration, without

epimerization. The isocyanate **220** is highly reactive towards nucleophiles and can therefore be used for the synthesis of different carbamate derivatives. Usually, the high excess of water present in the hypochlorite solution leads to the formation of a carbamic acid of type **223**, which decarboxylates to the free amine of type **194** or its hydrochloride (see **Figure 148**).

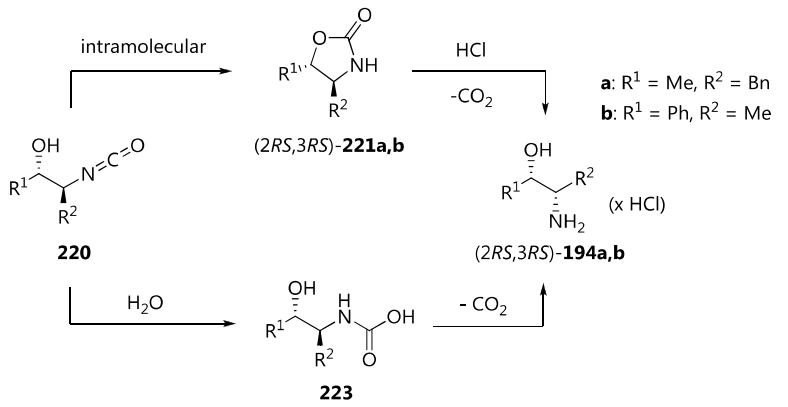


Figure 148. Nucleophilic addition derivatizing the isocyanate **220** by an inter- or intramolecular reaction to obtain the corresponding β-amino alcohol.

Since the β-hydroxy amides in this work are all diastereomers, an unreported epimerization would be observable by chiral HPLC analysis but also in ^{1}H-NMR spectra. Interestingly, the HOFMANN rearrangement yielded predominantly the intramolecular addition product of type **221**.

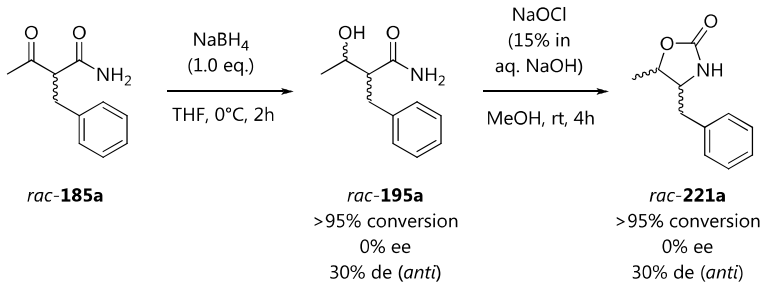


Figure 149. Borohydride reduction of racemic β-oxo amide and subsequent Hofmann rearrangement under retention of the diastereomeric excess.

This oxazolidinone **221** was analyzed with ^{1}H-NOESY spectra to identify the NMR shifts of its *syn*- and *anti*-diastereomers. Based on this data and the premiss of a complete retention of the absolute configuration, the *syn*- and *anti*-diastereomers of the β-hydroxy amide were also assigned. The diastereomeric excess was therefore determined by ^{1}H-NMR integration of the crude products. Even for the mainly racemic reference compounds, the diastereomeric excess of 30% de for the intermediate *anti*-β-hydroxy amide **195a** was unchanged in the oxazolidinone **221a**, which was obtained in

quantitative conversion and again with a diastereomeric excess of 30% de for its *anti*-diastereomer (see **Figure 149**). This is in accordance with the theoretical course of the HOFMANN degradation to proceed with complete retention of the absolute configuration.

4.5 SYNTHESIS OF β-LACTONE DERIVATIVES FOR THE PRODUCTION OF NOVEL INHIBITORS OF BACTERIAL VIRULENCE FACTORS

Another pharmacologically relevant structure is a β-lactone for the inhibition of bacterial virulence factors.[9293] These β-lactones function essentially like β-lactams, inhibiting proteins by irreversible acylation. However, antibiotics based

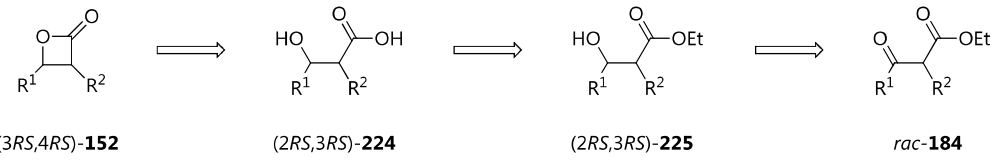


Figure 150. Retrosynthetic approach for the production of enantio- and diastereomerically pure β-lactones from racemic β-oxo esters.

on β-lactams are no universal remedies and the number of pathogens being immune to them is increasing rapidly. These bacteria are widely known a Multi-Drug-Resistant Organisms (MDROs) and are usually classified by their ability to counter certain antibiotics, e.g. penicillin and vancomycin, like the methicillin-resistant *Staphylococcus aureus* (MRSA). This rise in resistance is the driving force behind new drug motifs, like β-lactones of type **152** (see **Figure 150**).

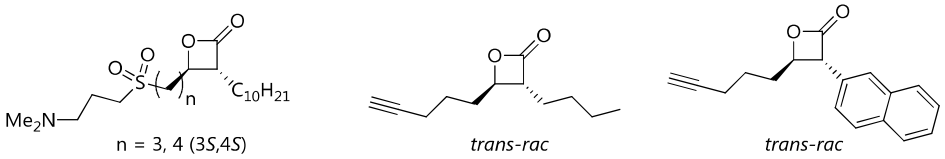


Figure 151. Lead structures of antibiotic β-lactones determined by the SIEBER group.[92]

Initial studies from the SIEBER group determined a series of β-lactone structures which were potent inhibitors for bacterial virulence factors like the caseinolytic protease ClpP (see **Figure 151)**.[92] A proof of concept for the chemoenzymatic synthesis of related structures, containing an enantio- and diastereoselective

reduction with alcohol dehydrogenases, was investigated in the present work (see **Figure 150**).

4.5.1 State of the scientific knowledge on β-lactone syntheses

The lead structures of antibacterial β-lactones were determined by Böttcher and Sieber, who synthesized the inhibitors by a condensation reaction of thioesters **226** with aldehydes of type **228** (see **Figure 152**). [94,95]

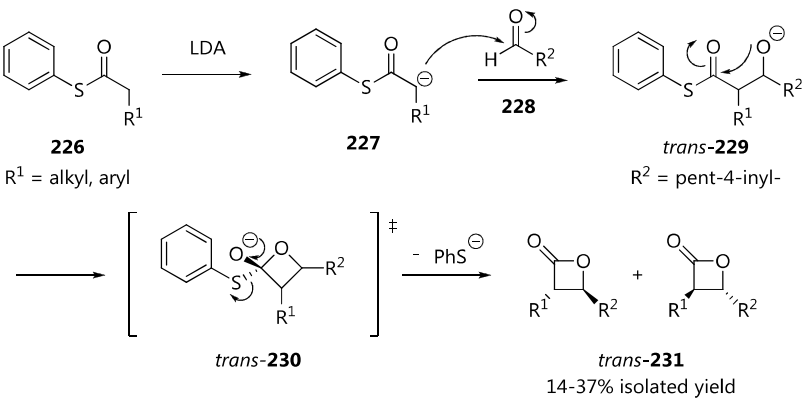


Figure 152. Synthetic approach for racemic *anti*-β-lactones by aldol addition and thioester substitution by Böttcher and Sieber.[95]

Abbreviations: Lithium *N,N*-diisopropylamide (LDA).

The aldolate **229** substitutes the thioester intramolecularly under formation of the β-lactone **231** in low to moderate isolated yields of 14-37%. Notably, only racemic mixtures of *anti*-lactones could be prepared by this method. Chandra *et al.* on the other hand successfully synthesized *syn*-β-lactones from achiral precursors by utilizing chiral alkaloids of type **232** and **235** in optically pure

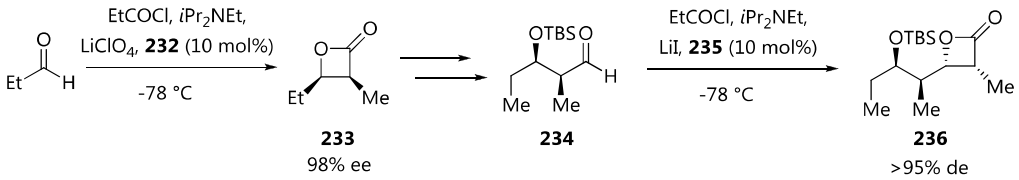


Figure 153. Synthesis of 3-methylated β-lactones as chiral precursors in the synthesis of Erythronolide B by Chandra *et al.*.[96]

form (see **Figure 153**).[96] The β-lactones **233** and **236** were used as activated precursors in a multi-step synthesis of Erythronolide B and were obtained in high yields of >95% and excellent diastereoselectivities of >95% de.

Another catalytic synthesis of chiral *syn*-β-lactones of type **239** was achieved by KRAMER *et al.* with insertion of carbon monoxide (CO) into *anti*-configured epoxides **237** using a chromium(III) tetracarbonylcobaltate complex salt **238** (see **Figure 154**).[97] Remarkably, this reaction takes place at already 1 bar of CO and room temperature, with excellent conversions and under full inversion of the sterically less hindered stereogenic center.

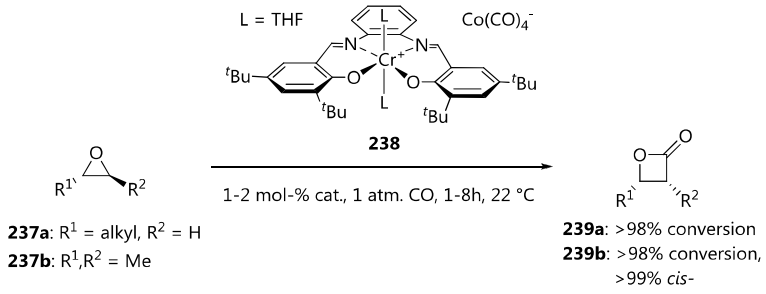


Figure 154. Diastereoselective *syn*-β-lactone synthesis through carbon monoxide insertion into chiral epoxides by KRAMER *et al.*[97]

For the production of pharmaceutically relevant *trans*-β-lactones, KULL and PETERS also applied a metal catalyst **242** for an intramolecular aldolate esterification of aldehydes **241** with carboxylic acid bromides **240** (see **Figure 155**).[98]

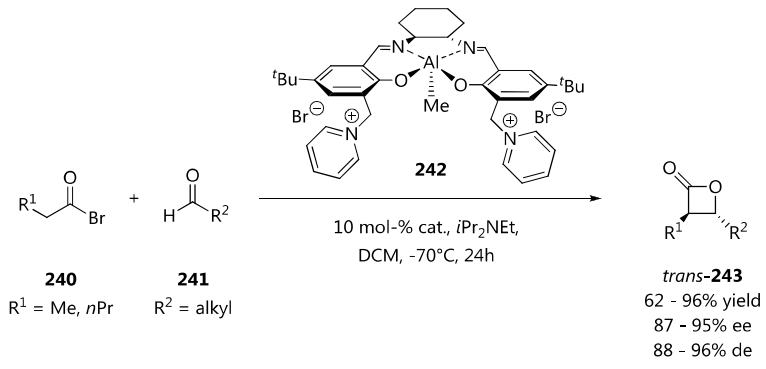


Figure 155. Enantio- and diastereoselective synthesis of *trans*-β-lactones *via* metal-catalyzed condensation of carboxylic acid bromides and aldehydes by KULL and PETERS.[98]

Despite using only short-chained alkyl substituents, the β-lactones **243** were prepared in moderate to high yields of 62-96%, with very good enantioselectivies of 87-95% ee and also excellent diastereoselectivities of up to 96% de. Although all of the described methods are highly versatile regarding the substitution pattern and commercially available starting

materials, high enantio- and diastereoselectivities were only achieved with complex catalysts or enantiomerically pure precursors. Thus, a biocatalytic alternative by enantio- and diastereoselective reductions of racemic β-oxo esters yielding all four possible isomers separately would be an attractive goal.

4.5.2 SUBSTRATE SYNTHESIS AND REFERENCE COMPOUNDS

Apart from the commercially available ethyl 2-benzyl-3-oxobutanoate (**184a**, see **Figure 156**), various β-oxo esters of type **184** were synthesized by alkylation (**Route A**) or in a two-step process combining a KNOEVENAGEL condensation with a C=C reduction (**Route B**). The lead compounds were chosen in regard of a general applicability of the process. In view of a practical synthesis, aromatic and heteroaromatic aldehydes of type **244** were condensed with α-unsubstituted β-oxo esters of type **183**. These reactions were catalyzed by a Bronsted acid/base pair of piperidine/acetic acid or pyrrolidine/trifluoroacetic acid. Subsequently, the α-acetyl β-arylacrylates **245** were reduced by different strategies. For instance, the chloroacetyl derivative **245e** dehalogenated during hydrogenation with palladium on charcoal and was therefore reduced with a stoichiometric amount of a HANTZSCH ester based

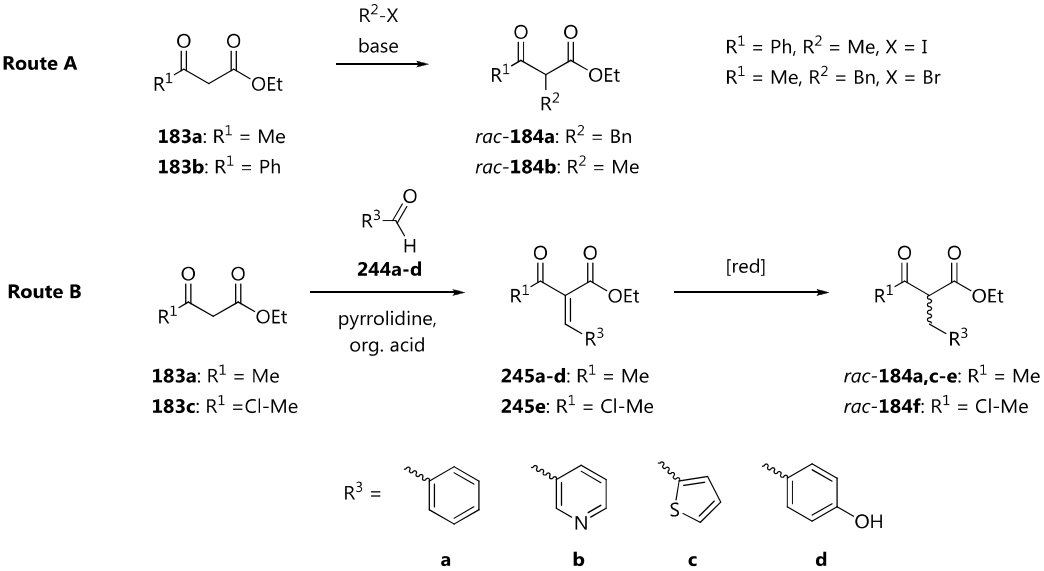
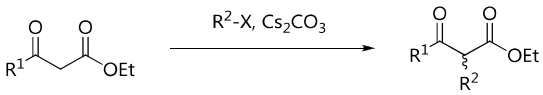


Figure 156. Synthetic strategies for the synthesis of racemic α,β-disubstituted β-oxo esters.

on altered literature procedures from OUELLET *et al.*.[99] The compounds **184** obtained on **Route A** by monoalkylation of α-unsubstituted β-oxo esters of

type **183** were already successfully prepared during the author's diploma thesis[90] and were used after purification *via* column chromatography (see **Table 31**). This was mandatory as the main impurities were the α,α-dialkyl β-oxo esters, which are unable to racemize and could not be used for a dynamic kinetic resolution. In addition, the products would lose their α-chirality.



183a,b **184a,b**

Entry	R^1	R^2	X	Conv.[a] [%]	Isol. Yield[b] [g(%)]
1	Me	Bn	Br	>95	1.63 (79.0)
2	Ph	Me	I	>95	0.75 (34.0)

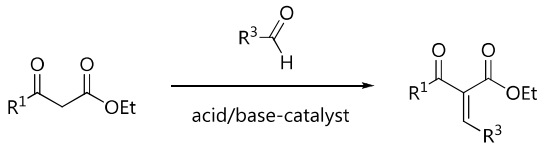
Table 31. Synthesis of α,β-disubstituted β-oxo esters *via* monoalkylation.

(a) The crude product contained mono- and dialkylated compounds in varying ratios (b) Determined after column chromategraphy on silica.

The substrate spectrum was extended to heteroatom-containing aromatic moieties, since these could be beneficial for substrate binding in the active side by interactions with protic peptide residues. Hence, two heteroaromatic compounds, i.e. the 2-thienyl and 3-pyridyl derivatives of substrate **184a**, were synthesized from the corresponding commercially available aldehydes and ethyl acetoacetate (see **Table 32**, page 142, entries 3 and 4). Furthermore, the already established phenyl group was extended to its 4-hydroxy derivative **184e** analogous to a phenylalanine – tyrosine comparison (entry 5). This synthesis was of particular success since the product precipitated in spectroscopically pure form from the reaction mixture when utilizing tert-butyl methyl ether (MTBE) as solvent, reducing the work-up to simple filtration and washing with MTBE. Finally, considering the implementation of β-lactones into larger molecular structures, the methyl substituent of **244a** was altered to its corresponding chloromethyl derivative (entries 2 and 6). The initial synthesis was conducted without solvent and in the presence of an ionic liquid, i.e. 3-methoxypropylamine acetate, which was reported by WANG *et al.* as catalyst for HENRY and KNOEVENAGEL reactions (entries 1 and 2).[100] Although the reaction proceeded with quantitative conversions and moderate isolated yields, the procedure was again changed to a different catalyst. This was due to the compatibility of the pyrrolidine/trifluoroacetic acid catalyst with both KNOEVENAGEL condensation and HANTZSCH reduction. Thus, this protocol was favored in view of a sequential one-pot process for this γ-halogenated β-oxo

ester. A tandem-reaction seems also possible but was not examined in the present work.

The KNOEVENAGEL products **244** were either purified or used as crude material for the subsequent C=C reduction to the actual substrates of type **184** (see **Table 33**). While most of the acrylates of type **244** were reduced by hydrogenation with palladium on charcoal at atmospheric pressure and room temperature (see **Table 33**, page 143, entries 1, 4 and 6), the 3-pyridyl derivative **244c** exhibited a lower reactivity and a non-quantitative conversion of 47.2% after 18 hours (entry 5). As mentioned before, the chloromethyl compound **244e** was dehalogenated under the same conditions, leading to the



183a,c *E/Z*-**244a-e**

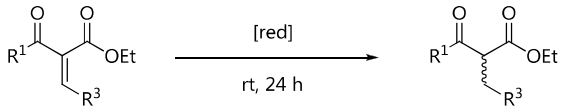
Entry	**183** [mmol]	R^1	R^3	Acid/base	Solvent	Conv.[a] [%]	Isol. Yield[b] [g(%)]
1	100	Me	Ph	MPA/AcOH	none	>95	12.2 (56.1)
2	50	Cl-Me	Ph	MPA/AcOH	none	>95	3.89 (51.3)
3	100	Me	2-Thienyl	Piperidine/AcOH	MTBE	>95	20.6 (n.d.)[a]
4	50	Me	3-Pyridyl	Piperidine/AcOH	MTBE	>95	6.45 (58.8)[b]
5	100	Me	4-OH-Ph	Piperidine/AcOH	MTBE	>95	22.9 (97.9)[c]
6	100	Cl-Me	Ph	Pyrrolidine/TFA	EtOAc	>95	19.8 (n.d.)[a]

Knoevenagel condensation of aromatic aldehydes with β-oxo esters.

(a) The crude product contained unreacted residual aldehyde and excess ethyl acetoacetate (b) Determined after purification by column chromatography, recrystallization or distillation, respectively.

respective substrate **184a**.

In contrast to that, the hydride reduction with a HANTZSCH ester was successful with a product-related conversion of 91.3% (entry 3). Unfortunately, the *E*-acrylate **245e** and the corresponding racemic β-oxo ester **184f** showed similar retention times on TLC analysis, aggravating the establishment of a purification method. Additionally, also preparative HPLC yielded always mixtures with residual *E*-**245e**. Hence, the biocatalytic experiments were conducted with mixtures.



E/Z-**245a-e** *E/Z*-**184a,c-f**

Entry	R^1	R^3	[red]	Solvent	Conv.[a] [%]	Isol. Yield[b] [g(%)]
1	Cl-Me	Ph	Pd/C, H_2	none	>95	n.d.[a]
2	Cl-Me	Ph	HANTZSCH ester	EtOAc	91.3	n.d.
3	Me	2-Thienyl	Pd/C, H_2	MTBE	>95	0.91 (91.3)
4	Me	3-Pyridyl	Pd/C, H_2	MTBE	47.2	n.d.
5	Me	4-OH-Ph	Pd/C, H_2	MTBE	>95	0.95 (94.8)

Table 33. Synthesis of racemic β-oxo esters by ene reduction of KNOEVENAGEL products.

(a) Determined by ^{1}H-NMR spectroscopy (b) Determined after purification.

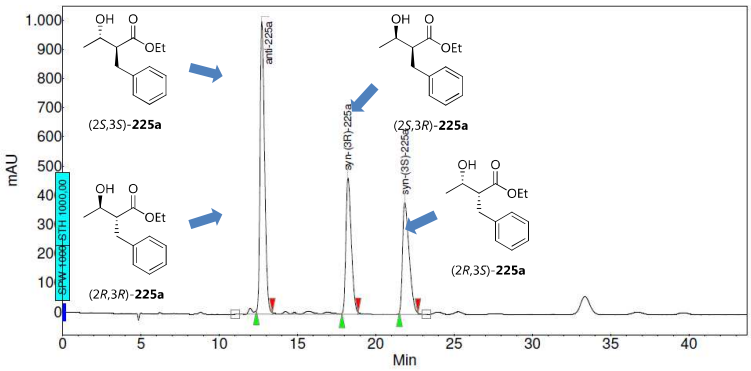
4.5.3 ANALYTICAL METHODS FOR THE DETERMINATION OF THE ENANTIO- AND DIASTEREOSELECTIVITY

The reference compounds of racemic β-hydroxy esters of type **225** were prepared from crude mixtures of hydrogenated KNOEVENAEGEL products by sodium borohydride reduction in tetrahydrofuran (THF) at 0-4°C. This solvent and temperature were mandatory as β-oxo esters can be further reduced to their corresponding 1,3-diols, in contrast to the expected chemoselectivity of sodium borohydride to reduce solely the ketone moiety. This was already observed during the own diploma thesis[90] but is also known in literature by the work of KIM *et al.*, who investigated the borohydride reduction comparing methanol and THF as solvents.[101] Since both β-oxo esters and activated C=C bonds can be reduced by hydrides, crude mixtures containing residual KNOEVENAGEL products **245** did not need to be purified to obtain spectroscopically pure racemic β-oxo esters of type **225**.

ETHYL (2RS,3RS)-2-BENZYL-3-HYDROXYBUTANOATE

This compound was chosen as a reference for the biocatalytic reduction with alcohol dehydrogenases (ADH) since it was already successfully used during

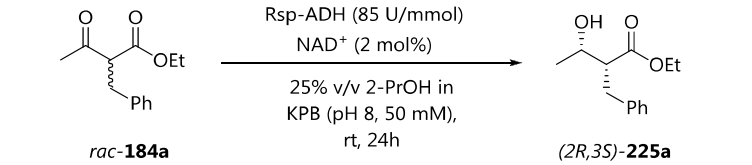
the own diploma thesis.[90] However, new analytical methods for its conversion needed to be established as a base for the novel substrates **184c-f**, which are literature-known in organic synthesis but have never been used with an ADH before. While the conversion could be determined *via* ^{1}H-NMR by integration of the benzylic protons due to the shifting after carbonyl reduction, the enantiomeric excess was calculated by integration of the enantiomeric peaks on chiral normal-phase HPLC (see **Figure 157**).

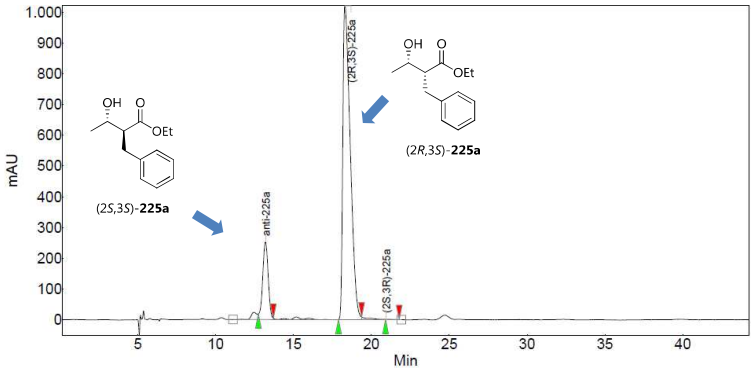


Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	anti-225a	12,72	46,99	1003,3	335,3	46,991
2	syn-(3R)-225a	18,21	26,53	467,0	189,3	26,529
3	syn-(3S)-225a	21,84	26,48	384,2	188,9	26,480
Total			100,00	1854,5	713,5	100,000

Figure 157. HPLC chromatogram of racemic **225a** for the determination of the enantio- and diastereomeric excess.

Although the four diastereomers could not be separated completely, the enantiomeric excess of the *syn*-β-hydroxy esters of **225a** was determined. Also, the diastereomeric excess was quantified by integration of *syn*- and *anti*-diastereomers on chiral HPLC and confirmed by ^{1}H-NMR integration. In contrast to enantiomers, diastereomers have altered magnetic environments and can be differentiated by NMR spectroscopy.For exemplary purposes, the HPLC analysis of the product of a biocatalytic reduction with an alcohol dehydrogenase from *Rhodococcus* sp., is presented (see **Figure 158**). This ADH is predominantly (*S*)-selective for the carbonyl reduction. Hence, the enantiomerically enriched peak of the *syn*-enantiomers was assigned with the (2*R*,3*S*)-configured β-hydroxy ester **225a**. Based on the integration, the enantioselectivity for the *syn*-product (2*R*,3*S*)-**225a** was higher than 99.8% ee. In addition, the diastereoselectivity for the *syn*-product was 68.9% de.





Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	anti-225a	13,20	15,52	253,1	104,0	15,519
2	(2R,3S)-225a	18,29	84,38	1030,7	565,6	84,382
3	(2S,3R)-225a	20,93	0,10	1,1	0,7	0,099
Total			100,00	1284,9	670,2	100,000

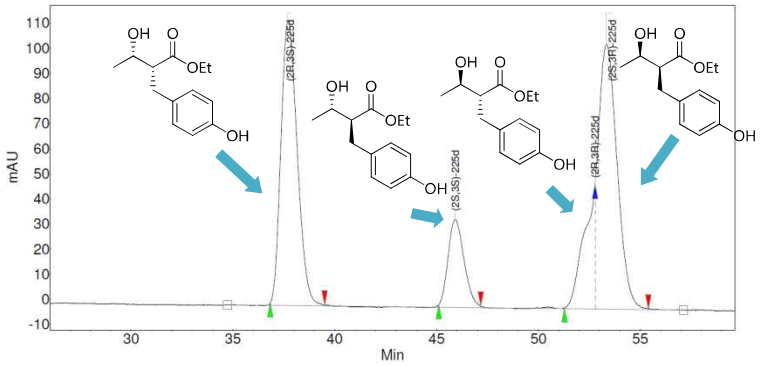
Figure 158. HPLC chromatogram of the biocatalytic reduction of **184a** with an alcohol dehydrogenase from *Rhodococcus* sp..

The values obtained for the other alcohol dehydrogenases were determined analogously. Nevertheless, a separation method for the *anti*-β-hydroxy esters would be essential for analyzing *anti*-selective biocatalysts in future experiments.

Ethyl (2RS,3RS)-2-(4-hydroxyphenylmethyl)-3-hydroxybutanoate

The *p*-hydroxyphenylmethyl substrate **184e** was synthesized in high purity due to its crystallization after Knoevenagel condensation and high reactivity for the palladium/charcoal-catalyzed hydrogenation. Spectroscopically pure reference compounds were obtained after borohydride reduction, facilitating the establishment of analytical methods (see **Figure 159**). In contrast to the heteroaromatic substrates, the 4-hydroxyphenylmethyl compound **225d** was separated on chiral HPLC, at least for highly enantioselective biocatalysts. To emphasize this, the biocatalytic reduction of **184e** with an alcohol dehydrogenase from *Rhodococcus* sp. is shown (see **Figure 160**). As stated before, this ADH is (*S*)-selective for the carbonyl reduction and showed *syn*-selectivity for the standard substrate **184a**.

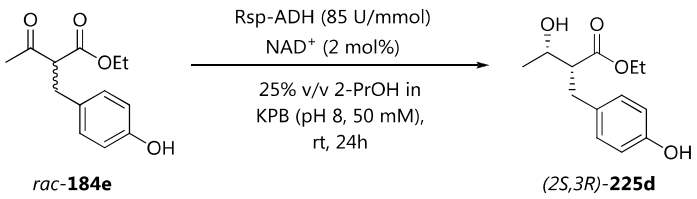
Figure 159. HPLC chromatogram of racemic **225d** for the determination of the enantio- and diastereomeric excess.



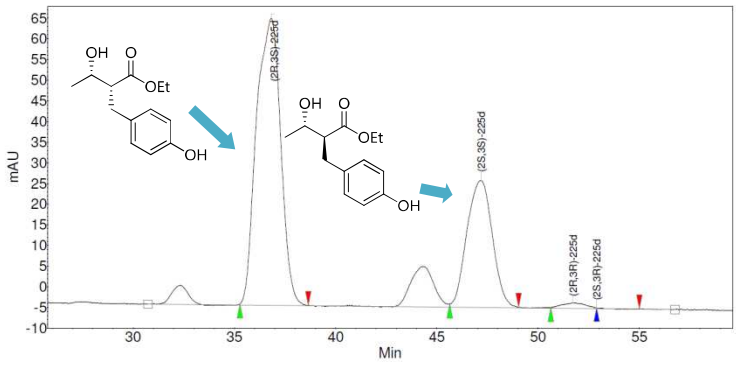
Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(2R,3S)-225d	37,70	37,55	113,4	104,9	37,551
2	(2S,3S)-225d	45,92	11,41	35,0	31,9	11,408
3	(2R,3R)-225d	52,78	11,14	52,2	31,1	11,138
4	(2S,3R)-225d	53,33	39,90	105,4	111,4	39,903
Total			100,00	306,0	279,3	100,000

It was therefore used to assign the absolute configuration of all diastereomers of **225d**. Although an integral for the corresponding (*R*)-carbinol can be observed, some impurities were produced during the biocatalytic reduction.

Figure 160. HPLC chromatogram of the biocatalytic reduction of **184e** with an alcohol dehydrogenase from *Rhodococcus* sp.



rac-**184e** *(2S,3R)*-**225d**



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(2R,3S)-225d	36,82	67,36	69,5	96,1	67,356
2	(2S,3S)-225d	47,17	31,36	30,8	44,8	31,358
3	(2R,3R)-225d	51,73	1,28	1,3	1,8	1,284
4	(2S,3R)-225d	52,90	0,00	0,1	0,0	0,002
Total			100,00	101,7	142,7	100,000

The corresponding absorption spectrum was also different. A possible explanation would be the pH of the aqueous reaction mixture. The enzymatic reaction was done at a pH of 8.0, which would certainly convert some of the free p-hydroxyphenyl products into their corresponding sodium phenolates. The slight yellow coloration during the reaction also confirmed this hypothesis due to its disappearance after extraction with tert-butyl methyl ether (MTBE). Nevertheless, even without purification the enantiomeric excess of the *syn*-(3*S*)-product **225d** can be calculated to >99.9% ee.

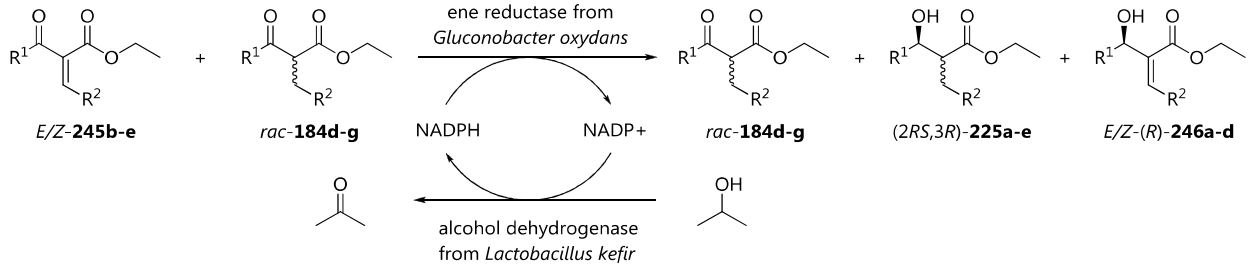
The other b-hydroxy esters **225b-c** and **225f** were analyzed in the same way.

4.5.4 ENOATE REDUCTASE-CATALYZED SYNTHESIS OF RACEMIC β-OXO ESTERS

From a conceptional point of view, the C=C reduction of α-acyl-β-arylacrylates of type **244** could also be achieved by a biocatalytic reduction, more specifically with an ene reductase. Therefore, the synthesized KNOEVENAGEL products **244** were investigated in a one-pot reaction with an ene reductase from *Gluconobacter oxydans* (Gox-ER) in combination with an alcohol

Table 34. Preparative screening for different activities of oxidoreductases on mixtures of unsaturated and saturated β-oxo esters.

(a) Determined by ^{1}H-NMR spectroscopy (b) Determined by chiral HPLC analysis.



Entry	R^1	R^3	Substrate E/Z-**245**	Product E/Z-**245**	Conv. to **184**	Conv. to **225**	Conv. to **246**
1	Cl-Me	Ph	62:38	59:41	n.d.[b]	n.d.[b]	6.3
2	Me	2-Thienyl	76:24	5:95	82.0	n.d.[b]	n.d.[b]
3	Me	3-Pyridyl	77:23	39:61	22.4	n.d.[b]	n.d.[b]
4	Me	4-OH-Ph	74:26	54:46	19.1	obs[c].	n.d.[b]

dehydrogenase from *Lactobacillus kefir* (LK-ADH, see **Table 34**, page 147).

This ADH was primarily implemented for an *in situ* cofactor regeneration with 2-propanol as cosolvent and cosubstrate. Due to the necessity of the cofactor NADPH for the ene reductase, alcohol dehydrogenases preferring NADH could not be used. In addition, previous experiments with the substrate **184a** indicated a very low activity of LK-ADH for β-oxo esters of this substitution pattern, minimizing the risk of undesired side-reactions. Thus, the C=C reduction to the racemic β-oxo ester should have been predominant and observable by chiral HPLC. Remarkably, the reaction of the chlorinated acrylate **244e** resulted in the carbonyl reduction to the β-hydroxycinnamic ester (see **Table 34**, page 147, entry 1). No ene reduction was observed, whether to the racemic β-oxo ester **184f** or the double-reduced β-hydroxy ester **225e**. Judging from the comparison with the other experiments, the chloromethyl substituent seems to enhance the activity of this substrate, at least for the biocatalytic reduction with LK-ADH. On the contrary, the heteroaromatic derivatives showed moderate to high activities for the ene reductase from Gluconobacter oxydans and no ADH-related carbonyl reduction. The 2-thienyl substrate **244b** was reduced with 82.0% conversion to the racemic β-oxo ester **184c** (entry 2). Also the 3-pyridyl derivative **244c** was reduced, however, with a lower activity, reaching a conversion of 22.4% under equal reaction conditions (entry 3). Also surprising was the formation of enantio- and diastereomerically enriched β-hydroxy ester of the 4-hydroxyphenyl substrate **244d** (entry 4), suggesting also enhanced reactivity for LK-ADH. Both enzymes showed activity for C=C or C=O reduction, respectively. Unfortunately, the experiment was done with a crude mixture of **244d** and contained an impurity overlaying two of the diastereomer peaks on the chiral HPLC. Nevertheless, the lack of one *anti*-enantiomer indicated a highly enantioselective carbonyl reduction to the (*3R*)-hydroxy ester **225d**. These initial experiments showed promising tendencies for the subsequent preparative screenings of alcohol dehydrogenases.

4.5.5 Enantio- and diastereoselective synthesis of β-hydroxy esters *via* alcohol dehydrogenases

Based on the established analytical methods for the β-oxo ester reduction, the substrates were preparatively screened with available alcohol dehydrogenases. The investigated biocatalysts were already discussed in chapters **4.2.2** (page 121) and **4.3.3.2** (page 133) for the reduction of β-oxo amides and β-oxo

nitriles, respectively. The separation of standard substrates obtained by monoalkylation and the compounds obtained by KNOEVENAGEL reaction is related to the purity of the substrates (see Table 35). In accordance with the experimental results from the reduction of β-oxo nitriles (chapter **4.3.3.2**, page 133), the α-benzylated β-oxo ester **184a** was converted with all of the investigated biocatalysts. Especially the alcohol dehydrogenase from *Rhodococcus* sp. was highly active with quantitative conversion and with an excellent enantioselectivity of >99% ee for the (3*S*)-enantiomer (see Table 35, page 149, entry 1). In addition, the diastereopreference for the *syn*-product was also very good, with a diastereomeric excess of up to 80% de. In contrast to this high activity, the 3-phenyl derivative **184b** was not converted at all, undermining the occasional limitation of alcohol dehydrogenases not to convert ketones with two bulky substituents.

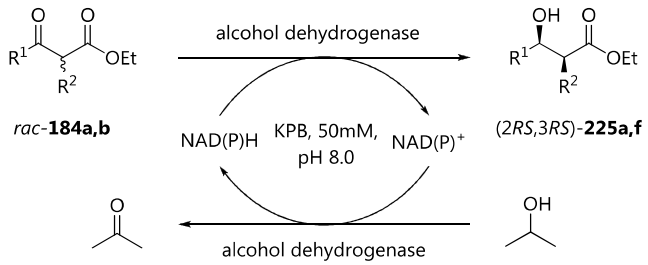
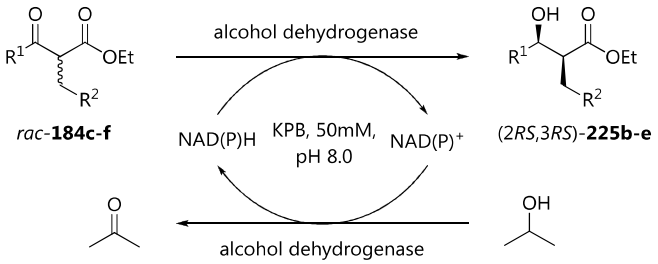


Table 35. Preparative screening of monoalkylated β-oxo esters with alcohol dehydrogenases

(a) Determined by ^{1}H-NMR spectroscopy (b) Determined by chiral HPLC analysis.

Entry	R^1	R^2	ADH (selectivity)	Conv.[a] [%]	ee[b] [%]	de[b] [%]
1	Me	Bn	*Rhodococcus sp. (S)*	>95	>99 (3*S*)	80 (*syn*)
2	Me	Bn	*evocatal 1.1.200 (R)*	68	>99 (3*R*)	95 (*syn*)
3	Me	Bn	*Lactobacillus* sp. (*R*)	24	>99 (3*R*)	33 (*anti*)
4	Ph	Me	*Rhodococcus sp. (S)*	-	-	-
5	Ph	Me	*evocatal 1.1.200 (R)*	-	-	-
6	Ph	Me	*Lactobacillus* sp. (*R*)	-	-	-

The preparative screening with other 2-arylmethyl substrates was confined to the 2-thienylmethyl (**184c**) and the 4-hydroxyphenyl (**184e**) derivative. For these cases, the crude products were analyzed for enantio- and diastereopreference of the enzymes. Unfortunately, an unidentified impurity overlaid the integrated diastereomers in the reactions with the bacterial ADH evocatal 1.1.200.



Entry	**184**	R^1	R^2	ADH (selectivity)	Conv.[a] [%]	ee[b] [%]	de[b] [%]
1[c]	**a**	Me	Ph	*Rhodococcus sp. (S)*	>95	>99 (3*S*)	79.8 (*syn*)
2[c]	**a**	Me	Ph	*evocatal 1.1.200 (R)*	68	>99 (3*R*)	94.4 (*syn*)
3	**c**	Me	2-Thienyl	*Rhodococcus sp. (S)*	>95	>99 (3*S*)	30.4 (*syn*)
4	**c**	Me	2-Thienyl	*evocatal 1.1.200 (R)*	>95	>99 (3*R*)	n.d.[d]
5	**e**	Me	4-OH-Ph	*Rhodococcus sp. (S)*	>95	>99 (3*S*)	36.5 (*syn*)
6	**e**	Me	4-OH-Ph	*evocatal 1.1.200 (R)*	>95	>99 (3*R*)	n.d.[d]

Table 36. Preparative screening of heteroatom-containing α-arylmethyl β-oxo esters with alcohol dehydrogenases

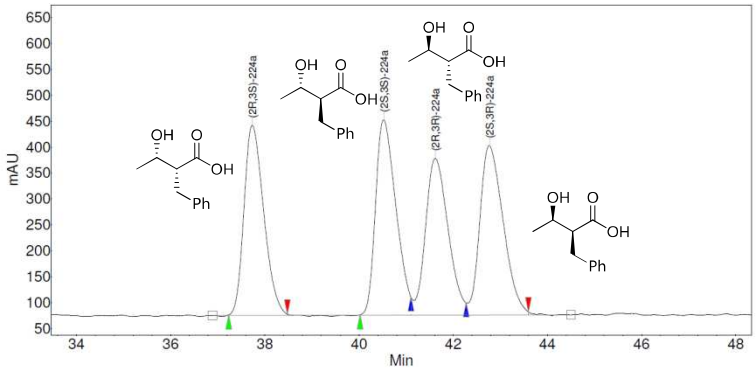
(a) Determined by ^{1}H-NMR spectroscopy (b) Determined by chiral HPLC analysis (c) Reference entries for comparison with standard substrate (d) Peaks overlaid by unidentified impurity.

Therefore, only the enantiomeric excess of the syn-products were successfully determined. The biocatalysts showed the expected excellent enantioselectivities of >99% ee for the corresponding (*R*)- or (*S*)-carbinol. The diastereomeric excess was moderate with 30.4% de for the 2-thienylmethyl β-oxo ester **184c** and 36.5% for the *p*-hydroxyphenylmethyl ester **184e**, respectively. This is nevertheless promising since the low diastereomeric excess is based on a controllable competition between the pH-induced racemization and the biocatalytic conversion. The high enzyme amount used in these initial experiments led to a faster overall conversion. By reducing the catalyst amount, the racemization could induce a more effective dynamic kinetic resolution.

4.5.6 Biocatalytic hydrolysis of beta-hydroxy esters and combination with an enzymatic reduction

The hydrolysis of enantio- and diastereomerically enriched β-hydroxy esters **225** to their corresponding β-hydroxy carboxylic acids **224** was achieved by rather simple hydrolysis with an ethanolic sodium hydroxide solution (1.25 M) and stirring at 60 °C. Notably, in contrast to the β-oxo esters, the β-hydroxy esters did not epimerize during hydrolysis in basic aqueous medium.

Nevertheless, during storage at 4-8 °C for several hours, diastereomerically enriched crystals of **224a** were formed in the crude product. Recrystallization of the product in MTBE yielded spectroscopically pure **224a** with an altered diastereomeric excess of 70% de. The racemic β-hydroxy ester **225a** was also hydrolyzed for establishing chiral HPLC methods (see **Figure 161**).



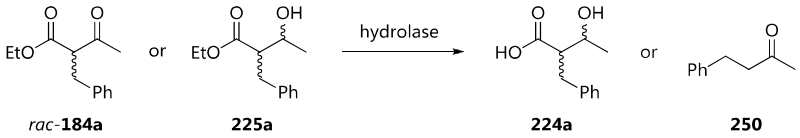
Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(2R,3S)-224a	37,73	24,61	367,4	178,4	24,607
2	(2S,3S)-224a	40,53	26,02	377,1	188,7	26,024
3	(2R,3R)-224a	41,62	23,40	302,3	169,7	23,403
4	(2S,3R)-224a	42,77	25,97	327,1	188,2	25,966
Total			100,00	1373,9	725,0	100,000

Figure 161. HPLC chromatogram of racemic **224a** for the determination of the enantio- and diastereomeric excess.

The racemic mixture was almost completely separated. In addition, the absolute configurations of the corresponding peaks were assigned by comparison with the product of a biocatalytic hydrolysis with porcine liver esterase (PLE). This enzyme was used after a photometric screening assay identified potent hydrolases for the saponification of the racemic β-hydroxyester **225a** (see **Figure 162**). In view of a bienzymatic tandem process with alcohol dehydrogenases, the racemic β-oxo ester **184a** was also screened. Unfortunately, all active hydrolases showed even higher activity for the hydrolysis of the starting material. Figure X shows the results of the photometric screening with the acid-base indicator bromothymol blue. Due to the formation of a carboxylic acid, the pH of the reaction mixture decreases, leading to a yellow absorption for the protonated indicator species. This coloration is measured at 432 nm or 615 nm, respectively. The latter was used in this example. The highest activity, observed with the strongest coloration to yellow, was measured for the porcine liver esterase (PLE, row E). However, the b-oxo ester 184a was more active and led to the formation of an unstable b-oxo carboxylic acid which decarboxylates to the achiral ketone **250**. In the presence of alcohol dehydrogenases this ketone would certainly be reduced to the corresponding carbinol, which itself would remain as impurity. To get a deeper insight in the respective reactivities of the b-oxo ester **184a** and the b-

hydroxy ester **225a**, both substrates were tested in a preparative screening with PLE and in comparison with a parallel alcohol dehydrogenase-catalyzed reduction.

Figure 162. Photometric screening assay for the identification of potent hydrolases for the saponification of β-oxo and β-hydroxy esters.



rac-**184a** **225a** **224a** **250**

	184a	184a	225a	225a	BL
A	0.83	0.83	0.94	0.91	0.90
B	1.60	1.23	1.29	1.48	1.37
C	0.88	0.91	1.00	1.03	1.00
D	0.89	0.94	1.06	1.01	0.99
E	0.44	0.45	0.60	0.60	0.88
F	0.90	0.93	1.03	1.03	0.95
G	0.83	0.74	1.01	0.94	0.91
H	1.39	1.30	1.49	1.07	1.58

	184a	184a	225a	225a	BL
I	0.83	0.84	0.95	0.96	0.92
J	0.93	0.89	1.01	1.02	1.05
K	0.71	0.72	0.86	0.85	0.85
L	0.97	0.97	1.08	1.13	0.99
M	0.87	0.89	1.05	1.02	0.94
N	0.89	0.92	1.02	0.94	0.91
O	0.87	0.90	1.04	1.03	0.98
P	0.93	0.94	1.04	1.08	0.99

A	Protease from *Rhizopus* sp.	**I**	Pronase from *Streptomyces griseus*
B	Alcalase ® CLEA	**J**	Protease from *Aspergillus saitoi*
C	Protease from *Aspergillus* sp. (ABCR)	**K**	Protease from bovine pancreas
D	Amano Acylase (*Aspergillus melleus*)	**L**	Protease S, *Pyrococcus furiosus*
E	Porcine liver esterase (PLE, crude)	**M**	Amano Lipase M, *Mucor javanicus*
F	Protease from *Bacillus amilolyquefaciens*	**N**	Protease from *Bacillus* sp. (Sigma)
G	α-Chymotrypsin (Biozym CHY-03)	**O**	Protease from *Bacillus licheniformis*
H	Amano Lipase PS (*Pseudomonas cepacia*)	**P**	Protease from *Aspergillus oryzae*

Remarkably, the biocatalytic hydrolysis of *rac*-**225a** with porcine liver esterase stopped at about 47% conversion. Also the one-pot process with racemic β-oxo ester **184a** in the presence of an alcohol dehydrogenase from *Rhodococcus* sp. stopped at this state. A possible product inhibition was subsequently ruled out with the addition of further substrate (see **Figure 163**, page 153, green line) and the observation of the same reaction behavior. Due to the formation of a carboxylic acid, the reaction was continuously titrated with diluted sodium hydroxide (1.0M) and the consumption was recorded).

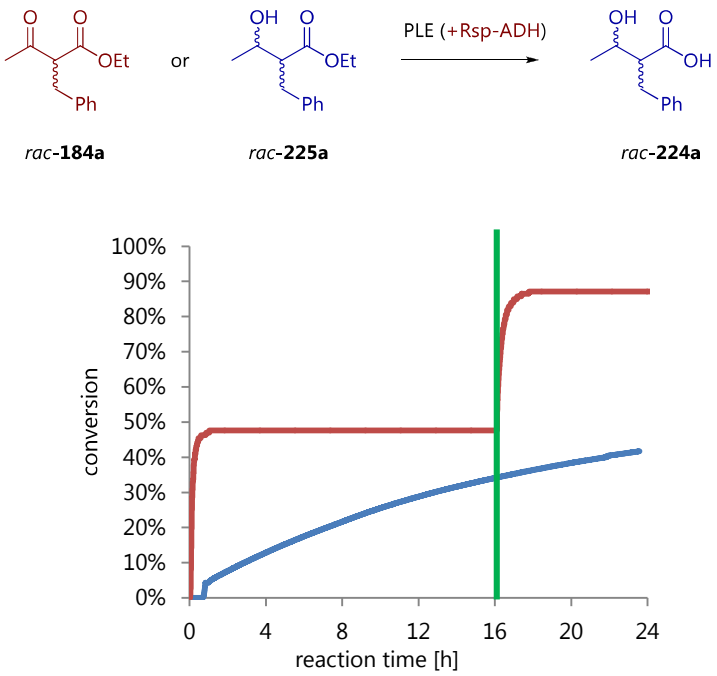


Figure 163. Titer consumption of the biocatalytic hydrolysis with porcine liver esterase (PLE) in a two-step (blue) or one-pot process (red) for the synthesis of enantio- and diastereomerically enriched β-hydroxy carboxylic acids. After 16 hours and reaching a stationary progress, more substrate 184a was added to the one-pot process (green line).

The tremendous difference in activity can be explained by the parallel hydrolysis of racemic β-oxo ester **184a** to its carboxylic acid and subsequent decarboxylation, a side-reaction which was also predominant in the photometric screening assay.

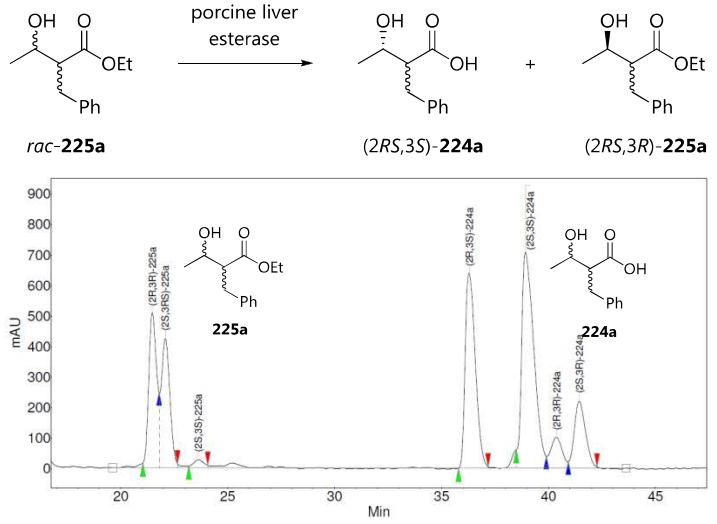


Figure 164. HPLC chromatogram of the biocatalytic hydrolysis of racemic **225a** by porcine liver esterase (PLE).

Analysis of the crude product on chiral HPLC revealed an enantioselectivity of porcine liver esterase for the (3*S*)-diastereomers (see Figure 164, page 153).

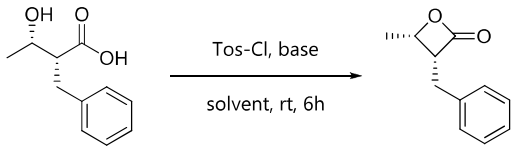
This (*S*)-selectivity for β-hydroxy esters was also observed in literature reports for the desymmetrization of dialkyl 3-hydroxyglutarates by SANTANIELLO *et al.*[102]

4.5.7 LACTONIZATION OF ENANTIO- AND DIASTEREOMERICALLY ENRICHED β-HYDROXY CARBOXYLIC ACIDS

The final step of an intramolecular esterification for the synthesis of β-lactones of type **152** was done by a chemical reaction, since β-lactones are potent inhibitors of hydrolases and are thus not formed by an e.g. lipase-catalyzed transesterification. Common literature procedures for the synthesis of β-lactones from β-hydroxy carboxylic acids use derivatizing reagents activating the carboxylic ester, so it can be substituted by the free carbinol. Mostly, the carboxylate is activated by the addition of benzenesulfonyl chloride[103] or toluenesulfonyl chloride in the presence of pyridine[104]. This way the resulting acidic by-products are neutralized. In addition, the weak nucleophilic carboxylic acid is activated as carboxylate for predominant activation.

Table 37. Chemical derivatization of β-hydroxy carboxylic acids to their corresponding β-lactones.

(a) Determined by ^{1}H-NMR spectroscopy (b) Determined by chiral HPLC analysis.

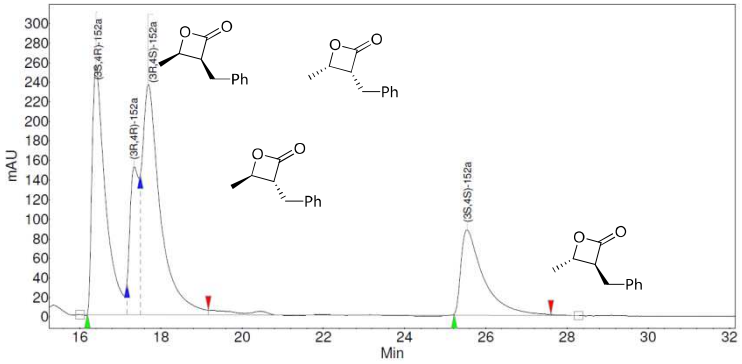


(2*RS*,3*S*)-**224a**
99% ee (3*S*)
70% de (2*R*)

(3*RS*,4*S*)-**152a**

Entry	Base	Solvent	Conv. [%]	ee[b] [%]	de[b] [%]
1	Na_2CO_3	Cyclohexane	>95	>99 (3*S*)	70 (*syn*)
2	Pyridine	Pyridine	>95	>99 (3*S*)	83 (*syn*)

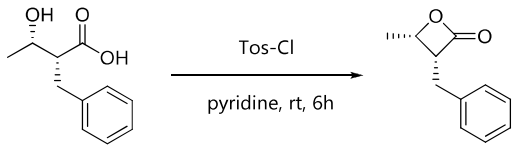
Nevertheless, under these reaction conditions, a possible side-reaction could be the activation of the carbinol as leaving group and S_N2 reaction by the carboxylate under complete inversion of the stereogenic center. Parallel activations would therefore result in lowered diastereoselectivities. Within this work, two reagent combinations were examined (see **Table 37**, page 154).



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(3S,4R)-152a	16,40	30,97	255,5	97,9	30,967
2	(3R,4R)-152a	17,33	12,79	151,3	40,4	12,789
3	(3R,4S)-152a	17,68	38,35	235,7	121,2	38,345
4	(3S,4S)-152a	25,54	17,90	87,2	56,6	17,899
Total			100,00	729,7	316,0	100,000

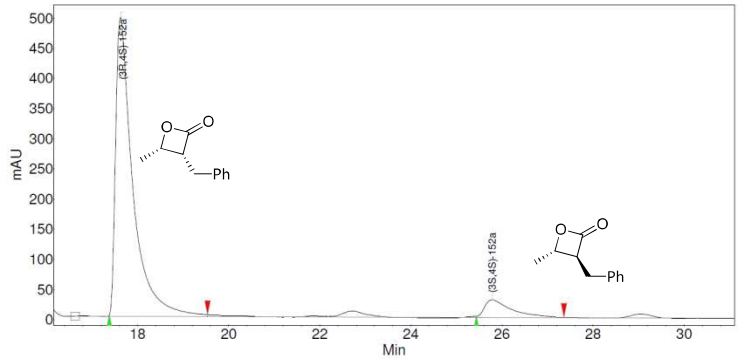
Figure 165. HPLC chromatogram of racemic **152a** for the determination of the enantio- and diastereomeric excess.

On the one hand, the lactonization with toluenesulfonyl chloride (TosCl) in the presence of anhydrous sodium carbonate proceeded smoothly without epimerization of the stereogenic centers. Nevertheless, a 6-fold excess of TosCl and a 12-fold excess of hardly soluble inorganic base seemed exaggerating. Therefore, another reaction was done by reacting **224a** with two equivalents of TosCl in pyridine as solvent.



(2*RS*,3*S*)-**224a**
99% ee (3*S*)
70% de (2*R*)

(3*RS*,4*S*)-**152a**



Index	Name	Time [Min]	Quantity [% Area]	Height [mAU]	Area [mAU.Min]	Area % [%]
1	(3R,4S)-152a	17,63	91,52	496,2	217,6	91,517
2	(3S,4S)-152a	25,78	8,48	29,2	20,2	8,483
Total			100,00	525,4	237,7	100,000

Figure 166. HPLC chromatogram of enantio- and diastereomerically enriched β-lactones with base-induced epimerization towards diastereomeric enrichment.

After extractive work-up, the crude product showed quantitative conversion of the starting material, but also a change in the diastereomeric excess compared to the β-hydroxy carboxylic acid **224a** (see **Figure 166**, page 155). This was presumably related to the high excess of pyridine epimerizing the α-stereogenic center over a prolonged reaction time of 24 hours. Although the epimerization did arbitrarily enrich the desired diastereomer (3*R*,4*S*)-**152a**, the epimerization itself is counteracting the intended retention of the absolute configuration implemented by the biocatalytic reduction. Thus, additional experiments will be necessary to find a suitable lactonization reaction for β-hydroxy carboxylic acids of type **224a**.

4.6 CONCLUSION AND OUTLOOK

The enantio- and diastereoselective reduction of β-oxo carbonyl compounds was successfully achieved for α,β-disubstituted β-oxo nitriles of type **197a** to circumvent the lack of activity for the corresponding β-oxo amides. Three alcohol dehydrogenases were tested for their reduction. All of them showed high activities with quantitative conversion of the substrate, excellent enantioselectivities for the carbonyl reduction and also high diastereoselectivities for the vicinal stereogenic center. By using racemizable β-oxo nitriles **197a**, a dynamic kinetic resolution process for the preparation of enantiomerically pure and diastereomerically enriched β-hydroxy nitriles **196a** was successfully established, with three of four possible diastereomers directly addressable (see **Figure 167**, page 156).

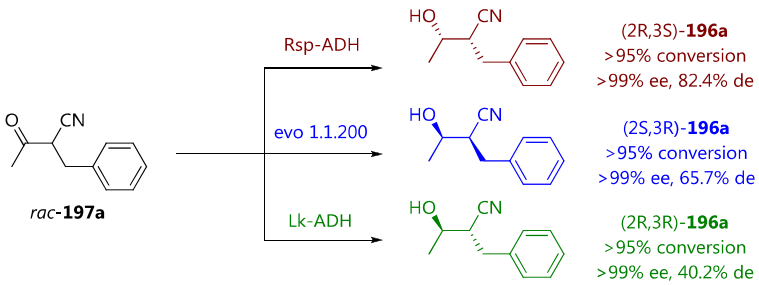
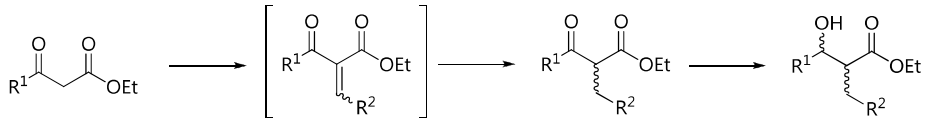


Figure 167. Enantio- and diastereoselective synthesis of three of four possible diastereomers of β-hydroxy nitrile 196a by dynamic kinetic resolution of racemic β-oxonitrile 197a.

Although the subsequent hydration to the corresponding β-hydroxy amides was not investigated in the present work, the chemical degradation of diastereomerically enriched racemic β-hydroxy amides was successfully established under complete retention of the diastereomeric excess. The obtained 4,5-disubstituted oxazolidin-2-ones are excellent precursors for the



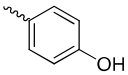
	R^1 = Me, R^2 = 				R^1 = Cl-Me, R^2 = Bn
245:	56.1%	n.d.	58.8%	97.9%	n.d.
184:	-	91.3%	47.2% conv.	94.8%	91.3% conv.
225:	>95% conv. >99% ee 95.0% de	>95% conv. >99% ee 30.4% de	-	>95% conv. >99% ee 36.5% de	-

Figure 168. Synthesis of new racemic β-oxo ester substrates and biocatalytic reduction with alcohol dehydrogenases in a dynamic kinetic resolution process.

synthesis of β-amino alcohols and the cyclic urethane structure represents an intramolecular protecting group for the regioselective monoalkylation of the corresponding amino alcohol.

Besides nitrogen-containing β-oxo carbonyl compounds, also β-oxo esters **184** have been successfully reduced by the use of alcohol dehydrogenases (see **Figure 168**). The stereoselectivities were in good agreement with those obtained for the β-oxo nitriles **197**. Furthermore, a two-step synthesis of β-arylmethyl substrates was realized by combination of a BRÖNSTED acid/base-catalyzed KNOEVENAGEL condensation to obtain the intermediates of type **245** and a subsequent ene reduction with palladium charcoal or a hydride reduction with a Hantzsch ester.

Additionally, a chloromethyl moiety was inserted in view of a subsequent insertion of the β-lactone moiety into larger molecular structures by, for example, a WITTIG reaction. Furthermore, an ene reductase from *Gluconobacter oxydans* showed high activities for the C=C reduction of heteroatom-containing β-arylacrylates. This could be favorable in regard of a bienzymatic tandem reaction for the synthesis of enantio- and diastereomerically pure β-hydroxy esters starting from α-unsubstituted β-oxo esters and aldehydes (see **Figure 169**).

The chemical saponification of the β-hydroxy esters was investigated for a biocatalytic alternative. A photometric screening assay identified porcine liver esterase as potent hydrolase. The enzymatic hydrolysis was successfully achieved in a sequential two-step process after ADH reduction or in a one-pot process starting from racemic β-oxo esters in the presence of an ADH. PLE revealed itself to be highly enantioselective for the hydrolysis of the (3*S*)-β-hydroxyester, thereby further increasing the diastereomeric excess from the biocatalytic reduction with an alcohol dehydrogenase from *Rhodococcus* sp.

Finally, a chemical lactonization protocol was achieved. By using different inorganic or organic bases, an epimerization of the α-stereogenic center was either minimized or draw towards enrichment of the predominant *syn*-diastereomer.

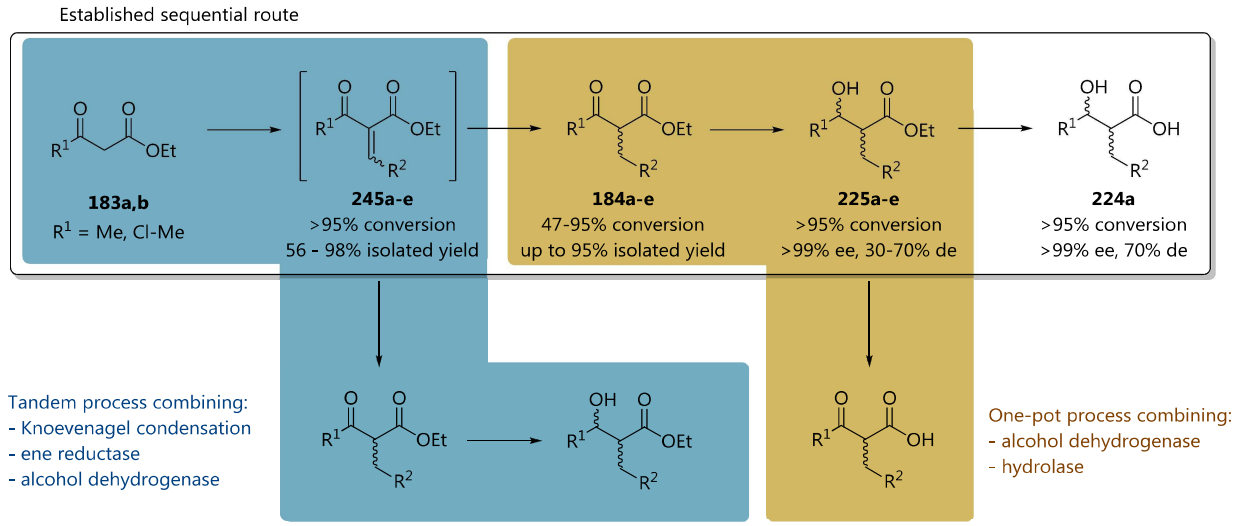


Figure 169. Multi-step synthesis of β-hydroxy carboxylic acids. Sequential vs. combinatorial routes.

As these results demonstrate the versatility of the described chemoenzymatic routes to β-amino alcohols and β-lactones, many reactions were still in the initial stage and could surely be optimized for an enhanced diastereoselectivity. Also, the biocatalytic hydration of β-hydroxy nitriles, as well as the enzymatic hydrolysis of β-hydroxy esters represent a challenge for future experiments.

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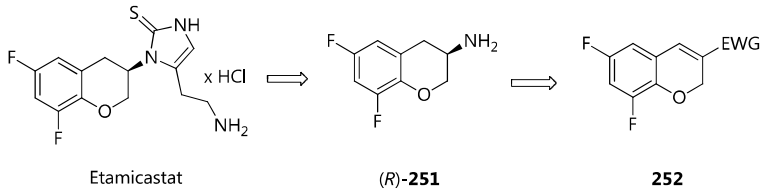
5 CHEMOENZYMATIC SYNTHESIS OF AN ETAMICASTAT PRECURSOR BY ENOATE REDUCTASE-CATALYZED REDUCTION OF 3-SUBSTITUTED 2*H*-CHROMENES

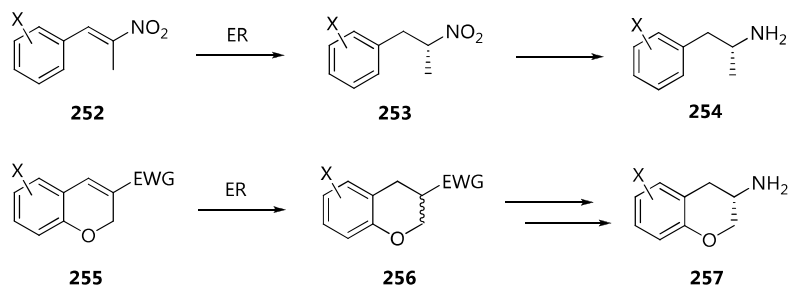
This project was based on a recent publication of a multi-kg scale process for Etamicastat, a dopamine β-hydroxylase inhibitor for the treatment of hypertension and heart failure, by BIAL Portela.[105] The key building block of this compound, the enantiomerically pure 3-aminochroman **251**, can be synthesized from the corresponding 3-substituted 2*H*-chromene by enantioselective C=C reduction and derivatization (see **Figure 176**).



Etamicastat (*R*)-**251** **252**

Figure 176. Retrosynthetic approach to Etamicastat by an enan-tioselective C=C reduction and derivatization to the chiral 3-aminochroman.

This methodology was based on the extensive work of M.Sc. TINA RESS on the enantioselective reduction of α-methylated nitroalkenes of type **252**.[106] The author is grateful for her great experience and expertise in this field and for many fruitful discussions regarding ene reductases (ER).



252 **253** **254**

255 **256** **257**

Figure 177. Literature-known a-methyl nitro-alkene reduction and intended route to the chiral 3-aminochroman building block of Etamicastat by an ene reductase (ER)-catalyzed C=C reduction.

5.1 ENE REDUCTASES FOR THE ENANTIOSELECTIVE SYNTHESIS OF CHIRAL CARBON-CARBON BONDS

The previous chapters comprised of the use of alcohol dehydrogenases for an enantio- or diastereoselective carbonyl reduction and the synthesis of corresponding chiral secondary carbinols. In addition to these C=O reductions, C=C reductions are also achievable with biocatalysis. Ene reductases have become of great interest in the last couple of years and their applicability for bulk and fine chemicals increases steadily.[107] Belonging to the family of Old Yellow Enzymes (OYE) these proteins catalyze the *trans*-selective reduction of activated alkenes by means of a cofactor-dependent enantioselective hydride addition to the β-position and a trans-selective protonation.

Recently, the industrial application of ene reductases was successfully demonstrated by DEBARGE and his coworkers from Pfizer during an evaluation of different synthetic routes to Pregabalin (Lyrica).[108] The enzymatic route is based on the ene reductase-catalyzed reduction of the *E*-3-cyanoacrylic ester **258** to the (*S*)-3-cyano ester **259** (see **Figure 178**). In addition, the biocatalytic reduction of the corresponding β-cyano carboxylic acid **261** is currently under investigation. Interestingly, by using *E*- or *Z*-alkenes, the substrate binding is also inverted, yielding the opposite enantiomer of the product. Nevertheless,

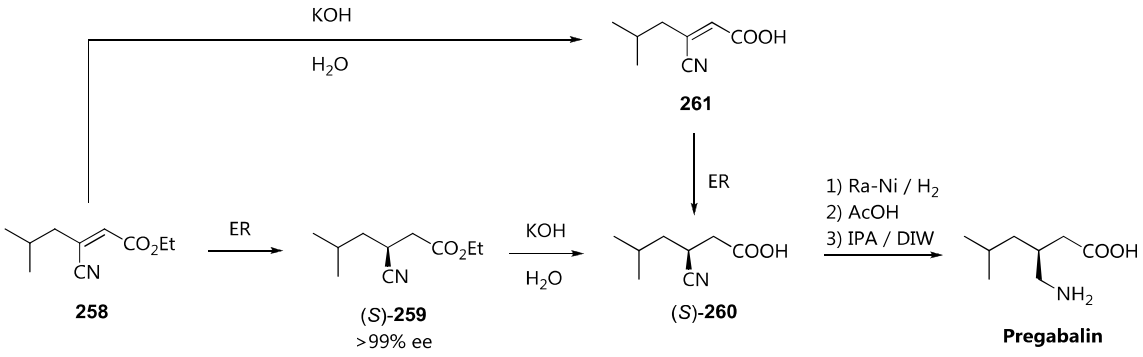


Figure 178. Ene reduct-ase-catalyzed route to Pregabalin (Lyrica) by DEBARGE and coworkers from Pfizer.[108]

the reduction of the pure *E*-acrylic ester yielded the desired product in excellent enantiomeric purities of >99% ee (*S*). Notably, the substitution pattern of the α- and β-position is strongly influencing the enantiopreference of the enzyme. YANTO *et al.* screened various activated alkenes of type **262** for an enantioselective reduction with ene reductases from *Kluyveromyces lactis* (KYE-1) and *Yersinia bercovieri* (Yers-ER) (see **Figure 178**, page 167) .[109] Two closely-related substrates, more specifically α- and β-methyl nitrostyrene, were

reduced with either excellent or no selectivity, depending on the position of the methyl substituent.

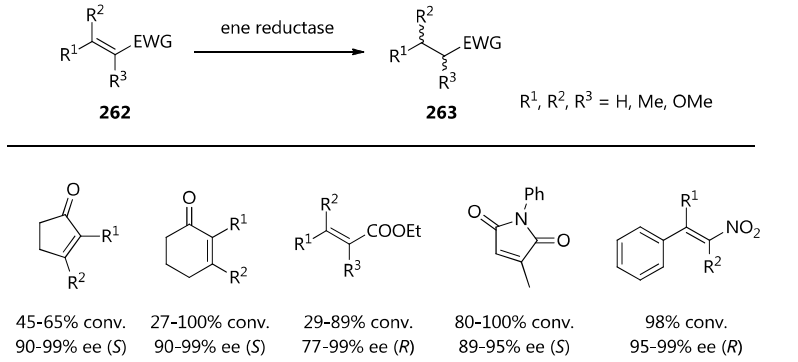


Figure 179. Substrate spectrum for ene reductases demonstrating the versatility of these biocatalysts (YANTO *et al.*).[109]

While the investigated biocatalysts were only selective for the β-methyl substrate, BURDA *et al.* applied an ene reductase from *Gluconobacter oxydans* (Gox-ER) for the highly enantioselective reduction of α-methyl β-aryl nitroalkenes.[110] During their studies, the substitution pattern of the aromatic moiety also affected the enantiomeric excess. Substrates with para-substituents were converted with significantly lower enantioselectivity than meta-substituted α-methyl β-aryl nitroethylenes. Nevertheless, the conversions were predominantly very high and had excellent enantioselectivities of up to 95% ee.

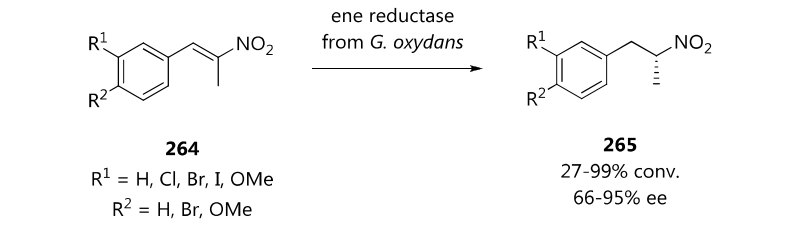


Figure 180. Enantioselective β-aryl α-methyl nitroalkene reduction with an ene reductase from *Glucono-bacter oxydans* by BURDA *et al.*[110]

However, it should also be pointed out that the planar substrates with ortho- and meta-substituents can also have conformers, which could probably influence the binding orientation in the active site.

5.2 SYNTHESIS OF 3-SUBSTITUTED 2*H*-CHROMENES BY A ROBINSON ANNULATION TYPE CONDENSATION OF SALICYLIC ALDEHYDE WITH ACTIVATED ETHYLENES

From a conceptional point of view the chiral 3-aminochroman building block **251** of Etamicastat could be produced from various amine precursors like nitroalkanes (**266a**), carboxylic acids **266b** and amides **266c**, nitriles (**266d**) and aldehydes (**266e**) (see **Figure 181**).

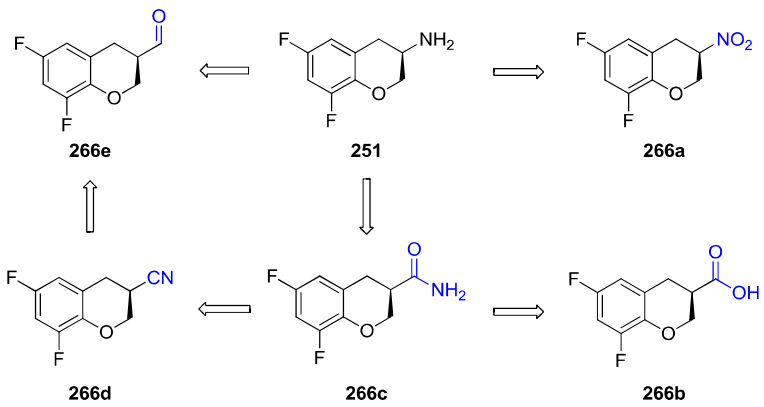


Figure 181. Retrosynthetic approach to chiral amine precursors based on an electron-withdrawing group (blue).

These precursors have an α-chiral carbon atom with alkyl substituents and can therefore be synthesized asymmetrically by a C=C reduction of the corresponding α,β-unsaturated compounds, leading to 3-substituted 2*H*-chromene structures of type **266**. These chromenes, especially the 3-nitrochromene **266a**, have a very similar structure compared to the previously discussed α-methyl β-aryl nitroalkenes **264**, which have been successfully reduced before. Furthermore, only one aromatic conformation is possible, oppressed by the annulated dihydro-2*H*-pyran heterocycle. The synthesis of these 3-substituted 2*H*-chromenes was also extensively studied by Merck as organic compounds for new liquid crystal media.[111] Interestingly, one of the industrial pathways to Etamicastat also incorporated this methodology of a Robinson annulation-type ring condensation of the corresponding salicyl aldehyde with acrylonitrile, leading to the unsaturated analogue of the 3-cyanochroman **266d**.[112] Based on these syntheses, four 3-substituted 2*H*-chromenes were prepared in the present study. At first, commercially available 2,4-difluorophenol (**270**) is *ortho*-formylated *via* DUFF reaction with

hexamethylenetetramine (**267**) under strongly acidic conditions (see **Figure 182**).

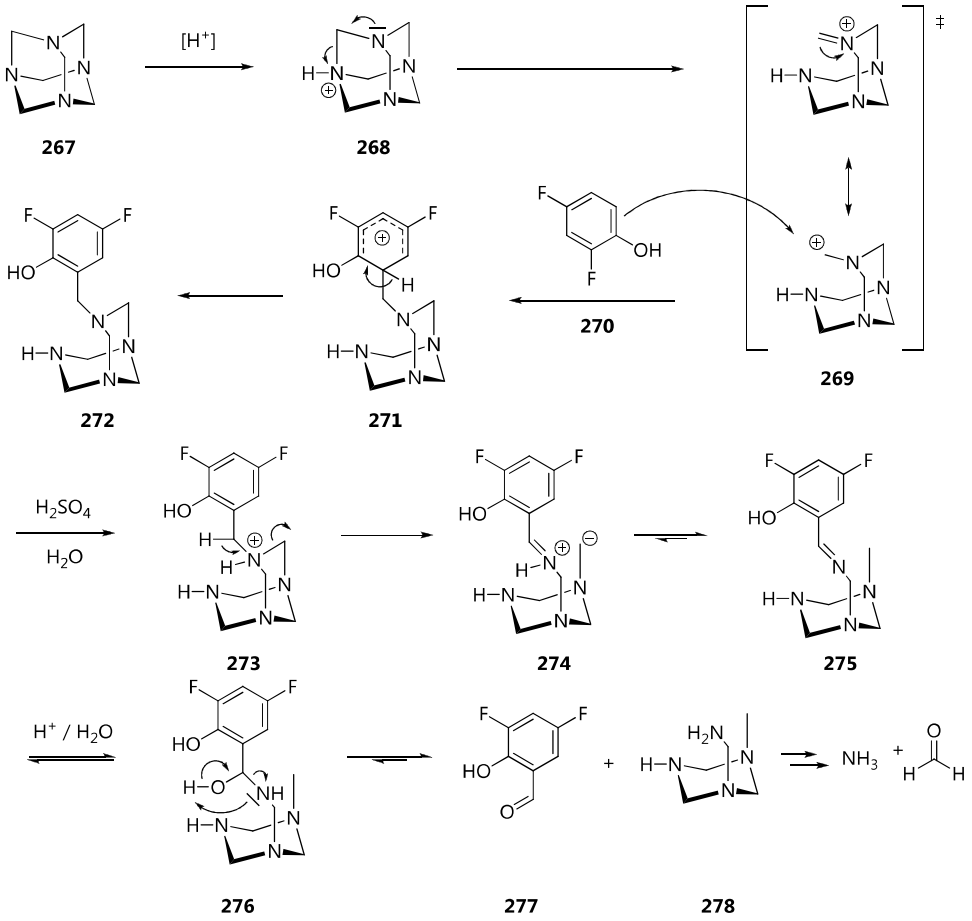
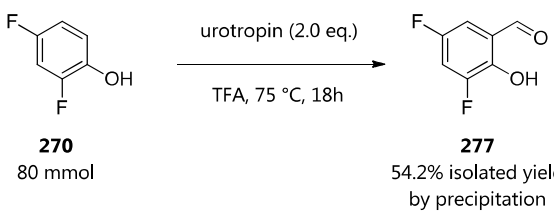


Figure 182. Proposed mechanism for the DUFF formylation of 2,4-difluorophenol.

The protonation of urotropine (**267**) leads to the formation of an iminium **269**, which undergoes an electrophilic aromatic substitution reaction ($S_{E,Ar}$) with the phenol **270**. After rearomatization of the MEISENHEIMER complex **271**, the aminomethyl compound **272** is fairly stable.

Figure 183. DUFF formylation of 2,4-difluorophenol in trifluoroacetic acid (TFA).



270
80 mmol

277
54.2% isolated yield
by precipitation

Hydrolysis with sulfuric acid (24%) induces a second iminium formation and proton transfer to the stable aromatic benzimine derivative **275**. Addition of

water leads to the metastable aminal **276**, which eliminates the amine **278** under formation of the desired salicylic aldehyde **277**. Under these aqueous acidic conditions, the byproduct **278** is completely degraded to ammonia and formic acid derivatives. Due to the low solubility of the product **277** in aqueous medium, the purification is achieved by simple filtration (see **Figure 183**, page 170). The moderate yield of 54.2% is presumably due to the high volume of the aqueous acidic medium (300 mL). The mother liquor was not evaporated but showed further crystallization while standing at room temperature.

The subsequent ROBINSON annulation-type condensation starts with the MICHAEL addition of the phenolate **279** to the β-position of the activated ethylene reagent **280** (see **Figure 184**). This enolate **281** further reacts intramolecular with the aldehyde to the aldol derivative **282**. Given the basicity of the reaction and the stabilization by the electron-withdrawing group, this adduct eliminates hydroxide in an E_{1cb} mechanism to yield the desired 3-substituted 2*H*-chromene **252**. As straightforward as this reaction may seem, the product-related conversion was in most cases only moderate (15-40%, see **Table 38**, page 172) and the synthesis with acrylamide yielded no product at

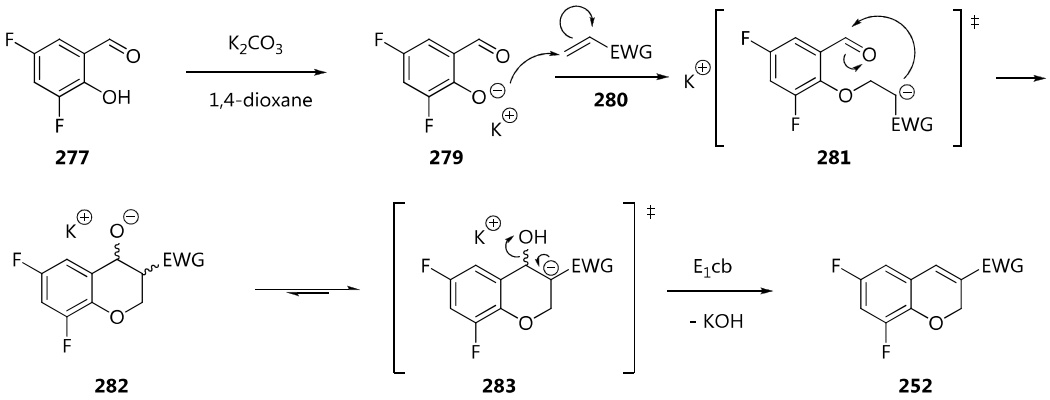
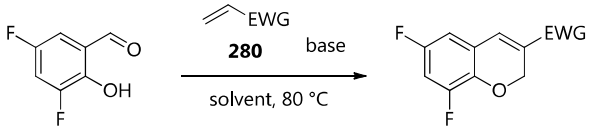


Figure 184. Proposed mechanism for the ROBINSON annulation-type condensation to 3-substituted 2*H*-chromenes.

all. Invariably, the salicylic aldehyde **277** was recycled by extraction from the acidified reaction mixture and did not show degradation to its corresponding salicylic acid. Thus, the reason for the low conversion was found in the polymerization of the activated ethylene reagent **280**. Among the examined reagents, acrylamide had the highest tendency to polymerize under the basic reaction conditions, solidifying the mixture to a hardly soluble sponge. Alteration of the experimental procedure was made by the addition of *p*-methoxyphenol as stabilizer, however, with only minor effect on the experimental results.

Table 38. Substrate syn-thesis of various 3-substituted 2*H*-chromenes.

(a) carbaldehyde (CHO), carbox-amide ($CONH_2$), carbonitrile (CN) nitro (NO_2) (b) 1,4-Diazabi-cyclo[2.2.2]octane (c) Determined after purification by column chromatography on silica.



277 **252a-d**

Entry	**252**	EWG[a]	Base[b]	Solvent	Isol. Yield[c] [g (%)]
1	**a**	CHO	K_2CO_3	Dioxane	0.15 (15.5)
2	**b**	$CONH_2$	Bu_2NH	Toluene	0.38 (39.3)
3	**c**	CN	DABCO	-	0.87 (40.9)
4	**d**	NO_2	Bu_2NH	Toluene	n.d. (-)

A special case was the preparation of the 3-nitro-2*H*-chromene **252a**. The necessary nitroethylene (**280a**) is hardly available as a commercial reagent. Therefore, the reagent was freshly prepared by elimination reaction from 2-nitroethanol (**284**, see **Figure 185**, page 173). Although this compound is manufactured commercially, the compound can also be produced from the bulk chemicals nitromethane and paraformaldehyde via HENRY reaction. A literature procedure for the preparation of nitroethylene from 2-nitroethanol (**284**) was examined. The starting material **284** was mixed with phthalic acid anhydride and heated to melting temperature. The product **280a** was directly distilled from the reaction mixture and condensed to solidification. Residual water was separated *via* extraction with dichloromethane. Nevertheless, another impurity, *i.e.* nitromethane, resulted from a retro-HENRY reaction and was also distilled into the product. Keeping the polymerization in mind, a second strategy was also pursued, activating the free carbinol to a better leaving group and alkylation of the salicylate. This *O*-nitroethyl derivative could then be basified to undergo the intramolecular cyclization without polymerization.

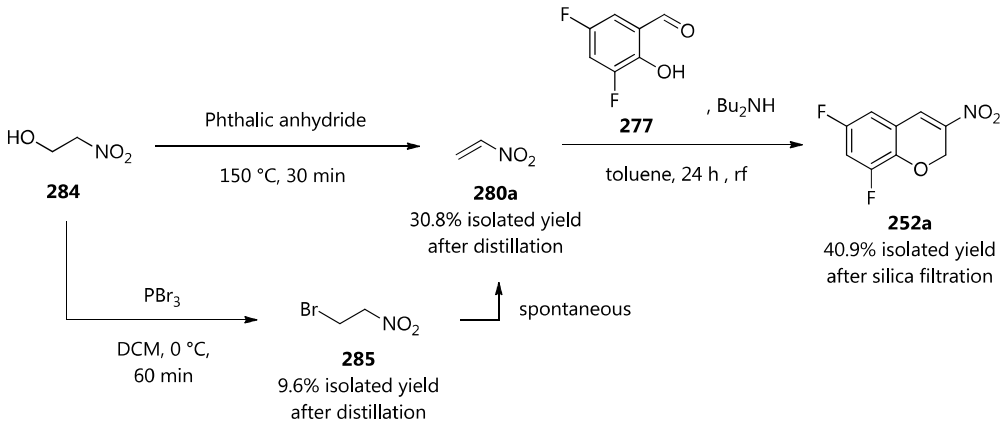


Figure 185. Synthetic routes to the heterocyclic nitroalkene.

Abbreviations: dichloromethane (DCM).

While the preparation of the corresponding 2-bromo-1-nitroethane **285** was successfully achieved by mixing 2-nitroethanol with phosphorous tribromide, the subsequent alkylation predominantly yielded the elimination product **280a** (see **Figure 185**). Therefore, another literature procedure was tested, again with phthalic acid anhydride, but now in the presence of the salicylic aldehyde to immediately condensate the nitroethylene (**280a**) to the desired chromene **252a**. This way, the concentration of ethylene reagent is minimized related to the other reagents, preventing polymerization. This one-pot procedure, which was later also found in literature[113], yielded around 40% of analytically pure 3-nitro-2*H*-chromene **252a** after silica filtration of the reaction mixture without additional work-up. Notably, phthalic acid anhydride was identified to be irrelevant for product-related conversion. The same reaction yielded again around 40% of pure product without addition of anhydride, demonstrating the tendency of 2-nitroethanol to form nitroethylene under basic conditions at elevated temperatures.

5.3 Reference compounds and analytical methods for the determination of product-related conversion and a possible enantioselectivity

The successful synthesis of 6,8-difluoro-3-nitro-2*H*-chromene **252a** also focused the subsequent analysis on this compound. The racemic 3-nitrochroman **251** was synthesized by reduction with sodium borohydride and was also separated by chiral HPLC analysis (see **Figure 186**). Since these nitro-compounds are unknown to literature, no reference data was available.

The absolute configuration of the two enantiomers could therefore not be assigned.

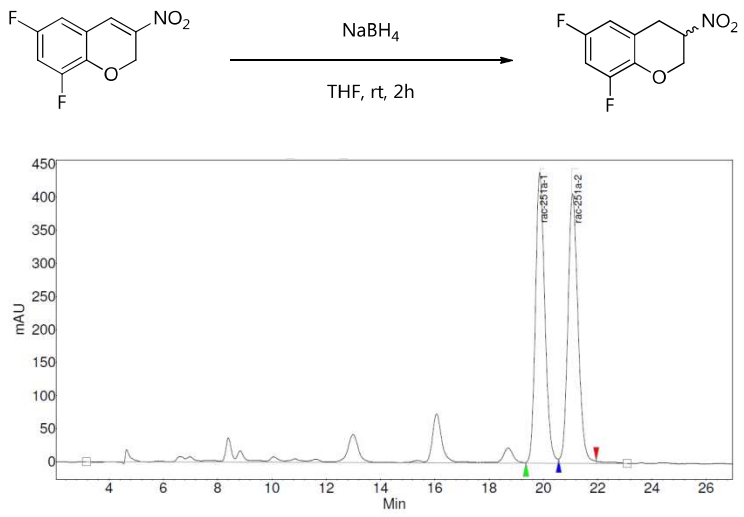


Figure 186. HPLC chromatogram of racemic 3-nitrochroman as analytical reference.

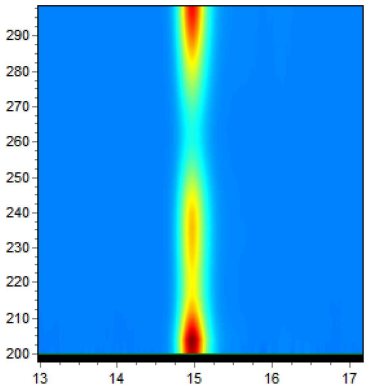


Figure 187. HPLC absorption spectra of the nitrochromene **252a** suggesting absorption bands in the visible light region.

However, judging from the photodiode array (PDA) spectrum of the substrate measurement (200-300 nm), the compound had multiple absorption maxima, also in the visible spectrum region (see **Figure 187**). This becomes relevant when analyzing enzyme kinetics for substrates. For NAD(P)H-dependent oxidoreductases, the literature assay for measuring the enzymatic activity is based on the absorption maximum of the reduced cofactor at λ = 340 nm. By consumption of cofactor for the biocatalytic reduction, the absorbance decreases in proportion to the amount of reduced substrate. With an extinction coefficient of 6.2 x 10^3 L/mol*cm, this decrease can be used to determine the volumetric activity of the biocatalyst for the given substrate in units (U) corresponding to umol of substrate converted per minute. With a substrate and/or product having an own absorption at 340 nm, the calculation would be flawed. For this reason, a new photometric assay for the conversion of 6,8-difluoro-3-nitro-2*H*-chro-mene **252a** was established.

5.4 Establishment of a photometric assay for the reduction of 2H-chromenes to their corresponding chromans

The use of NAD(P)H absorption for the kinetic measurement is based on its high extinction coefficient of 6.2 x 10^3 L/mol*cm at 340 nm. In addition, most substrates bearing an aromatic moiety have no absorption above 300 nm and

would therefore not distort the measurement. The PDA spectrum of the substrate **252a** however, indicated significant absorption above 300 nm. Therefore, the reaction components and the reaction mixture of the ene reductase-catalyzed reduction of 3-nitro-2*H*-chromene to its 3-nitrochroman were analyzed photometrically (see **Figure 188**).

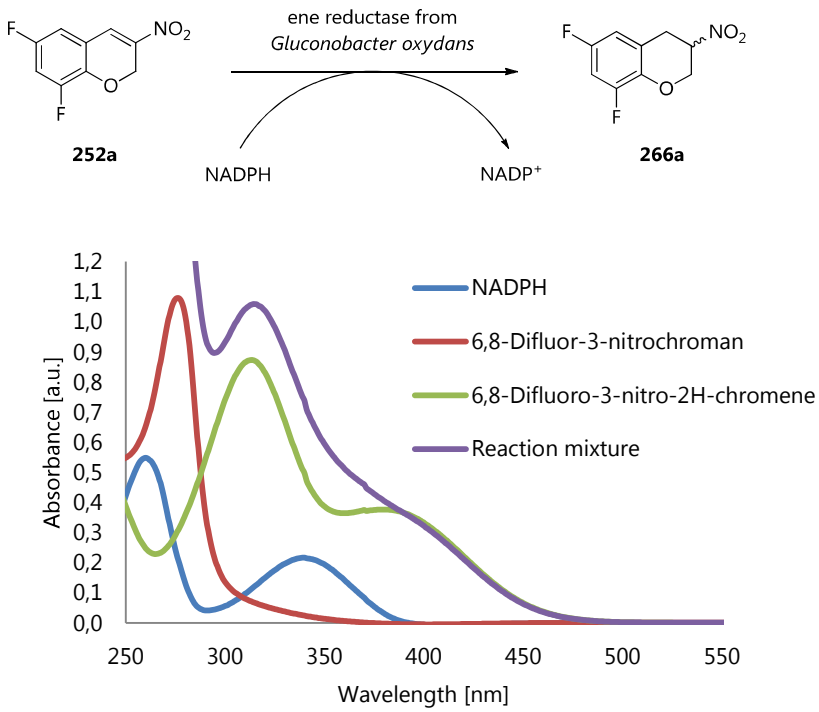


Figure 188. Absorption spectra of reaction components and the reaction mixture of the nitrochromene reduction.

Abbreviations: Nicotinamide adenine dinucleotide phosphate (NAD(P)H).

It is clearly shown that the substrate **252a** has a significantly higher fraction of the overall absorption at 340 nm. Even the product has a slight absorption in this region. Measuring the kinetics at this wavelength would comprise of the decrease of cofactor, decrease of substrate and an increase of product. Hence, the spectrum was examined for a wavelength specifically related to the decrease of only one component, e.g. the substrate itself. At 400 nm and above, only the substrate is responsible for the absorption of the reaction mixture. Neither the cofactor nor the product would interfere at this wavelength. The molar extinction coefficient of 6,8-difluoro-3-nitro-2*H*-chromene was determined by measuring the absorbance of four different weigh-ins of substrate, each diluted to three different concentrations. By this, a total of 12 concentrations were recorded and the molar extinction coefficient was calculated according to LAMBERT-BEER's law (see **Table 39**).

Table 39. Determination of the molar extinction coefficient of substrate **252a** at 400 nm.

Abbreviations: Standard deviation (Std dev.), relative deviation (Rel. dev.)

$\varepsilon = E / (c*d)$ E = Measured extinction [a.u.]
c = Analyst concentration [mol/L]
l = Path length [cm]

ε = Molar extinction coefficient [L/(mol*cm)]

Conc. **252a** [mol/L]	E [a.u.]	l [mm]	ε [L/(mol*cm)]
4.69E-05	0.168	10	3,57E+03
3.98E-05	0.133	10	3,34E+03
6.64E-05	0.170	10	2,56E+03
3.15E-05	0.097	10	3,09E+03
9.38E-05	0.324	10	3,46E+03
7.96E-05	0.251	10	3,16E+03
1.33E-04	0.397	10	2,99E+03
6.31E-05	0.194	10	3,08E+03
1.88E-04	0.643	10	3,43E+03
1.59E-04	0.526	10	3,31E+03
2.66E-04	0.818	10	3,08E+03
1.26E-04	0.418	10	3,32E+03
		Average ε	**3,20E+03**
		Std dev.	**2,57E+02**
		Rel. dev.	8,05%

With a molar extinction coefficient of $\varepsilon = 3.2 \pm 0.3 * 10^3$ L/(mol*cm), the biocatalytic reduction of **252a** was measured directly for substrate-related conversion. This is also important for the determination of kinetics under actual reaction conditions. Without using the cofactor absorption, an *in situ* cofactor regeneration can be implemented and the effect of additives can be measured directly by photometric analysis.

5.5 BIOCATALYTIC REDUCTION WITH AN ENE REDUCTASE FROM *GLUCONOBACTER OXYDANS* (GOX-ER)

In parallel to the establishment of analytical methods, the synthesized substrates of type **252** were preparatively screened with an enzyme-coupled

cofactor regeneration. As described before, the ene reductase from *Gluconobacter oxydans* uses NADPH as cofactor. Thus, an alcohol dehydrogenase from *Lactobacillus kefir* was used for cofactor regeneration under consumption of stoichiometric amounts of 2-propanol. With an alcohol content of 25% v/v, this was also beneficial for a more homogenous reaction mixture. The low solubilities of nitroalkenes were already extensively studied in the work of M.SC. TINA RESS.[114]

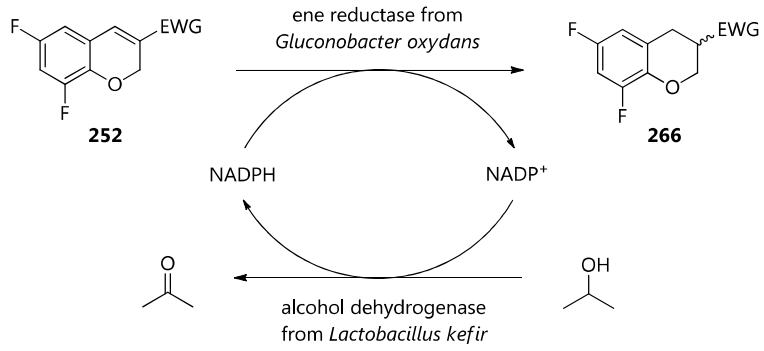


Table 40. Preparative screening of 3-substituted 2*H*-chromenes **252** for the biocatalytic reduction with an ene reductase from *Gluconobacter oxydans*.

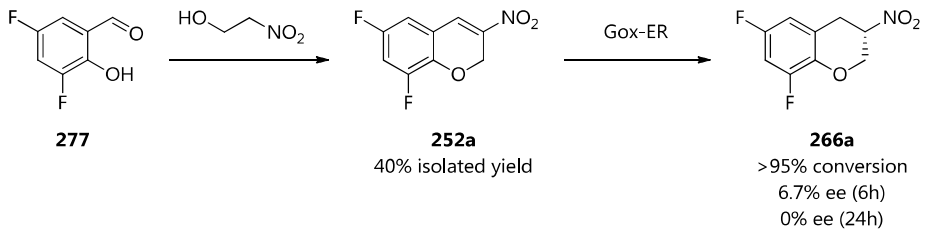
(a) carbaldehyde (CHO),carbonitrile (CN) nitro (NO_2) (b) LK-ADH-catalyzed reduction of carb-aldehyde; 2H-chromene-3-carbinol and 3-hydroxymethylchroman as by-products (c) racemic product after 24 hours, however, 6.7% ee after 6 hours reaction time.

Entry	**252** / **266**	EWG[a]	Conversion [%]	Yield [mg (%)]	ee [%]
1	**e**	CHO	>95.0[b]	n.d.	n.d.
2	**d**	CN	<1.0	-	-
3	**a**	NO_2	>95.0	n.d.	< 1.0 (6.7)[c]

The biocatalytic reduction was successfully achieved with the 3-carbaldehyde derivative **252e** and the 3-nitrochromene **252a** (see **Table 40**). The corresponding carbonitrile **252d** was inert as substrate (entry 2). Unfortunately, the otherwise low activity observed for the alcohol dehydrogenase from *Lactobacillus kefir* during this study was enhanced for the substrate **252e** and led to significant amounts of side-products by carbonyl reduction, *i.e.* the allylic alcohol and the hydroxymethylchroman.. The crude product was not further analyzed. In contrast to that, the nitrochromene **252a** was converted chemoselectively, without observation of literature-known side-activities like a biocatalytic Nef reaction.[115] Unfortunately, the formed nitrochroman showed only a slight enantiomeric excess of 6.7% ee after 6 hours reaction time, continuously decreasing to reach a racemic mixture after 24 hours. Due to the lack of additional enzymes, this project was not further investigated.

5.6 DISCUSSION AND OUTLOOK

A facile synthesis of 3-substituted 2*H*-chromenes was successfully adapted for the preparation of a literature-unknown 3-nitro-2*H*-chromene as prochiral precursor of Etamicastat (see **Figure 189**). Unfortunately, the investigated biocatalyst, an ene reductase from Gluconobacter oxydans, showed only minor enantioselectivity for the reduction, even leading to a racemic product after prolonged reaction times, indicating a slow racemization process. Nevertheless, during the search for chiral HPLC methods, a new photometric assay was established, specifically related to the actual product formation and not the error-prone cofactor consumption. By this alternative photometric assay, *in situ* kinetics can be measured in the presence of a cofactor regeneration, thus giving a more detailed insight into the actual reaction progress. This can also be useful for examination of additive effects on the activity or stability of ene reductases. Although the product was obtained as a racemate, this project clearly substantiated the potential of biocatalysis for pharmaceutical syntheses.



277 | **252a** 40% isolated yield | **266a** >95% conversion 6.7% ee (6h) 0% ee (24h)

Figure 189. Synthetic route to the chiral 3-nitrochroman **266a** as precursor of Etamicastat.

5.7 REFERENCES

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6 ABSTRACT

The present work on chemoenzymatic syntheses of pharmaceutically relevant building blocks consists of four separate studies. One of two main projects was the establishment of a biocatalytic desymmetrization route within a multi-step synthesis of the blockbuster drug Rosuvastatin, which had an annual world market sale of 6.253 billion US dollars in 2012. An initial route was adjusted several times to circumvent chemical as well as biocatalyst limitations. After extensive analysis of the reaction parameters and subsequent optimization of the biocatalytic process, the final route to the enantiomerically enriched key intermediate, the chiral phosphoranylidene **93b**, was successfully realized with high yields, excellent enantioselectivities and only one necessary product isolation within nine consecutive reaction steps. The most notable fact is that a bienzymatic process for the production of ethyl (3*R*)-hydroxyglutarate (**60**) was realized to circumvent a low enantioselectivity of the biocatalyst for the starting material (see **Figure 190**). By this method, the enantioselectivity of the hydrolase α-chymotrypsin was increased from moderate 60% ee to excellent >97% ee. In addition, the reaction proceeded smoothly even at a substrate concentration of 4.0 mol/L of **22a**, leading to a binary mixture of aqueous reaction buffer and organic substrate phase of around 1:1 volumetric ratio. By combining the highly enantioselective desymmetrization with a subsequent chemoselective deacetylation by the hydrolase cephalosporin C acetylesterase (CAE), the enantiomerically enriched ethyl (3*R*)-hydroxyglutarate (**60**) was produced in more than 350 g/L, which is remarkable for a biocatalytic process.

The subsequent derivatization to the enantiomerically enriched ylide (*3R*)-**93b** (see **Figure 191**, page 181), was established using literature-known protective-group chemistry and activated carboxylic acid derivatives. Although the desired ylide **93b** could not be isolated in pure crystalline form, the content and enantiomeric excess were determined by the conversion of aldehyde in the following WITTIG reaction and chiral HPLC analysis of a preparative TLC

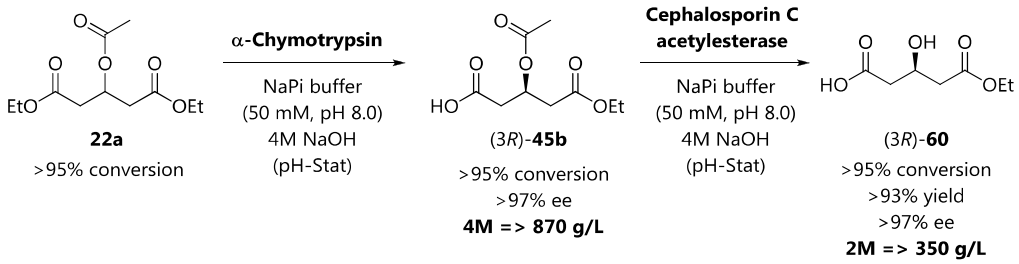


Figure 190. Bienzymatic process for the production of the enantiomerically enriched monoester (3*R*)-60 at high substrate concentrations.

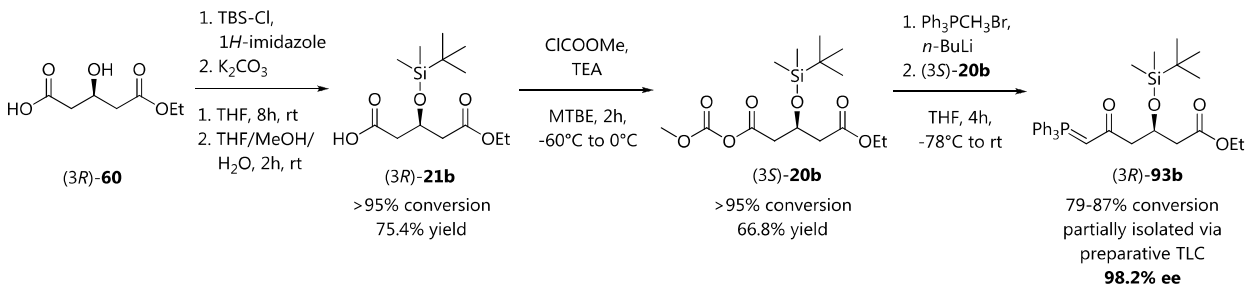


Figure 191. Chemical derivatization of enantiomerically pure monoester (3*R*)-**60** to the desired phosphorous ylide (3*R*)-**93b** in the chemoenzymatic synthesis of Rosuvastatin.

purification of the crude product, respectively. With respect to the already established WITTIG reaction with the commercial ylide and the need for 1.1 equivalents of reagent, the conversion of aldehyde revealed an ylide content of 78-87%. The enantiomeric excess was determined to be 98% ee, an optical purity which was considered to be sufficient for an industrial scale-up. A patent application for this chemoenzymatic multi-step synthesis is currently pending.

The other main project, the biocatalytic synthesis of chiral nitriles, started from basically no literature regarding an enantioselectivity of aldoxime dehydratases (*Oxd*s), a recently discovered and characterized enzyme belonging to the class of lyases. Within this work, the first scientific evidence for an enantioselective dehydration of racemic aldoximes was produced. By reducing the activity of the biocatalyst, *i.e.* an aldoxime dehydratase from *Bacillus* sp. OxB-1 (*OxdB*) overexpressed in recombinant *E. coli* HB101 / pOxD-9OF, and the free energy of the system by lowering the reaction temperature, a kinetic resolution of the racemic *E*-aldoxime of 2-phenylpropionaldehyde (*E*-**133a**) was observed for the first time (see **Figure 192**).

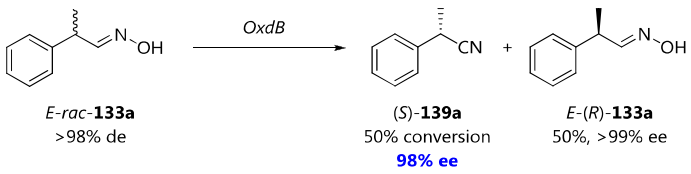


Figure 192. Synthesis of enantiomerically pure (*S*)-2-phenylpropionitrile by an enzymatic dehydration of the racemic *E*-aldoxime **133a** by means of a kinetic resolution.

The progress of the enzymatic reaction was analyzed from a mixture of two diastereomers of *E*- and *Z*-aldoxime consisting of a racemate. By identifying an inert substrate, namely *E*-(*R*)-**133a**, as well as assigning the absolute configuration of the enantiomerically enriched nitrile (*S*)-**139a**, the

enantiospecificity of *OxdB* for this substrate was clarified. The key to this enantioselective nitrile synthesis was found by separating *E*- and *Z*-aldoximes from their mixture. Essentially pure (*S*)-**139a** was synthesized in a kinetic resolution starting from racemic *E*-**133a** (>99% isomeric purity), with 50% conversion and an excellent enantioselectivity of 98% ee (see **Figure 192**, page 181), while the corresponding Z-aldoxime resulted in a racemic product. This unusual enantiopreference for *E*-aldoximes was also observed for four other chiral substrates bearing aliphatic, cyclic, bicyclic and heterocyclic substituents, demonstrating the high value of this methodology.

The third project consisted of two target structures, namely β-amino alcohols related to HIV-1 protease inhibitors and β-lactones as potent antibiotic drugs. Both compound classes were synthesized by a dynamic kinetic resolution starting from racemic α,β-disubstituted β-oxo carbonyl compounds. In all cases, the applied biocatalysts were oxidoreductases, more precisely alcohol dehydrogenases from *Rhodococcus* sp., *Lactobacillus* sp., a commercial ADH, evocatal 1.1.200, and an ene reductase from *Gluconobacter oxydans*. The latter was primarily used for substrate synthesis by C=C reduction of the corresponding α-acyl γ-aryl acrylates. The enantio- and diastereoselective reduction of β-oxo carbonyl compounds was successfully achieved for α,β-disubstituted β-oxo nitriles of type **197a** to circumvent a lack of activity observed for the corresponding β-oxo amides. Three alcohol dehydrogenases were tested for their reduction. All of them showed high activities with quantitative conversion of the substrate, excellent enantioselectivities for the carbonyl reduction and also high diastereoselectivities for the vicinal stereogenic center (see **Figure 193**).

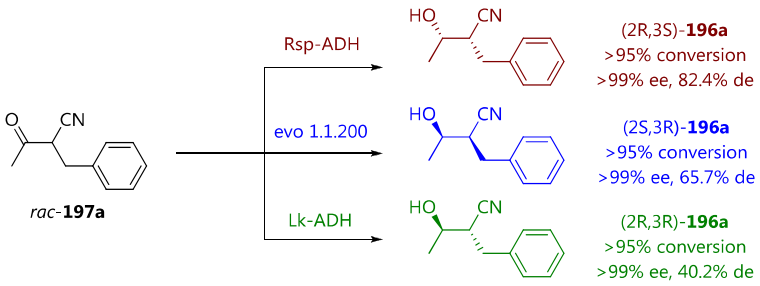


Figure 193. Enantio- and diastereoselective synthesis of three of four possible diastereomers of β-hydroxy nitrile **196a** by dynamic kinetic resolution of racemic β-oxonitrile **197a**.

By using the racemizable β-oxo nitrile **197a**, a dynamic kinetic resolution process for the preparation of enantiomerically pure and diastereomerically enriched β-hydroxy nitrile **196a** was successfully established, with three of four possible diastereomers directly addressable.

Although the subsequent hydration to the corresponding β-hydroxy amides was not investigated in this work, the chemical degradation of diastereomerically enriched racemic β-hydroxy amides were successfully established by means of a HOFMANN rearrangement under complete retention

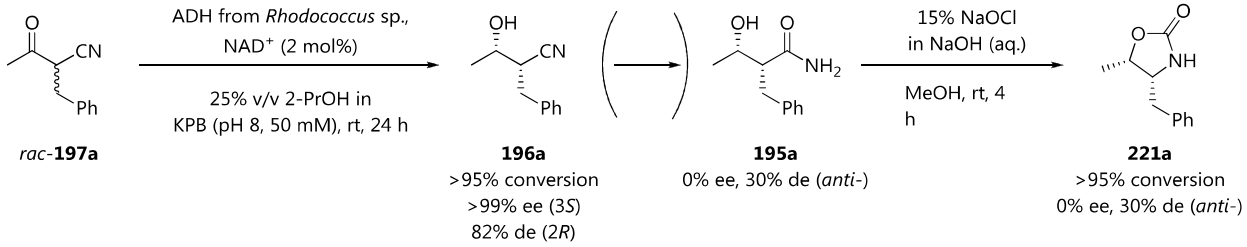
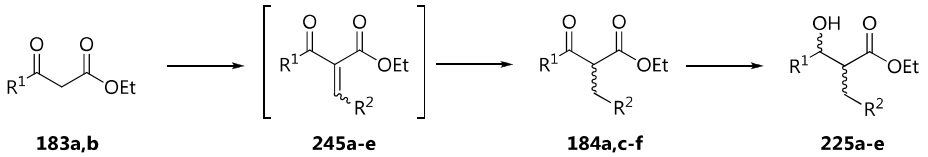


Figure 194. Synthesis of enantio- and diastereomerically pure 4,5-disubstituted oxazolidin-2-ones from racemic β-oxo nitriles.

of the diastereomeric excess (see **Figure 194**). The obtained 4,5-disubstituted oxazolidin-2-ones are excellent precursors for the synthesis of β-amino alcohols and the cyclic urethane structure represents an intramolecular protecting group for the regioselective monoalkylation of the corresponding amino alcohol.

Besides nitrogen-containing β-oxo carbonyl compounds, also β-oxo esters **184** were successfully reduced by the use of alcohol dehydrogenases (see **Figure 195**). The stereoselectivities were in good agreement with those obtained for the β-oxo nitriles **197**. Furthermore, a two-step synthesis of β-arylmethyl substrates was realized by combination of a BRÖNSTED acid/base-catalyzed KNOEVENAGEL condensation to obtain the intermediates of type **245** and a subsequent ene reduction with palladium charcoal or a hydride reduction with a HANTZSCH ester. Additionally, a chloromethyl moiety was

Figure 195. Synthesis of new racemic β-oxo ester substrates and biocatalytic reduction with alcohol dehydrogenases in a dynamic kinetic resolution process.



	R^1 = Me, R^2 =				R^1 = Cl-Me, R^2 = Bn
245:	56.1%	n.d.	58.8%	97.9%	n.d.
184:	-	91.3%	47.2% conv.	94.8%	91.3% conv.
225:	>95% conv. >99% ee 95.0% de	>95% conv. >99% ee 30.4% de	-	>95% conv. >99% ee 36.5% de	-

inserted in view of a subsequent insertion of the β-lactone moiety into larger molecular structures by, for example, a WITTIG reaction. The chemical saponification of the β-hydroxy esters was investigated for a biocatalytic alternative. A photometric screening assay identified porcine liver esterase as potent hydrolase. The enzymatic hydrolysis was successfully achieved in a sequential two-step process after ADH reduction or in a one-pot process starting from racemic β-oxo esters in the presence of an ADH. PLE revealed itself to be highly enantioselective for the hydrolysis of the (3*S*)-β-hydroxy ester, thereby further increasing the diastereomeric excess from the biocatalytic reduction with an alcohol dehydrogenase from *Rhodococcus* sp.. In addition, a chemical lactonization protocol was achieved (see **Figure 196**). By using different inorganic or organic bases, an epimerization of the α-stereogenic center was either minimized or drawn towards enrichment of the predominant

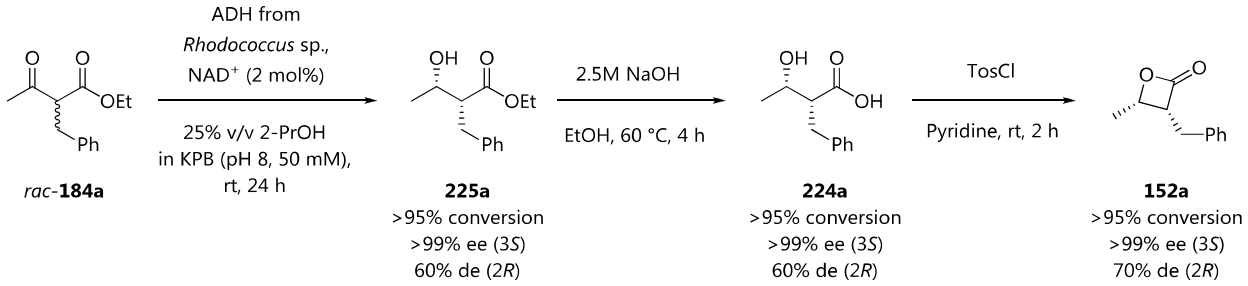


Figure 196. Synthesis of enantio- and diastereomerically pure 3,4-disubstituted β-lactones from racemic β-oxo esters.

syn-diastereomer.

Last but not least, a facile synthesis of 3-substituted 2*H*-chromenes was successfully adapted for the preparation of a literature-unknown 3-nitro-2*H*-chromene as prochiral precursor of the dopamine β-hydroxylase inhibitor Etamicastat. Despite the fact that the investigated biocatalyst, an ene reductase from *Gluconobacter oxydans,* showed only low enantioselectivity for the desired reduction, a new photometric assay was established, specifically related to the actual product formation and not the error-prone cofactor consumption, which is often used for oxidoreductase activity measurements. By this alternative photometric assay, *in situ* kinetics can be measured in the presence of a cofactor regeneration, thus giving a more detailed insight into the actual reaction progress. This will be useful as a general assay for examination of additive effects on the activity or stability of ene reductases.

The investigated processes clearly demonstrate the impact of biocatalytic alternative syntheses in organic chemistry, especially by their combination in chemoenzymatic synthesis of pharmaceutically relevant, enantiomerically pure building blocks.

7 ZUSAMMENFASSUNG

Die hier vorgestellte Arbeit im Bereich der chemoenzymatischen Synthese von pharmazeutisch relevanten Synthesebaustainen besteht aus vier Einzelstudien. Eines der zwei Hauptprojekte war die Etablierung einer biokatalytischen Desymmetrisierung in Verbindung mit einer Mehrstufensynthese des Blockbuster-Wirkstoffs Rosuvastatin, der im Jahr 2012 mit einem Weltmarktanteil von 6.253 Milliarden US-Dollar zu Buche schlug. Die geplante Syntheseroute musste aufgrund von chemischen als auch biokatalytischen Einschränkungen mehrmals verändert werden. Nach intensiver Analyse der Reaktionsbedingungen und anschließender Optimierung des biokatalytischen Prozesses konnte die enantiomerenreine Schlüsselverbindung, das chirale Phosphor-Ylid **93** in hohen Ausbeuten, exzellenten Enantioselektivitäten und ausschließlich einer einzigen Aufreinigung über sechs aufeinanderfolgende Stufen synthetisiert werden. Am bemerkenswertesten war hierbei die Realisierung eines bienzymatischen Prozesses für die Darstellung des

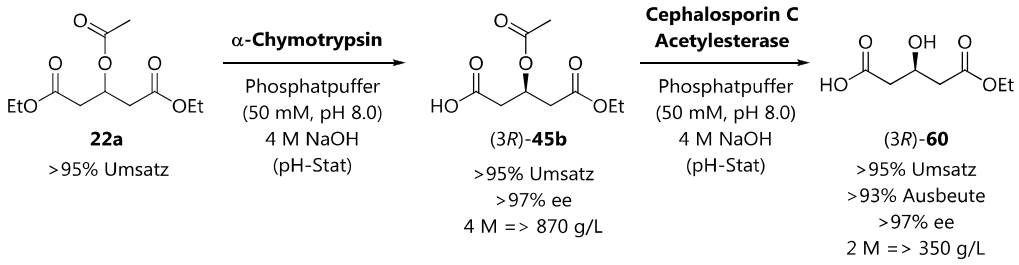


Abbildung 1. Bienzymatischer Prozess für die Darsellung von enantiomerenangereichertem Monoester **60** bei hohen Substratkonzentrationen.

enantiomerenreinen (3*R*)-Hydroxyglutarsäuremonoethylesters (**60**) mit Umgehung einer niedrigen Enantioselektivität des Biokatalysators. (siehe **Abbildung 1**). Hierdurch konnte die Enantioselektivität der Hydrolase α-Chymotrypsin von mäßigen 60% ee auf hervorragende >97% ee gesteigert warden. Hinzu kommt, dass die Reaktion selbst bei einer Substratkonzentration von 4.0 mol/L bezüglich **22a** noch problemlos lief und dabei einer binären 1:1-Mischung aus wäßriger Pufferlösung und organischer Substratphase entsprach. Durch die Kombination der hochenantioselektiven Desymmetrisierung mit einer nachfolgenden, chemoselektiven Deacetylierung unter Verwendung der Hydrolase Cephalosporin C Acetylesterase (CAE) konnte enantiomerenangereicherter Monoester **60** mit einer Produktkonzentration von mehr als 350 g/L synthetisiert werden. Eine solche hohe Produktkonzentration ist herausragend für biokatalytische Prozesse. Die anschließende Derivatisierung zum enantiomerenangereicherten Phosphor-Ylid

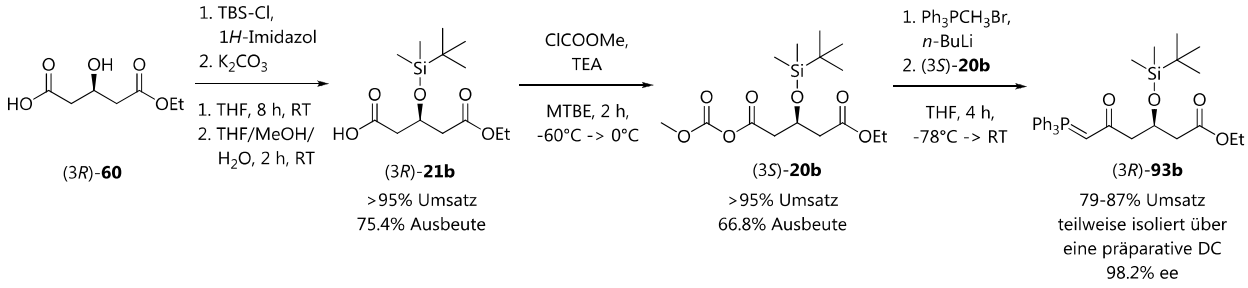


Abbildung 2. Chemische Derivatisierung von enantiomerenreinem Monoester **60** zum angestrebten Phosphor-Ylid **93b** für die Synthese von Rosuvastatin.

93b wurde mit Hilfe von literaturbekannten Schutzgruppen und Aktivestern etabliert. (siehe **Abbildung 2**), Obwohl das angestrebte Ylid nicht in kristalliner Reinform gewonnen werden konnte, wurde sein Gehalt in Rohprodukten und sein Enantiomerenüberschuss bestimmt. Ersteres erfolgte über die anschließende Wittigsynthese mit dem hetereoaromatischen Aldehyd von Rosuvastatin, letzteres mit Hilfe einer chiralen HPLC-Analyse nach teilweiser Aufreinigung über eine präparative DC. Unter Berücksichtigung der gängigen Wittigsynthese mit dem kommerziell erhältlichen Ylid und dem Bedarf von 1.1 Äquivalenten, die Umsetzung des Aldehyds bestätigte einen Gehalt im Bereich von 78-87% im Rohprodukt der Ylidsynthese. Der Enantiomerenüberschuss wurde auf 98% ee bestimmt, gleichbedeutend mit einer optischen Reinheit, die für eine industrielle Anwendung ausreichend waren. Eine diesbezügliche Patentanmeldung ist im Gange.

Das zweite Hauptorjekt, die enantioselective Synthese von chiralen Nitrilen, hatte nahezu keine Literaturgrundlage bezüglich einer Enantioselektivität von Aldoximdehydratasen (Oxds), einem kürzlich entdeckten und charakterisierten Enzym, das zur Klasse der Lyasen gehört. Innerhalb der hier vorgestellten Arbeit wurde erstmals eine enantioselective Dehydratation von racemischen Aldoximen wissenschaftlich belegt. Durch Reduzierung der Enzymaktivität des Biokatalysators, d.h. der Aldoximdehydratase aus *Bacillus* sp. OxB-1 (OxdB), die in recombinanten *E. coli* HB101 / pOxD-9OF überexprimiert wurde, und der Absenkung der freien Energie des Systems durch Abkühlung auf 8 °C wurde eine kinetische Racematspaltung des chiral *E*-Aldoximes von 2-Phenylpropionaldehyd erstmals beobachtet.

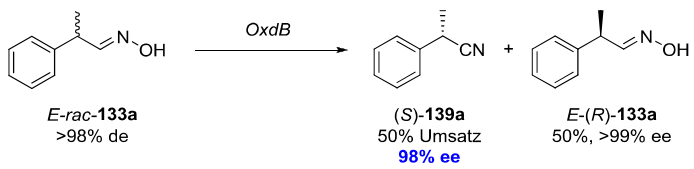


Abbildung 3. Synthese von enantiomerenreinem (S)-2-Phenylpropionitril **139a** durch enzymatische Dehydratation des racemischen E-Aldoxims **133a** im Sinne einer kinetischen Racematspaltung.

Der Verlauf der Enzymreaktion wurde mit einer Diastereomerenmischung des *E*- und *Z*-Aldoxims analysiert, die aus Racematen bestand. Durch Identifizierung eines inerten Substrats, genauergesagt *E*-(*R*)-**133a**, und der Zuordnung der absoluten Konfiguration des enantiomerenangereicherten Nitrils **139a**, konnte die Enantiospezifität des Enzyms für dieses Substrat abschließend geklärt werden. Der Schlüssel für diese enantioselektive Nitrilsynthese lag in der Separierung von *E*- und *Z*-Aldoximes aus ihrer Mischung. Nahezu optisch reines (*S*)-**139a** wurde ausgehend von racemischem *E*-**133a** (>99% Isomerenreinheit) durch eine kinetische Racematspaltung gewonnen, mit 50% Umsatz und einem hervorragenden Enantiomerenüberschuss von 98% ee (siehe **Abbildung 3**, Seite 186). Die Synthese basierend auf dem entsprechendem Z-Aldoxim führte zu einem racemischen Produkt. Diese ungewöhnliche Enantiopreferenz für *E*-Aldoxime wurde ebenfalls bei vier weiteren, chiralen Substraten, beobachtet. Diese beinhalten aliphatische, zyklische, bizyklische und heterozyklische Strukturen und verdeutlichen den hohen Wert dieser Synthesemethode.

Das dritte Projekt war auf zwei Leitstrukturen ausgerichtet, genauergesagt β-Aminoalkoholen, die strukturverwandt mit HIV-1 Proteaseinhibitoren sind, und β-Lactonen als mögliche Antibiotika. Beide Strukturklassen wurden über eine dynamisch-kinetische Racematspaltung ausgehend von racemischen β-Oxocarbonylverbindungen hergestellt. In jedem Fall waren die verwendeten Biokatalysatoren Oxidoreduktasen, genauergesagt Alkoholdehydrogenasen (ADH) aus *Rhodococcus* sp., *Lactobacillus* sp., einer kommerziellen ADH, evocatal 1.1.200, und einer En-Reduktase aus *Gluconobacter oxydans*. Letztgenannte wurde primär für die Substratsynthese genutzt, über eine C=C-Reduktion der entsprechenden α-Acyl-γ-arylacrylsäureester. Die enantio- und diastereoselektive Reduktion von β-Oxocarbonylverbindungen wurde erfolgreich für α,β-disubstituted β-Oxonitrile wie **197a** durchgeführt, um die fehlende Aktivität der ADHs gegenüber den entsprechenden β-Oxocarbonsäureamiden zu kompensieren. Drei Alkoholdehydrogenasen wurden im Zuge dessen getestet.

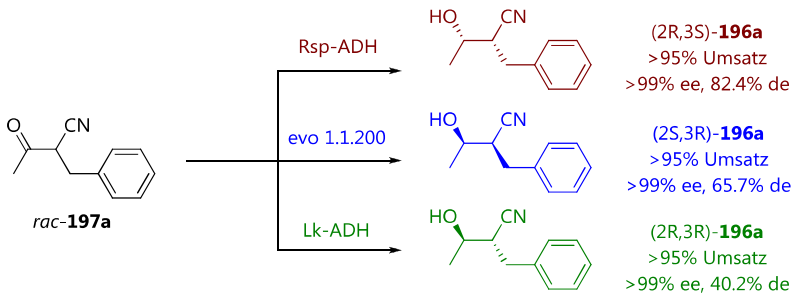
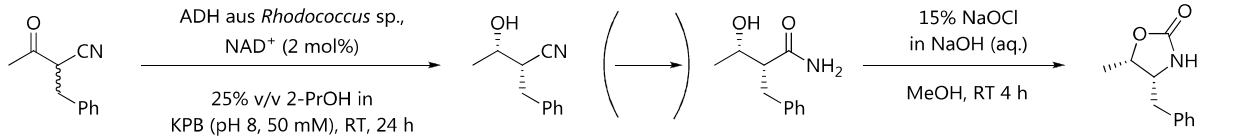


Abbildung 4. Enantio- und diastereoselektive Synthese der drei von vier möglichen Diastereomeren des β-hydroxynitrils **196a** mittels dynamisch-kinetischer Racematspaltung.



rac-**197a**

196a
>95% Umsatz
>99% ee (3*S*)
82% de (2*R*)

195a
0% ee, 30% de (*anti*-)

221a
>95% Umsatz
0% ee, 30% de (*anti*-)

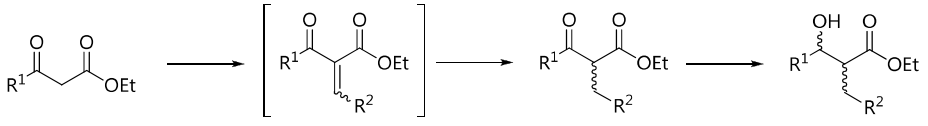
Abbildung 5. Synthese von enantio- und diastereomerenreinen 4,5-disubstituierten Oxazolidin-2-onen basierend auf racemischen β-Oxonitrilen.

Alle zeigten hohe Aktivitäten mit quantitativen Umsätzen, ausgezeichneten Enantioselektivitäten für die Carbonylreduktion und ebenfalls hohe Diastereoselektivitäten für das benachbarte Stereozentrum. Bei der Verwendung von racemisierbarem **197a** konnte eine dynamisch-kinetische Racematspaltung (DKR) für die Darstellung von enantiomerenreinem und diastereomerenangereichertem β-Hydroxynitril **196a** erfolgreich etabliert werden. Drei von vier möglichen Diastereomeren waren durch geeignete Wahl des Biokatalysators direkt synthetisierbar (siehe **Abbildung 4**, Seite 187).

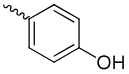
Der später durchgeführte Hofmann-Carbonsäureabbau verlief unter vollständiger Retention der absoluten Konfiguration. Die dadurch zugänglichen 4,5-disubstituierten Oxazolidin-2-one sind hervorragende Vorstufen bei der Synthese von β-Aminoalkoholen und das zyklische Urethan enspricht einer intramolekularen Schutzgruppe für die regioselektive Monoalkylierung des jeweiligen Aminoalkohols.

Abbildung 6. Synthese von neuen racemischen β-Oxoester-Substraten und deren biokatalytische Reduktion in einer DKR.

Neben stickstoffhaltigen β-Oxocarbonylverbindungen wurden auch β-Oxocarbonsäureester **184** erfolgreich mit ADHs umgesetzt. (siehe **Abbildung 6**). Die Stereoselektivitäten waren dabei in guter Übereinstimmung mit den Versuchen zu β-Oxonitrilen **197**. Ebenfalls realisiert

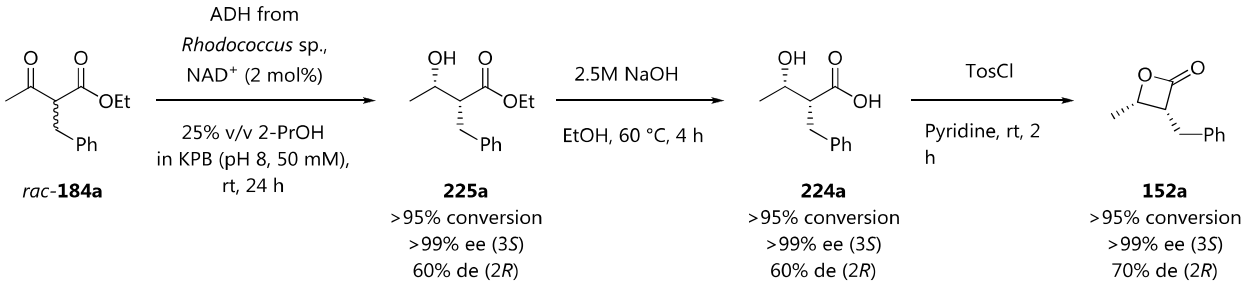


183a,b **245a-e** **184a,c-f** **225a-e**

	R^1 = Me, R^2 = 				R^1 = Cl-Me, R^2 = Bn
245:	56.1%	n.d.	58.8%	97.9%	n.d.
184:	-	91.3%	47.2% conv.	94.8%	91.3% conv.
225:	>95% conv. >99% ee 95.0% de	>95% conv. >99% ee 30.4% de	-	>95% conv. >99% ee 36.5% de	-

wurde eine zweistufige Synthese von β-Arylmethylsubstraten durch Kombination einer BRÖNSTED säure-/basenkatalysierten KNOEVENAGEL Kondensation zu den Verbindungen des Typs **245** und einer darauffolgenden Alkenreduktion mit Palladium/Aktivkohle bzw. einer HANTZSCH-Ester-Reduktion. Zusätzlich wurde eine Chlormethylgruppe eingeführt, um das β-Lacton **152a** z.B. über eine WITTIG-Reaktion in größere Molekülstrukturen einzubinden.
Desweiteren wurde eine chemische Lactonisierung erzielt (siehe **Abbildung 7**). Durch die Verwendung von verschiedenen anorganischen und organischen Basen wurde eine Epimerisierung entweder vermindert oder in Richtung einer weiteren Diastereomerenanreicherung des *syn*-Diastereomers gedrängt.
Zu guter Letzt wurde eine simple Synthese von 3-substituierten 2*H*-Chromenen erfolgreich auf die Darstellung eines literaturunbekannten 3-Nitro-2*H*-chromens übertragen. Dieses stellt eine prochirale Vorstufe des Dopamin-β-

Abbildung 7. Synthese von enantio- und diastereomerenreinen 3,4-disubstituierten β-Lactonen basierend auf racemischen β-Oxonitrilen.



hydroxylaseinhibitors Etamicastat dar. Abgesehen davon, dass der untersuchte Biokatalysator, die En-Reduktase aus *Gluconobacter oxydans*, nur eine sehr niedrige Enantioselektivität bezüglich des Nitrosubstrats aufweist, wurde ein neuartiger Photometertest etabliert. Dieser misst nun spezifisch die Produktbildung der En-Reduktase-katalysierten Reaktion anstatt die fehlerbehaftete Cofaktorabnahme, die Gang und Gäbe bei photometrischen Aktivitätsbestimmungen von Oxidoreduktasen ist. Durch diesen alternativen Photometertest lässt sich die Reaktionskinetik in der Gegenwart einer Cofaktor-Regenerierung bestimmen. Dies dürfte sich als nützlich erweisen, wenn es darum geht, den Einfluss von Additiven auf die Aktivität und Stabilität von En-Reduktasen zu untersuchen.

Die untersuchten Prozesse zeigen deutlich den Einfluss von biokatalytischen Alternativsynthesen in der organischen Synthesechemie, vor allem in Bezug auf deren Kombination in chemoenzymatischen Synthesen von pharmazeutisch relevanten, enantiomerenreinen Synthesebausteinen.

8 EXPERIMENTAL PROCEDURES - ROSUVASTATIN

8.0 MATERIALS AND METHODS

Solvents and reagents

Unless stated otherwise all chemicals and solvents (reagent and HPLC grades) were purchased from commercial manufacturers (Sigma-Aldrich, ABCR, Alfa Aesar, Tokyo Chemical Industry Co., Ltd., Carl Roth GmbH & Co. KG) and used without further purification. Methyl (3*R*)-(*tert*-butyldimethylsilyl)-oxy-5-oxo-6-(triphenylphosphor-anylidenyl)-hexanoate, *n*-(4-(4-fluorophenyl)-5-formyl-6-isopropylpyrimidin-2-yl)-*N*-methylmethane-sulfonamide, Rosuvastatin methyl ester and Rosuvastatin calcium salt were provided by Sandoz GmbH, Kundl, Austria.

Enzymes

Samples of α-chymotrypsin or mixtures with trypsin were purchased from BBI enzymes, Biozym, Biocon and Sigma-Aldrich. Cephalosporin C acetylesterase was provided by Sandoz GmbH, Kundl, Austria. Lipases Amano AYS, Amano PS SD, Amano P were provided from Amano Enzymes Inc, Nagoya, Japan. Dehydroabietylamine was purified according to chapter 8.0.3. Deuterated solvents chloroform-d (>99.9%, stabilized with silver foil, 0.03% v/v TMS) and dimethylsulfoxid-d6 (>99.8%) were purchased from Acros Organics, New Jersey, USA and Alfa Aeser, Lancashire, UK, respectively.

Photometric measurements

Photometric assays for active hydrolases were measured on 96-well plates (Thermo Scientific Nunc*) in a Multiskan GO microplate spectrophotometer from Thermo Fisher Scientific Inc., Waltham, MA, USA. The absorbance at wavelengths of 433 nm and 618 nm, respectively, were plotted against reference solutions for relative changes. Absorbance spectra of compounds, molar extinction coefficients and time courses of reactions were measured on a V-630 spectrophotometer, equipped with a PSC-763 air-cooled automatic 6-position Peltier cell-changer, from JASCO Inc., Easton, MD, USA. The data was analyzed with Spectra Manager 2 (Vers. 2.08.04).

Incubators

The microplates containing hydrolase screening solutions were incubated in a Heidolph Titramax 1000 microplate shaker combined with a Heidolph Inkubator 1000. The 96-well plates were shaken for 2-24 hours at 25-60 °C.

High-Performance Liquid Chromatography (*HPLC*)

The conversion and composition of aqueous reaction phases was measured on a LC-2000 HPLC system equipped with PU2080 Plus pumps, a DG2080-53 degasser, a MD-2010 Plus multiwavelength detector, a CO-2060 Plus column thermostat, an AS-2059-SF Plus autosampler and a RI-2031 Plus refractive index detector from JASCO Inc., Easton, MD, USA. The samples were separated on reversed-phase HPLC columns Nucleosil 100-5 C18 from Macherey-Nagel GmbH & Co. KG, Düren, Germany, or SunFire C18 from Waters GmbH, Eschborn, Germany, respectively. Mixtures of water and acetonitrile, methanol or aqueous buffers at various pH ranges were used as eluents. The composition and optical purity of extracted samples were determined on another LC-2000 HPLC system with identical equipment (excl. RI detector) and additional modules for the use of supercritical carbon dioxide as apolar mobile phase, i.e. a F250 cryostat from Julabo GmbH, Seelbach, Germany, and a BP-2080 Plus back pressure generator from JASCO Inc., Easton, MD, USA, respectively. The samples were separated on normal-phase chiral HPLC columns, i.e Chiralpak AD-H, Chiralcel OD-H, Chiralcel OJ-H, Chiralcel OB-H and Chiralpak IC, respectively, from Chiral Technologies Europe SAS, Illkirch, France, with mixtures of 2-propanol in *n*-hexane and/or SC-CO_2 as mobile phase. The data was analyzed with the chromatography software Galaxie (Vers. 1.10.0.5590) from Agilent / Varian, Inc., Walnut Creek, CA, USA.

Gas chromatography (*GC*)

The enantiomeric excess of (2*RS*)-phenylpropanol was measured on a GC-2010 equipped with an AOC-20i autoinjector from Shimadzu Corp., Kyoto, Japan, and a chiral GC column Rt-bDEXm™ from Restek Corp., Bellefonte, PA, USA, or a GC-2010 Plus equipped with an AOC-20i autoinjector, an AOC-20s autosampler, and a chiral GC column Rxi®-5ms from the same manufacturers. The compounds were detected by a flame ionization detector (FID) and the data was analyzed with LabSolutions / GCsolution (Vers. 2.41.00).

Nuclear Magnetic Resonance (*NMR*)

All ^{1}H-, ^{13}C-, COSY-, NOESY- and HMQC-NMR spectra were measured on either a DRX 500 MHz NMR or AV 300 MHz NMR from Bruker Corp., Billerica, MA, USA. All ^{19}F- and ^{31}P-NMR spectra were measured on an AV 500 MHz NMR from the same manufacturer with capillaries containing $CFCl_3$ (δ = 0.0 ppm) and Me_3PO_4 (δ = 4.0 ppm), dissolved in acetone-d_6, as internal standards. The measurements were processed with MestReNova (Vers. 7.0.2-8636) from Mestrelab Research S.L., Santiago de Compostela, Spain. The shifts were measured in chloroform-d, acetone-d_6 and dimethylsulfoxide-d_6, respectively, and will be given in relation to tetramethylsilane (TMS, δ = 0.0 ppm) or a residual solvent peak according to literature values[116]. The frequencies used for ^{1}H- and ^{13}C- measurements were 500 MHz and 126 MHz (DRX500), 300 MHz and 75 MHz (AV300), respectively. The frequencies for ^{19}F- and ^{31}P-measurements were 470 MHz and 202 MHz (AV500), respectively. Unless stated otherwise all samples were measured at room temperature.

Mass spectrometry (*GC-MS, ESI-MS*)

For GC-MS analysis the sample was separated in a GC-17A (Version 3)/MS QP 5050A from Shimadzu Corp., Kyoto, Japan, equipped with an AOC-20i autoinjector and a capillary column HP-5ms from Agilent Technologies, Inc., Loveland, Colorado. The spectra were recorded with helium carrier gas (0.95 bar) and an ionization energy of 70 eV. Integrals were not corrected. The data was analyzed with LabSolutions GC/MS solution (Version 1.02) from Shimadzu Corp., Kyoto, Japan.

Polarimetry

For the identification of enantiomers their specific optical rotary power was measured on a Model 341 polarimeter from Perkin Elmer Instruments, Waltham, MA, USA. All values were measured at least five times in a 10-mm cell at 589.3 nm (Sodium D-Line). The direction of polarization was given as "+" (dextrorotary) or "-" (levorotary), respectively.

Infrared spectroscopy

Infrared spectra were recorded with a NICOLET 380 FT-IR spectrometer from Thermo Electron Corp., now Thermo Fisher Scientific, Inc., Waltham, MA, USA, and analyzed with the OMNIC (Vers. 7.3) software.

pH Stat

For maintaining the pH during hydrolysis of carboxylic esters in aqueous reaction media, a titrino apparatus, i.e. Titroline alpha plus from SI analytics

(Schott AG), Mainz, Germany, and Titrino 702 SM from METROHM GmbH & Co. KG, Filderstadt, Germany, was used. The data was recorded and analyzed with the software TitriSoft (Vers. 2.73) and/or tiamo (Vers. 1.2.1) from the same manufacturers, respectively.

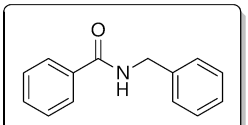
Melting points

The melting points of solid compounds were measured at least three times on a B-540 melting point analyzer from BUCHI Labortechnik AG, Flavil, Switzerland.

Mixing and centrifugation

Samples in 1.5-mL-, 15-mL- and 50-mL-vials were mixed on a Vortex Genie 2 from Scientific Industries, Inc., Bohemia, NY, USA. Heterogeneous mixtures were separated by centrifugation in a himac CT15RE (T15A61-1929 rotor) from Hitachi Koki Co., Ltd., Tokyo, Japan, or a HERAEUS Multifuge 3S-R from Thermo Fisher Scientific, Inc., Waltham, MA, USA, respectively.

8.0.1 *N*-BENZYL BENZOATE



$C_{14}H_{13}NO$
M = 211.26 g/mol

A mixture of benzoic acid (1.22 g, 10.0 mmol) and triethylamine (2.09 mL, 15.0 mmol) was dissolved in ethyl acetate (10 mL). After the addition of benzylamine (1.09 mL, 10.0 mmol) and *tris*-*n*-propylphosphonic anhydride (T3P, 50 w% in EtOAc, 5.90 mL, 10.0 mmol) the resulting solution was stirred for five hours at room temperature. The reaction was quenched by the addition of hydrochloric acid (1M, 10 mL). The phases were separated and the organic phase was washed with saturated sodium bicarbonate solution (10 mL) and brine (10 mL). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo*. The crude product was recrystallized from ethanol/*n*-hexane and dried in high vacuum to obtain colorless needles.

Isolated yield: 1.04 g (4.94 mmol, 49.4%).

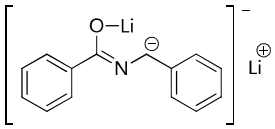
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.79 (d, *J* = 6.9 Hz, *o*-Bz-*H*), 7.53 – 7.48 (t, *J* = 7.4 Hz, 1H, *p*-Bz-*H*), 7.46 – 7.41 (t, *J* = 7.5 Hz, 2H, *m*-Bz-*H*), 7.36 (m, 4H, Ar-*H*), 7.33 – 7.28 (m, 1H, Ar-*H*), 4.65 (d, *J* = 5.6 Hz, 2H, CH_2).

^{13}C-NMR (126 MHz, $CDCl_3$): δ [ppm] = 167.43, 138.26, 134.39, 131.53, 128.76, 128.57, 127.89, 127.57, 127.01, 44.10.

Mp: 104-106 °C (Lit. 107–108 °C (EtOH))[117].

The data corresponds to literature values.[117]

8.0.2 DETERMINATION OF THE REACTIVE CONCENTRATION OF COMMERCIAL *N*-BUTYLLITHIUM SOLUTIONS



deep blue color

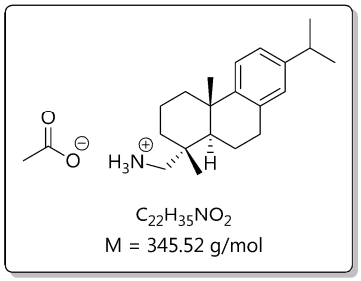
The following titration was conducted based on a literature procedure from BURCHAT *et al.*.[118] A solution of *n*-benzyl benzoate (212 mg, 1.00 mmol) in abs. THF (10 mL) was cooled to -78 °C (acetone/dry ice). The corresponding *n*-butyllithium solution (1.6 M in *n*-hexane) was added dropwise *via* syringe (1.0 mL max.). The volume for stoichiometric deprotonation of the starting material, thus corresponding to 1.0 mmol active *n*-butyllithium, was determined by an intense blue coloration of the solution, resulting from the dianionic species of *n*-benzyl benzoate.

Consumed volume: 0.85 mL[i]

Titer concentration: 1.18 M (based on result).

8.0.3 PURIFICATION OF (+)-DEHYDROABIETYLAMINE

8.0.3.1 (+)-DEHYDROABIETYLAMINE ACETATE



$C_{22}H_{35}NO_2$
M = 345.52 g/mol

Commercially available dehydroabietylamine[ii] (100 g, 350 mmol max.) was diluted with toluene (150 mL). A solution of glacial acetic acid (20.0 mL, 350 mmol) in toluene (50 mL) was added dropwise and the mixture was stirred at room temperature. On congelation of the mixture, toluene (100 mL) was added and the suspension was heated to reflux (120 °C) until homogenization. The solution was slowly cooled to room temperature until precipitation occurs. The precipitate was filtered off and washed with cold toluene (50 mL). The residue was recrystallized in toluene to obtain the product as colorless solid.

Isolated yield: 42.8 g (123.87 mmol, 64.2 %).

Mp: 139-141 °C (Lit: 140-143 °C[119]).

Specific rotation: a = +28.5° (c = 5, MeOH, 28 °C) (Lit: +30.2° (c = 5, MeOH, 25 °C))

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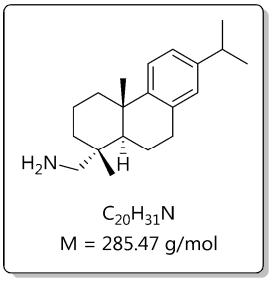
[i] This volume was necessary under the given conditions (considering dryness of solvent, dryness of inert gas and contact to humidity during transfer *via* syringe). The resulting concentration does not correspond to the actual concentration of the commercial solution. The titration can be used to determine the necessary amount of base for complete deprotonation prior to each experiment.

[ii] Commercial purity of 55%.

8.0.3.2 (+)-Dehydroabietylamine



(+)-Dehydroabiethylamine acetate (10.0 g, 28.9 mmol) was suspended in dist. H_2O (40 mL) and heated until homogenization. Subsequently, an aqueous solution of sodium hydroxide (10 w%, 15 mL, 37.5 mmol) was added and the solution was slowly cooled to room temperature. After extraction with MTBE (3x 50 mL) the combined organic phase was washed with dist. H_2O (50 mL) and dried over anhydrous potassium carbonate. All volatile compounds were removed in vacuo to obtain the product as colorless oil which crystallizes under storage at 4 °C.

Isolated yield: 8.10 g (28.4 mmol, 98.3%).

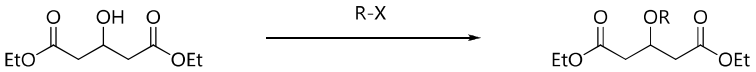
Mp: 39 - 41 °C (Lit: 43-45 °C[119]).

8.1 Standard operating procedure 1 (SOP1): Substrate synthesis for the hydrolase-coupled desymmetrization of O-protected diethyl 3-hydroxyglutarates

The following syntheses were based on literature-known procedures from various authors. The corresponding reference will be given in parenthesis for each substrate.

If not stated otherwise, a solution of catalyst (0.1 - 10 mol-%) and the corresponding protective group reagent (1.05 eq.) was mixed with diethyl 3-hydroxyglutarate (4.00 mmol) and stirred for 2 - 48 hours at 20 - 60 °C. The reaction was quenched by addition of saturated sodium bicarbonate solution (5 mL/mmol) and stirred for additional 30 minutes at room temperature. The mixture was extracted with ethyl acetate (3 x 20 mL) and the combined organic phases were dried over $MgSO_4$. All volatile compounds were removed *in vacuo* and the crude product was further dried in high vacuum before being analyzed by NMR spectroscopy. If necessary, the crude product was purified by column chromatography or distillation, respectively.

Table 41. Substrate synthesis of *O*-protected diethyl 3-hydroxyglutarates

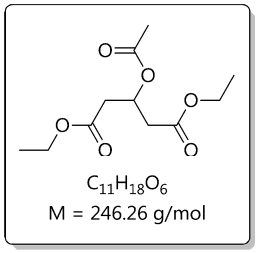


1a **22**

Entry	R	X	Cat. (mol%)	Time [h]	Temp. [°C]	Conv. [%][a]	Yield [g (%)]
1	Ac	OAc	$Zn(ClO_4)_2$ (0.1)	2	20	>95.0	0.84 (85.3)
2	Boc	OBoc	$Zn(OAc)_2$ (10.0)	6	60	>95.0	1.11 (91.2)
3	TFA	OTFA	$Zn(ClO_4)_2$ (1.0)	2	20	>95.0	1.07 (89.1)
4	PhAc	Cl	-	16	0-20	>95.0	5.30 (82.2)
5	MeOAc	Cl	Pyridine (110)	24	0-20	92.1	3.54 (64.0)
6	TBS	Cl	1*H*-imidazole	24	20	>95.0	0.58 (36.4)
7	THP	-	PPTS	21	20	>95.0	1.68 (58.3)

(a) Determined by integration of ^{1}H-NMR signals for 3-DHG and products.

8.1.1 DIETHYL 3-(ACETYL)-OXYGLUTARATE



$C_{11}H_{18}O_6$
M = 246.26 g/mol

The synthesis was conducted according to BARTOLI *et al.*.[120] A mixture of zinc perchlorate hexahydrate (1.5 mg, 0.004 mmol, 0.1 mol%) and acetic anhydride (397 µL, 4.2 mmol, 1.05 eq.) was mixed with diethyl 3-hydroxyglutarate (741 µL, 4.0 mmol) and stirred for two hours at room temperature. The work-up was done as described in SOP1. The crude product was obtained as colorless oil and used without further purification.

Isolated yield: 0.84 g (3.41 mmol, 85.3 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.51 (m, 1H, C*H*-O), 4.13 (dq, *J* = 7.2 Hz, 4H, O-C*H*$_2$-CH$_3$), 2.69 (d, *J* = 6.3 Hz, 4H, C*H*$_2$-CH-C*H*$_2$), 2.02 (s, 3H, C(=O)-C*H*$_3$), 1.24 (t, *J* = 7.2 Hz, 6H, CH$_2$-C*H*$_3$).

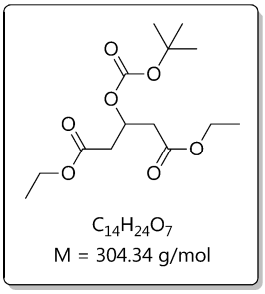
^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 170.06 (1C, (O=)*C*-CH$_3$), 170.00 (2C, *C*O$_2$Et), 67.06 (1C, *C*H-OAc), 60.93 (2C, O-*C*H$_2$-CH$_3$), 38.60 (2C, *C*H$_2$-CH-*C*H$_2$), 21.07 (1C, (O=)C-CH$_3$) 14.28 (2C, CH$_2$-*C*H$_3$).

The data corresponds to literature-known values.[121]

Table 42. Scale-up for the synthesis of diethyl 3-(acetyl)-oxyglutarate

Entry	Amount DHG [mmol]	Conversion [%]	Isolated yield [g (%)]
1	4.00	>95.0	0.84 (85.0)
2	20.0	>95.0	4.70 (95.4)
3	200	>95.0	43.4 (88.1)
4	500	>95.0	111 (90.5)

8.1.2 DIETHYL 3-(*TERT*-BUTYLOXYCARBONYL)-OXYGLUTARATE



The synthesis was conducted according to BARTOLI *et al.*.[120] Diethyl 3-hydroxy-glutarate (741 µL, 4.00 mmol) and boc anhydride (916.7 mg, 4.2 mmol, 1.05 eq.) were mixed. After addition of zinc acetate dihydrate (87.8 mg, 0.4 mmol, 0.10 eq.) the mixture was stirred for six hours at 60 °C. The work-up was conducted as described in SOP1. The crude product was obtained as colorless oil and used without further purification.

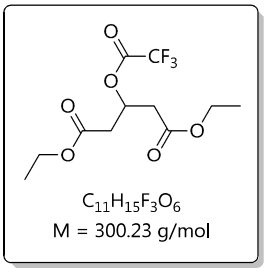
Isolated yield: 1.11 g (3.65 mmol, 91.2 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.36 (m, 1H, C*H*-O), 4.19 – 4.09 (m, 4H, O-C*H*$_2$-CH$_3$), 2.71 – 2.70 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.47 (s, 9H, C-(C*H*$_3$)$_3$), 1.25 (t, *J* = 7.2 Hz, 6H, CH$_2$-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 169.89, 152.56, 82.70, 69.77, 60.95, 38.76, 27.86, 14.26.

The compound is unknown to literature.

8.1.3 DIETHYL 3-(TRIFLUORACETYL)-OXYGLUTARATE



The synthesis was conducted according to BARTOLI *et al*[120]. Diethyl 3-hydroxyglutarate (741 µL, 4.0 mmol) and trifluoroacetic anhydride (584 µL, 4.2 mmol, 1.05 eq.) were mixed. After the addition of zinc perchlorate hexahydrate (14.9 mg, 0.04 mmol, 0.01 eq.) the mixture was stirred for two hours at room temperature. The work-up was done as described in SOP1. The crude product was obtained as colorless oil and used without further purification.

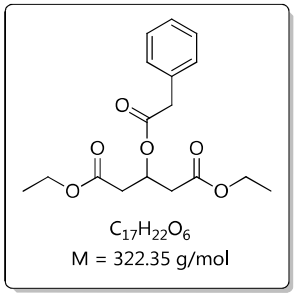
Isolated yield: 1.07 g (3.56 mmol, 89.1%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.75 (m, 1H, C*H*-O), 4.20 – 4.14 (m, 4H, O-C*H*$_2$-CH_3), 2.80 (d, *J* = 6.6 Hz, 4H, C*H*$_2$-CH-C*H*$_2$), 1.25 (t, *J* = 7.3 Hz, 6H, CH_2-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 169.10, 156.54, 114.50, 71.05, 61.50, 38.23, 14.25.

The compound is unknown to literature.

8.1.4 DIETHYL 3-(PHENYLACETYL)-OXYGLUTARATE



$C_{17}H_{22}O_6$
M = 322.35 g/mol

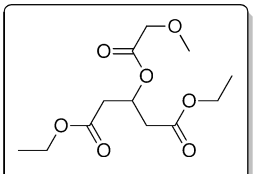
The synthesis was conducted according to BARTOLI *et al*[120]. Diethyl 3-hydroxyglutarate (741 µL, 4.0 mmol) and phenylacetyl chloride (555 µL, 4.2 mmol, 1.05 eq.) were mixed at 0-4 °C. The mixture was stirred for 16 hours while thawing up to room temperature. The work-up was done as described in SOP1. The crude product was obtained as a colorless oil and used without further purification.

Isolated yield: 5.30 g (16.4 mmol, 82.2%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.32-7.24 (m, 5H, Ar-*H*), 5.53 (m, 1H, C*H*-O), 4.10-4.07 (m, 4H, O-C*H*$_2$-CH_3), 3.59 (s, 2H, C*H*$_2$-Ph), 2.69 (d, 4H, *J* = 6.3 Hz, C*H*$_2$-CH-C*H*$_2$), 1.20 (t, 6H, *J* = 7.1 Hz, CH_2-C*H*$_3$).

The compound is unknown to literature.

8.1.5 DIETHYL 3-(METHOXYACETYL)-OXYGLUTARATE



$C_{12}H_{20}O_7$
M = 276.28 g/mol

The synthesis was conducted according to a procedure of ÖHRLEIN and BAISCH[122]. Diethyl 3-hydroxyglutarate (3.70 ml, 20.0 mmol) was diluted with dichloromethane (1 mL) and cooled to 0-4 °C. Pyridine (1.78 ml, 22.0 mmol, 1.1 eq.) and methoxyacetyl chloride (2.00 ml, 22.0 mmol, 1.1 eq.) were added dropwise. The mixture was brought to room temperature and further stirred for 12 hours. The reaction was quenched by the addition of ethyl acetate (20 mL) and saturated sodium bicarbonate solution. The phases were separated and the organic phase was washed once with each hydrochloric acid (1N), saturated bicarbonate solution and brine. After drying over $MgSO_4$ the solvent and all volatile compounds were removed *in vacuo*. The crude product was purified by distillation (0.15 mbar, 126-128 °C) to obtain the product as colorless oil.

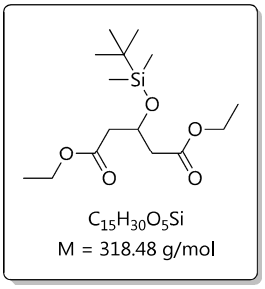
Isolated yield: 3.54 g (12.8 mmol, 64.0%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.63 (m, 1H, C*H*-O), 4.15 (q, 4H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 4.00 (s, 2H, C*H*$_2$-O-CH$_3$), 3.43 (s, 3H, CH$_2$-O-C*H*$_3$), 2.73 (d, 4H, *J* = 6.3 Hz, C*H*$_2$-CH-C*H*$_2$), 1.26 (t, 6H, *J* = 7.2 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 169.65, 169.33, 69.64, 67.41, 60.90, 59.36, 38.45, 14.15.

The data corresponds to literature-known values.[123]

8.1.6 DIETHYL 3-(*TERT*-BUTYLDIMETHYLSILYL)-OXYGLUTARATE



$C_{15}H_{30}O_5Si$
M = 318.48 g/mol

The synthesis was conducted based on a procedure of HEATHCOCK *et al.*[124] Diethyl 3-hydroxyglutarate (974 µL, 5.0 mmol) and 1*H*-imidazole (0.51 g, 7.5 mmol, 1.5 eq.) were dissolved in THF (15 mL). After addition of *tert*-butyldimethylchlorosilane (0.83 g, 5.5 mmol, 1.1 eq.) the suspension was stirred for 24 hours at room temperature. The work-up was conducted according to SOP1. The crude product was analyzed by NMR spectroscopy and shows quantitative conversion. The crude product was purified by column chromatography on silica (15% v/v ethyl acetate in cyclohexane) to obtain a colorless oil.

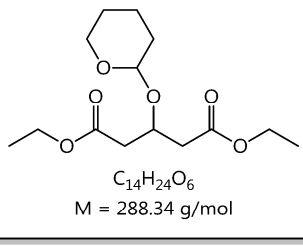
Isolated yield[i]: 0.58 g (1.82 mmol, 36.4%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.55 (m, 1H, C*H*-O), 4.16-4.11 (m, 4H, O-C*H*$_2$-CH$_3$), 2.54 (dd, 4H, *J* = 6.1 Hz, C*H*$_2$-CH-C*H*$_2$), 1.26 (t, 6H, *J* = 7.2 Hz, CH$_2$-C*H*$_3$), 0.85 (s, 9H, C-(C*H*$_3$)$_3$), 0.07 (s, 6H, Si-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 171.10, 66.39, 60.49, 42.65, 25.67, 17.91, 14.19, -4.89.

The data corresponds to literature-known values.[124]

8.1.7 DIETHYL 3-(TETRAHYDROPYRAN-2-YL)-OXYGLUTARATE



$C_{14}H_{24}O_6$
M = 288.34 g/mol

Pyridinium *p*-toluenesulfonate (0.25 g, 1.00 mmol, 0.1 eq.) was added to a solution of diethyl 3-hydroxyglutarate (2.04 g, 10.0 mmol) and dihydropyrane

[i] Following fractions were contaminated with different contents of silanol and therefore discarded.

(1.26 g, 15.0 mmol, 1.5 eq.) in dichloromethane (70 mL). The mixture was stirred for 21 hours at room temperature. The reaction was quenched with addition of a saturated sodium bicarbonate solution (50 mL). The phases were separated and the aqueous phase was extracted with dichloromethane (3x 50 mL). The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude extract was purified by column chromatography (10% v/v ethyl acetate in cyclohexane) to obtain the product as colorless oil.

Isolated yield: 1.68 g (5.83 mmol, 58.3%).

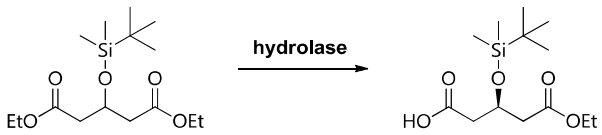
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.48 (m, 1H, C*H*-O), 4.13 (m, 5H, O-CH_2-CH_3, O-C*H*-O), 3.38-3.88 (m, 2H, H_2C-CH_2-O), 2.54-2.82 (m, 4H, CH_2-CH-CH_2), 1.51-186 (m, 6H, CH_2-CH_2-CH_2), 1.26 (dt, *J* = 7.1 Hz, 6H, CH_2-CH_3).

The data corresponds to literature-known values.[125]

8.2 STANDARD OPERATING PROCEDURE 2 (SOP2): COLORIMETRIC SCREENING ASSAY OF HYDROLASES FOR THE DESYMMETRIZATION OF *O*-PROTECTED DIETHYL 3-HYDROXYGLUTARATES

The colorimetric assay was based on an already published acid-base-assay from BAUMANN[126]. A solution of a hydrolase (180 µL, 1 mg/mL) in KPB (pH 7.3, 50 mM) was pipetted into a 96-well-plate. The corresponding substrate (20 µL, 20 mM in DMSO containing 0.75 M bromothymol blue) was added and the resulting color changes observed by time-dependent measurement in a plate reader at 418 nm and 626 nm, respectively. The obtained values were compared with hydrolase solutions (180 µL, 1 mg/mL) without substrate containing bromothymol blue (20 µL, 0.75 M in DMSO) and with substrate solutions in the absence of biocatalyst.

Table 43. Photometric assay for potential hydrolases in the desymmetrisation of TBS-DHG



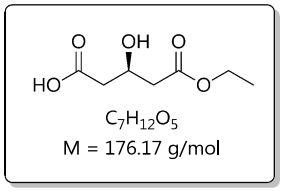
Hydrolase	Activity (Abs)		Rel. activity	
	DHG	TBS-DHG	DHG	TBS-DHG
α-Chymotrypsin (Biozym CHY-03)	0.106	0.131	100%	100%
Alcalase CLEA	0.101	0.067	95%	51%
Protease from *Rhizopus* sp.	0.097	0.080	92%	61%
Protease from *Aspergillus* sp.	0.082	0.074	77%	56%
Amano Acylase	0.079	0.094	75%	72%
Protease from *Aspergillus saitoi*	0.023	0.031	22%	24%
α-Chymotrypsin (AppliChem Grade I)	0.028	0.048	26%	37%
Protease S from *Pyrococcus furiosus*	0.051	0.089	48%	68%
Protease from *Aspergilus oryzae*	0.053	0.053	50%	40%
Protease from *Bacillus licheniformis*	0.016	0.018	15%	14%
α-Chymotrypsin (AppliChem Grade II)	0.034	0.053	32%	40%
Protease from bovine pancreas	0.075	0.166	71%	127%
α-Chymotrypsin (Amresco)	0.016	0.021	15%	16%
Pronase	0.062	0.042	58%	32%
Protease from *Bacillus* sp.	0.024	0.014	23%	11%
Protease from *B. amyloliquefaciens*	0.087	0.171	82%	131%

8.3 STANDARD OPERATING PROCEDURE 3 (SOP3): PREPARATIVE DESYMMETRIZATION OF DHG AND ITS *O*-PROTECTED DERIVATIVES BY THE USE OF HYDROLASES

An adequate amount of hydrolase (1.6 – 18 kU/mmol) was dissolved in KPB (pH 8.0, 50mM) to reach the desired substrate concentration (0.05 – 6.00 M) and titrated with aqueous sodium hydroxide solution (2.0 N) to pH 8.0. After addition of the corresponding amount of DHG or its O-protected derivatives the resulting emulsion was stirred vigorously at room temperature. The progress of the reaction was monitored by the volume of titer (aq. sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. After reaching the end of the reaction the solution was acidified (pH 1-2)

with hydrochloric acid (12 N) and extracted with ethyl acetate (3x 1:1 v/v). The combined organic phases were dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The conversion was determined witH-NMR spectroscopy. Unless otherwise stated the crude extract was further used without purification.

8.3.1 (3*R*)- 5-ETHOXY-3-HYDROXY-5-OXOPENTANOIC ACID



$C_7H_{12}O_5$
M = 176.17 g/mol

The synthesis was conducted according to SOP3. A solution of α-chymotrypsin (2.50 g, Biozym CHY-03) in KPB (50.0 mL, 50mM, pH 8.0) was titrated with aqueous sodium hydroxide solution (1.0M) to pH 8.0. After addition of diethyl 3-hydroxyglutarate (9.26 mL, 50.0 mmol) to reach a substrate concentration of 1.0 M the resulting emulsion was vigorously stirred for 48 hours at room temperature. The progress of the reaction was monitored by the volume of titer (1.0 M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. The work-up was conducted as described in SOP3.

Yield: 6.65 g (37.7 mmol, 75.4 %).

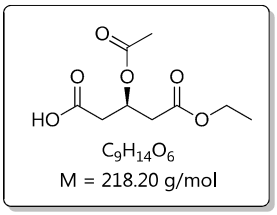
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.47 (m, 1H, C*H*-OH), 4.18 (q, 2H, *J* = 7.2 Hz, O-CH_2-CH_3), 2.62 – 2.54 (m, 4H, CH_2-CH-CH_2), 1.28 (t, 3H, *J* = 7.1 Hz, CH_2-CH_3).

The data corresponds to literature-known values.[127]

Table 44. Scale-up for the synthesis of (3*R*)-5-ethoxy-3-hydroxy-5-oxo-pentanoic acid

Entry	Substrate [mmol]	Biocatalyst [mg]	Sub. conc. [M]	Conversion [%]	Yield [g (%)]
1	50.0	2500	1.00	>95	6.65 (75.4)
2	100	7500	2.00	>95	8.73 (49.5)
3	100	7500	2.00	>95	9.65 (54.8)

8.3.2 (3*R*)-5-ETHOXY-3-(ACETYL)-OXY-5-OXOPENTANOIC ACID



The synthesis was conducted according to SOP3. A solution of α-chymotrypsin (300 mg, Biozym CHY-03) in KPB (10.0 mL, 50 mM, pH 8.0) was titrated with aqueous sodium hydroxide solution (4.0 M) to pH 8.0. After addition of diethyl 3-(acetyl)-oxyglutarate (9.85 g, 40.0 mmol) to reach a substrate concentration of 4.0 M the resulting emulsion was vigorously stirred for 6-24 hours at room temperature. The progress of the reaction was monitored by the volume of titer (4.0 M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. The work-up was done as described in SOP3.

Yield: 9.12 g (91% product content[i], 38.2 mmol, 95.4 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.50 (m, 1H, C*H*-O), 4.16 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.79 (dd, 2H, *J* = 6.0 Hz, C*H*$_2$-COOH), 2.72 (d, 2H, *J* = 6.3 Hz, C*H*$_2$-COOEt), 2.05 (s, 1H, (O=)C-C*H*$_3$), 1.26 (t, 6H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR (126 MHz, $CDCl_3$): δ [ppm] = 175.13, 170.08, 169.84, 66.56, 60.93, 38.29, 37.93, 20.95, 14.15.

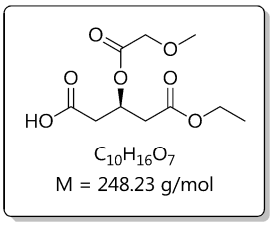
The data corresponds to literature-known values.[121]

Table 45. Scale-up for the synthesis of (3*R*)-(acetyl)-oxy-5-ethoxy-5-oxo-pentanoic acid

Entry	Substrate [mmol]	Biocatalyst [mg]	Sub. conc. [M]	Conversion [%]	Yield [g(%)]
1	15.0	49.3	1.00	>95	3.37 (97.2)
2	40.0	300	4.00	>95	9.12 (95.5)
3	50	2500	5.00	>95	8.58 (78.6)
4	60	2500	6.00	>95	9.91 (75.7)
5	100	7500	2.00	>95	21.8 (95.4)

[i] Determined by quantitative NMR integration against internal standard (see chapter 2.4.3, page 16).

8.3.3 (3*R*)-5-ETHOXY-3-(METHOXYACETYL)-OXY-5-OXOPENTANOIC ACID



$C_{10}H_{16}O_7$
M = 248.23 g/mol

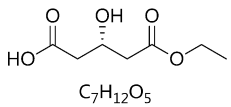
The synthesis was conducted according to SOP3. A solution of α-chymotrypsin (100 mg, Biozym CHY-03) in KPB (10.0 mL, 50mM, pH 8.0) was titrated with aqueous sodium hydroxide solution (1.0M) to pH 8.0. After addition of diethyl 3-(methoxyacetyl)-oxyglutarate (2.76 g, 10.0 mmol) to reach a substrate concentration of 1.0 M the resulting emulsion was vigorously stirred for 6-24 hours at room temperature. The progress of the reaction was monitored by the volume of titer (1.0 M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. The work-up was done as described in SOP3. NMR analysis shows a conversion of >95% (74% product-related, 21% hydrolysis of methoxyacetyl ester).

Yield: 2.09 g (74% product content[i], 6.23 mmol, 62.3%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.64 (m, 1H, C*H*-O), 4.17 (q, 2H, ^{J} = 7.1 Hz, O-CH_2-CH_3), 4.04 (s, 2H, CH_2-O-CH_3), 3.45 (s, 3H, O-CH_3), 2.84 (d, 2H, *J* = 6.3 Hz, CH_2-COOH), 2.77 (d, 2H, *J* = 6.3 Hz, CH_2-COOEt), 1.28 (t, 3H, *J* = 7.2 Hz, CH_2-*CH_3*).

The data corresponds to literature-known values.[128]

8.3.4 (3*S*)-5-ETHOXY-3-HYDROXY-5-OXOPENTANOIC ACID



$C_7H_{12}O_5$
M = 176.17 g/mol

The synthesis was conducted according to SOP3. *Candida antarctica* lipase B (350 mg, immobilized on acrylic resin) was suspended in KPB (15.0 mL, 50 mM, pH 8.0) and titrated with aqueous sodium hydroxide solution (1.0 M) to pH 8.0. After addition of diethyl 3-hydroxyglutarate (2.04 g, 10.0 mmol) to reach a substrate concentration of 0.7 M the resulting heterogeneous mixture was vigorously stirred for two hours at room temperature. The progress of the reaction was monitored by the volume of titer (1.0 M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. The work-up was conducted as described in SOP3. NMR analysis shows quantitative conversion. The product was used without further purification.

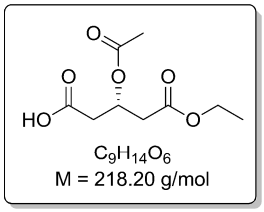
Isolated yield: 643 mg (3.65 mmol, 36.5%).

[i] Determined by quantitative NMR integration after identification of all impurities.

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.47 (m, 1H, C*H*-OH), 4.18 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.62 – 2.54 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.28 (t, 3H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

The data corresponds to literature-known values.[129]

8.4 (3*S*)-5-Ethoxy-3-(acetyl)-oxy-5-oxopentanoic acid



Acetic anhydride (3.96 mL, 42.0 mmol, 2.1 eq.) and zinc perchlorate hexahydrate (14.9 mg, 0.04 mmol, 0.002 eq.) were mixed and stirred for 10 minutes at room temperature. After the addition of (3*S*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid (3.52 g, 20.0 mmol) the solution was vigorously stirred for four hours at room temperature. A saturated sodium bicarbonate solution (50 mL) was added to hydrolyze the excess of anhydride. The solution was stirred for another 30 minutes and extracted with ethyl acetate (3x 50 mL). The organic layer was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo* to obtain the product as colorless oil.

Isolated yield: 3.46 g (15.9 mmol, 79.3 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.50 (m, 1H, C*H*-O), 4.16 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.79 (dd, 2H, *J* = 6.0 Hz, C*H*$_2$-COOH), 2.72 (d, 2H, *J* = 6.3 Hz, C*H*$_2$-COOEt), 2.05 (s, 1H, (O=)C-C*H*$_3$), 1.26 (t, 6H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR (126 MHz, $CDCl_3$): δ [ppm] = 175.13, 170.08, 169.84, 66.56, 60.93, 38.29, 37.93, 20.95, 14.15.

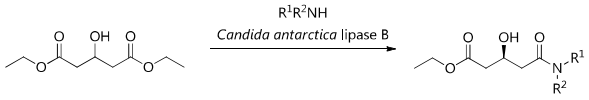
The data corresponds to literature-known values.[121]

8.5 Standard operating procedure 4 (SOP4): Preparative desymmetrization by lipase-catalyzed aminolysis of diethyl 3-hydroxyglutarate

The synthesis was conducted based on a procedure by Sánchez *et al.*.[130] *Candida antarctica* lipase B (immobilized on acrylic resin) was suspended in an adequate solvent (10 mL) or the corresponding amine itself. Diethyl 3-hydroxyglutarate (2.00 – 10.0 mmol) and the primary or secondary amine (1.00 – 50.0 eq.) were added and the suspension was stirred for 24 hours at 20-

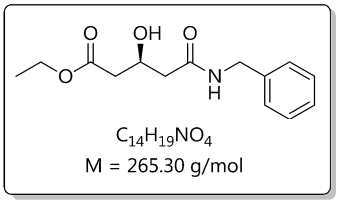
60 °C. The biocatalyst was filtered off and washed with the same solvent or dichloromethane (2x 10 mL). The filtrate was concentrated *in vacuo* and the crude product was purified by column chromatography or recrystallization, respectively.

Table 46. Lipase-catalyzed aminolysis of DHG with various primary and secondary amines



Entry	R^1	R^2	DHG [mmol]	CAL-B [mg]	Temp. [°C]	Solvent	Conv. [%]	Yield [g (%)]
1	H	Bn	2.00	180	20	Dioxane	>95	0.52 (98.9)
2	H	Bn	2.00	180	20	THF	>95	0.47 (89.3)
3	H	Bn	2.00	180	20	MTBE	>95	0.49 (93.1)
4	H	Bn	2.00	180	20	Toluene	84.0	n.d.
5	H	Bn	10.0	907	20	MTBE	>95	2.20 (83.1)
6	Me	Bn	10.0	900	reflux	MTBE	7.2	0.16 (5.6)
6	Et	Et	2.00	180	reflux	MTBE	0	-
7	morpholine		10.0	900	reflux	MTBE	n.d.	0.86 (35.0)
8	pyrrolidine		5.00	110	20	MTBE	55.9	n.d.
9	piperidine		5.00	113	reflux	MTBE	32.0	n.d.

8.5.1 ETHYL (3*S*)-5-(*N*-BENZYLAMINO)-3-HYDROXY-5-OXOPENTANOATE



The synthesis was conducted according to SOP4. To a suspension of *Candida antarctica* lipase B (907 mg, immobilized on acrylic resin) in MTBE (30 mL) were added benzylamine (1.10 mL, 10.0 mmol, 1.00 eq.) and diethyl 3-hydroxyglutarate (1.86 mL, 10.0 mmol) in the given order. The mixture was stirred for 17 hours at room temperature. The biocatalyst was filtered off and washed with MTBE (3 x 30 mL). The filtrate was concentrated *in vacuo* and the crude product recrystallized in MTBE to obtain a colorless solid. The enantiomeric excess could not be determined yet.

Isolated yield: 2.20 g (8.31 mmol, 83.1 %).

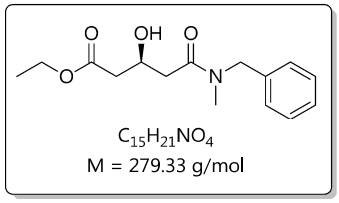
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.33-7.25 (m, 5H, Ar-*H*), 6.67 (s, 1H, *n*-*H*), 4.43-4.37 (m, 3H, C*H*-O, CH_2-Ph), 4.14 (q, 2H, *J* = 7.1 Hz, O-CH_2-CH_3), 2.55-2.38 (m, 4H, CH_2-CH-CH_2), 1.26 (t, 3H, *J* = 7.1 Hz, CH_2-CH_3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 172.12, 171.23, 138.07, 128.71, 127.69, 127.51, 65.31, 60.85, 34.44, 42.02, 40.85, 14.15

.

The data corresponds to literature-known values.[131]

8.5.2 Ethyl (3*S*)-5-(*N*-benzyl-*N*-methylamino)-3-hydroxy-5-oxo-pentanoate



The synthesis was conducted according to SOP4. To a suspension of *Candida antarctica* lipase B (900 mg, immobilized on acrylic resin) in MTBE (10 mL) were added *n*-benzylmethylamine (1.30 mL, 10.0 mmol, 1.00 eq.) and diethyl 3-hydroxy-glutarate (1.86 mL, 10.0 mmol) in the given order. The mixture was stirred for 17 hours at 60 °C. The biocatalyst was filtered off and washed with MTBE (3x 30 mL). The filtrate was concentrated *in vacuo* and the crude product was purified *via* column chromatography (50-100% v/v ethyl acetate in cyclohexane) to obtain a 3:2 *E/Z*-mixture as yellow oil. The enantiomeric excess could not be determined.

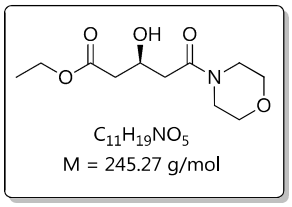
Isolated yield: 157 mg (0.56 mmol, 5.6%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.45 – 7.13 (m, 10H, Ar-*H*), 4.68 – 4.58 (d, *J* = 3.2 Hz, 2H, *n*-CH_2-Ph), 4.57 – 4.48 (m, 2H, C*H*-OH), 4.46 – 4.43 (dd, *J* = 5.9, 3.4 Hz, 2H, *n*-CH_2-Ph), 4.22 – 4.17 (q, *J* = 7.1 Hz, 2H, O-CH_2-CH_3), 4.17 – 4.12 (q, *J* = 7.2 Hz, 2H, O-CH_2-CH_3), 3.03 – 2.96 (s, 3H, *n*-CH_3), 2.96 – 2.89 (s, 3H, *n*-CH_3), 2.76 – 2.46 (m, 8H, CH_2-CH-CH_2), 1.32 – 1.28 (t, *J* = 7.2 Hz, 3H, CH_2-CH_3), 1.28 – 1.23 (t, *J* = 7.1 Hz, 3H, CH_2-CH_3).

^{13}C NMR (125 MHz, $CDCl_3$): δ [ppm] = 172.31, 171.98, 171.61, 171.53, 136.81, 135.95, 129.04, 128.73, 128.69, 127.96, 127.80, 127.71, 127.52, 126.26, 65.29, 65.28, 65.18, 60.87, 60.63, 60.60, 53.19, 50.66, 43.48, 41.99, 40.97, 40.71, 38.84, 38.42, 34.74, 33.77, 14.20, 14.16.

The compound is unknown to literature.

8.5.3 ETHYL (3*S*)-HYDROXY-5-(*N*-MORPHOLINO)-5-OXOPENTANOATE



$C_{11}H_{19}NO_5$
M = 245.27 g/mol

The synthesis was conducted according to SOP4. To a suspension of *Candida antarctica* lipase B (900 mg, immobilized on acrylic resin) in MTBE (10 mL) were added morpholine (0.87 mL, 10.0 mmol, 1.00 eq.) and diethyl 3-hydroxyglutarate (1.86 mL, 10.0 mmol) in the given order. The mixture was stirred for 17 hours at reflux temperature. The biocatalyst was filtered off and washed with MTBE (3x 10 mL). The filtrate was concentrated *in vacuo* and the crude product was purified by column chromatography on alkaline aluminum oxide (50-100 % v/v ethyl acetate in cyclohexane) to obtain a colorless solid. The enantiomeric excess could not be determined.

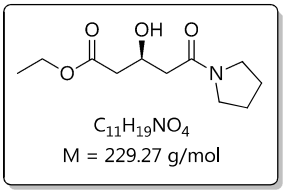
Isolated yield: 0.860 g (3.50 mmol, 35.0%).

^{1}H-NMR ($CDCl_3$, 500 MHz): δ [ppm] = 4.52 – 4.41 (m, 1H, C*H*-O), 4.18 – 4.12 (m, 2H, O-C*H*$_2$-CH$_3$), 3.72 – 3.46 (m, 8H, C*H*$_2$-CH-C*H*$_2$, *n*-C*H*$_2$-C*H*$_2$), 2.66 – 2.36 (m, 4H, *N*-C*H*$_2$-C*H*$_2$), 1.28 (t, 3H, *J* = 7.2 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR ($CDCl_3$, 125 MHz): δ [ppm] = 171.6, 170.3, 66.8, 66.5, 65.0, 60.6, 45.9, 41.7, 41.0, 38.5, 14.2.

The data corresponds to literature-known values.[131]

8.5.4 ETHYL (3*S*)-HYDROXY-5-(*N*-PYRROLIDINO)-5-OXOPENTANOATE



$C_{11}H_{19}NO_4$
M = 229.27 g/mol

The synthesis was conducted according to SOP4. To a suspension of *Candida antarctica* lipase B (110 mg, immobilized on acrylic resin) in MTBE (10 mL) were added pyrrolidine (418 µL, 5.0 mmol, 1.00 eq.) and diethyl 3-hydroxyglutarate (1.0 mL, 5.0 mmol) in the given order. The mixture was stirred for 12 hours at room temperature. The biocatalyst was filtered off and washed with MTBE (3 x 10 mL). The filtrate was concentrated *in vacuo* and the crude product was analyzed by NMR spectroscopy. The crude product was partially purified by a silica filtration (MTBE) and analyzed by chiral HPLC (Daicel ChiralPak AD-H, 91:9 SC-CO_2/IPA, 0.7 mL/min, 216 nm, 10 MPa back pressure) to determine an enantiomeric excess of 59.1% ee.

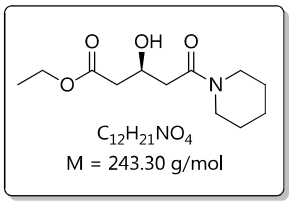
Conversion: 55.9 %.

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 4.51 – 4.40 (m, 1H, C*H*-OH), 4.15 (q, *J* = 7.1 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.51 – 3.35 (m, 4H, C*H*$_2$-*N*-C*H*$_2$), 2.69 – 2.35 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 2.03 – 1.80 (m, 4H, C*H*$_2$-C*H*$_2$), 1.31 – 1.22 (m, 3H, CH$_2$-C*H*$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$): δ [ppm] = 170.75, 169.61, 64.43, 59.69, 45.98, 44.83, 40.57, 39.37, 25.23, 23.65, 13.50.

The data corresponds to literature-known values.[131]

8.5.5 Ethyl (3*S*)-hydroxy-5-(*N*-piperidino)-5-oxopentanoate



The synthesis was conducted according to SOP1. To a suspension of *Candida antarctica* lipase B (113 mg, immobilized on acrylic resin) in MTBE (10 mL) was added piperidine (495 µL, 5.0 mmol, 1.00 eq.) and diethyl 3-hydroxyglutarate (1.0 mL, 5.0 mmol) in the given order. The mixture was stirred for four hours at reflux temperature. The biocatalyst was filtered off and washed with MTBE (3x 10 mL). The filtrate was concentrated *in vacuo* and the crude product analyzed by NMR spectroscopy. The enantiomeric excess could not be determined yet.

Conversion: 32.0%

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 4.45 (m, 1H, C*H*-OH), 4.15 (q, *J* = 7.2, 2H, O-C*H*$_2$-CH_3), 3.62 – 3.47 (m, 2H, *n*-C*H*$_2$), 3.40 – 3.33 (m, 2H, *n*-C*H*$_2$), 2.54 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.64 – 1.49 (m, 6H, C*H*$_2$-C*H*$_2$-C*H*$_2$), 1.26 (t, J=7.1, 3H, CH_2-C*H*$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$): δ [ppm] = 171.2, 169.5, 64.8, 60.2, 46.1, 42.1, 40.7, 38.1, 26.0, 25.2, 24.0, 13.8.

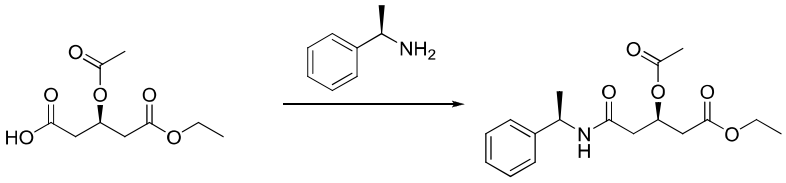
The data corresponds to literature-known values.[131]

8.6 Standard operating procedure 5 (SOP5): Determination of an enantiomeric excess of chiral carboxylic acids by coupling with enantiopure (*R*)- and (*S*)-1-phenylethylamine

The desymmetrized monoester (2.00 mmol) and triethylamine (4.00 mmol, 2.0 eq.) were dissolved in ethyl acetate (10 mL) and cooled to 0-4 °C. Subsequently, enantiopure (*R*)- or (*S*)-1-phenylethylamine (2.00 mmol, 1.00 eq.) and *tris*-*n*-propylphosphonic anhydride (T3P, 2.30 mmol, 1.15 eq.) were added dropwise and in the given order. The mixture was stirred at 0-20 °C and the progress of reaction was monitored *via* TLC. The reaction was quenched with addition of saturated sodium bicarbonate solution and stirring for additional 10 minutes at room temperature. After separation of the phases the aqueous phase was extracted with ethyl acetate (2x 10 mL) and the combined organic phase was washed once with hydrochloric acid (0.5 M, 30 mL), saturated

sodium bicarbonate solution (30 mL) and brine (30 mL). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo*. The crude product was used without further purification and analyzed by NMR and chiral HPLC. The enantiomeric excess of the previous desymmetrization was determined by the diastereomeric excess of the corresponding 1-phenylethylamide.

Table 47. Determination of the enantiomeric excess by derivatization to a diastereomeric amide

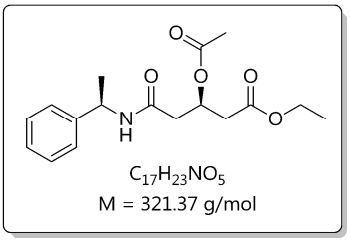


HPLC method:

Column: Daicel ChiralPak OJ-H
Eluent: SC-CO2/2-PrOH 95:5 v/v
Back pressure: 10 MPa
Flowrate: 0.8 mL/min
Detection wavelength: 208 nm
Column temperature: 20 °C
Sample concentration: 2 mg/mL
Injection volume: 10 µL
R_t: 14.0 min (3*R*,7*R*), 15.5 min (3*S*,7*R*)

Entry	Description / additive	Conv. [%]	de [%]	STDEV de [+- %]
1	Benchmark DHG	>95	60.7	0.4
2	Benchmark 3-acetyl-DHG (1.0M)	>95	97.6	0.6
3	1.2M sodium acetate	>95	97.6	1.0
4	1.2M sodium chloride	>95	98.1	0.4
5	2.4M sodium acetate	>95	96.8	0.2
6	0.1 mol% zinc perchlorate	>95	97.0	0.1
7	3-Acetyl-DHG (2.0M)	>95	97.1	0.5
8	1.0M ammonium sulfate	>95	97.3	0.0
9	Reaction medium: dest. H_2O	>95	96.0	0.9
10	Purified α-chymotrypsin (fraction 1)	>95	97.0	n.d.
11	Purified α-chymotrypsin (fraction 2)	>95	97.2	n.d.
12	Purified α-chymotrypsin (fraction 3)	>95	97.5	n.d.
13	3-Acetyl-DHG (3.0M)	>95	96.8	n.d.
14	3-Acetyl-DHG (4.0M)	>95	96.7	n.d.
15	Chymotrypsin / Trypsin 1:1	>95	96.8	n.d.

8.6.1 (3*R*,1'*R*)-Ethyl 5-(*N*-(1-phenylethyl)amino)-3-(acetyl)-oxy-5-oxopentanoate



$C_{17}H_{23}NO_5$
M = 321.37 g/mol

The synthesis was conducted according to SOP5. A solution of (3*R*)-5-ethoxy-3-(acetyl)-oxy-5-oxopentanoic acid (436 mg, 2.00 mmol) and triethylamine (558 µL, 4.00 mmol, 2.0 eq.) in ethyl acetate (10 mL) was cooled

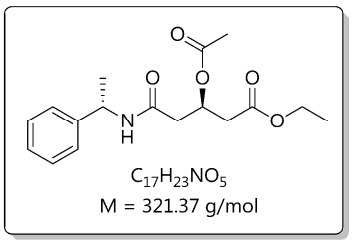
to 0-4 °C. Subsequently, (*R*)-1-phenylethylamine (318 µL, 2.00 mmol, 1.00 eq.) and *tris*-*n*-propylphosphonic anhydride (T3P, 50 wt-% in EtOAc, 1.45 mL, 2.30 mmol, 1.15 eq.) were added dropwise and in the given order. The mixture was stirred at 0-20 °C and the progress of reaction was monitored *via* TLC. The work-up was done as described in SOP5. The crude product was analyzed by chiral HPLC and showed an enantiomeric excess of 98.1+- 0.4% ee. The product was purified by a short column chromatography on silica (MTBE) to obtain a colorless solid.

Isolated yield: 592 mg (1.84 mmol, 92.1%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.38 – 7.22 (m, 5H, Ar-*H*), 6.02 (d, 1H, *J* = 8.0 Hz, *n*-*H*), 5.45 (m, 1H, C*H*-O), 5.12 (m, 1H, C*H*-NH), 4.13 (qd, *J* = 7.2, 6.1 Hz, 2H, O-CH_2-CH_3), 2.78 (dd, *J* = 15.9, 5.4 Hz, 1H, HN-CO-CH_2^a), 2.64 (dd, *J* = 15.9, 6.8 Hz, 1H, HN-CO-CH_2^b), 2.58 (d, *J* = 6.3 Hz, 2H, O-CO-CH_2), 1.98 (s, 3H, CO-CH_3), 1.49 (d, *J* = 6.9 Hz, 3H, CH-CH_3), 1.25 (t, *J* = 7.1 Hz, 3H, CH_2-CH_3).

The data corresponds to literature-known values.[132]

8.6.2 (3*R*,1'*S*)-Ethyl 5-(*N*-(1-phenylethyl)amino)-3-(acetyl)-oxy-5-oxopentanoate



The synthesis was conducted according to SOP5. A solution of (3*R*)-5-ethoxy-(acetyl)-oxy-5-oxopentanoic acid (436 mg, 2.00 mmol) and triethylamine (558 µL, 4.00 mmol, 2.0 eq.) in ethyl acetate (10 mL) was cooled to 0-4 °C. Subsequently, (*R*)-1-phenylethylamine (318 µL, 2.00 mmol, 1.0 eq.) and *tris*-*n*-propylphosphonic anhydride (T3P, 50w% in EtOAc, 1.45 mL, 2.30 mmol, 1.15 eq.) were added dropwise and in the given order. The mixture was stirred at 0-20 °C and the progress of reaction was monitored *via* TLC. The work-up was done as described in SOP5. The crude product was analyzed by chiral HPLC and showed an enantiomeric excess of 97.6+- 0.6% ee. The product was purified by recrystallization (*n*-hexane/2-propanol 1:1 v/v) to obtain a colorless solid.

Isolated yield: 487 mg (1.52 mmol, 75.8%).

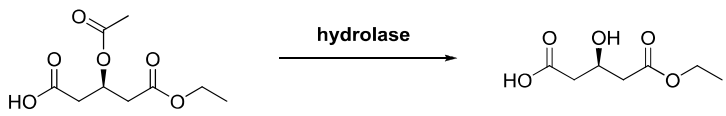
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.38 – 7.22 (m, 5H, Ar-*H*), 6.02 (d, *J* = 8.0 Hz, 1H, N*H*), 5.45 (m, 1H, C*H*-O), 5.12 (m, 1H, C*H*-NH), 4.13 (qd, *J* = 7.2, 6.1 Hz, 2H, O-CH_2-CH_3), 2.78 (dd, *J* = 15.9, 5.4 Hz, 1H, HN-CO-CH_2^a), 2.64 (dd, *J* = 15.9, 6.8 Hz, 1H, HN-CO-CH_2^b), 2.58 (d, *J* = 6.3 Hz, 2H, O-CO-CH_2), 1.98 (s, 3H, CO-CH_3), 1.49 (d, *J* = 6.9 Hz, 3H, CH-CH_3), 1.25 (t, *J* = 7.1 Hz, 3H, CH_2-CH_3).

The data corresponds to literature-known values.[132]

8.7 STANDARD OPERATING PROCEDURE 6 (SOP6): BIOCATALYTIC REMOVAL OF *O*-ACETYL GROUP BY THE USE OF HYDROLASES

The crude or isolated product from the previous desymmetrization with chymotrypsin (10.0 – 50.0 mmol) was dissolved in KPB (50mM, pH 8.0) or an organic solvent to reach a certain substrate concentration (1.0 – 4.0M) and, in the case of aqueous medium, titrated to pH 8.0 with aqueous sodium hydroxide solution (1.0 - 4.0M). An adequate amount of hydrolase was added and the resulting mixture was stirred at room temperature. The progress of the reaction was monitored by the volume of titer (1.0 – 4.0M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. After reaching the end of the reaction the solution was acidified (pH 1-2) with hydrochloric acid (12M) and extracted with ethyl acetate (3x 1:1 v/v). The combined organic phases were dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The conversion was determined by NMR spectroscopy. Unless otherwise stated the crude extract was further used without purification.

Table 48. Removal of O-acetyl protective group by the use of hydrolases

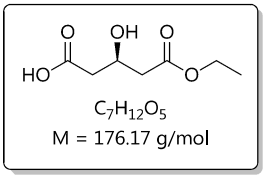


Entry	Amount substrate [mmol]	Biocatalyst (g)	Additive (eq.)	Conc. substrate [M]	Conversion [% (h)]	Yield [g(%)]
1	10.0	CAL-B (1.00)	EtOH (2.00)	1.43	22.0 (15.5)	n.d.
2	10.0	CAL-B (1.00)	EtOH (10.0)	1.43	30.1 (76.5)	n.d.
3	1.25	Amano PS SD (0.05)	-	0.05	4.76 (24.0)	n.d.
4	1.25	Amano AK (0.05)	-	0.05	7.41 (24.0)	n.d.
5	1.25	Amano AYS (0.05)	-	0.05	4.76 (24.0)	n.d.
6	5.00	CAL-B (0.50)	EtOH (17.1)	0.50	5.66 (24.0)	n.d.
7	5.00	CAL-B (0.50)	EtOH (34.2)	0.50	<1.00 (24.0)	n.d.
8	4.58	CAL-B	MeOH	0.76	<1.00	n.d.

		(0.20)	(5.38)		(24.0)	
9	5.00	CCAH (0.05)	-	1.00	12.3[a] (18.5)	n.d.
10	10.0	CCAH (0.05)	-	1.00	89.5 (5.5)	1.47 (74.8)
11	40.0	CCAH (0.50)	-	0.80	>95.0 (4.5)	5.43 (77.0)
12	48.5	CCAH (0.50)	-	4.00	>95.0 (8.0)	4.28 (50.1)

(a) pH decreased to 3-4 which could have deactivated the enzyme

8.7.1 (3*R*)-5-Ethoxy-3-hydroxy-5-oxopentanoic acid



$C_7H_{12}O_5$
M = 176.17 g/mol

The synthesis was conducted according to SOP6. A solution of (3*R*)-5-ethoxy-3-acetoxy-5-oxopentanoic acid (10.6 g, 48.5 mmol) in KPB (12.5 mL, 50mM, pH 8.0) was titrated to pH 8.0 with aqueous sodium hydroxide solution (4.0 M). Cephalosporin C acetylesterase (CAE, immobilized, 500 mg) was added and the resulting suspension was stirred at room temperature. The progress of the reaction was monitored by the volume of titer (1.0 – 4.0 M sodium hydroxide) consumed to neutralize the free carboxylic acid and maintaining pH 8.0. The biocatalyst was filtered off and the work-up was done as described in SOP6. The conversion and the product content were determined by NMR spectroscopy.

Yield: 6.09 g (70.2% product content[i], 24.3 mmol, 50.1 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.47 (m, 1H, C*H*-OH), 4.18 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.62 – 2.54 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.28 (t, 3H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

The data corresponds to literature-known values.[127]

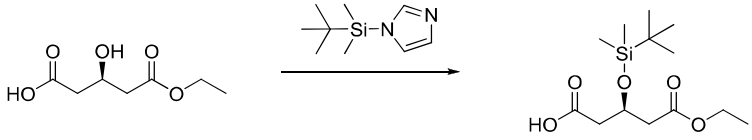
8.8 Standard operating procedure 7 (SOP7): Protection of beta-hydroxy carbonacids as *tert*-butyldimethylsilylethers

The synthesis was based on a procedure from Schoenherr *et al*..[129] A solution of 5-ethoxy-3-hydroxy-5-oxopentanoic acid (5.00 – 117 mmol) and 1*H*-imidazole (2.0-6.0 eq.) in an adequate solvent was stirred at room temperature. After the addition of *tert*-butyldimethylchlorosilane (1.1-2.2 eq.) the mixture

[i] Determined by quantitative NMR integration against internal standard (see chapter 2.4.3, page 16).

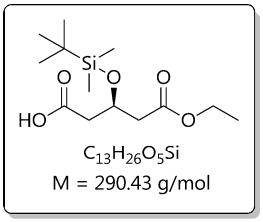
was stirred for 6-72 hours at 20-80 °C. The progress of the reaction was monitored *via* TLC. The reaction was quenched by addition of methanol (50 % v/v) and a solution of potassium carbonate (0.5 eq.) in dist. H_2O (1:2 v/v). The progress was again monitored *via* TLC. Brine (1:2 v/v) was added to enhance phase separation The aqueous phase was separated and extracted with MTBE (2 x 1:1 v/v). The combined organic phase was washed once with brine (2:3 v/v), dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The main impurity was *tert*-butyldimethylsilanol which can be removed by evaporation of its hydrate or silica filtration (10 % v/v ethyl acetate in cyclohexane).

Table 49. TBS-protection of β-hydroxy carboxylic acids



Entry	Educt [mmol]	TBS-Cl (eq.)	1*H*-imidazole (eq.)	Solvent (°C)	Conversion [% (h)]	Yield [g (%)]
1	5.00	2.20	6.00	THF (20)	>95.0 (17.0)	2.26 (75.4)
2	10.0	1.10	3.00	MTBE (20)	43.2 (26.0)	n.d.
3	5.00	1.50	2.50	MTBE (reflux)	59.2 (12.0)	n.d.
4	5.00	2.00	3.00	MTBE (reflux)	89.3 (20.0)	1.82 (51.9)
5	30.8	2.20	2.20	MTBE (reflux)	87.0 (6.0)	10.5 (50.5)
6	40.0	2.20	2.20	2-Me-THF (80)	31.8 (18.0)	n.d.
7	100	2.00	2.00	THF (20)	>95.0 (21.0)	9.80 (23.6)
8	117	2.00	2.00	EtOAc (20)	78.1 (19.5)	n.d.
9	100	2.00	2.00	THF (20)	>95.0 (16.5)	16.8 (57.8)

8.8.1 (3*R*)-5-ETHOXY-3-(*TERT*-BUTYLDIMETHYLSILYL-)OXY-5-OXO-PENTANOIC ACID



The synthesis was conducted according to SOP7. To a solution of (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid (17.6 g, 0.10 mol) in dry THF

(100 mL) was added 1*H*-imidazole (30.1 g, 0.20 mol, 2.0 eq.). *tert*-Butyldimethyl-chlorosilane (13.6 g, 0.2 mol, 2.0 eq.) was dissolved in dry THF (50 mL) and added in one portion. The slurry was vigorously stirred at room temperature until TLC shows complete conversion[i]. The reaction was diluted with methanol (75 mL) and subsequently mixed with a potassium carbonate solution (6.91 g, 0.05 mol, 0.5 eq.) in dist. H_2O (75 mL). The resulting emulsion was stirred until TLC shows complete hydrolysis of the side-product[ii]. The work-up was done as described in SOP7. The crude product was contaminated by excess silanol (20-60 %) which was completely removed by silica filtration (10 % v/v ethyl acetate in cyclohexane) to obtain the product as colorless oil.

Isolated yield: 16.8 g (57.8 mmol, 57.8 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 4.63 – 4.47 (m, 1H, C*H*-O), 4.21 – 4.07 (dd, *J* = 7.2, 1.4 Hz, 2H, O-C*H*$_2$-CH$_3$), 2.84 – 2.40 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.38 – 1.12 (t, *J* = 7.2 Hz, 3H, CH$_2$-C*H*$_3$), 0.92 – 0.76 (s, 9H, C-(C*H*$_3$)$_3$), 0.13 – 0.04 (d, *J* = 1.8 Hz, 6H, Si-C*H*$_3$).

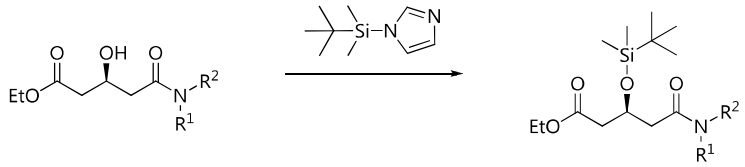
The spectroscopic data corresponds to literature-known values.[129]

8.9 STANDARD OPERATING PROCEDURE 8 (SOP8): PROTECTION OF BETA-HYDROXYAMIDES AS *TERT*-BUTYLDIMETHYLSILYL ETHERS

The corresponding amide (2.00 mmol) and 1*H*-imidazole (3.0 eq.) were dissolved in the given organic solvent and *tert*-butyldimethylchlorosilane (1.1 – 2.0 eq.) was added in portions at room temperature. The suspension was stirred for 2-6 hours at 20-60 °C. The mixture was diluted with ethyl acetate (10 – 50 mL) and washed with dist. H_2O (1:2 v/v), saturated sodium bicarbonate solution (1:2 v/v) and brine (1:2 v/v). The organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was purified by column chromatography on silica (33% v/v ethyl acetate in cyclohexane).The product is analyzed *via* NMR spectroscopy.

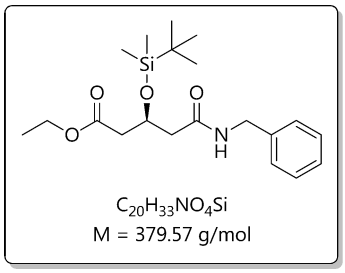
[i] The reaction is monitored by TLC (cyclohexane/ethyl acetate 1:1) on silica with bromocresol green as acid/base indicator and terminated after disappearance of the educt.
[ii] The hydrolysis of the TBDMS-ester is monitored with phosphomolybdic acid solution as plunge reagent and terminated after complete disappearance of the side-product.

Table 50. TBS-protection of β-hydroxy amides



Entry	R^1	R^2	TBS-Cl (eq.)	1*H*-Imidazole (eq.)	Conv. [%]	Isol. yield [g(%)]
1	H	Bn	1.1	3.0	>95.0	0.49 (64.5)
2	Me	Bn	2.0	3.0	>95.0	0.20 (66.0)
3	Morpholine		2.0	3.0	>95.0	0.12 (83.4)

8.9.1 ETHYL (3*S*)-5-(*N*-BENZYLAMINO)-3-((*TERT*-BUTYLDIMETHYLSILYL)-OXY-5-OXOPENTANOATE



$C_{20}H_{33}NO_4Si$
M = 379.57 g/mol

The following synthesis was conducted according to SOP8. To a solution of ethyl (3*S*)-5-(*N*-benzylamino)-3-hydroxy-5-oxopentanoate (0.53 g, 2.00 mmol) and 1*H*-imidazole (0.41 g, 6.00 mmol, 3.0 eq.) in THF (6 mL) was added *tert*-butyldimethylchlorosilane (0.33 g, 2.20 mmol, 1.1 eq.). The resulting suspension was stirred for two hours at room temperature. The work-up was done as described in SOP8. The crude product was purified by column chromatography on silica (33 % v/v ethyl acetate in cyclohexane) to obtain a colorless oil.

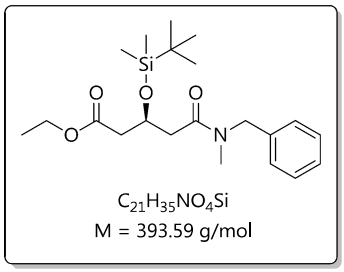
Isolated yield: 0.49 g (1.29 mmol, 64.5 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.70 (s, 1H, *n-H*), 7.27-7.34 (m, 5H, Ar-*H*), 4.47-4.53 (m, 2H, C*H*$_2$-Ph), 4.38 (q, 2H, *J* = 7.1 Hz, O-C*H*$_2$-CH$_3$), 4.12 (m, 1H, C*H*-O), 2.53-2.57 (m, 2H, C*H*$_2$-CONH), 2.43-2.47 (m, 2H, C*H*$_2$-COOEt), 1.26 (t, 3H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$), 0.84 (s, 9H, C-(C*H*$_3$)$_3$), 0.07 (s, 6H, Si-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 171.05, 138.10, 128.70, 127.95, 127.51, 66.73, 60.61, 43.90, 43.63, 41.72, 25.67, 17.80, 14.17, -5.04.

The compound is unknown to literature.

8.9.2 Ethyl (3*S*)-5-(*N*-benzyl-*N*-methyl)-amino)-3-((*tert*-butyldimethylsilyl)-oxy-5-oxopentanoate



$C_{21}H_{35}NO_4Si$
M = 393.59 g/mol

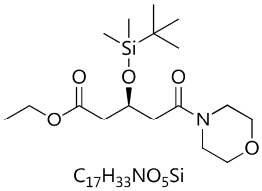
The following synthesis was conducted according to SOP8. To a solution of ethyl (3*S*)-5-(*N*-benzyl-*N*-methyl)-amino)-3-hydroxy-5-oxopentanoate (216 mg, 0.77 mmol) and 1*H*-imidazole (162 mg, 2.32 mmol, 3.0 eq.) in MTBE (5 mL) was added *tert*-butyldimethylchlorosilane (247 mg, 1.55 mmol, 2.0 eq.). The resulting suspension was stirred for six hours at reflux temperature. The work-up was done as described in SOP8. The crude product was purified by column chromatography on silica (33 % v/v ethyl acetate in cyclohexane) to obtain a colorless oil with an *E/Z*-ratio of 3:2.

Isolated yield: 0.20 g (0.51 mmol, 66.0 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.39-7.14 (m, 5H, Ar-*H*), 4.61-4.41 (m, 3H, C*H*$_2$-Ph, C*H*-O), 4.19-4.10 (q, *J* = 7.1 Hz, 2H, O-C*H*$_2$-CH_3), 2.96, 2.91 (s, 3H, *n*-C*H*$_3$), 2.70-2.45 (m, 4H, *CH*$_2$-CH-C*H*$_2$), 1.28, 1.23 (t, 3H, *J* = 7.1 Hz, CH_2-C*H*$_3$), 0.85 (s, 9H, C-(C*H*$_3$)$_3$), 0.08, 0.05 (2s, 6H, Si-C*H*$_3$).

The compound is unknown to literature.

8.9.3 Ethyl (3*S*)-5-*N*-morpholino-3-((*tert*-butyldimethylsilyl)-oxy-5-oxopentanoate



$C_{17}H_{33}NO_5Si$
M = 359.53 g/mol

The following synthesis was conducted according to SOP8. To a solution of ethyl (3*S*)-hydroxy-5-*N*-morpholino-5-oxopentanoate (102 mg, 0.40 mmol) and 1*H*-imidazole (81.7 mg, 1.20 mmol, 3.0 eq.) in MTBE (5 mL) was added *tert*-butyldimethylchlorosilane (121 mg, 1.55 mmol, 2.0 eq.). The resulting suspension was stirred for six hours at reflux temperature. The work-up was done as described in SOP8. The crude product was purified by column chromatography on silica (33 % v/v ethyl acetate in cyclohexane) to obtain a colorless oil.

Isolated yield: 0.12 g (0.33 mmol, 83.4 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.62 (m, 1H, C*H*-O), 4.12 (m, 2H, O-C*H*$_2$-CH_3), 3.72-3.46 (m, 8H, C*H*$_2$-C*H*$_3$), 2.69-2.45 (m, 4H, *CH*$_2$-CH-C*H*$_2$), 1.26 (t, 3H, *J* = 7.1 Hz, CH_2-C*H*$_3$), 0.85 (s, 9H, C-(C*H*$_3$)$_3$), 0.08, 0.05 (2s, 6H, Si-C*H*$_3$).

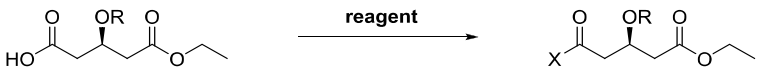
^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 171.19, 169.34, 66.94, 66.86, 66.69, 60.42, 46.72, 42.53, 41.90, 39.99, 25.73, 17.92, 14.20, -4.67, -5.03.

The compound is unknown to literature.

8.10 STANDARD OPERATING PROCEDURE 9 (SOP9): ACTIVATION OF *O*-PROTECTED BETA-HYDROXYCARBONACIDS

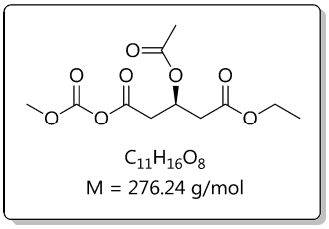
The following syntheses were based on literature procedures from different sources. The corresponding references were given in parenthesis. In general, the free O-protected carboxylic acid (1.60 – 15.0 mmol) was dissolved in an organic solvent (5 – 30 mL) and deprotonated with a non-nucleophilic base, e.g. triethylamine. The carboxylate was then converted into active esters or an acid chloride by the use of different reagents. The reaction was quenched with the addition of aqueous saturated sodium bicarbonate solution (1:1 v/v), the phases were separated and the aqueous phase was extracted with additional organic solvent (2 x 1:1 v/v). In the case of water-miscible solvents, the mixture was brought to saturation with neat sodium chloride. The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was analyzed by NMR spectroscopy and directly used without further purification, if not stated otherwise.

Table 51. Synthesis of activated carbonacid derivatives



Entry	R (mmol)	Reagent (eq.)	Additive (eq.)	Solvent (T [°C])	Conv. (t) [% (h)]	Yield [g (%)]
1	Ac (1.60)	ClCOOMe (1.25)	TEA (1.50)	Toluene (-40)	86.3 (0.5)	0.29 (60.6)
2	Ac (5.00)	ClCOOMe (1.25)	TEA (1.50)	MTBE (-40)	>95.0 (0.5)	0.94 (68.1)
3	Ac (4.00)	$C_2O_2Cl_2$ (1.30)	DMF (0.01)	DCM (0-20)	>95.0 (4.0)	n.d.
4	TBS (3.77)	$(H_3C)_3CCOCl$ (1.03)	TEA (1.03)	THF (0)	0.0 (3.0)	-
5	TBS (3.44)	TosCl (1.10)	TEA (1.10)	MTBE (20)	0.0 (24.0)	-
6	TBS (2.00)	ClCOOMe (1.65)	TEA (1.25)	MTBE (-60)	>95.0 (1.0)	0.28 (33.8)
7	TBS (15.0)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	78.7 (2.0)	5.64 (n.d.)
8	TBS (12.6)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	91.7 (2.0)	3.17 (66.1)
9	TBS (5.00)	ClCOOMe (1.50)	TEA (2.00)	MTBE (-70)	91.0 (16.5)	1.16 (66.8)

8.10.1 METHYL (3*S*)-5-ETHOXY-3-(ACETYL)-OXY-5-OXOPENTANOYL-CARBONATE



The synthesis was based on a patent procedure from Teva Pharmaceuticals.[133] A mixture of (3*R*)-5-ethoxy-3-(acetyl)-oxy-5-oxopentanoic acid (1.09 g, 5.0 mmol) and triethylamine (1.04 mL, 7.50 mmol, 1.5 eq.) in MTBE (30 mL) was cooled to -40 °C (ethanol/dry ice) under argon atmosphere. A solution of methyl chloroformate (484 µL, 6.25 mmol, 1.25 eq.) in MTBE (5 mL) was added dropwise and the mixture was stirred for 20 minutes while thawing up to 0 °C. The reaction was quenched with addition of dist. H_2O (20 mL) and the phases were separated immediately. The organic phase was washed with dist H_2O (20 mL), saturated sodium bicarbonate solution (2 x 10 mL) and brine (10 mL). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo* and the crude product was analyzed by NMR spectroscopy. No impurities were observed.

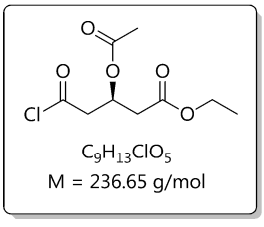
Isolated yield: 940 mg (3.40 mmol, 68.1 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.51 (m, 1H, C*H*-O), 4.16 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 3.91 (s, 3H, O-C*H*$_3$), 2.97-2.85 (m, 2H, *CH*$_2$-COOMoc), 2.78-2.69 (m, 2H, *CH*$_2$-COOEt), 2.05 (s, 1H, (O=)C-C*H*$_3$), 1.26 (t, 6H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 169.90, 169.55, 164.31, 149.15, 66.00, 60.99, 56.09, 38.11, 37.96, 20.85, 14.14.

The compound is unknown to literature.

8.10.2 (3*S*)-5-ETHOXY-3-(ACETYL)-OXY-5-OXOPENTANOYL CHLORIDE



The synthesis was conducted according to a patent procedure from OEHRLEIN *et al.*.[122] A solution of (3*R*)-5-ethoxy-3-(acetyl)-oxy-5-oxopentanoic acid (0.87 g, 4.00 mmol) and a catalytic amount of DMF (2 µL) in dry dichlormethane (5 mL) was cooled to 0-4 °C. Oxalyl dichloride (440 µL, 5.20 mmol, 1.3 eq.) was added dropwise and the mixture was stirred for 30 minutes at 0 °C and additional 3.5 hours at room temperature. The reaction was quenched with addition of saturated sodium bicarbonate solution (5 mL) and the phases were separated immediately. The aqueous phase was extracted with DCM (2 x 10 mL) and the combined organic phase was washed with brine (10 mL). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo* and the crude product

was analyzed by NMR spectroscopy. The ^{13}C-NMR shows complete conversion to the corresponding acid chloride.

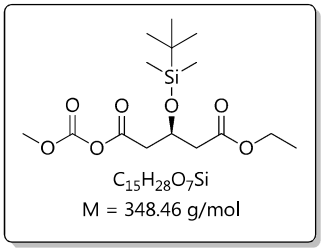
Isolated yield: 619 mg (2.62 mmol, 65.4%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.50 (m, 1H, C*H*-O), 4.16 (q, 2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.79 (dd, 2H, J= 6.0 Hz, *CH*$_2$-COCl), 2.72 (d, 2H, *J* = 6.3 Hz, *CH*$_2$-COOEt), 2.05 (s, 1H, (O=)C-C*H*$_3$), 1.26 (t, 6H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$).

^{13}C-NMR (125 MHz, $CDCl_3$): δ [ppm] = 175.36, 170.08, 169.84, 66.56, 60.93, 38.29, 37.93, 20.95, 14.15.

The data corresponds to literature-known values.[133]

8.10.3 METHYL (3*S*)-5-ETHOXY-3-(*TERT*-BUTYLDIMETHYLSILYL)-OXY-5-OXOPENTANOYLCARBONATE



$C_{15}H_{28}O_7Si$
M = 348.46 g/mol

Triethylamine (1.40 mL, 10.0 mmol, 2.00 eq.) was added to a solution of (3*R*)-5-ethoxy-3-(*tert*-butyldimethylsilyl)-oxy-5-oxopentanoic acid (1.46 g, 5.00 mmol) in MTBE (30 mL) and the mixture was cooled to -70 °C (acetone/dry ice). Methyl chloroformate (580 µL, 7.5 mmol, 1.50 eq.) was dissolved in MTBE (20 mL) and added dropwise. After stirring for two hours at -70 °C the reaction was warmed up to 0 °C and filtrated. All volatile compounds were removed *in vacuo*. NMR analysis of the crude product shows a conversion of 91.0% with 9 % starting material as the sole impurity.

Yield: 1.28 g (91.0% product content, 3.34 mmol, 66.8 %).

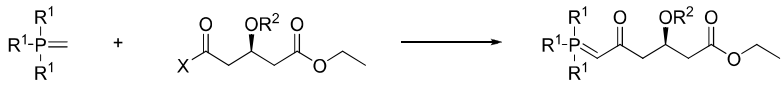
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 4.63 – 4.47 (m, 1H, C*H*-O), 4.21 – 4.07 (dd, *J* = 7.2, 1.4 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.91 (s, 3H, O-C*H*$_3$), 2.84 – 2.40 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.38 – 1.12 (t, *J* = 7.2 Hz, 3H, CH$_2$-C*H*$_3$), 0.92 – 0.76 (s, 9H, C-(C*H*$_3$)$_3$), 0.13 – 0.04 (d, *J* = 1.8 Hz, 6H, Si-C*H*$_3$).

The compound is unknown to literature.

8.11 STANDARD OPERATING PROCEDURE 10 (SOP10): SYNTHESIS OF STABILIZED PHOSPHOR YLIDES AS WITTIG AND HORNER-WADSWORTH-EMMONS REAGENTS

The following syntheses were based on literature procedures from different sources. The corresponding references will be given in parenthesis. In general, a methylated triphenylphosphine or dimethyl methylphosphonate, respectively, was dissolved in abs. THF and deprotonated by dropwise addition of *n*-butyllithium at -50 to -78 °C (ethanol or acetone/dry ice). This methylene solution was stirred for 1-3 hours while thawing up to room temperature. An activated carbonacid derivative was dissolved in MTBE and added dropwise to the again cooled methylene solution. The mixture was stirred for two hours while thawing up to room temperature. The reaction was quenched with the addition of aqueous saturated sodium bicarbonate solution (1:1 v/v), the phases were separated and the aqueous phase was extracted with additional organic solvent (2 x 1:1 v/v). In the case of water-miscible solvents, the mixture was brought to saturation with neat sodium chloride. The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was analyzed by NMR spectroscopy and directly used without further purification, if not stated otherwise.

Table 52. Synthesis of phosphor ylides as Wittig and HWZ reagents

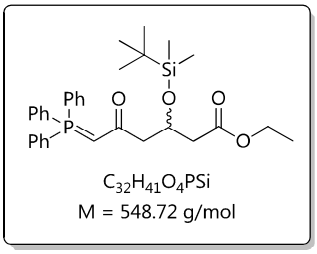


Entry	Reagent (eq)	R1 (mmol)	X	Solvent (T[°C])	Conv. (t) [% (h)]	Yield [g(%)]
1	Ph_3PCH_3Br (2.0)	H (3.60)	BnNH[a]	THF/toluene (-55)	n.d.[b]	-
2	Ph_3PCH_3Br (2.0)	2-THP (2.30)	BnNH[a]	THF (-55)	n.d.[b]	-
3	Ph_3PCH_3Br (2.0)	Ac (2.50)	Cl	THF (-55)	n.d.[c]	-
4	Ph_3PCH_3Br (2.0)	Ac (2.30)	MeOCO	THF (-55)	n.d.[c]	-
5	Ph_3PCH_3Br (2.0)	TBS (1.00)	EtO	THF (-15)	27.0[d] (3.0)	n.d.
6	$(MeO)_2POCH_3$ (4.0)	TBS (2.00)	EtO	THF (-78)	74.0[e] (2.5)	0.32 (n.d.)
7	Ph_3PCH_3Br	TBS	MeOC	THF/toluene	61-69[f]	0.34

	(2.0)	(0.80)	O	(-70)	(3.0)	(n.d.)
8	Ph_3PCH_3Br (2.5)	TBS (3.67)	MeOC O	THF (-78)	n.d.[g]	-
9	Ph_3PCH_3Br (2.5)	TBS (9.00)	MeOC O	THF (-78)	n.d.[g]	-

[a](*S*)-enantiomer through lipase-catalyzed aminolysis [b]not determined; the lower pKa of protic moieties (NH < OH < methylene) led to various side-products which could neither be isolated nor identified [c]not determined; the strongly basic methylene compound led to major elimination of acetic acid with the loss of chirality [d]reactivity of ethyl ester too low [e]quantitative conversion of the starting material, mixture of at least four products incl. elimination products with loss of chirality, 26% product-related conversion [f]conversion was determined by the conversion of aldehyde in the subsequent Wittig reaction, incl. correction of 1.1 eq. aldehyde and 97% conversion of the reference reaction [g] not determined, isolation methods were tested but unsuccessful

8.11.1 ETHYL *RAC*-3-(*TERT*-BUTYLDIMETHYLSILYL)-OXY-5-OXO-6-(TRIPHENYL-PHOSPHORANYLIDENYL)-HEXANOATE[i]



$C_{32}H_{41}O_4PSi$
M = 548.72 g/mol

Methyltriphenylphoshonium bromide (714 mg, 2.0 mmol) was suspended in abs. THF (5.0 mL) and cooled to -50 °C (ethanol/dry ice). After addition of *n*-butyllithium (1.25 mL, 1.6 M in hexanes, 2.0 mmol) the mixture was stirred for 60 minutes while thawing up to room temperature. The solution was again cooled to -40 °C and diethyl 3-(*tert*-butyldimethylsilyl)-oxyglutarate (318 mg, 1.0 mmol), dissolved in abs. THF (5.0 mL), was added dropwise *via* syringe. The work-up was done as described in SOP9. Analysis of the crude product by NMR spectroscopy showed a product-related conversion of 27%. The product was not further purified.

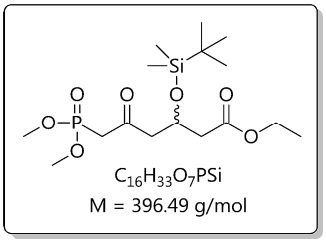
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.53 – 7.41 (m, 15H, Ar-*H*), 4.61 – 4.51 (m, 1H, C*H*-O), 4.21 – 4.07 (q, *J* = 7.4 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.69 (d, *J* = 15.9 Hz, 1H, P=C*H*), 2.76 – 2.42 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.25 (t, *J* = 7.3 Hz, 3H, CH$_2$-C*H*$_3$), 0.84 (s, 9H, C-(C*H*$_3$)$_3$), 0.08, 0.06 (2s, 6H, Si-C*H*$_3$).

ESI-MS: m/z [fragment] = 549.74 $[M+H]^+$.

The compound was known in literature. However, no NMR data was available.

[i] This experiment gave a good insight into the reactivity of the ethyl ester moiety under the given reaction conditions, a side reaction which could lead to the opposite enantiomer of the desired synthetic route.

8.11.2 ETHYL *RAC*-3-((*TERT*-BUTYLDIMETHYLSILYL)-OXY-6-(DIMETHOXY-PHOSPHORYL)-5-OXOHEXANOATE



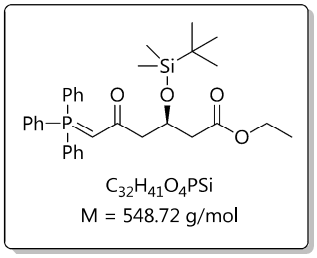
$C_{16}H_{33}O_7PSi$
M = 396.49 g/mol

Dimethyl methylphosphonate (1.03 mL, 9.6 mmol) was dissolved in abs. THF (10 mL) and cooled to -50 °C (ethanol/dry ice). After the dropwise addition of *n*-butyllithium (5.25 mL, 1.6M in hexanes, 8.4 mmol) the solution was stirred for 60 minutes at -50 °C. A solution of diethyl 3-(*tert*-butyldimethylsilyl)-oxyglutarate (720 mg, 2.0 mmol) in abs. THF (10 mL) was added dropwise and the resulting mixture was stirred for additional two hours at -50 °C. The work-up was done as described in SOP9. Analysis of the crude product by NMR spectroscopy showed a product-related conversion of 26 %. The product was not further purified.[i]

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 4.58 (m, 1H, C*H*-O), 4.23 – 4.15 (m, 2H, O-CH_2-CH_3), 3.79, 3.78 (2q, *J* = 11.0 Hz, 6H, P-O-CH_3), 3.11 (d, *J* = 23.6 Hz, 2H, P-CH_2-CO), 2.88 (d, *J* = 6.0 Hz, 2H, CH_2), 2.56 (dd, *J* = 15.0, 5.8 Hz, 1H, CH^a_2), 2.46 (dd, *J* = 15.0, 6.4 Hz, CH^b_2), 1.27 (t, *J* = 7.3 Hz, 3H, CH_2-CH_3), 0.84 (s, 9H, C-(CH_3)$_3$), 0.07 (s, 3H, Si-CH_3), 0.06 (s, 3H, Si-CH_3).

The data corresponds to literature values (*methyl ester*).[124]

8.11.3 ETHYL (3*R*)-(*TERT*-BUTYLDIMETHYLSILYL)-OXY-5-OXO-6-(TRIPHENYL-PHOSPHORANYLIDENYL)-HEXANOATE



$C_{32}H_{41}O_4PSi$
M = 548.72 g/mol

Methyltriphenylphosphonium bromide (572 mg, 1.6 mmol) was suspended in abs. THF (20 mL) and cooled to -70 °C (acetone/dry ice). After dropwise addition of *n*-butyllithium (1.10 mL, 1.5 M in hexanes, 1.6 mmol) the mixture was stirred for 60 minutes while thawing up to room temperature. The solution was cooled to -50 °C and a solution of methyl (3*S*)-5-ethoxy-3-(*tert*-butyldimethyl-silyl)-oxy-5-oxopentanoylcarbonate (279 mg, 0.8 mmol) in dry toluene (5 mL) was added dropwise. The mixture was stirred for additional two hours at -50 °C to room temperature. The work-up was done as described in SOP9. The product-related conversion of 61-69% was determined by the subsequent Wittig reaction and the conversion of the heteroaromatic aldehyde with respect to its excess of 1.1 eq. and the conversion of 97% of the reference

[i] Comparison with the triphenylphosphorylide shows an enhanced reactivity but also enhanced side-product formation; the starting material is converted in 74% to at least three side-products, two of them identified as the corresponding acrylates after silanol elimination

reaction with the commercial methylester ylide. The enantiomeric excess was calculated to be 98.1% ee. (see **Figure 46**, page 43).

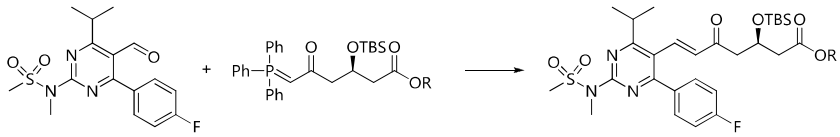
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.53 – 7.41 (m, 15H, Ar-*H*), 4.61 – 4.51 (m, 1H, C*H*-O), 4.21 – 4.07 (q, *J* = 7.4 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.69 (d, *J* = 15.9 Hz, 1H, P=C*H*), 2.76 – 2.42 (m, 4H, C*H*$_2$-CH-C*H*$_2$), 1.25 (t, *J* = 7.3 Hz, 3H, CH$_2$-C*H*$_3$), 0.84 (s, 9H, C-(C*H*$_3$)$_3$), 0.08, 0.06 (2s, 6H, Si-C*H*$_3$).

The compound was known in literature. However, no NMR data was available.

8.12 Standard operating procedure 11 (SOP11): Wittig reaction of different phosphor ylides with the heteroaromatic core of Rosuvastatin

The following synthesis was based on a literature procedure from Watanabe *et al.*.[134] The corresponding *O*-protected triphenylphosphoranylide (1.15 eq.) and *N*-(4-(4-fluorophenyl)-5-formyl-6-isopropylpyrimidin-2-yl)-*N*-methylmethane-sulfonamide (0.49 - 29.0 mmol) were dissolved in various organic and aqueous media and stirred for 6-24 hours at 90 °C or the corresponding reflux temperature. After cooling to room temperature the mixture was diluted with dist. H_2O (3:1 v/v) and extracted with an appropriate organic solvent (3 x 1:1 v/v). The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. If not stated otherwise the product was purified by column chromatography on silica (10 % v/v ethyl acetate in cyclohexane).

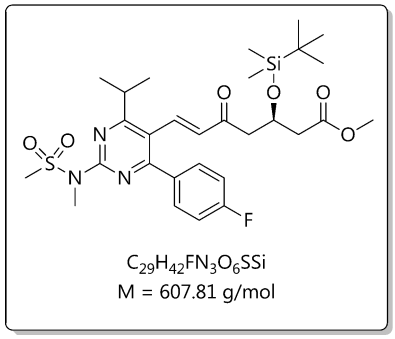
Table 53. Wittig reaction with phosphoranylidenes and heteroaromatic aldehyde



Entry	R	Ald [mmol]	Solvent	Conv. (time) [%(h)]	Isolated yield[a] [g (%)]
1	Me	0.90	MeCN	69.2 (12)	n.d.
2	Me	0.90	H_2O	68.5 (12)	n.d.
3	Me	0.90	KPB/*i*PA	97.2 (12)	0.43 (78.6)
4	Me	2.85	*i*PA	97.4 (8.5)	1.43 (82.6)
5	Et	0.49	*i*PA	62.8 (6.0)	n.d.
6	Me	28.9	MTBE	98.0 (18.0)	n.d.

(a) n.d. = not determined

8.12.1 METHYL *E*-(3*R*)-((*TERT*-BUTYLDIMETHYLSILYL)-OXY-7-(4-(4-FLUOROPHENYL)-6-ISOPROPYL-2-(*N*-METHYLMETHYLSULFONAMIDO)-PYRIMIDIN-5-YL)-5-OXOHEPT-6-ENOATE



$C_{29}H_{42}FN_3O_6SSi$
M = 607.81 g/mol

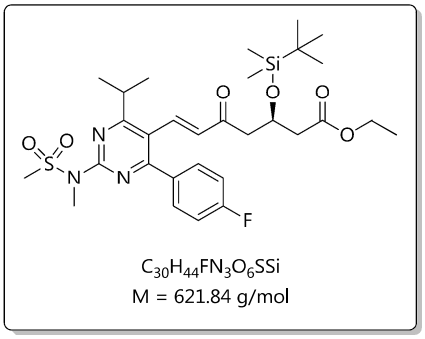
The synthesis was conducted according to SOP11. Methyl (3*R*)-(*tert*-butyldimethylsilyl)-oxy-5-oxo-6-(triphenylphosphoranylidenyl)-hexanoate (1.80 g, 3.28 mmol, 1.15 eq.) and *N*-(4-(4-fluorophenyl)-5-formyl-6-isopropylpyrimidin-2-yl)-*N*-methylmethane-sulfonamide (1.00 g, 2.85 mmol) were dissolved in 2-propanol (10 mL) and stirred for 8.5 hours at 90 °C. The work-up was done as described in SOP11. The crude product was purified by column chromatography on silica (10 % v/v ethyl acetate in cyclohexane).

Isolated yield: 1.43 g (2.35 mmol, 82.6 %).

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.70-7.60 (m, 3H, Ar-C*H*=C, *m*-Ar-*H*), 7.12 (t, *J* = 8.6 Hz, 2H, *o*-Ar-*H*), 6.16 (d, *J* = 16.4 Hz, 1H, C=C*H*-CO), 4.60 (m, 1H, C*H*-O), 3.66 (s, 3H, O-CH_3), 3.59 (s, 3H, S-CH_3), 3.51 (s, 3H, *n*-CH_3), 3.36 (m, 1H, H_3C-C*H*-CH_3), 2.75 (dd, *J* = 5.7 Hz, 2H, (O=)C-CH_2), 2.49 (dd, *J* = 5.7 Hz, 2H, CH_2-COOMe), 1.29 (d, *J* = 6.6 Hz, 6H, H_3C-CH-CH_3), 0.81 (s, 9H, C-(CH_3)$_3$), 0.06, 0.01 (2s, 6H, Si-CH_3).

The data corresponds to literature-known values.

8.12.2 ETHYL *E*-(3*R*)-((*TERT*-BUTYLDIMETHYLSILYL)-OXY-7-(4-(4-FLUOROPHENYL)-6-ISOPROPYL-2-(*N*-METHYLMETHYLSULFONAMIDO)-PYRIMI-DIN-5-YL)-5-OXOHEPT-6-ENOATE



$C_{30}H_{44}FN_3O_6SSi$
M = 621.84 g/mol

The synthesis was conducted according to SOP11. Ethyl (3*R*)-(*tert*-butyldimethylsilyl)-oxy-5-oxo-6-(triphenylphosphoranylidenyl)-hexanoate (309 mg, 0.56 mmol, 1.15 eq.) and *N*-(4-(4-fluorophenyl)-5-formyl-6-isopropyl-pyrimidin-2-yl)-*N*-methyl-methanesulfonamide (172 mg, 0.49 mmol) were dissolved in 2-propanol (2 mL) and stirred for 6.0 hours at 90 °C. The work-up was done as described in SOP11. NMR analysis of the crude product showed 87% conversion of the aldehyde. It was not further purified.

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.70-7.60 (m, 3H, Ar-C*H*=C, *m*-Ar-*H*), 7.12 (t, 2H, *J* = 8.6 Hz, *o*-Ar-*H*), 6.16 (d, 1H, *J* = 16.4 Hz, C=C*H*-CO), 4.60 (m, 1H,

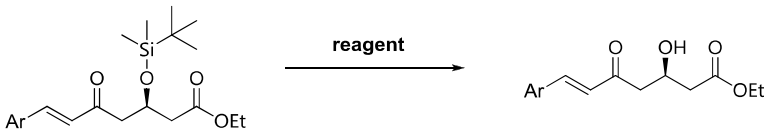
C*H*-O), 4.21 – 4.07 (q, *J* = 7.4 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.59 (s, 3H, S-C*H*$_3$), 3.51 (s, 3H, *n*-C*H*$_3$), 3.36 (m, 1H, H$_3$C-C*H*-CH$_3$), 2.75 (dd, 2H, *J* = 5.7 Hz, (O=)C-C*H*$_2$), 2.49 (dd, 2H, *J* = 5.7 Hz, C*H*$_2$-COOMe), 1.29 (d, 6H, *J* = 6.6 Hz, *H*$_3$C-CH-C*H*$_3$), 1.25 (t, *J* = 7.3 Hz, 3H, CH$_2$-C*H*$_3$), 0.81 (s, 9H, C-(C*H*$_3$)$_3$), 0.06, 0.01 (2s, 6H, Si-C*H*$_3$).

The data corresponds to literature-known values (*methyl ester*).

8.13 STANDARD OPERATING PROCEDURE 12 (SOP12): REMOVAL OF *TERT*-BUTYLDIMETHYLSILYL PROTECTIVE GROUP

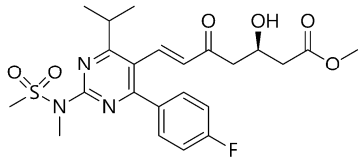
The following syntheses were conducted according to literature procedures. The corresponding references will be given in parenthesis. In general, the *O*-TBS protected enone (0.69 - 2.30 mmol) was dissolved in an appropriate organic solvent (18 - 20 mL) and a protonating or fluorinating reagent was added. The reaction was quenched by the addition of saturated aqueous salt solution. The aqueous phase was extracted with MTBE or ethyl acetate (3 x 1:1 v/v) and the combined organic phase was washed with brine (1:2 v/v). After drying over MgSO$_4$ all volatile compounds were removed *in vacuo*. Unless stated otherwise the product was used without further purification.

Table 54. TBS-deprotection of Wittig reaction products



Entry	Enone [mmol]	Reagent	Solvent	Conversion [%]	Yield [g(%)]
1	0.69	TBAF	THF	>95.0	n.d.
2	2.30	HCl	EtOH	>95.0	1.00 (88.1)

8.13.1 METHYL *E*-(3*R*)-7-(4-(4-FLUOROPHENYL)-6-ISOPROPYL-2-(*N*-METHYL-METHANESULFONAMIDO)-PYRIMIDIN-5-YL)-3-HYDROXY-5-OXOHEPT-6-ENOATE BY TBAF DEPROTECTION



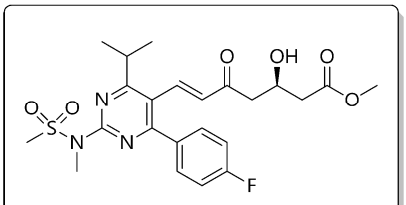
$C_{23}H_{28}FN_3O_6S$
M = 493,55 g/mol

The synthesis was conducted according to a literature procedure from TOUATI *et al.*.[135] Tetra-*n*-butylammonium fluoride (0.75 mL, 1.0 M in THF, 0.75 mmol) was added dropwise to a solution of ethyl *E*-(3*R*)-((*tert*-butyldimethyl-silyl)-oxy-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethyl-sulfonamido)-pyri-midin-5-yl)-5-oxo-hept-6-enoate (377 mg, 0.69 mmol) in THF (20 mL). The mixture was stirred for 14 hours at room temperature. The reaction was quenched by the addition of saturated ammonium chloride solution (10 mL) and the aqueous solution was extracted with ethyl acetate (3 x 20 mL). The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The conversion of >95.0 % was determined by analysis with ^{1}H-NMR spectroscopy. Due to the toxicity of hydrofluoric acid and therefore strict avoidance of acidic conditions, this methodology was considered inferior to the acidic hydrolysis.[i] The crude product was not further purified.

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.70-7.60 (m, 3H, Ar-C*H*=C, *m*-Ar-*H*), 7.12 (t, 2H, *J* = 8.6 Hz, *o*-Ar-*H*), 6.16 (d, 1H, *J* = 16.4 Hz, C=C*H*-CO), 4.56 (m, 1H, C*H*-O), 3.66 (s, 3H, O-CH_3), 3.59 (s, 3H, S-CH_3), 3.51 (s, 3H, *n*-CH_3), 3.36 (m, 1H, H_3C-C*H*-CH_3), 2.75 (dd, 2H, *J* = 5.7 Hz, (O=)C-CH_2), 2.49 (dd, 2H, *J* = 5.7 Hz, CH_2-COOMe), 1.29 (d, 6H, *J* = 6.6 Hz, H_3C-CH-CH_3).

The data corresponds to literature-known values.

8.13.2 METHYL *E*-(3*R*)-7-(4-(4-FLUOROPHENYL)-6-ISOPROPYL-2-(*N*-METHYL-METHANESULFONAMIDO)-PYRIMIDIN-5-YL)-3-HYDROXY-5-OXOHEPT-6-ENOATE BY ACIDIC HYDROLYSIS



$C_{23}H_{28}FN_3O_6S$
M = 493,55 g/mol

The synthesis was conducted according to a patent procedure from Teva Pharmaceuticals.[136] To a solution of ethyl *E*-(3*R*)-((*tert*-butyldimethylsilyl)-oxy-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonami-do)-pyrimidin-5-yl)-5-oxohept-6-enoate (1.14 g, 2.30 mmol) in EtOH (18 mL) was added dropwise hydrochloric acid (2.0 mL, 2 M). The mixture was stirred for six hours

[i] However, TBS fluoride should be easier to remove by distillation as the corresponding silanol or its hydrate.

at room temperature. The work-up was conducted with saturated sodium bicarbonate solution and MTBE as described in SOP12. Analysis with ^{1}H-NMR spectroscopy revealed quantitative conversion. The crude product was purified by column chromatography on silica (10 % v/v ethyl acetate in cyclohexane) to obtain a pale yellow oil.

Isolated yield: 1.00 g (2.02 mmol, 88.1 %).

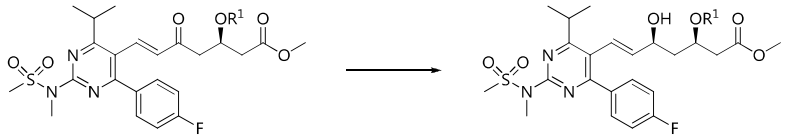
^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.70-7.60 (m, 3H, Ar-C*H*=C, *m*-Ar-*H*), 7.12 (t, 2H, *J* = 8.6 Hz, *o*-Ar-*H*), 6.16 (d, 1H, *J* = 16.4 Hz, C=C*H*-CO), 4.56 (m, 1H, C*H*-O), 3.66 (s, 3H, O-CH_3), 3.59 (s, 3H, S-CH_3), 3.51 (s, 3H, *n*-CH_3), 3.36 (m, 1H, H_3C-C*H*-CH_3), 2.75 (dd, 2H, *J* = 5.7 Hz, (O=)C-CH_2), 2.49 (dd, 2H, *J* = 5.7 Hz, CH_2-COOMe), 1.29 (d, 6H, *J* = 6.6 Hz, H_3C-CH-CH_3).

The data corresponds to literature-known values (*methyl ester*).

8.14 Standard operating procedure 13 (SOP13): Diastereoselective reduction of beta-chiral enones by sodium borohydride

Sodium borohydride (1.3 – 2.0 eq.) was dissolved in an appropriate dry organic solvent (20 mL) and cooled to the given temperature. After the addition of any additives, e.g. diethyl methoxyborane (0.5 – 1.0 eq.), a solution of β-chiral enone (0.06 – 2.30 mmol) in a mixture of methanol and THF (2:7 v/v) was added dropwise while maintaining low temperature. The resulting mixture was stirred at cold temperature until TLC control shows quantitative conversion. Subsequently, the cold solution was poured onto a mixture of aqueous sodium bicarbonate solution (1.0 wt-%, 1:3 v/v) and ethyl acetate (2:3 v/v). The phases were separated and the aqueous phase was extracted with ethyl acetate (2 x 1:2 v/v). The combined organic phase was washed with brine (1:3 v/v) and concentrated *in vacuo*. In the case of added borane the organic phase was concentrated to 2/3 of the extraction volume and heated to 50 °C before hydrogen peroxide (35 % in H_2O, 3 eq.) was added dropwise. The mixture was stirred for 90 minutes at 45 °C. The phases were separated and the organic phase was washed with brine (1:1 v/v) before all volatile compounds were removed *in vacuo*. The crude product was analyzed by ^{1}H-NMR spectroscopy. Unless stated otherwise the product was used without further purification.

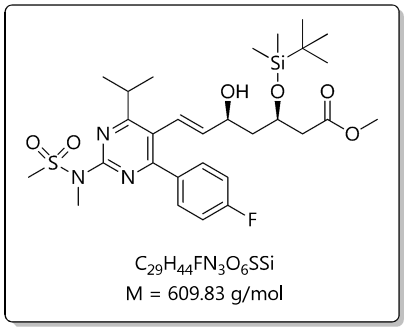
Table 55. Diastereoselective reduction of β-chiral enone with $NaBH_4$



Entry	R^1 [mmol]	Additive (eq.)	Solvent (°C)	Conv. (time) [%(h)]	d.r. (*syn/anti*)	Yield [g(%)]
1	TBS (0.06)	-	THF (0)	>95.0 (1.0)	80:20	n.d.
2	H (2.30)	Et_2BOMe (0.5)	THF (-15)	>95.0 (1.0)	66:34	1.20 (n.d.)
3	H (2.00)	Et_2BOMe (0.5)	THF (-70)	>95.0 (2.0)	65:35	0.92 (n.d.)
4	H (1.00)	Et_2BOMe (1.0)	THF (-15)	>95.0 (1.0)	80:20	n.d. (-)
5	H (1.00)	Et_2BOMe (1.0)	THF (-70)	>95.0 (1.0)	>98:2	n.d. (-)

8.14.1 Methyl *E*-(3*R*,5*S*)-3-((*tert*-butyldimethylsilyl)-oxy-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)-pyrimidin-5-yl)-5-hydroxyhept-6-enoate

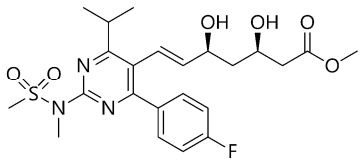
The synthesis was based on a literature procedure from Kim *et al.*[137] A solution of ethyl *E*-(3*R*)-((*tert*-butyldimethylsilyl)-oxy-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)-pyrimidin-5-yl)-5-oxohept-6-enoate (38.0 mg, 0.06 mmol) in THF (2.0 mL) was cooled to 0-4 °C. Sodium borohydride (4.4 mg, 0.12 mmol, 2.0 eq.) was added and the mixture was stirred for 60 minutes at 0-4 °C. The work-up was conducted as described in SOP13. NMR analysis showed quantitative conversion of the starting material and a diastereomeric ratio of 80:20 for the *major*-diastereomer. Due to its low amount the product was not further purified.



$C_{29}H_{44}FN_3O_6SSi$
M = 609.83 g/mol

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.66-7.63 (m, 2H, *m*-Ar-*H*), 7.09 (t, 2H, *J* = 8.8 Hz, *o*-Ar-*H*), 6.64 (d, 1H, *J* = 16.0 Hz, Ar-C*H*=C), 5.45 (dd, 1H, *J* = 5.3, 16.0 Hz, C=C*H*-C), 4.46 (m, 1H, H_2C-C*H*-OH), 4.21 (m, 1H, CH-C*H*-OH), 3.74 (s, 3H, O-CH_3), 3.68 (s, 1H, OH), 3.57 (s, 3H, S-CH_3), 3.52 (s, 3H, *n*-CH_3), 3.51 (s, 1H, OH), 3.36 (m, 1H, H_3C-C*H*-CH_3), 2.49-2.47 (m, 2H, CH_2-COOMe), 1.59-1.45 (m, 2H, CH-CH_2-CH), 1.27 (d, 6H, *J* = 6.6 Hz, H_3C-CH-CH_3), 0.81 (s, 9H, C-(CH_3)$_3$), 0.06, 0.01 (2s, 6H, Si-CH_3).

8.14.2 METHYL *E*-(3*R*,5*S*)-7-(4-(4-FLUOROPHENYL)-6-ISOPROPYL-2-(*N*-ME-THYLMETHANESULFON-AMIDO)-PYRIMIDIN-5-YL)-3,5-DIHYDROXYHEPT-6-ENOATE (ROSUVASTATIN METHYL ESTER)



$C_{23}H_{30}FN_3O_6S$
M = 495.56 g/mol

The synthesis was conducted according to SOP13. Sodium borohydride (115 mg, 3.0 mmol, 1.3 eq.) was suspended in dry THF (15 mL) and cooled to -70 °C (acetone/dry ice). Diethyl methoxyborane (2.3 mL, 1.0 M in THF, 2.3 mmol, 1.0 eq.) was added dropwise and the resulting solution was degased with argon. Subsequently, a solution of methyl *E*-(3*R*)-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfon-amido)-pyrimidin-5-yl)-3-hydroxy-5-oxohept-6-enoate (1.17 g, 2.3 mmol) in a mixture of methanol and THF (2:7 v/v, 23 mL) was added dropwise while maintaining -70 °C. The reaction progress was observed *via* TLC control (silica, 50% ethyl acetate in *n*-heptane). The reaction was stirred for 60 minutes until TLC shows quantitative conversion of starting material. The work-up was done as described in SOP13. Analysis with ^{1}H-NMR spectroscopy confirms quantitative conversion and shows a diastereomeric ratio of >98:2 for the *major* diastereomer. Comparison with the reference data identified the *major* diastereomer as the desired *syn*-1,3-diol. The product was not further purified.

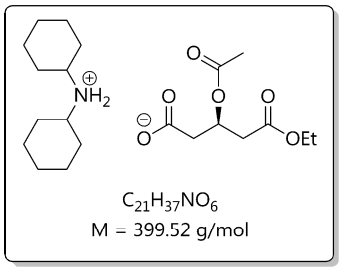
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.66-7.63 (m, 2H, *m*-Ar-*H*), 7.09 (t, *J* = 8.8 Hz, 2H, *o*-Ar-*H*), 6.64 (d, *J* = 16.0 Hz, 1H, Ar-C*H*=C), 5.45 (dd, *J* = 5.3, 16.0 Hz, 1H, C=C*H*-C), 4.46 (m, 1H, H_2C-C*H*-OH), 4.21 (m, 1H, CH-C*H*-OH), 3.74 (s, 3H, O-CH_3), 3.68 (s, 1H, OH), 3.57 (s, 3H, S-CH_3), 3.52 (s, 3H, *n*-CH_3), 3.51 (s, 1H, OH), 3.36 (m, 1H, H_3C-C*H*-CH_3), 2.49-2.47 (m, 2H, CH_2-COOMe), 1.59-1.45 (m, 2H, CH-CH_2-CH), 1.27 (d, *J* = 6.6 Hz, 6H, H_3C-CH-CH_3).

The data corresponds to the commercial reference compound.

8.15 PROCESS DEVELOPMENT A: PURIFICATION OF INTERMEDIATES AVOIDING COLUMN CHROMATOGRAPHY

Since most of the compounds have a high boiling point of above 200 °C, distillation was not investigated. Most of the intermediates were carboxylic acids and were therefore tested for crystallization as free acid, sodium salt and ammonium salt. The first two forms were investigated for crystallization from the crude product. None of the experiments were successful. However, with some primary and secondary amines, crystallization can be easily achieved.

8.15.1 DICYCLOHEXYLAMINE (3*R*)-5-ETHOXY-3-(ACETYL)-OXY-5-OXO-PENTANOATE



$C_{21}H_{37}NO_6$
M = 399.52 g/mol

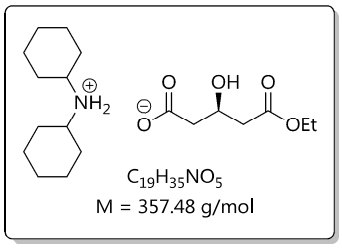
A mixture of (3*R*)-5-ethoxy-3-(acetyl)-oxy-5-oxopentanoic acid (1.00 g crude product, 4.58 mmol max.) and dicyclohexylamine (916 µL, 4.60 mmol) was dissolved in MTBE (4.0 mL) and stirred at reflux temperature. *n*-Hexane (2.0 mL) was added dropwise and the turbid solution was cooled slowly to room temperature with stirring and to -20 °C while resting. The precipitate was filtered off, washed with cold *n*-hexane (8.0 mL) and dried in high vacuum to obtain spectroscopically pure product as colorless, amorphous solid.

Isolated yield: 697 mg (1.75 mmol, 38.1%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 5.57 – 5.47 (m, 1H, C*H*-O), 4.20 – 4.04 (dq, *J* = 7.1 Hz, 2H, O-CH_2-CH_3), 3.01 – 2.89 (m, 2H, C*H*-*N*-C*H*), 2.86 – 2.77 (dd, *J* = 15.5 Hz, 1H, C$H^a{}_2$), 2.68 – 2.54 (m, 2H, CH_2), 2.50 – 2.38 (dd, *J* = 15.3 Hz, 1H, C$H^b{}_2$), 2.08 – 1.91 (m, 7H, CH_2, (O=)C-CH_3), 1.86 – 1.72 (m, 4H, CH_2), 1.69 – 1.58 (m, 1H, CH_2), 1.49 – 1.34 (m, 5H, CH_2), 1.32 – 1.09 (m, 9H, CH_2, CH_2-CH_3).

The compound is unknown to literature.

8.15.2 DICYCLOHEXYLAMINE (3*R*)-5-ETHOXY-3-HYDROXY-5-OXOPENTAN-OATE



$C_{19}H_{35}NO_5$
M = 357.48 g/mol

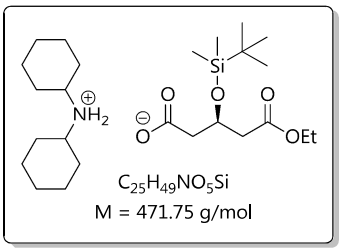
A mixture of (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid (810 mg crude product, 4.60 mmol max.) and dicyclohexylamine (916 µL, 4.60 mmol) was dissolved in MTBE (4.0 mL) and stirred at reflux temperature. *n*-Hexane (2.0 mL) was added dropwise and the turbid solution was cooled slowly to room temperature with stirring and to -20 °C while resting. The precipitate was filtered off, washed with cold *n*-hexane (8.0 mL) and dried in high vacuum to obtain spectroscopically pure product as colorless, amorphous solid.

Isolated yield: 952 mg (2.66 mmol, 57.9%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.47 (m, 1H, C*H*-O), 4.20 – 4.04 (dq, *J* = 7.1 Hz, 2H, O-CH_2-CH_3), 3.01 – 2.89 (m, 2H, C*H*-*N*-C*H*), 2.86 – 2.77 (dd, *J* = 15.5 Hz, 1H, C$H^a{}_2$), 2.68 – 2.54 (m, 2H, CH_2), 2.50 – 2.38 (dd, *J* = 15.3 Hz, 1H, C$H^b{}_2$), 2.08 – 1.91 (m, 4H, CH_2), 1.86 – 1.72 (m, 4H, CH_2), 1.69 – 1.58 (m, 1H, CH_2), 1.49 – 1.34 (m, 5H, CH_2), 1.32 – 1.09 (m, 9H, CH_2, CH_2-CH_3).

The compound is unknown to literature.

8.15.3 DICYCLOHEXYLAMINE (3*R*)-5-ETHOXY-3-(TERT-BUTYLDIMETHYL-SILYL)-OXY-5-OXOPENTANOATE



$C_{25}H_{49}NO_5Si$
M = 471.75 g/mol

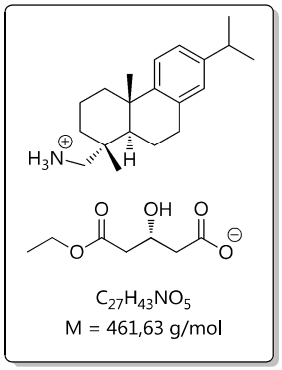
A mixture of (3*R*)-5-ethoxy-3-(*tert*-butyldimethylsilyl)-oxy-5-oxopentanoic acid (1.34 g crude product, 4.60 mmol max.) and dicyclohexylamine (916 µL, 4.60 mmol) was dissolved in MTBE (8.0 mL) and stirred at reflux temperature. *n*-Hexane (4.0 mL) was added dropwise and the turbid solution was cooled slowly to -20 °C while stirring. The precipitate was filtered off and dried in high vacuum to obtain spectroscopically pure product as colorless, amorphous solid.

Isolated yield: 1.15 g (2.44 mmol, 53.0%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 4.57 – 4.50 (m, 1H, C*H*-O), 4.16 – 4.05 (m, 2H, O-C*H*$_2$-CH$_3$), 2.88 – 2.82 (m, 2H, C*H*-*N*-C*H*), 2.75 – 2.71 (dd, *J* = 3.5, 14.8 Hz, 1H, C*H*^{a}$_2$), 2.52 – 2.41 (m, 2H, C*H*$_2$), 2.37 – 2.32 (dd, *J* = 8.5, 15.1 Hz, 1H, C*H*^{b}$_2$), 2.01 – 1.94 (m, 4H, CH$_2$), 1.80 – 1.74 (m, 4H, CH$_2$), 1.66 – 1.60 (m, 1H, C*H*$_2$), 1.39 – 1.12 (m, 14H, C*H*$_2$, CH$_2$-C*H*$_3$), 0.85 (s, 9H, C-(C*H*$_3$)$_3$), 0.08, 0.06 (2s, 6H, Si-C*H*$_3$).

The compound is unknown to literature.

8.15.4 (+)-DEHYDROABIETYLAMINE (3*R*)-5-ETHOXY-3-HYDROXY-5-OXO-PENTANOATE



$C_{27}H_{43}NO_5$
M = 461,63 g/mol

A mixture of (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid (1.00 g crude product, 5.67 mmol max.) and (+)-dehydroabietylamine (1.62 g, 5.70 mmol) was dissolved in MeOH (12.5 mL) and stirred at reflux temperature. Dist. H_2O (10 mL) was added dropwise and the turbid solution was cooled slowly to room temperature with stirring and to 0-4 °C while resting. The precipitate was filtered off, washed with cold dist. H_2O (10 mL) and dried in high vacuum to obtain spectroscopically pure product as colorless, amorphous solid.

Isolated yield: 1.89 g (4.10 mmol, 71.3%).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.20 (d, *J* = 8.1 Hz, 1H, 6-Ar-*H*), 7.05 – 6.98 (m, 1H, 5-Ar-*H*), 6.94 – 6.89 (m, 1H, 3-Ar-*H*), 4.47 (m, 1H, C*H*-OH), 4.18 (q,

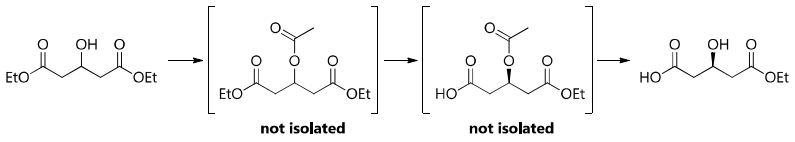
2H, *J* = 7.2 Hz, O-C*H*$_2$-CH$_3$), 2.97 – 2.78 (m, 3H, C*H*$_2$-Ar, C*H*-(CH$_3$)$_2$), 2.64 – 2.54 (m, 5H, C*H*$_2$-CH-C*H*$_2$, H$_3$N-C*H*$_2^a$), 2.45 (d, *J* = 13.2 Hz, 1H, H$_3$N-C*H*$_2^b$), 2.38 – 2.25 (m, 1H, C*H*$_2^a$), 1.86 – 1.27 (m, 13H, CH$_2^b$, C*H*$_2$), 1.28 – 1.22 (m, 12H, Ar-C-C*H*$_3$, CH-(C*H*$_3$)$_2$, CH$_2$-C*H*$_3$), 0.92 (s, 3H, C*H*$_3$).

The compound is unknown to literature. The spectroscopical data corresponds to the respective separate components.

8.16 Process Development B: Combination of reaction steps to reduce isolation procedures

The following procedures are combinations of already described procedures with the purpose of shortening the synthetic route to a minimum of isolation steps. The solvents of various reactions were also exchanged to reduce the work-up steps.

8.16.1 Combination of acetylation, biocatalytic desymmetrization and deacetylation



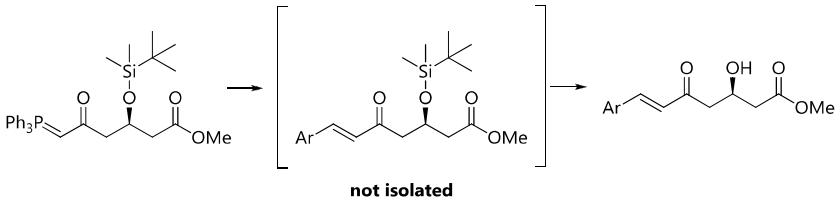
Zinc perchlorate hexahydrate (37.2 mg, 0.10 mmol, 0.01 eq.) was dissolved in acetic anhydride (990 µL, 10.5 mmol, 1.05 eq.) at room temperature. After the addition of diethyl 3-hydroxyglutarate (2.04 g, 10.0 mmol) the reaction was stirred for two hours at room temperature.[i] Aqueous potassium phosphate buffer (KPB, 10.0 mL, 50 mM, pH 8.0) was added and the resulting emulsion was neutralized by the addition of neat sodium carbonate (583 mg, 5.50 mmol, 0.55 eq.) in portions. After the addition of α-chymotrypsin (100 mg, Biozym CHY-03) the reaction was stirred for 24 hours at room temperature while maintaining the pH of 8.0 by titration with sodium hydroxide (4.0M). The biocatalyst was removed *via* µLtrafiltration (Merck Millipore, PLGC, 10,000 NMWC[ii], regenerated cellulose) and a suspension of Cephalosporin C

[i] Due to the absence of solvent the reaction progress can be directly monitored by NMR spectroscopy.
[ii] NMWC = nominal molecular weight cut-off

acetylesterase (CAE, immobilized, 500 mg) in KPB (7.5 mL, 50 mM, pH 8.0) was added. The reaction was stirred for 5.5 hours while maintaining the pH of 8.0 by titration with sodium hydroxide (4.0 M). The immobilized biocatalyst was removed by filtration (glass frit Por4, 10-16 µm) and the filtrate was brought to pH 1 with hydrochloric acid (12 M). After saturation with neat sodium chloride the aqueous phase was extracted with ethyl acetate (2 x 25 mL). The combined organic phase was washed with brine (25 mL) and dried over $MgSO_4$. The product was isolated from remaining enzyme debris by filtration over a short silica column. All volatile compounds were removed *in vacuo* to obtain spectroscopically pure product (1.68 g, 9.52 mmol, 95.2 % over 3 steps) as colorless oil.

The spectroscopical data is identical with chapter 8.3.1.

8.16.2 COMBINATION OF WITTIG REACTION AND TBS DEPROTECTION

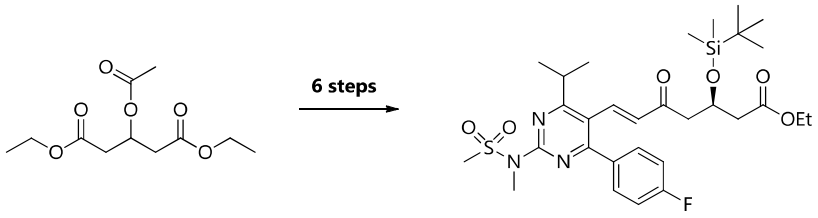


A suspension of methyl (3*R*)-(*tert*-butyldimethylsilyl)-oxy-5-oxo-6-(triphenylphos-phoranylidenyl)-hexanoate (19.9 g, 32.8 mmol, 1.15 eq.) and *n*-(4-(4-fluorophenyl)-5-formyl-6-isopropylpyrimidin-2-yl)-*N*-methylmethane-sulfonamide (10.0 g, 28.5 mmol) in 2-propanol (10 mL) was stirred for six hours at 90 °C. The solvent was removed *in vacuo* and the residue taken up in MTBE (30 mL) and cooled to -20 °C for 12 hours. Precipitated TPPO was filtered off and washed with MTBE (-20 °C, 20 mL) before the filtrate was concentrated *in vacuo*. The residue was taken up in ethanol (174 mL) and hydrochloric acid (2M, 26.0 mL) was added dropwise. After complete addition the mixture was stirred for additional six hours at room temperature, until TLC[i] shows complete deprotection. Ethyl acetate (500 mL) and aqueous sodium bicarbonate solution (2%, 350 mL) was added under vigorous stirring. The phases were separated and the aqueous phase was again extracted with ethyl acetate (100 mL). The organic phase was dried over $MgSO_4$ and concentrated *in vacuo*. The crude product was used without further purification.

The spectroscopical data is in accordance with chapter 8.13.2.

[i] Silica, 50% v/v ethyl acetate in n-heptane, UV detection

8.16.3 Elimination of all isolation steps between biocatalytic desymmetrization and Wittig reaction



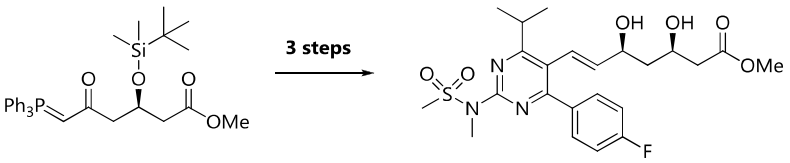
To a solution of α-chymotrypsin (300 mg, Biozym CHY-03) in KPB (10.0 mL, 50 mM, pH 8.0) was added diethyl 3-(acetyl)-oxyglutarate (9.85 g, 40.0 mmol) to a substrate concentration of 4.0 M. The pH was adjusted to 8.0 by titration with sodium hydroxide (4.0 M). The emulsion was stirred for 24 hours at room temperature.[i] The homogenously dissolved biocatalyst was removed *via* ultrafiltration (Merck Millipore, PLGC, 10,000 NMWC, regenerated cellulose). A suspension of Cephalosporin C acetylesterase (CAE, immobilized, 500 mg) in KPB (5.0 mL, 50 mM, pH 8.0) was added to the filtrate and the resulting mixture was stirred for 7.6 hours at room temperature.[i] The immobilized biocatalyst was subsequently removed *via* filtration over a glass frit (Por4, 10-16 µm) and the filtrate was brought to pH 1 by the addition of hydrochloric acid (12 M) and saturated with neat sodium chloride. The solution was extracted with ethyl acetate (2 x 50 mL) and the combined organic phase was dried over $MgSO_4$. All volatile compounds were removed *in vacuo*. Quantitative ^{1}H-NMR analysis with *p*-chlorobenzyl alcohol as internal standard revealed a product content of 6.84 g (38.8 mmol, 97.0%). The major part of this product (5.90 g, 33.5 mmol) was used for the following reaction.[ii] The intermediate was taken up in ethyl acetate (85 ml) before 1*H*-imidazole (16.1 g, 73.8 mmol, 2.2 eq.) was added in portions. To the turbid mixture was added a solution of *tert*-butyldimethylchlorosilane (11.1 g, 73.8 mmol, 2.2 eq.) in ethyl acetate (50 mL) and the resulting suspension was stirred for 72 hours[iii] at room temperature. The solvent and part of the main impurity *tert*-butyldimethylsilanol were removed *in vacuo*. The residue was taken up in THF (20 mL) and methanol (10 mL). A solution of potassium carbonate (2.31 g, 16.75 mmol, 0.5 eq.) in dist. H_2O (10 mL) was added and the turbid mixture was stirred for two hours at room temperature. The reaction was quenched by the addition of hydrochloric acid (0.5 M, 60.0 mL, pH 3-4) and saturated with neat sodium chloride. The organic phase was separated and the aqueous phase was extracted with ethyl acetate (2 x

[i] The reaction progress is monitored by the consumption of titer (4.0M sodium hydroxide) to neutralize the product or byproduct and maintaining pH 8.0 during the conversion of diester.
[ii] The remaining product was used for purification procedures and the determination of optical purity (in this case 97.2% *ee,* see Table 8, page 21)
[iii] The reaction progress could not be controlled before 72 hours, however, other experiments with TLC control indicate quantitative conversion within the first 10 hours.

60 mL). The combined organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo* to obtain the crude product (13.2 g). Quantitative ^{1}H-NMR analysis shows a product content of 7.85 g (27.0 mmol, 80.7%). The residue was taken up in MTBE (70 mL) and mixed with triethylamine (7.48 mL, 54.0 mmol, 2.0 eq.). The mixture was cooled to -70 °C (acetone/dry ice) and a solution of methyl chloroformate (3.13 mL, 40.5 mmol, 1.5 eq.) in MTBE (30 mL) was added dropwise while maintaining -70 °C. The reaction was stirred for 120 minutes at -70 °C and subsequently warmed up to 0 °C. Dist. H_2O (50 mL) was added and the phases were immediately separated. The organic phase was washed with saturated sodium bicarbonate solution (50 mL) and dried over $MgSO_4$ before all volatile compounds were removed *in vacuo*. The crude product (12.1 g) was quantified by ^{1}H-NMR spectroscopy with *p*-chlorobenzyl alcohol as internal standard to contain the mixed anhydride (9.03 g, 25.9 mmol, 96.0%) in a mixture with *tert*-butyldimethylsilanol as main impurity. The residue was taken up in MTBE (30 mL) and used without purification for the next step. Methyltriphenylphosphonium bromide (23.2 g, 65.0 mmol, 2.5 eq.) was suspended in abs. THF (150 mL) and cooled to -50 °C (acetone/dry ice) before *n*-butyllithium (1.5 M in hexanes, 43.3 mL, 65.0 mmol, 2.5 eq.) was added dropwise. The mixture was stirred for two hours while thawing up to room temperature. The resulting ylide solution was again cooled to -50 °C and the mixed anhydride solution was added dropwise. The mixture was stirred for three hours while thawing up to room temperature. The reaction was quenched by the addition of dist. H_2O (50 mL) and MTBE (50 mL) and was further stirred for 30 minutes at room temperature. The phases were separated and the aqueous phase was extracted with MTBE (2 x 50 mL). The combined organic phase was washed with saturated sodium bicarbonate solution (2x 50 mL) and brine (50 mL) before drying over $MgSO_4$. All volatile compounds were removed *in vacuo* and the crude product (18.4 g) was used without purification for the following Wittig reaction. *N*-(4-(4-fluorophenyl)-5-for-myl-6-isopropylpyrimidin-2-yl)-*N*-methylmethane-sulfonamide (8.27 g, 23.5 mmol) was added and the mixture was taken up in 2-propanol (50 mL). The resulting suspension was stirred for 19 hours at 90 °C. The solvent was removed *in vacuo* before the residue was dissolved in MTBE (50 mL) and cooled to -20 °C. Precipitated triphenylphosphine oxide (TPPO) was removed *via* filtration and the filtrate was concentrated *in vacuo*. Analysis *via* 1H-NMR spectroscopy revealed a product-related conversion of 78%, corresponding to an overall yield of 12.2 g (19.6 mmol, 58.6% over 6 steps). Due to the remaining impurities the product could not be purified by crystallization.

The spectroscopical data is in accordance with chapter 8.12.2.

8.16.4 Elimination of all isolation steps between phosphor ylide and Rosuvastatin methyl ester



A suspension of methyl (3*R*)-(*tert*-butyldimethylsilyl)-oxy-5-oxo-6-(triphenylphos-phoranylidenyl)-hexanoate (8.53 g, 16.0 mmol, 1.10 eq.) and *N*-(4-(4-fluorophenyl)-5-formyl-6-isopropylpyrimidin-2-yl)-*N*-methylmethanesulfonamide (5.10 g, 14.5 mmol) in MTBE/EtOH (20 mL, 1:1 v/v) was stirred for 19 hours at 80 °C.[i] The mixture was diluted with ethanol (100 mL) and hydrochloric acid (2.0 M, 13.0 mL) was added dropwise.[ii] After stirring for six hours at room temperature the solution was diluted with MTBE (100 mL) and subsequently washed with saturated sodium bicarbonate solution (100 mL) and brine (100 mL). The organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The residue was taken up in MTBE/EtOH (50 mL, 1:1 v/v) and cooled to -70 °C (acetone/dry ice). Diethyl methoxyborane (1.0M in THF, 14.5 mL, 14.5 mmol, 1.0 eq.) was added dropwise to the stirring solution. Sodium borohydride (0.71 g, 18.9 mmol, 1.3 eq.) was added in portions and the mixture was stirred for additional three hours at -70 °C.[iii] An aqueous sodium bicarbonate solution (2.0 M, 25 mL) was added and the phases were separated. The aqueous phase was extracted with MTBE (50 mL) and the combined organic phase was washed with brine (50 mL). The organic phase was separated and hydrogen peroxide (33 % in H_2O, 15 mL) was added dropwise at 50 °C. The mixture was stirred for additional 90 minutes at 50 °C and subsequently diluted with brine (30 mL). After additional 40 minutes stirring while cooling down to room temperature, the phases were separated and the organic phase was washed with brine (75 mL). The solution was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was analyzed by ^{1}H-NMR spectroscopy. Comparison with an internal standard[iv] revealed a product content of 5.03 g (10.2 mmol, 70.3 % overall yield) for Rosuvastatin methyl ester.

The spectroscopical data is in accordance with chapter 8.14.2.

[i] ^{1}H-NMR analysis shows a product-related conversion of 97% for the aldehyde.
[ii] The solution decolorizes from orange to light yellow during TBS deprotection. TLC control is done on silica (50 % v/v ethyl acetate in *n*-heptane)
[iii] The reaction progress is monitored via TLC control (silica, 50 % ethyl acetate in *n*-heptane)
[iv] *p*-Chlorbenzyl alcohol

8.17 PROCESS DEVELOPMENT C: RECYCLING OF BIOCATALYSTS

8.17.1 RECYCLING PROCEDURE FOR ALPHA-CHYMOTRYPSIN FROM HOMOGENOUS REACTION

A solution of α-chymotrypsin (500 mg, CHY-03) in KPB (12.5 mL, 50 mM, pH 8.0) was stirred at room temperature. Diethyl 3-(acetyl)-oxyglutarate (12.3 g, 50.0 mmol) was added to a substrate concentration of 4.0 M. The resulting emulsion was stirred vigorously at room temperature while maintaining pH 8.0 by titration with sodium hydroxide (4.0 M). After the complete conversion of starting material the clear reddish-colored solution was transferred to an ultrafiltration stirring cell (100 mL) equipped with an ultrafiltration membrane (Merck Millipore, PLGC, 10,000 NMWC, regenerated cellulose)) *via* pipette. The cell was pressurized with argon (2.0 bar) while stirring at 500 rpm. The clear filtrate was collected and directly used for the following step. After depressurizing the cell the remaining enzyme slurry was taken up in KPB (12.5 mL, 50 mM, pH 8.0) and transferred to the titration vessel for the second reaction cycle. Diethyl 3-(acetyl)-oxyglutarate (12.3 g, 50.0 mmol) was added and the reaction was stirred at room temperature while maintaining pH 8.0 by titration with sodium hydroxide (4.0 M). This procedure was continued until five reaction cycles were complete. The productivity was calculated with the reaction time necessary to reach quantitative conversion for each reaction batch, *i.e.* the consumption of the minimum theoretical amount of sodium hydroxide (12.5 mL, 4.0 M), and interpolation to 1.0 g of intermediate within 24 hours (see **Table 56**, page 241).

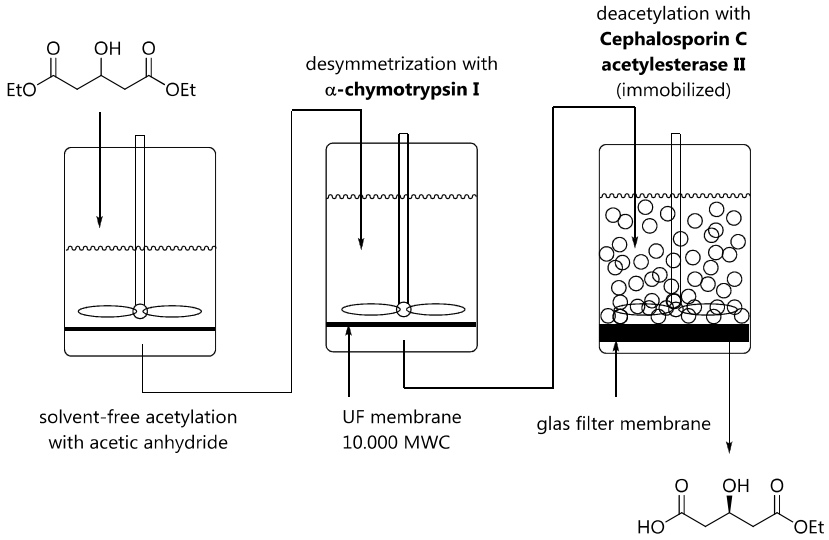
8.17.2 RECYCLING PROCEDURE FOR CEPHALOSPORIN C ACETYLESTERASE (CAE) FROM HETEROGENOUS REACTION

The filtrate of the first batch of the desymmetrization described in chapter **8.16.1**, *i.e.* KPB (25 mL, 25 mM, pH 8.0) containing sodium (3*R*)-5-ethoxy-3-(ace-tyl)-oxy-5-oxopentanoate (12.0 g, 50.0 mmol), was collected in a titration vessel and stirred at room temperature. Cephalosporin C acetylesterase (CAE, immobilized, 500 mg) was added and the resulting suspension was stirred at room temperature while maintaining pH 8.0 by titration with sodium hydroxide (4.0 M). After the complete conversion of intermediate the suspension was filtered over a glass frit (Por4, 10-16 µm). The filtrate was collected for extraction and the biocatalyst was transferred to the

titration vessel with a scoop. It was taken up with the filtrate of the second cycle from the desymmetrization reaction and again stirred at room temperature while maintaining pH 8.0 by titration with sodium hydroxide (4.0 M). This procedure was continued until five reaction cycles were complete. The productivity was calculated with the reaction time necessary to reach quantitative conversion for each reaction batch, *i.e.* the consumption of the minimum theoretical amount of sodium hydroxide (12.5 mL, 4.0M), and interpolation to 1.0 g of intermediate within 24 hours (see **Table 56**, page 241).

After saturation with neat sodium chloride the work-up of the filtrates was conducted as described in SOP6.

Table 56. Recycling of homogenous and heterogenous biocatalysts



Cycle	Conversion **I**	Conversion **II**	α-Chymotrypsin **I**	CAE[a] **II**
	[%]	[%]	[mg/(g_{Pr}*d)]	[mg/(g_{Pr}*d)]
1	>95.0	>95.0	31.51	44.35
2	>95.0	>95.0	32.12	44.64
3	>95.0	>95.0	38.27	49.36
4	>95.0	>95.0	68.34	49.65
5	>95.0	>95.0	124.43	49.91

(a) Cephalosporin C acetyl hydrolase (CAE), immobilized

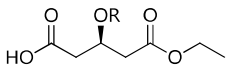
8.18 PROCESS DEVELOPMENT D: OPTIMIZATION OF EXTRACTION YIELDS BY PHYSICOCHEMICAL AND TECHNICAL MEANS

8.18.1 CONTINUOUS LIQUID – LIQUID EXTRACTION BY PERFORATION IN A KUTSCHER - STEUDEL APPARATUS

The following procedure was used for the extraction of aqueous reaction phases of the biocatalytic desymmetrization with *α*-chymotrypsin (chapter 8.3) and the biocatalytic deacetylation with Cephalosporin C acetylesterase (chapter 8.7.1), respectively. Both biocatalysts were present in the solution, leading to significant emulsification during usual work-up in a separation funnel.

In general, the acidified aqueous phase of a biocatalytic reaction was placed in a KUTSCHER – STEUDEL apparatus for the continuous extraction with organic solvents that have a lower density as the buffered reaction mixture, e.g. ethyl acetate[i] and *tert*-butyl methylether (MTBE). The aqueous solution was diluted to 50 vol-% of the extraction tube and overlayed by 50 vol-% of the corresponding organic solvent. A down-pipe was immersed and the receiver was filled with 100 vol-% of the same organic solvent. The receiver solution was stirred for 4-12 hours at reflux temperature. The boiling solvent was distilled into a DIMROTH cooler above the down-pipe and was directly flowing to the bottom of the extraction tube, bubbling through the aqueous phase and continuously extracting organic compounds. The surface of the organic solvent rises until it streams back into the receiver. With this methodology the product was continuously enriched in the receiver without additional solvent. Due to the low perturbation of the aqueous phase, emulsification was also significantly reduced.

Table 57. Continous liquid-liquid extraction of aqueous reaction media



Entry	R (mmol)	Solvent	Perforation [h]	Yield [g (%)]
1	Ac (40.0)	AcOEt	4	8.45 (96.8)
2	Ac (40.0)	MTBE	4	7.94 (90.9)
3	H (40.0)	MTBE	12	6.63 (94.1)

[i] Ethyl acetate showed better extraction yields than MTBE. However, there were also significant boiling delays which do not occur in MTBE.

8.18.2 DETERMINATION OF THE DISTRIBUTION COEFFICIENTS IN BINARY PHASES FOR MINIMIZING THE AMOUNT OF ORGANIC SOLVENTS IN EXTRACTIVE WORK-UPS

The continuous liquid-liquid extraction depends on the availability of technical equipment, in this case, a perforator. As it was not an option for the industrial process, the distribution coefficient for (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid in various binary extraction mixtures was determined empirically.

In general, a crude product of β-oxycarboxylic acid was weight into a vial and dissolved in hydrochloric acid (5 mL, 2.0 M), mimicking the acidified aqueous reaction mixture prior to extraction. The solution was overlaid with the same volume of an immiscible organic solvent (see **Table 58**, page 243) and was vigorously shaken for 30 seconds. The mixture was resting for ten minutes while the phases separated. Three aliquots of each 1.0 mL of the organic phase were transferred to flasks and all volatile compounds were removed *in vacuo*. The residue was weighed out and analyzed by ^{1}H-NMR spectroscopy. By comparison with an internal standard, *p*-chlorobenzyl alcohol, the product content was determined and extrapolated to the total volume of organic solvent. As it was common practice to salt out water-soluble organic compounds with a partition coefficient of P < 10, the experiments were also done with a solution of hydrochloric acid in brine (2.0 M) as aqueous phase.

Table 58. Determination of the distribution coefficients (D) of (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid in various extraction mixtures

Ethyl acetate		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		477,10	3,78	69,5%	1,22
Sample 2		474,45	3,68	71,0%	1,26
Sample 3		469,60	3,51	71,8%	1,27
Weigh-in [mg]	888,51	**Average D**	3,66		1,25
Content (NMR) [mg]	603,30	**STDEV D**	0,11		0,02

Ethyl acetate with NaCl saturation		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		550,90	5,99	93,4%	4,01
Sample 2		560,55	6,81	87,6%	3,24

Sample 3		564,00	7,16	94,1%	4,74
Weigh-in [mg]	890,31	**Average D**	6,66		3,99
Content (NMR) [mg]	642,80	**STDEV D**	0,49		0,61

***tert*-Butyl methylether**		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		325,15	1,19	63,4%	0,52
Sample 2		320,30	1,15	65,2%	0,54
Sample 3		321,65	1,16	64,1%	0,52
Weigh-in [mg]	882,37	**Average D**	1,16		0,53
Content (NMR) [mg]	599,13	**STDEV D**	0,02		0,005

***tert*-Butyl methylether with NaCl saturation**		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		430,55	2,08	88,7%	1,49
Sample 2		443,35	2,28	89,9%	1,67
Sample 3		425,45	2,01	94,5%	1,71
Weigh-in [mg]	882,87	**Average D**	2,12		1,62
Content (NMR) [mg]	637,43	**STDEV D**	0,12		0,09

Isopropyl acetate		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		249,50	0,64	91,2%	0,55
Sample 2		255,10	0,66	87,8%	0,54
Sample 3		246,60	0,63	86,4%	0,50
Weigh-in [mg]	886,92	**Average D**	0,64		0,53
Content (NMR) [mg]	640,36	**STDEV D**	0,01		0,02

Isopropyl acetate with NaCl saturation		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		462,75	2,57	95,7%	2,22
Sample 2		457,35	2,47	95,8%	2,14
Sample 3		453,55	2,40	95,2%	2,05
Weigh-in [mg]	889,89	**Average D**	2,48		2,14
Content (NMR) [mg]	642,50	**STDEV D**	0,07		0,07

Methyl-THF		**Weigh-out**	**D**	**Content (NMR)**	**D (NMR)**
		[mg]			
Sample 1		483,40	2,56	90,8%	1,88

Sample 2		484,25	2,57	91,1%	1,91
Sample 3		506,40	3,05	91,0%	2,18
Weigh-in [mg]	885,95	**Average D**	2,73		1,99
Content (NMR) [mg]	672,44	**STDEV D**	0,23		0,13
Methyl-THF with NaCl saturation		**Weigh-out** [mg]	**D**	**Content (NMR)**	**D (NMR)**
Sample 1		641,90	24,22	86,2%	4,81
Sample 2		639,25	21,93	86,3%	4,73
Sample 3		611,10	10,67	95,6%	6,94
Weigh-in [mg]	880,63	**Average D**	18,94		5,49
Content (NMR) [mg]	668,40	**STDEV D**	5,93		1,02

Table 59. Calculations of the number of necessary extractions for quantitative extraction of (3*R*)-5-ethoxy-3-hydroxy-5-oxopentanoic acid from aqueous reaction media

			Extr. #	>95%	1M conc.			
Organic solvent	**D**	**STDEV (+-)**	1	2	3	4	5	6
Ethyl acetate	1,25	0,02	55,6%	80,2%	96,1%	99,8%	100,0%	100,0%
Ethyl acetate + NaCl	3,99	0,61	80,0%	96,0%	99,8%	100,0%	100,0%	100,0%
Isopropyl acetate	0,53	0,02	34,6%	57,3%	81,8%	96,7%	99,9%	100,0%
Isopropyl acetate + NaCl	1,62	0,07	61,8%	85,4%	97,9%	100,0%	100,0%	100,0%
Methyl-THF	1,99	0,13	66,6%	88,8%	98,7%	100,0%	100,0%	100,0%
Methyl-THF + NaCl	5,49	1,02	84,6%	97,6%	99,9%	100,0%	100,0%	100,0%
tert-Butyl methylether	0,53	0,005	34,6%	57,3%	81,8%	96,7%	99,9%	100,0%
tert-Butyl methylether + NaCl	2,14	0,09	68,2%	89,9%	99,0%	100,0%	100,0%	100,0%

9 EXPERIMENTAL PROCEDURES – ALDOXIME DEHYDRATASES

9.0 MATERIALS AND METHODS

Chemicals

Unless stated otherwise all chemicals and solvents (reagent and HPLC grades) were purchased from commercial manufacturers (Sigma-Aldrich, Nacalai Tesque, Tokyo Chemical Industry Co., Ltd., Wako Pure Chemical Industries, Ltd., Kanto Chemical Co. Inc.) and used without further purification. Phenylacetaldehyde, 2-phenyl-propionaldehyde, cyclohex-3-ene-1-carboxaldehyde, bicyclo[2.2.1]hept-5-ene-2-carboxaldehyde, tetrahydrofuran-3-carboxaldehyde (50 w% in H_2O) and 3-phenylbutyraldehyde were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo, Japan. Tetrahydrofuran-3-carboxaldehyde was purified by extraction with ethyl acetate and subsequent Kugelrohr distillation. Hydroxylammonium chloride (Wako 1st grade) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Bacto Yeast Extract, Bacto Tryptone and agar powder were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan and Nacalai Tesque, Shiga, Japan, respectively. Deuterated solvents chloroform-d (>99.9%, stabilized with silver foil, 0.03% v/v TMS) and dimethylsulfoxide-d6 (>99.8%) were purchased from Acros Organics, New Jersey, USA and Alfa Aeser, Lancashire, UK, respectively.

Photometric measurements

The protein content of purification fractions was determined by staining with "Bio-Rad Protein Assay Dye Reagent Concentrate" from Bio-Rad Laboratories GmbH, Munich, Germany, in a 1:5 dilution. The absorbance at 595 nm was measured on 96-well plates (Thermo Scientific Nunc*) in a TECAN infinite® M200 Pro and plotted against a linear standard curve of bovine serum albumin (BSA, Cohn Fraction V, pH 7.0) purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The cell growth (OD) of cultivation experiments was measured at 610 nm in 10mm cuvettes in a JASCO V-630Bio spectrophotometer against the corresponding pure growth medium.

Incubators

The 0.5 mL-scaled screening experiments were conducted in a TAITEC BioShaker M-BR-024 at 15 °C and 30 °C, respectively. The biotransformations at 8 °C were reciprocally shaken in a TAITEC PersonalLt-10F equipped with a freezing unit (5 - 200 mL scale) or a constant temperature bath with magnetic stirrer EYELA PSL-2000 (100 - 500 mL scale), respectively. The cell cultures

(250 - 1000 mL) were incubated in a New Brunswick Scientific innova® 44, Eppendorf, Enfield, USA.

Centrifugation

Removal of whole-cells for work-up and HPLC analysis was done in a Eppendorf Centrifuge 5415R or HITACHI CT15RE (15,000 rpm, 4 °C, 5 min, 1.5 mL microtubes), a HITACHI CR22GIII (12,000 rpm, 4 °C, 5 min, 5 - 50 mL falcon tubes) and a HITACHI CR 21G (6,000 rpm, 4 °C, 10 min, 500 mL PPCO bottles), respectively. The latter was also used for the work-up and concentration of cell cultures. Pooled purification fractions of *OxdB* were concentrated in Millipore Centriprep centrifuge filtration units equipped with ULtracel® 10K membranes (10,000 Da MWC) at 3,000 x g and 4 °C.

Nutrient media and agar plates

Overexpression of *Oxd* in recombinant *E. coli* was carried out in LB medium (1.0 % Bacto Tryptone, 0.5 % Bacto Yeast Extract, 1.0 % sodium chloride, pH 7.0) or MMI medium (1.25 % Bacto Tryptone, 2.5 % Bacto Yeast Extract, 0.85 % sodium chloride, 20 mM Tris-HCl [pH 7.5], 0.4 % glycerol), respectively, containing 100 mg/mL ampicillin. Induction of *lac* expression was initiated with the addition of 1 mM isopropyl-β-thiogalactopyranoside (IPTG) either from the beginning or after reaching the exponential cell growth (A^{610} = 0.5 - 1.0). For the cultivation of already expressed vectors 2 μL of a glycerol stock solution (1:1 v/v) of *HB101/pOxD-9OF* and *BL21 Star (DE3)/pOxD-S17*, respectively, were spread over LB agar plates containing 100 µg/mL ampicillin. The plates were incubated at 37 °C for 12-16 hours.

High-Performance Liquid Chromatography (*HPLC*)

The conversion and composition of the aqueous reaction phase was measured on a Shimadzu LC20-AD equipped with a SPD-20A UV/Vis detector, a CTO-20AC column oven (40 °C), a SIL-20AC autosampler and a reversed-phase HPLC column COSMOSIL 5C18-MS-II (4.6 ID x 150 mm) with isocratic mixtures of acetonitrile in DIW (10-30 %) as mobile phase. The enantiomeric excess of 2-phenylpropionitrile, bicycle[2.2.1]-hept-5-ene-2-carbonitrile and 3-phenylbutyronitrile was measured on a Shimadzu LC-10AT equipped with a FLOM Co. Ltd. GASTORR degasser unit, a WatersTM 486 UV/Vis detector, a Waters 717 plus autosampler, a Sugai U-620 V50 column heater and a Shimadzu C-R6A Chromatopac printer. The enantiomers were separated on normal-phase Daical Chiralcel OJ-H, OD-H and Chiralpak AD-H columns, respectively, with isocratic mixtures of 2-propanol in *n*-hexane (1-5 % v/v) as mobile phase.

Gas chromatography (*GC*)

The enantiomeric excess of cyclohex-3-ene-1-carbonitrile and tetrahydro-furan-3-carbonitrile were measured on a Shimadzu GC-14B equipped with an AOC-20i autoinjector, an AOC-20s autosampler, a C-R8A Chromatopac printer and a flame ionization detector (FID). The enantiomers were separated on a chiral GC column BGB-174 (0.25 ID x 30 m, 0.25 µm film) from BGB Analytik AG, Switzerland, at isocratic column temperatures of 120 °C or 150 °C.

Nuclear Magnetic Resonance (*NMR*)

All ^{1}H-, ^{13}C-, COSY-, NOESY- and HMQC-NMR spectra were measured on a Bruker AVANCE III ULtrashield 400 Plus and processed with the software TopSpin (Version 3.2). The shifts were measured in chloroform-d and dimethylsulfoxide-d_6, respectively, and given in relation to tetramethylsilane (TMS, δ = 0.0 ppm). The frequencies used for ^{1}H- and ^{13}C- measurements were 400 MHz and 100 MHz, respectively. Unless otherwise stated all measurements were done at room temperature.

Polarimeter

For the identification of enantiomers the specific optical rotary power of 2-phenylpropionitrile and *E*-2-phenylpropionaldoxime were measured on an ATAGO AP-300 automatic polarimeter. All values were measured at least ten times in a 10.01-mm cell at 589.3 nm (sodium D-Line). The direction of polarization is given as "+" (dextrorotary) or "-" (levorotary).

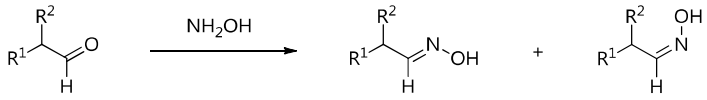
9.1 STANDARD OPERATING PROCEDURE 14 (SOP14): SYNTHESIS OF RACEMIC ALDOXIMES THROUGH CONDENSATION OF CHIRAL ALDEHYDES WITH HYDROXYLAMINE SALTS

Hydroxylamine salt (e.g. hydroxylamine hydrochloride, hydroxylamine phosphate, 1.0 - 1.5 eq.) and a soluble base (e.g. sodium acetate, potassium carbonate, pyridine, 1.3 - 2.0 eq.) were mixed and suspended in deion. H_2O (DIW) or ethanol (0.10 - 0.45 M) and vigorously stirred for five minutes at 0 °C to room temperature.[i] After the addition of racemic aldehyde (45.0 - 120.0 mmol) the mixture was vigorously stirred for at least two hours at room

[i] The salts are completely dissolved (hydroxylamine hydrochloride) or suspended (hydroxylamine phosphate), respectively. Regardless of isomerism at elevated temperatures there was nearly no effect of low temperature (even -20 °C in ethanol) on the isomeric ratio of *E/Z*-aldoxime.

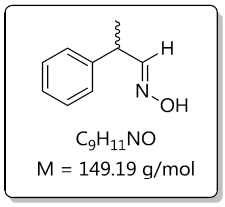
temperature, until TLC shows complete conversion.[i] The mixture was extracted three times with ethyl acetate (1:1 v/v), the organic phases were combined and dried over anhydrous $MgSO_4$. After filtration the solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica or Kugelrohr distillation, respectively. The conformation and *E*/*Z*-ratio were determined *via* ^{1}H-NMR spectroscopy and the enantiomeric excess was determined *via* chiral HPLC or GC, respectively.

Table 60. Substrate synthesis of racemic aldoximes by condensation of chiral aldehydes with hydroxylamine



Entry	R^1	R^2	Conv. (time) [% (h)]	Isol. Yield [g (%)]
1	Ph	Me	>95.0 (2.0)	8.46 (94.5)
2	1-Cyclohex-3-ene		>95.0 (2.0)	3.63 (96.8)
3	2-Norborn-5-ene		>95.0 (2.0)	2.40 (44.1)
4	3-Tetrahydrofuran		>95.0 (2.0)	3.93 (75.9)
5	2-Ph-Pr	H	>95.0 (2.0)	3.03 (82.5)

9.1.1 *RAC*-*E*/*Z*-2-PHENYLPROPIONALDEHYDE OXIME



$C_9H_{11}NO$
M = 149.19 g/mol

The synthesis was conducted according to SOP14. Hydroxylamine hydrochloride (6.25 g, 90.0 mmol, 1.5 eq.) and sodium carbonate (9.54 g, 90.0 mmol, 1.5 eq.) were suspended in DIW (100 mL). After the addition of *rac*-2-phenylpropionaldehyde (8.05 g, 60.0 mmol) the mixture was vigorously stirred at room temperature for at least two hours until TLC shows complete conversion. The work-up was done as described in SOP 14 to obtain the product as colorless oil (*E*/*Z* = 72:28). An aliquot of 0.20 g was separated into the corresponding *E*- and *Z*-isomer by column chromatography on silica (10% v/v ethyl acetate in *n*-hexane, Ø = 1.5 cm, h = 40 cm, 60-200 µm, pH 6-8) to yield 0.12 g *rac*-*E*-2-phenylpropionaldehyde oxime (98 % isomeric purity), 0.02 g of an *E*/*Z*-mixture (1:1) and 0.06 g *rac*-*Z*-2-phenylpropionaldehyde oxime (92 % isomeric purity).

[i] The conversion is determined by TLC with different *n*-hexane/ethyl acetate eluents and phosphomolybdic acid (5 % in ethanol, heating to 300 °C).

Reversed-phase HPLC: Nacalai Tesque COSMOSIL C18-MS-II, 30 % v/v acetonitrile in water, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*), R_{t2} = 12.4 min (*Z*).

Normal-phase chiral HPLC: Daicel Chiralcel OJ-H, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 20.4 min (*Z*-2*S*), R_{t1} = 26.7 min (*E*-2*S*), R_{t1} = 30.0 min (*Z*-2*R*), R_{t1} = 31.5 min (*E*-2*R*).

Isolated yield: 8.46 g (56.7 mmol, 94.5 %).

E-rac-2-Phenylpropionaldehyde oxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.52 (d, *J* = 6.1 Hz, 1H, *H*C=NOH), 7.38 – 7.19 (m, 5H, Ar-*H*), 3.67 (m, *J* = 6.8 Hz, 1H, C*H*-CH=NOH), 1.46 (d, *J* = 7.0 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] =155.09, 142.11, 128.74, 127.39, 126.95, 40.37, 18.75.

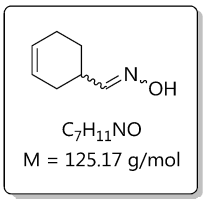
Z-rac-2-Phenylpropionaldehyde oxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.38 – 7.19 (m, 5H, Ar-*H*), 6.80 (d, *J* = 7.3 Hz, 1H, *H*C=NOH), 4.45 (m, 1H, C*H*-CH=NOH), 1.42 (d, *J* = 7.0 Hz, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] =154.62, 141.78, 128.69, 127.19, 126.82, 34.99, 18.28.

The data corresponds to literature-known values.[138]

9.1.2 *RAC-E/Z*-CYCLOHEX-3-ENECARBALDEHYDE OXIME



$C_7H_{11}NO$
M = 125.17 g/mol

The following synthesis was performed according to SOP 14. Hydroxylamine hydrochloride (3.13 g, 45.0 mmol) and sodium carbonate (4.77 g, 45.0 mmol) were suspended in dist. H_2O (50 mL) and vigorously stirred at room temperature. After the addition of 3-cyclohexene-1-carboxaldehyde (3.30 g, 30.0 mmol) the mixture was vigorously stirred for at least two hours until TLC shows complete conversion. The work-up was done as described in SOP14. After filtration the solvent was removed *in vacuo* and the crude product was purified *via* column chromatography on silica (h = 15 cm, Ø = 6 cm, 9 % v/v ethyl acetate in *n*-hexane) to obtain the product as colorless oil in several isomeric mixtures (*E/Z* = 9:1, 4:1, 2:1).

The chiral GC analysis for the corresponding nitrile shows the aldoxime at R_{t1} = 18.9 min (*Z*) and R_{t2} = 19.2 min (*E*), regardless of configuration.

Isolated yield: 3.63 g (29.0 mmol, 96.8 %).

E-rac-3-Cyclohexene-1-carbaldehyde oxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.42 (d, *J* = 6.0 Hz, 1H, *H*C=NOH), 5.69 (m, 2H, *H*C=C*H*), 2.60 – 2.45 (m, 1H, C*H*-CH=NOH), 2.32 – 1.78 (m, 5H, CH_2), 1.65 – 1.46 (m, 1H, CH_2-CH_2-CH).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 155.33, 126.99, 125.10, 34.45, 28.50, 26.10, 24.11.

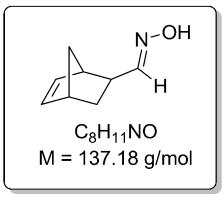
Z-rac-3-Cyclohexene-1-carbaldehyde oxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 6.65 (d, *J* = 7.3 Hz, 1H, *H*C=NOH), 5.69 (m, 2H, *H*C=C*H*), 3.33 – 3.18 (m, 1H, C*H*-CH=NOH), 2.32 – 1.78 (m, 5H, CH_2), 1.65 – 1.46 (m, 1H, CH_2-CH_2-CH).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 156.11, 127.06, 125.10, 29.76, 27.87, 25.16, 23.75.

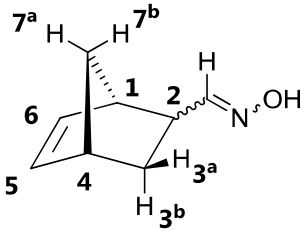
The data corresponds to literature-known values.[139]

9.1.3 *RAC-E/Z*-5-NORBORNENE-2-CARBALDEHYDE OXIME



The following synthesis was performed according to SOP 14. Hydroxylamine hydrochloride (4.17 g, 60.0 mmol) and sodium carbonate (6.36 g, 60.0 mmol) were suspended in DIW (40 mL) and vigorously stirred at room temperature. After the addition of 5-norbornene-2-carboxaldehyde (5.0 mL, 39.7 mmol) the mixture was vigorously stirred for at least two hours until TLC shows complete conversion. The work-up was done according to SOP 14. The crude product was purified by column chromatography on silica (h = 12 cm, Ø = 6 cm, 15% v/v ethyl acetate in *n*-hexane) to obtain a diastereomeric mixture of the product as colorless oil (*E*-(*R*)/*E*-(*S*)/*Z*-(*R*)/*Z*-(*S*) = 0.47 : 0.08 : 0.35 : 0.10).

Isolated yield: 2.40 g (17.5 mmol, 44.1 %).



E-(*1S,2S,4S*)-Bicyclo[2.2.1]hept-5-ene-2-carbaldoxime:

1H-NMR (400 MHz, $CDCl_3$) δ 7.45 (d, *J* = 7.4 Hz, 1H, *H*C=NOH), 6.12 (m, 2H, **5** and **6**), 2.92 (m, 1H, **4**), 2.82 (m, 1H, **1**), 2.23 (m, 1H, **2**), 1.62 (m, 1H, **3a**), 1.45 (m, 1H, **7a**), 1.38 (m, 1H, **3b**), 1.35 (m, 1H, **7b**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 156.02, 137.42, 135.57, 49.50, 46.27, 41.78, 38.72, 30.45.

E-(*1S,2R,4S*)-Bicyclo[2.2.1]hept-5-ene-2-carbaldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.05 (d, *J* = 8.0 Hz, 1H, *H*C=NOH), 6.21 (m, 1H, **5**), 5.98 (m, 1H, **6**), 2.98 (m, 1H, **1**), 2.90 (m, 1H, **2**), 2.89 (m, 1H, **4**), 1.99 (m, 1H, **3a**), 1.46 (m, 1H, **7a**), 1.30 (m, 1H, **7b**), 1.05 (m, 1H, **3b**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 156.47, 138.04, 132.26, 49.40, 46.69, 42.55, 38.91, 30.74.

Z-(*1S,2S,4S*)-Bicyclo[2.2.1]hept-5-ene-2-carbaldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$) δ 6.72 (d, *J* = 8.0 Hz, 1H, *H*C=NOH), 6.12 (m, 2H, **5** and **6**), 2.93 (m, 1H, **4**), 2.85 (m, 1H, **1**), 2.77 (m, 1H, **2**), 1.50 (m, 1H, **3a**), 1.45 (m, 1H, **7a**), 1.41 (m, 1H, **3b**), 1.35 (m, 1H, **7b**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 157.00, 137.67, 135.71, 49.50, 46.21, 42.09, 33.84, 31.88.

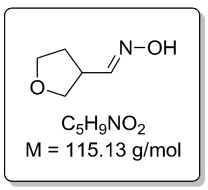
Z-(*1S,2R,4S*)-Bicyclo[2.2.1]hept-5-ene-2-carbaldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$) 6.37 (d, *J* = 7.4 Hz, 1H, *H*C=NOH), 6.21 (m, 1H, **5**), 6.03 (m, 1H, **6**), 3.46 (m, 1H, **2**), 3.08 (m, 1H, **1**), 2.92 (m, 1H, **4**), 2.11 (m, 1H, **3a**1.46 (m, 1H, **7a**), 1.30 (m, 1H, **7b**), 0.91 (m, 1H, **3b**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 156.96, 138.14, 132.67, 49.40, 45.47, 42.55, 34.18, 32.12.

The compound is literature-known. However, no NMR data is available. The isomers were distinguished by ^{1}H-COSY analysis of the diastereomers.

9.1.4 *RAC*-*E/Z*-TETRAHYDROFURAN-2-CARBALDEHYDE OXIME



The following synthesis was conducted according to SOP14. Hydroxylamine phosphate (2.96 g, 15.0 mmol) and sodium acetate (4.92 g, 60.0 mmol) were suspended in dist. H_2O (40 mL) and vigorously stirred at room temperature.

After the addition of tetrahydrofuran-3-carboxaldehyde (4.10 mL, 45.0 mmol) the mixture was vigorously stirred for at least two hours until TLC shows complete conversion. The work-up was dome as described in SOP14. After filtration the solvent was removed *in vacuo* and the crude product was purified *via* Kugelrohr distillation (0.5 mbar, 110 °C to give the product as colorless oil (*E/Z* 50:50).

The chiral GC analysis for the corresponding nitrile shows the aldoxime at R_t = 9.7 min, regardless of conformation or configuration.

Isolated yield: 3.93 g, (34.1 mmol, 75.9 %).

E-rac-Tetrahydrofuran-3-carbaldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.32 (d, *J* = 6.7 Hz, 1H, *H*C=NOH), 3.95 (m, 1H, O-C*H*$_2$-CH), 3.82 (m, 2H, O-C*H*$_2$-CH$_2$), 3.69 (m, 1H, O-C*H*$_2$-CH), 3.08 (m, 1H, CH$_2$-C*H*-CH$_2$), 2.18 (m, 1H, CH-C*H*$_2$-CH$_2$), 1.90 (m, 1H, CH-C*H*$_2$-CH$_2$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 152.02, 70.61, 68.03, 39.45, 30.58.

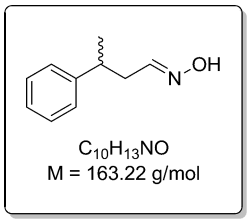
Z- rac-Tetrahydrofuran-3-carbaldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 6.63 (d, *J* = 5.4 Hz, 1H, *H*C=NOH), 4.02 (m, 1H, O-C*H*$_2$-CH), 3.92 (m, 2H, O-C*H*$_2$-CH$_2$), 3.65 (m, 1H, O-C*H*$_2$-CH), 3.64 (m, 1H, CH$_2$-C*H*-CH$_2$), 2.25 (m, 1H, CH-C*H*$_2$-CH$_2$), 1.82 (m, 1H, CH-C*H*$_2$-CH$_2$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 153.27, 70.86, 67.94, 35.05, 30.66.

The compound is literature-known. However, no NMR data is available.

9.1.5 *RAC-E/Z*-3-PHENYLBUTYRALDEHYDE OXIME



$C_{10}H_{13}NO$
M = 163.22 g/mol

The following synthesis was conducted according to SOP14. Hydroxylamine phosphate (1.48 g, 7.5 mmol) and sodium acetate (2.46 g, 30.0 mmol) were suspended in dist. H_2O (40 mL) and vigorously stirred at room temperature. After the addition of *rac*-3-phenylbutyraldehyde (3.35 mL, 22.5 mmol) the mixture was vigorously stirred for at least two hours until TLC shows complete conversion. The work-up was done as described in SOP14. After filtration the solvent was removed *in vacuo* and the crude product was purified by column chromatography on silica (Ø = 6cm, h = 8cm, 20% v/v ethyl acetate in cyclohexane) to obtain the product as colorless oil (*E/Z* = 50:50).

Reversed-phase HPLC: Nacalai Tesque COSMOSIL C18-MS-II, 30 % v/v acetonitrile in water, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 14.9 min (*E*), R_{t2} = 17.2 min (*Z*).

Normal-phase chiral HPLC: Daicel Chiralcel OJ-H, 2 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 33.2 min (*Z*-2*S*), R_{t1} = 34.8 min (*E*-2*S*), R_{t1} = 42.0 min (*Z*-2*R*), R_{t1} = 45.6 min (*E*-2*R*).

Isolated yield: 3.03 g (18.6 mmol, 82.5 %).

E-rac-3-Phenylbutyraldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.40 (d, *J* = 7.3 Hz, 1H, *H*C=NOH), 7.32 – 7.18 (m, 5H, Ar-*H*), 2.94 (m, 1H, Ph-C*H*-CH_3), 2.53 – 2.41 (m, 2H, CH_2), 1.31 (d, *J* = 6.4 Hz, 3H, CH_3).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 151.02, 145.61, 128.61, 126.87, 126.46, 38.00, 37.74, 22.22.

Z-rac-3-Phenylbutyraldoxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.32 – 7.18 (m, 5H, Ar-*H*), 6.75 (d, *J* = 5.8 Hz, 1H, *H*C=NOH), 3.00 (m, 1H, Ph-C*H*-CH_3), 2.77 – 2.58 (m, 2H, CH_2), 1.29 (d, *J* = 6.4 Hz, 3H, CH_3).

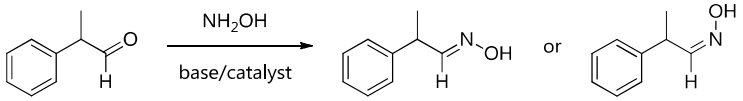
^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 151.26, 145.75, 128.61, 126.87, 126.46, 37.20, 33.10, 21.67.

The compound is literature-known. However, no NMR data is available.

9.2 Synthesis of isomerically enriched *E*-2-phenylpropionaldoxime

The following procedures were conducted according to literature procedures leading specifically to *E*- or *Z*-aldoximes. However, none of the tested methods could be adapted for aliphatic aldoximes. The crude products were not further purified.

Table 61. Stereoselective condensation with hydroxylamine for *E*- or *Z*-aldoximes



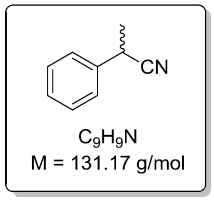
Entry	Catalyst	Solvent	Temp.	Conv.	ie (E/Z)
1	none	H_2O	20	>95%	60.3 (E)
2	none	H_2O	60	>95%	31.2 (E)
3	none	EtOH	-20	>95%	60.1 (E)
4	Mol. Sieves 3A	none	20	n.d.[a]	n.d.[a]
5	Pyridine	EtOH	20	>95%	42.4 (E)
6	$Cu(OAc)_2$	EtOH	-40	>95%	23.2 (E)

(a) Decomposition of reagents, thus discarded.

9.3 STANDARD OPERATING PROCEDURE 15 (SOP15): SYNTHESIS OF RACEMIC NITRILES BY TPPO-CATALYZED OXIDATION OF ALDOXIMES

Oxalyl dichloride (1.5 eq.) and triphenylphosphine oxide (TPPO, 5 mol-%) were dissolved in dry dichloromethane (0.5 M) and stirred for 5 minutes at 0 °C. Racemic aldoxime (2.24 - 5.00 mmol) was dissolved in dry dichloromethane (10 mL) and added dropwise to the reaction solution. The reaction progress was monitored qualitatively by the emission of gaseous byproducts (CO_2, CO, HCl). After two hours at 0 °C to room temperature the reaction was quenched by the addition of saturated aqueous sodium bicarbonate solution (20 mL). The aqueous phase was separated and extracted twice with dichloromethane (2 x 50 mL). The organic phase was unified and washed once with brine (50 mL). The crude products were purified by column chromatography on silica (MTBE) or *via* Kugelrohr distillation, respectively, to remove remaining TPPO. The enantiomeric excess was determined by normal-phase chiral HPLC or GC.

9.3.1 *RAC*-2-PHENYLPROPIONITRILE



Oxalyl dichloride (1.29 mL, 7.5 mmol) and triphenylphosphine oxide (69.6 mg, 0.25 mmol) were dissolved in dry dichloromethane (10 mL) and stirred for 5 minutes at 0 °C. *rac-E/Z*-2-phenylpropionaldehyde oxime (746 mg, 5.0 mmol) was dissolved in dry dichloromethane (10 mL) and added dropwise to the reaction solution. The work-up was done as described in SOP15. The crude

product was purified by filtration over a small silica column (MTBE) to obtain a colorless oil.

Normal-phase chiral HPLC: Daicel Chiralcel OJ-H, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 13.8 min (*R*), R_{t2}= 14.3 min (*S*).

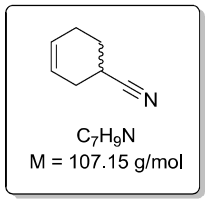
Isolated yield: 501 mg (3.82 mmol, 76.4%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.38 – 7.28 (m, 5H, Ar-*H*), 3.87 (q, *J* = 7.2 Hz, 1H, C*H*-CN), 1.60 (d, *J* = 7.3 Hz, 3H, C*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 137.17, 129.17, 128.06, 126.74, 121.66, 31.22, 21.46.

The data corresponds to literature-known values.[140]

9.3.2 *RAC*-CYCLOHEX-3-ENECARBONITRILE



Oxalyl dichloride (0.29 mL, 3.36 mmol) and triphenylphosphine oxide (31.3 mg, 0.11 mmol) were dissolved in dry dichloromethane (10 mL) and stirred for 5 minutes at 0 °C. Cyclohex-3-enecarboxaldehyde oxime (281 mg, 2.24 mmol) was dissolved in dry dichloromethane (10 mL) and added dropwise to the reaction solution. The work-up was conducted according to SOP15. The crude product was purified by Kugelrohr distillation (0.5 mbar, 100 °C). to obtain a colorless oil.

Chiral GC (FID): BGB-174 (0.25 ID x 30 m, 0.25 µm film), 120 °C (isocratic), R_{t1} = 22.5 min (*major*), R_{t2} = 23.4 min (*minor*).

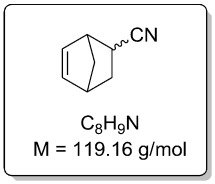
Isolated yield: 140 mg (1.31 mmol, 58.3 %).

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 5.79 – 5.73 (m, 1H, =C*H*-CH_2-CH_2), 5.67 – 5.62 (m, 1H, =C*H*-CH_2-CH), 2.86 – 2.79 (m, 1H, C*H*-CN), 2.45 – 2.32 (m, 2H, =CH-C*H*$_2$-CH), 2.31 – 2.04 (m, 2H, =CH-C*H*$_2$-CH_2), 2.03 – 1.84 (m, 2H, =CH-CH_2-C*H*$_2$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 127.01, 123.28, 122.45, 28.19, 25.34, 24.65, 22.92.

The compound is literature-known. However, no NMR data is available.

9.3.3 *RAC*-5-NORBORNENE-2-CARBONITRILE

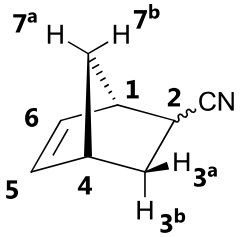


Oxalyl dichloride (0.47 mL, 5.48 mmol) and triphenylphosphine oxide (50.7 mg, 0.18 mmol) were dissolved in dry dichloromethane (30 mL) and stirred for 5 minutes at 0 °C. *E/Z*-5-norbornene-2-carbaldoxime (500 mg, 3.65 mmol) was dissolved in dry dichloromethane (10 mL) and added dropwise to the reaction solution. The work-up was conducted according to SOP15. The crude product was purified *via* column chromatography on silica (MTBE) to obtain a colorless oil. ^{1}H-NMR analysis shows a diastereomeric excess of 66.4 % de.

Normal-phase chiral HPLC: Daicel Chiralpak AD-H, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 7.8 min (*S*), R_{t2}= 8.6 min (*R*).

Isolated yield: 320 mg (2.69 mmol, 73.6 %).

(*1S,2R,*4S)-Bicyclo[2.2.1]-hept-5-ene-2-carbonitrile:



^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 6.34 (dd, *J* = 5.7 Hz, 3.1 Hz, 1H, **5**), 6.21 (dd, *J* = 5.7 Hz, 2.9 Hz, 1H, **6**), 3.24 (m, 1H, **1**), 3.04 (m, 1H, **4**), 2.86 (dt, *J* = 9.4 Hz, 3.8 Hz, 1H, **2**), 2.13 (qd, *J* = 11.1 Hz, 3.5 Hz, 1H, **3a**), 1.55 – 1.51 (m, 1H, **7b**), 1.37 – 1.32 (m, 1H, **3b**), 1.23 – 1.19 (dm, 1H, **7a**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 138.86, 132.77, 123.12, 48.55, 45.78, 42.40, 32.50.

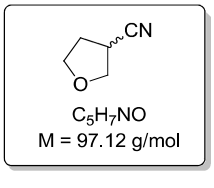
(*1S,2S,*4S)-Bicyclo[2.2.1]-hept-5-ene-2-carbonitrile:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 6.17 (dd, *J* = 5.7 Hz, 2.9 Hz, 1H, **5**), 6.05 (dd, *J* = 5.7 Hz, 3.1 Hz, 1H, **6**), 3.24 (m, 1H, **4**), 3.06 (m, 1H, **1**), 2.20 (m, 1H, **3a**), 1.97 (dt, *J* = 12.3 Hz, 4.0 Hz, 1H, **2**), 1.58 – 1.56 (m, 1H, **3b**), 1.55 – 1.51 (m, 1H, **7b**), 1.23 – 1.19 (m, 1H, **7a**).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 138.16, 134.08, 123.64, 47.20, 45.78, 41.85, 32.50.

The compound is literature-known. However, no ^{1}H-NMR data is available.

9.3.4 *RAC*-TETRAHYDROFURAN-3-CARBONITRILE



Oxalyl dichloride (0.56 mL, 6.51 mmol) and triphenylphosphine oxide (60.4 mg, 0.22 mmol) were dissolved in dry dichloromethane (30 mL) and stirred for

5 minutes at 0 °C. Tetrahydrofuran-3-carboxaldehyde oxime (500 mg, 4.34 mmol was dissolved in dry dichloromethane (10 mL) and added dropwise to the reaction solution. The work-up was conducted according to SOP15. The crude product was purified *via* Kugelrohr distillation (0.5 mbar, 80 °C) to obtain a colorless oil.

Chiral GC (FID): BGB-174 (0.25 ID x 30 m, 0.25 µm film), 120 °C (isocratic), R_{t1} = 11.4 min (*major*), R_{t2} = 12.3 min (*minor*).

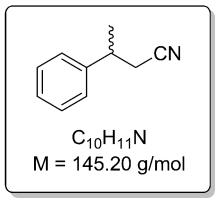
Isolated yield: 360 mg (3.71 mmol, 74.1 %).

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 4.05 – 3.84 (m, 4H, C*H*$_2$-O-C*H*$_2$), 3.09 (m, 1H, C*H*-CN), 2.36 – 2.19 (m, 2H, C*H*$_2$-CH$_2$-O).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 120.72, 70.53, 67.77, 30.87, 28.24.

The compound is literature-known. However, no NMR data is available.

9.3.5 *RAC*-3-PHENYLBUTYRONITRILE



$C_{10}H_{11}N$
M = 145.20 g/mol

Oxalyl dichloride (0.40 mL, 4.59 mmol) and triphenylphosphine oxide (44.8 mg, 0.15 mmol) were dissolved in dry dichloromethane (30 mL) and stirred for 5 minutes at 0 °C. *rac*-3-Phenylbutyraldehyde oxime (500 mg, 3.06 mmol), diluted with dry dichloromethane (5 mL) was added. The work-up was conducted according to SOP15. The crude product was purified by column chromatography on silica (MTBE) to obtain a colorless oil.

Normal-phase chiral HPLC: Daicel Chiralcel OJ-H, 2 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 23.3 min (*major*), R_{t2}= 24.6 min (*minor*).

Isolated yield: 420 mg (2.89 mmol, 94.5 %).

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.40 – 7.26 (m, 5H, Ar-*H*), 3.19 (m, 1H, Ph-C*H*-CH$_3$), 2.61 (qd, *J* = 7.5 Hz, 6.5 Hz, 2H, C*H*$_2$-CN), 1.48 (d, *J* = 7.0 Hz, 3H, C*H*$_3$).

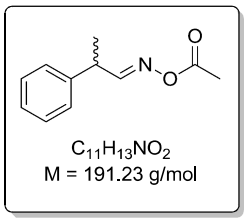
^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 128.88, 127.34, 126.56, 118.58, 36.54, 26.35, 20.68.

The data corresponds to literature-known values.[141]

9.4 STANDARD OPERATING PROCEDURE 16 (SOP16): SYNTHESIS OF O-ACYLATED CHIRAL ALDOXIMES BY SCHOTTEN-BAUMANN REACTION

To a solution of aldoxime in an appropriate organic solvent (e.g. *n*-hexane, MTBE) was added triethylamine (2.0 eq.). The mixture was stirred for five minutes at room temperature. The desired acyl chloride (1.5 eq.) was added dropwise. The reaction was stirred for 2 – 6 hours at 0 °C to room temperature. Insoluble side products were filtered off and the filtrate was washed with saturated sodium bicarbonate solution (1:1 v/v). The aqueous phase was extracted with MTBE (2 x 1:1 v/v) and the combined organic phase was washed with brine (1:3 v/v). After drying over $MgSO_4$ and evaporation of all volatile compounds the crude product was obtained as colored oil. It was purified by column chromatography on silica (MTBE).

9.4.1 *RAC*-*E*-2-PHENYLPROPIONALDOXIME ACETATE



To a solution of *E/Z*-2-phenylpropionaldoxime (746 mg, 5.0 mmol) in *n*-hexane (50 mL) was added triethylamine (1.40 mL, 10.0 mmol). The mixture was stirred for five minutes at room temperature before acetyl chloride (533 µL, 7.5 mmol) was added dropwise. The reaction was stirred at room temperature for two hours. The work-up was done as described in SOP16. NMR analysis shows complete conversion of the free aldoxime to the *E*-oxime ester[i] and 19.9 % elimination to the corresponding nitrile. The compound was not further purified.

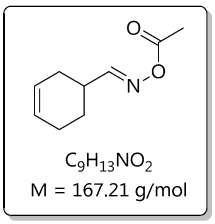
Yield: 910 mg.

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.75 (d, *J* = 7.3 Hz, 1H, *H*C=*N*-O), 7.43 – 7.23 (m, 5H, Ar-*H*), 3.90 (m, 1H, Ph-C*H*-CH_3), 2.16 (s, 3H, (O=)C-CH_3), 1.55 (d, *J* = 7.1 Hz, 3H, CH-CH_3).

The compound is literature-unknown.

[i] The observed effect for E-conformational preference of the esterification is maximized in the chemical reaction. The reason for this behavior is yet to be identified.

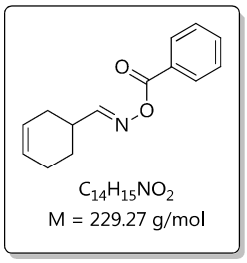
9.4.2 *E-RAC*-CYCLOHEX-3-ENE-1-CARBALDOXIME ACETATE



$C_9H_{13}NO_2$
M = 167.21 g/mol

A solution of *E/Z*-cyclohex-3-ene-1-carbaldoxime (E/Z 72:28, 626 mg, 5.0 mmol) and triethylamine (1.4 mL, 10.0 mmol, 2.0 eq.) in MTBE (25 mL) was cooled to 0-4°C. Acetyl chloride (533 uL, 7.5 mmol, 1.5 eq.) was added dropwise and the mixture was stirred for two hours at room temperature. The work-up was done as described in SOP16. The crude product was analyzed by ^{1}H-NMR spectroscopy and showed an *E/Z* ratio of 100:0 for the *E*-aldoxime acetate. The crude product was not purified.

9.4.3 *E-RAC*-CYCLOHEX-3-ENE-1-CARBALDOXIME BENZOATE



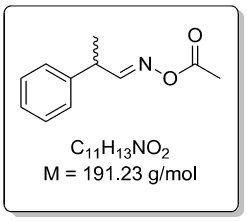
$C_{14}H_{15}NO_2$
M = 229.27 g/mol

A solution of *E/Z*-cyclohex-3-ene-1-carbaldoxime (E/Z 72:28, 626 mg, 5.0 mmol) and triethylamine (1.4 mL, 10.0 mmol, 2.0 eq.) in MTBE (25 mL) was cooled to 0-4°C. Benzoyl chloride (865 uL, 7.5 mmol, 1.5 eq.) was added dropwise and the mixture was stirred for two hours at room temperature. The work-up was done as described in SOP16. The crude product was analyzed by ^{1}H-NMR spectroscopy and showed an *E/Z* ratio of 6:1 for the aldoxime benzoate.

9.5 STANDARD OPERATING PROCEDURE 17 (SOP17): SYNTHESIS OF O-ACYLATED CHIRAL ALDOXIMES BY LIPASE-CATALYZED TRANSESTERIFICATION

The synthesis was conducted based on a literature procedure from SALUNKHE and NAIR.[142] A mixture of aldoxime (1.1 mmol) and the desired acyl ester (3.0 eq.) in an appropriate solvent (e.g. n-hexane) was stirred for 10 minutes at room temperature. After the addition of a lipase, the suspension was stirred for 12 hours at room temperature. The biocatalyst was removed *via* filtration and all volatile compounds were removed in vacuo. The crude products were analyzed by NMR spectroscopy and chiral HPLC, if an adequate method was found.

9.5.1 (2*RS*)-*E/Z*-2-PHENYLPROPIONALDOXIME ACETATE



The following procedure is based on SOP17. A mixture of *E/Z*-2-phenyl-propionaldoxime (3:2 E/Z, 164 mg, 1.1 mmol) and vinyl acetate[i] (277 µL, 3.0 mmol) in *n*-hexane (10 mL) was stirred for 10 minutes at room temperature. After addition of lipase Amano PS-D (immobilized on diatomite, 50 mg) the suspension was stirred for 12 hours at room temperature. The work-up was done according to SOP17. The crude product was analyzed by NMR spectroscopy. It shows 70.6 % conversion to a mixture of *E/Z*-aldoxime ester (4:1) and remaining *E/Z*-aldoxime (2:1).[ii] The crude product was not further purified.

Yield: 220 mg

<u>*E-rac*-*O*-Acetyl-2-phenylpropionaldoxime</u>

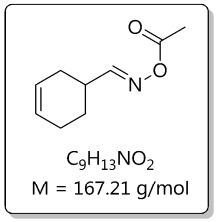
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.75 (d, *J* = 7.3 Hz, 1H, *H*C=*N*-O), 7.43 – 7.23 (m, 5H, Ar-*H*), 3.90 (m, 1H, Ph-C*H*-CH_3), 2.16 (s, 3H, (O=)C-C*H*$_3$), 1.55 (d, *J* = 7.1 Hz, 3H, CH-C*H*$_3$).

<u>*Z-rac*-*O*-Acetyl-2-phenylpropionaldoxime</u>

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.20 (d, *J* = 7.6 Hz, 1H, *H*C=*N*-O), 7.43 – 7.23 (m, 5H, Ar-*H*), 4.40 (m, 1H, Ph-C*H*-CH_3), 2.21 (s, 3H, (O=)C-C*H*$_3$), 1.50 (d, *J* = 7.3 Hz, 3H, CH-C*H*$_3$).

The compound is unknown to literature.

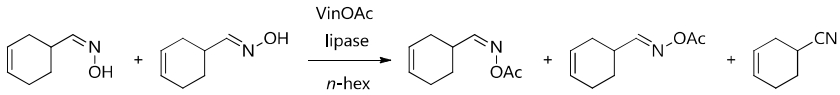
9.5.2 (2*RS*)-*E/Z*-3-CYCLOHEXENE-1-CARBALDOXIME ACETATE



The following procedure is based on SOP17. A mixture of *E/Z*-cyclo-hex-3-ene-1-carbaldoxime (3:2 *E/Z*, 164 mg, 1.1 mmol) and vinyl acetate[iii] (277 µL, 3.0 mmol) in *n*-hexane (10 mL) was stirred for 10 minutes at room temperature. After addition of the corresponding lipase (immobilized) the suspension was stirred for 18 - 72 hours at room temperature. The work-up was done according to SOP17. The crude product was analyzed by NMR spectroscopy. It was not further purified.

[i] The same experiment with ethyl acetate as acyl donor led to <2 % conversion after 24 hours.
[ii] It is clearly shown that the *E*-isomer is being enriched during the reaction. This could be explained by thermodynamical effects preferring *E*-conformation.
[iii] The same experiment with ethyl acetate as acyl donor led to <2 % conversion after 24 hours.

Table 62. Biocatalytic acetylation of racemic *E/Z*-cyclohex-3-ene-1-carbaldoxime



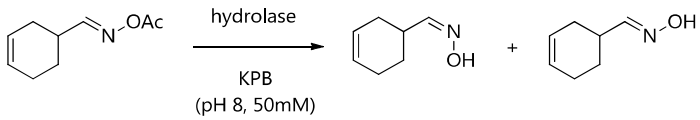
Entry	Lipase	Time [h]	Conversion[a] [%]	E/Z[b]	Nitrile[b] [%]
1	Amano PS-D	18	45.0	88:12	34.4
2	Amano PS-D	72	57.8	92:8	31.1
3	CAL-B	72	98.4	88:12	43.2

(a) Conversion of starting material (b) Determined by ^{1}H-NMR integration.

9.6 STANDARD OPERATING PROCEDURE 18 (SOP18): BIOCATALYTIC DEACETYLATION OF ALDOXIME ACETATES BY HYDROLASE-CATALYZED HYDROLYSIS

The corresponding aldoxime acetate (1.0 mmol) and a hydrolase are added to KPB (8.0, 50 mM, 10 mL) in a Titrino apparatus. The formed acetic acid is neutralized by the addition of aqueous sodium hydroxide solution (1.0M) to maintain the weak basic pH of 8.0. The reaction is stopped after the consumption of the theoretical amount of base and the biocatalyst is filtered off. The aqueous filtrate is extracted with MTBE (3x 25 mL) and the combined organic phase is washed with brine (25 mL). After drying over MgSO4 all volatile compounds are removed in vacuo (4°C). The crude product is analyzed with ^{1}H-NMR spectroscopy. The initial experiments were not further purified.

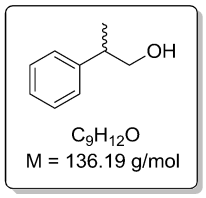
Table 63. Biocatalytic hydrolysis of *E*-cyclohex-3-ene-1-carbaldoxime acetate



Entry	Hydrolase (amount)	Time [h]	Conversion [%]	E/Z[a]
1	Cephalosporin C acetylesterase (1.5 mL)	7.5	>95.0	72:28
2	Amano PS-D (50 mg)	19.5	>95.0	82:18

(a) Determined by ^{1}H-NMR integration

9.7 *RAC*-2-PHENYLPROPANOL *VIA* $NaBH_4$ REDUCTION



A solution of DL-2-phenylpropionaldehyde (1.34 mL, 10.0 mmol) in THF (20 mL) was cooled to 0-4 °C. Sodium borohydride (378 mg, 10.0 mmol) was added in portions over five minutes. The mixture was stirred for additional two hours at 0-20 °C. The reaction was quenched by the addition of saturated sodium bicarbonate solution. The mixture was extracted with MTBE (3x 25 mL) and the combined organic phase was washed once with brine. After filtration over a short silica column and drying over $MgSO_4$ all volatile compounds were removed *in vacuo*. The product was obtained as colorless oil.

Normal-phase chiral HPLC: Chiralpak AD-H, 80:18:2 v/v SC-CO_2/*n*-hexane/ 2-propanol, 0.8 mL/min, 10 MPa back pressure, 216 nm): R_{t1} = 25.0 min (*S*), R_{t2} = 30.9 min (*R*).

Isolated yield: 1.26 g (9.25 mmol, 92.5 %).

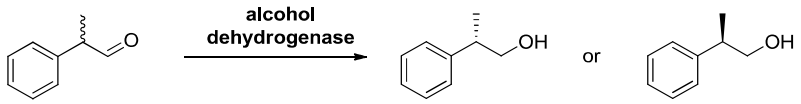
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.40 – 7.23 (m, 5H, Ar-*H*), 3.71 (d, *J* = 6.6 Hz, 2H, C*H*$_2$), 2.97 (h, *J* = 6.9 Hz, 1H, C*H*), 1.31 (d, *J* = 7.0 Hz, 3H, C*H*$_3$).

The data corresponds to literature-known values.[143]

9.8 STANDARD OPERATING PROCEDURE 19 (SOP19): ENANTIOSELECTIVE REDUCTION OF *RAC*-2-PHENYLPROPIONALDEHYDE BY ALCOHOL DEHYDROGENASES

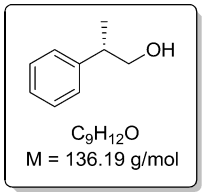
$NAD(P)^+$ (2 mol %) was dissolved in KPB (50 mM, pH 8.0, 3:1 v/v) and an adequate amount of alcohol dehydrogenase was added. DL-2-Phenylpropionaldehyde (10.0 mmol) was dissolved in 2-propanol (1:3 v/v) and added in one portion. The resulting emulsion was stirred for 24 hours at room temperature. The mixture was extracted with MTBE (3x 2:1 v/v) and the combined organic phase was washed once with brine (1:4 v/v). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo*. The crude product was analyzed by NMR spectroscopy and showed quantitative conversion in all cases. The enantiomeric excess was determined on a chiral HPLC column (Chiralpak AD-H, 80:18:2 v/v SC-CO_2/*n*-hexane/2-PrOH, 0.8 mL/min, 10 MPa back pressure, 216 nm): R_{t1} = 25.0 min (*S*), R_{t2} = 30.9 min (*R*).

Table 64. Biocatalytic reduction of DL-2-phenylpropionaldehyde by different ADH



Entry	ADH (Batch-No.)	Sub [mmol]	ADH [U]	Conv. [%]	Yield [g(%)]	ee [%(*RS*)]
1	Rsp (327)	10.0	150	>95.0	1.34 (98.4)	15.5 (*S*)
2	evo-1.1.200 (365)	10.0	200	>95.0	1.31 (96.2)	56.1 (*R*)

9.8.1 (2*S*)-PHENYLPROPANOL



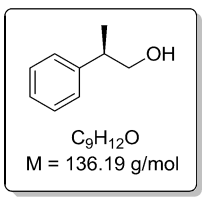
The following synthesis was conducted according to SOP16. NAD^+ (136.3 mg, 0.2 mmol, 2.0 mol-%) was dissolved in KPB (150 mL, 50 mM, pH 8.0) and an alcohol dehydrogenase from *Rhodococcus* sp. (2.0 mL, 1:1 v/v in glycerin, 75 U/mL) was added. DL-2-Phenylpropionaldehyde (1.34 mL, 10.0 mmol) was dissolved in 2-propanol (50mL) and added in one portion. The resulting emulsion was stirred for 24 hours at room temperature. The work-up was done as described in SOP16 to obtain the product as colorless oil with an enantiomeric excess of 15.5% ee (*S*).[i]

Isolated yield: 1.34 g (0.98 mmol, 98.4 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.40 – 7.23 (m, 5H, Ar-*H*), 3.71 (d, *J* = 6.6 Hz, 2H, C*H*$_2$), 2.97 (m, *J* = 6.9 Hz, 1H, C*H*), 1.31 (d, *J* = 7.0 Hz, 3H, C*H*$_3$).

The data corresponds to literature-known values.[143]

9.8.2 (2*R*)-PHENYLPROPANOL



The following synthesis was conducted according to SOP16. NAD^+ (136.3 mg, 0.2 mmol, 2.0 mol-%) was dissolved in KPB (150 mL, 50 mM, pH 8.0) and the alcohol dehydrogenase evo-1.1.200 (evocatal, 100 mg, 2.0 U/mg) was added. DL-2-Phenylpropionaldehyde (1.34 mL, 10.0 mmol) was dissolved in 2-propanol (50mL) and added in one portion. The resulting emulsion was stirred for 24 hours at room temperature. The work-up was done as described in

[i] This was most likely due to the high enzyme amount. The experiment will be reproduced with 10% of the biocatalyst amount.

SOP16 to obtain the product as colorless oil with an enantiomeric excess of 56.1% ee (*R*).[i]

Isolated yield: 1.31 g (0.96 mmol, 96.2 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.40 – 7.23 (m, 5H, Ar-*H*), 3.71 (d, *J* = 6.6 Hz, 2H, CH_2), 2.97 (m, *J* = 6.9 Hz, 1H, C*H*), 1.31 (d, *J* = 7.0 Hz, 3H, CH_3).

The data corresponds to literature-known values.[143]

9.9 STANDARD OPERATING PROCEDURE 20 (SOP20): OVEREXPRESSION AND PURIFICATION OF VARIOUS ALDOXIME DEHYDRATASES (*OXD*) IN RECOMBINANT *E. COLI*

A single colony of recombinant *E. coli HB101/pOxD-9OF* or *BL21 Star (DE3)/ pOxdS17*, respectively, was transferred from a LB agar plate into a 20 mL test tube containing 5 mL of LB medium, 100 µg/mL ampicillin and 1 mM IPTG. The solution was incubated at 37 °C and reciprocally shaken for 18 hours. The preculture was transferred into a 500-mL Erlenmeyer flask with different volumes of media containing 100 µg/mL ampicillin and incubated with shaking at 200 rpm and 37 °C. After the cells reached the exponential growth rate (OD = A^{610} = 0.5-1.0), IPTG was added to a final concentration of 1 mM[ii]. The suspension was shaken at 200 rpm and different temperatures. After centrifugation at 6,000 x g and 4 °C for 10 minutes, the supernatant was removed and the cells were washed and concentrated in 10 mM KPB containing 5 mM 2-mercaptoethanol and 1 mM dithiothreitol (DTT). The suspension was either disrupted by sonication to yield the cell-free extract of directly used for whole-cell biotransformations.

9.9.1 OVEREXPRESSION OF *OXD* FROM *BACILLUS SP.* (OXB-1) IN RECOMBINANT *E. COLI HB101/POXD-9OF*

The following procedure was conducted according to SOP17. The preculture was transferred into a 500-mL erlenmeyer flask with 245 mL LB medium[iii]

[i] This was most likely due to the high enzyme amount. The experiment will be reproduced with 10% of the biocatalyst amount.
[ii] Isopropyl β-thiogalactopyranoside (IPTG) initiates gene expression under the control of the *lac operon* or the *T7 promoter*.
[iii] The medium volume is based on literature procedures showing highest enzyme activity under these conditions.

containing 100 µg/mL ampicillin and incubated for another 5.5 hours[i] with shaking at 200 rpm and 37 °C. After the cells reached the exponential growth rate (OD = A^{610} = 0.5-1.0) IPTG was added to a final concentration of 1 mM[ii]. The suspension was shaken for additional 9 hours[iii] at 200 rpm and 30 °C[iv]. The work-up was done as described in SOP17.

Table 65. Time course of cell-growth of recombinant *E. coli HB101 / pOxd90F*

Entry	Time [h]	OD (A^{610}) [a.u.]	Spec. Act.[a] [U/L_{cult}]
1	2.0	0.04	n.d.
2	4.7	0.14	n.d.
3	5.0	0.25	n.d.
4	5.3	0.37	n.d.
5	8.5	1.30	402
6	12.5	1.70	700
7	14.0	1.82	705

(a) The specific activity was measured for *E/Z*-2-phenylpropionaldoxime as substrate

9.9.2 PURIFICATION OF ALDOXIME DEHYDRATASE (*OXD*) FROM RECOMBINANT *E.COLI HB101/POXD-9OF*

Unless stated otherwise 10 mM KPB (pH 7.0) containing 5 mM 2-mercaptoethanol was used throughout the purification. The temperature was maintained at 4 °C for the entire process. The total protein content of the solutions was determined by photometric analysis with Coomassie Brilliant Blue G-250 staining (Bradford assay).

The cells of 2 L culture (17.11 g wet weight) were washed and concentrated with 10 mM KPB and disrupted in a KUBOTA Insonator 201M. After centrifugation at 12,000 x g and 4 °C for 10 minutes the cell-free extract (1.4 g total protein content) was brought to 40 % saturation by addition of solid ammonium sulfate. The formed protein pellet was removed by centrifugation (12,000 x g, 4 °C, 10 min) and the supernatant was saturated to 70 % ammonium sulfate. The active protein pellet was separated, dissolved in 10 mM

[i] The lag phase of cell growth was previously measured in a time-dependent growth curve at 610 nm.
[ii] Isopropyl β-thiogalactopyranoside (IPTG) binds to the *lac repressor* and initiates protein expression under *lac operon* control.
[iii] The activity of a 1-mL aliquot did not increase further; hence the cell growth reached the stationary phase.
[iv] The temperature is changed according to literature procedures indicating that the *Oxd* gene is higher expressed at the given temperature.

KPB and dialyzed against the same buffer (2 x 5 L) for at least five hours. The solution was placed on a DEAE-Toyopearl column (24 mm x 150 mm, 15 mg/mL) equilibrated with 10 mM KPB. After washing the column with 10 mM KPB the enzyme was eluted with 320 mL of 100 mM KPB. The column was subsequently washed with 0.5 M sodium hydroxide solution, 2.0 M sodium chloride solution, deion. water, 20 % ethanol and was stored in 20 % ethanol for reuse. The active fractions were pooled and showed a total protein content of 244.0 mg. Ammonium sulfate was added to 30 % saturation and the solution was placed on a Butyl-Toyopearl column (25 mm x 110 mm, 15 mg/mL) equilibrated with 10 mM KPB at 30 % ammonium sulfate saturation. After washing the column with the same buffer the proteins were eluted with 10 mM KPB and a linear gradient of 30 - 0 % ammonium sulfate. The pooled active fractions showed a total protein content of 42.97 mg. The solution was dialyzed against 10 mM KPB (2 x 3 L) and concentrated to 2 mL in a centrifuge ultrafiltration unit. The concentrate was injected on a Superdex 200 column (16/600) equilibrated with 50 mM KPB containing 100 mM sodium chloride and eluted with 150 mL of the same buffer. This procedure yielded 5.69 mg of purified *Oxd* with a specific activity of 8.47 U/mg, which was in good accordance to the literature value of 8.35 U/mg.[144]

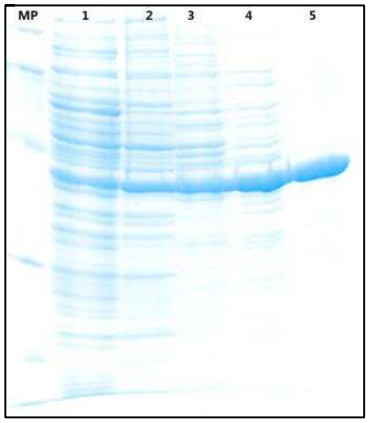


Figure 197. SDS-PAGE of *OxdB* from recombinant *E. coli HB101/ pOxD-9OF*

(MP) marker proteins (1) cell-free extract (2) ammo-ium sulfate precipitation (40-70% saturation) (3) DEAE-Toyopearl (4) Butyl-Toyopearl (5) Superdex 200

Table 66. Purification of *Oxd* from recombinant *E. coli HB101/pOxD-9OF*

Entry	Method	Total Protein (mg)	Specific activity (U/mg)	Total activity (U)	Recovery (%)	Purification (fold)
1	Cell-free extract	1357	0.21	279.2	100	1.0
2	40-70% $(NH_4)_2SO_4$	958.8	0.23	220.5	79.0	1.1
3	DEA*E*-Toyopearl	244.0	0.34	82.96	29.7	1.6
4	Butyl-Toyopearl	42.97	1.80	77.35	27.7	8.6
5	Superdex 200	5.690	8.47	48.19	17.3	40

9.9.3 OVEREXPRESSION OF OXD FROM RHODOCOCCUS GLOBERULUS (OXDRG) IN RECOMBINANT E. COLI BL21 STAR (DE3) / POXDS17[145]

An Aliquot of 50 µl of the preculture was transferred into a 500-mL Erlenmeyer flask with 375 mL TB medium[i] containing 100 µg/mL ampicillin and incubated for another 4.5 hours[ii] with shaking at 200 rpm and 37 °C. After the cells reached the exponential growth rate (OD = A^{610} = 0.5 - 1.0) IPTG was added to a final concentration of 1mM[iii]. The suspension was shaken for additional 12 hours[iv] at 200 rpm and 25 °C[v]. The work-up was done as described in SOP17. A cell-free extract was not prepared. All experiments were carried out with resting cells.

Table 67. Time course of cell-growth of recombinant *E. coli BL21 Star* (*DE3*)*/ pOxdS17*

Entry	Time [h]	OD (A^{610}) [a.u.]	Spec. Act.[a] [U/L_{cult}]
1	2.0	0.02	n.d.
2	3.0	0.06	n.d.
3	4.0	0.27	n.d.
4	4.5	0.50	n.d.
5	15.0	1.71	2590
6	16.5	1.74	2340

(a) The specific activity was measured for *E/Z*-2-phenylpropionaldoxime as substrate

9.10 STANDARD OPERATING PROCEDURE 21 (SOP21): GROWTH EXPERIMENTS WITH ADDITION OF CHIRAL SUBSTRATES

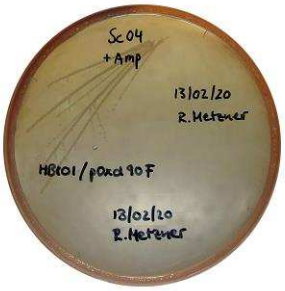


Figure 198. Agar plate containning 5mM tetra-hydrofuran-2-carbaldoxime with grown recombinant *E. coli HB101/pOxD-9OF* after 12 hours at 37 °C.

Agar plates containing 100 ug/mL ampicillin were prepared according to the previously described procedure, containing 5mM of different chiral aldoxime substrates. Resting cells of recombinant E. coli HB101 / pOxd90F were streaked over the plates and incubated for 12 hours at 37C. The growth was assessed qualitatively with the naked eye. One colony of each positive candidate was

[i] The medium volume is based on literature procedures showing highest enzyme activity under these conditions.
[ii] The lag phase of cell growth was previously measured in a time-dependent growth curve at 610 nm.
[iii] Isopropyl β-thiogalactopyranoside (IPTG) initiates protein expression under *T7 promoter* control.
[iv] The activity of a 1-mL aliquot did not increase further; hence the cell growth reached the stationary phase.
[v] The temperature is changed according to literature procedures indicating that the *Oxd* gene is higher expressed at the given temperature.

cultivated in 5 mL LB medium containing 100 ug/mL ampicillin and 1mM IPTG at 37C for 12 hours and measured for its specific activity towards *E/Z*-2-phenylpropionaldoxime.

Table 68. Growth and specific activity of *E. coli HB101/pOxD-9OF* in the presence of chiral substrates

Entry	Substrate additive (5 mM)	Growth	Spec. act. [U/L_{cult}]*
1	None	+++	572.7
2	*E/Z*-2-Phenylpropionaldoxime	-	n.d.
3	*E/Z*-3-Cyclohexene-1-carbaldoxime	-	n.d.
4	*E/Z*-5-Norbornene-2-carbaldoxime	-	n.d.
5	*E/Z*-Tetrahydrofuran-3-carbaldoxime	+++	457.5
6	*E/Z*-3-Phenylbutyraldoxime	-	n.d.

(a) Reaction temperature: 8 °C, reaction time: 10 min.

9.11 STANDARD OPERATING PROCEDURE 22 (SOP22): ASSAY PROCEDURE FOR THE DETERMINATION OF ENZYME ACTIVITY AND ENANTIOSELECTIVITY

The standard assay for the determination of *Oxd* activity was measured quantitatively for the product-related conversion of phenylacetaldoxime (PAOx) to phenylacetonitrile (PAN). 352.5 µL KPB (50 mM, pH 7.0), 125.0 µL flavin mononucleotide solution (FMN, 1 mM in DIW) and 10 µL of enzyme formulation (resting cell suspension or cell-free extract) were mixed in a 1.5 - mL microtube and incubated at 30 °C for 5 minutes. The reaction was started by the addition of 12.5 µL PAOx solution (200mM in DMSO) to a final volume of 500 µL. After 60 seconds incubation at 30 °C and shaking at 200 rpm the reaction was quenched with hydrochloric acid (100 µL, 0.1 M). The solution was further diluted with 400 µL DIW and centrifuged at 15,000 rpm and 4 °C for five minutes. 800 µL of the supernatant were transferred to another microtube and again centrifuged under the same conditions. The supernatant (750 µL) was transferred to a HPLC vial (1.5mL) and measured directly for product-related conversion. The specific activity was calculated in units per liter of culture according to the following equation:

$$\text{Specific activity} = \frac{[\text{PAN}] * \text{Vexp}}{\text{f} * \text{t} * \text{Vcat}} \quad [\text{U/L culture}]$$

[PAN]	concentration of phenylacetonitrile in [mmol/L]
Vexp	experiment volume in [L]
Vcat	volume of enzyme solution in [L]
f	dilution factor of concentrated enzyme solution
t	reaction time in [min]

The specific activity for chiral substrates was measured in the same manner. In case of very low activity, specifically *E/Z*-3-cyclohexene-1-carbaldoxime and *E/Z*-tetrahydrofuran-3-carbaldoxime, the assay was changed to 100 µL enzyme solution and 262.5 µL KPB (50 mM, pH 7.0). For the determination of an enantiomeric excess of 2-phenylpropionitrile, 5-norbornene-2-carbonitrile and 3-phenylbutyronitrile, the aqueous HPLC sample was further processed as follows. The solution was transferred into a 1.5-mL microtube and 750 µL MTBE was added. The extraction was done by using a vortexer at full speed for 15 seconds. After centrifugation at 15,000 rpm and 4 °C for 30 seconds, the organic phase (500 µL) was transferred to a 1.2-mL HPLC vial and diluted with MTBE (500 µL). The samples (4.0 µL) were measured at 210 nm. Reference spectra can be seen in chapter 3.X.X. In case of products with low absorbance, specifically 3-cyclohexene-1-carbonitrile and tetrahydrofuran-3-carbonitrile, the enantiomeric excess was determined by chiral GC and FID analysis. The previously measured HPLC sample was transferred to a microtube and extracted with MTBE (300 µL) on a vortexer. After centrifugation at 15,000 rpm and 4 °C for 5 minutes, residual DMSO and H_2O was removed by transferring 200 µL of the supernatant organic phase to another microtube and washing with an equal volume (200 µL) of brine by vortexing. After a second centrifugation at 15,000 rpm and 4 °C for 5 minutes, 100 µL of the organic phase was transferred to a GC vial (150 µL) and analyzed by FID. The parameters are given in the corresponding experiments.

9.12 Standard operating procedure 23 (SOP23): Preparation of acetone-dried cells of various *Oxd* in recombinant *E. coli* and comparison of formulations

The following procedure was based on literature procedures by ASANO *et al.*[146,147] The cultivation was conducted as described in chapters 9.8.1 and 9.8.3,

respectively. The washed cell suspension was poured into cold acetone (10:1 v/v, -20 °C) and immediately filtered under membrane vacuum. After washing with the same volume of cold acetone (-20 °C) the cells were dried in membrane vacuum and immediately stored at -20 °C until use. The cells showed a stable activity even after four months in storage, while cell-free extracts and resting cell suspensions significantly lost their activities.

Table 69. Stability of enzyme activity according to enzyme formulation

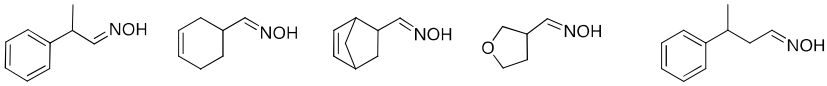
Entry	*Oxd* from	Formulation	Storage time [d]	Spec. Activity[a] [U/L_{cult} (%)]
1	*HB101 / pOxD-9OF*	resting cells	1	5625 (100)
2	*HB101 / pOxD-9OF*	resting cells	20	3566 (63.4)
3	*HB101 / pOxD-9OF*	acetone-dried cells	1	0.42[b] (100)
4	*HB101 / pOxD-9OF*	acetone-dried cells	20	0.42[b] (99.5)
5	*HB101 / pOxD-9OF*	acetone-dried cells	60	0.41[b] (96.2)
6	*HB101 / pOxD-9OF*	acetone-dried cells	119	0.38 (89.6)
7	*HB101 / pOxD-9OF*	cell-free extract	1	2016 (100)
8	*HB101 / pOxD-9OF*	cell-free extract	10	1092 (54.2)
9	*HB101 / pOxD-9OF*	cell-free extract	20	40 (1.98)
10	*BL21 Star* (*DE3*) */ pOxdS17*	acetone-dried cells	1	0.77[b] (100)
11	*BL21 Star* (*DE3*) */ pOxdS17*	acetone-dried cells	20	0.01[b] (1.3)

(a) Assay conditions: 500 µl-scale, 30 °C, 60 seconds incubation (b) Units per mg of dry cell weight.

9.13 STANDARD OPERATING PROCEDURE 24 (SOP24): ENANTIOSELECTIVE BIOTRANSFORMATION OF RACEMIC ALDOXIMES INTO ENANTIOMERICALLY ENRICHED NITRILES

A standard solution of aldoxime (200 mM in DMSO) was added to the aqueous reaction mixture consisting of flavin mononucleotide solution (FMN, 25 % v/v, 1 mM in DIW), an *Oxd* formulation and KPB (pH 7.0, 50 mM) to a final volume of 5.0 – 500 mL (5 mM substrate conc.). The mixture was stirred at 8 °C for 24

hours. After HPLC analysis for quantitative conversion the solution was centrifuged (12,000 rpm, 4 °C, 5 min) to remove the biocatalyst. The supernatant was extracted with ethyl acetate (3 x 1:1 v/v) and the organic phase was extracted twice with brine (2 x 1:4 v/v) to remove FMN and DMSO residues. After drying over $MgSO_4$ the solvent was removed *in vacuo*. The crude product was purified *via* column chromatography on silica (5:1 v/v ethyl acetate in *n*-hexane) to separate the enantiomerically enriched product from remaining aldoxime. The products were analyzed by NMR, HPLC and polarimetry, respectively.



A **B** **C** **D** **E**

9.13.1 (2*S*)-PHENYLPROPIONITRILE FROM AN *E/Z*-MIXTURE OF 4:1

The following procedure was conducted according to SOP21. A standard solution of *E/Z*-2-phenylpropionaldoxime (E/Z ratio 4:1, 978 µL, 1.02 M in DMSO) was added to the aqueous reaction mixture consisting of 50 mL flavin mononucleotide solution (FMN, 1 mM in DIW), 4.0 mL of resting cell suspension (*HB101/pOxD-9OF* with a specific activity of 7 U/mL, KPB pH 7.0, 50 mM) and KPB (pH 8.0, 50 mM) to a final volume of 200 mL (5 mM substrate concentration). The mixture was stirred at 8 °C for 24 hours. The work-up was done as described in SOP21. After purification via column chromatography the product and remaining aldoxime were obtained in spectroscopically pure form. The enantiomeric excess was determined by chiral normal-phase HPLC (Daicel Chiralcel OJ-H, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 13.8 min (*R*), R_{t2}= 14.3 min (*S*), R_{t3} = 20.4 min (*Z*-2*S*), R_{t4} = 26.7 min (*E*-2*S*), R_{t5} = 30.0 min (*Z*-2*R*), R_{t6} = 31.5 min (*E*-2*R*)).

Isolated yield ((2*S*)-phenylpropionitrile): 68.4 mg (0.52 mmol, 52.1 %).

Isolated yield (*E*-(2*R*)-phenylpropionaldehyde): 61.6 mg, (0.41 mmol, 41.2 %).

(2*S*)-Phenylpropionitrile:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.38 – 7.28 (m, 5H, Ar-*H*), 3.87 (q, *J* = 7.2 Hz, 1H, C*H*-CN), 1.60 (d, *J* = 7.3 Hz, 3H, CH_3).

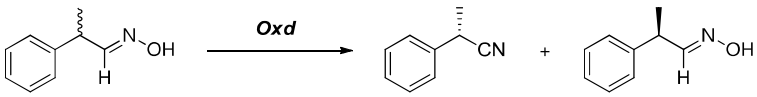
^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 137.17, 129.17, 128.06, 126.74, 121.66, 31.22, 21.46.

E-(2*R*)-Phenylpropionaldehyde oxime:

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.52 (d, *J* = 6.1 Hz, 1H, *H*C=NOH), 7.38 – 7.19 (m, 5H, Ar-*H*), 3.67 (m, *J* = 6.8 Hz, 1H, C*H*-CH=NOH), 1.46 (d, *J* = 7.0 Hz, 3H, C*H*$_3$).

^{13}C NMR (100 MHz, $CDCl_3$): δ [ppm] =155.09, 142.11, 128.74, 127.39, 126.95, 40.37, 18.75.

Table 70. Selectivity of *OxdB* for *E/Z*-2-phenylpropionaldoxime (4:1)



Entry	Product	Configuration	ee [%]	Isol. yield [mg(%)]
1	2-phenylpropionitrile	(*S*)[a]	65.2	68.4 (52.1)
2	*E*-2-phenylpropionaldoxime	(*R*)[b]	>99.8	61.6 (41.2)

(a) identified by its specific optical rotation of $[a]^{D}_{24}$ = - 4.4 (c 2.25, $CDCl_3$) (Lit. (*R*): $[a]^{D}_{20}$ = + 9.5 (c 2.4, $CHCl_3$) (b) identified by product configuration, specific optical rotation $[a]^{D}_{23}$ = - 7.57 (c 1.32, $CDCl_3$).

9.14 STANDARD OPERATING PROCEDURE 25 (SOP25): TIME COURSES FOR THE BIOTRANSFORMATION OF MIXTURES OR ISOMERICALLY ENRICHED *E*- AND *Z*-ALDOXIMES

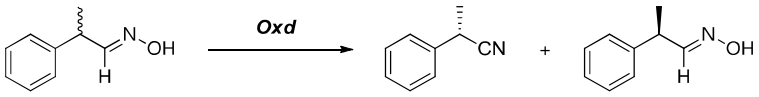
The corresponding aldoxime (0.025 mmol, 200 mM in DMSO, 0.125 mL) was added to the aqueous reaction mixture consisting of flavin mononucleotide (FMN, 1 M in H_2O, 1.25 mL), *OxdB* (resting cell suspension of recombinant *E.coli HB101/pOxD-9OF*, 7 U/mL, 0.10 mL) and KPB (50 mM, pH 7.0, 3.525 mL) at room temperature. The reaction was reciprocally shaken at 180 min^{-1} and 8.0 °C. To measure the time course of the reaction, aliquots of 0.50 mL were transferred to a 1.5-mL-microtube and diluted with dist. H_2O (0.50 mL). After centrifugation for five minutes at 15,000 rpm and 4 °C the supernatant (0.80 mL) was transferred to another microtube and centrifuged again (15,000 rpm, 4 °C, 5 min). The resulting supernatant (0.75 mL) was transferred to a HPLC vial and directly measured (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-1), R_{t2} = 12.4 min (*Z*-1), R_{t3} = 18.1 min (2)) for conversion. Subsequently, the sample was again transferred to a 1.5-mL-microtube and overlaid with *tert*-butyl methylether (MTBE, 0.50 mL). After mixing on a vortexer the phases were separated by

centrifugation (15,000 rpm, 4 °C, 5 min) and the organic phase (0.40 mL) was transferred to another microtube. Brine was added (0.40 mL) and the mixture was again mixed by vortexer and centrifuged. The organic phase (0.30 mL) was transferred to a 1.0-mL-HPLC vial and diluted with additional MTBE (0.45 mL). The sample was measured on normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-2), R_{t2} = 14.4 min ((*S*)-2), R_{t3} = 20.3 min (*Z*-(*S*)-1), R_{t4} = 26.7 min (*E*-(*S*)-1), R_{t5} = 30.0 min (*Z*-(*R*)-1), R_{t6} = 31.5 min (*E*-(*R*)-1)) for the enantiomeric excess of both product and remaining substrate.

9.14.1 (2*S*)-PHENYLPROPIONITRILE FROM RACEMIC *E*-SUBSTRATE

The synthesis was conducted according to SOP22 with *E-rac*-2-phenylpropion-aldoxime (3.73 mg, 0.025 mmol) at pH 7.0 and pH 8.0, respectively.

Table 71. Time course of *Oxd* dehydration for *E-rac*-2-phenylpropionaldoxime



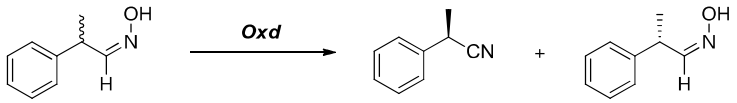
Entry	pH	Time [min]	ie[a] [%]	[E] in mM	[Z] in mM	[CN] in mM	ee [%]
1	7.0	9	98.9	3.828	-	1.171	95.9 (*S*)
2	7.0	32	98.9	2.748	-	2.183	98.0 (*S*)
3	7.0	62	98.9	2.332	-	2.667	97.9 (*S*)
4	7.0	402	98.9	2.177	-	2.823	97.5 (*S*)
5	7.0	1382	98.9	2.192	-	2.809	96.2 (*S*)
6	8.0	38	98.9 (E)	3.421	-	1.579	96.8 (*S*)
7	8.0	226	98.9 (E)	2.274	-	2.728	97.9 (*S*)
8	8.0	1361	98.9 (E)	2.181	-	2.818	96.1 (*S*)

(a) Isomeric excess

9.14.2 (2*R*)-PHENYLPROPIONITRILE FROM RACEMIC *Z*-SUBSTRATE

The synthesis was conducted according to SOP22 with *E-rac*-2-phenylpropion-aldoxime (6.15 mg, 0.025 mmol) at pH 7.0 and pH 8.0, respectively.

Table 72. Time courses of aldoxime dehydration for *Z*-2-phenylpropionaldoxime

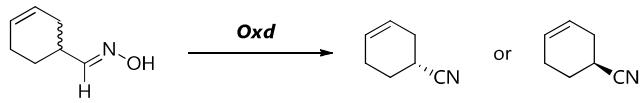


Entry	pH	Time	Ie [%]	[E] in mM	[Z] in mM	[CN] in mM	ee [%]
1	7.0	7	90.2	0.294	3.225	1.483	86.5 (*R*)
2	7.0	30	90.2	0.236	2.475	2.288	82.8 (*R*)
3	7.0	60	90.2	0.21	2.047	2.743	74.1 (*R*)
4	7.0	400	90.2	0.086	0.882	4.034	18.7 (*R*)
5	7.0	1380	90.2	0.035	-	4.966	-6.0 (*R*)
6	8.0	37	90.2 (Z)	0.264	4.055	0.682	97.5 (*R*)
7	8.0	225	90.2 (Z)	0.215	2.168	2.615	85.9 (*R*)
8	8.0	1360	90.2 (Z)	0.089	1.134	3.777	36.4 (*R*)

9.14.3 (*RS*)-3-CYCLOHEXENE-1-CARBONITRILE

The following procedure was conducted according to SOP22 with *E/Z*-3-cyclohexene-1-carbaldoxime (3.13 mg, 0.025 mmol) in DMSO (125 µL).

Table 73. Time courses of aldoxime dehydration of *E/Z*-3-cyclohexene-1-carb-aldoxime



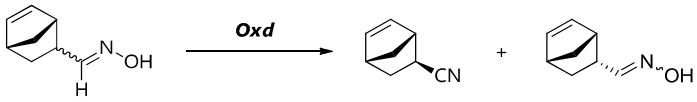
Entry	Substrate	Time [min]	ie[a] [%]	Conv. [%]	ee[b] [%]
1	*E/Z*-B	30	37.3 (E)	3.3	96.6
2	*E/Z*-B	60	37.3 (E)	13.6	83.1
3	*E/Z*-B	120	37.3 (E)	69.7	46.9
4	*E/Z*-B	240	37.3 (E)	75.9	17.2

(a) Isomeric excess (b) Determined by chiral HPLC (Daicel Chiralpak AD-H, 5% v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 6.4 min, R_{t2} = 7.5 min).

9.14.4 (*1R,2RS,3R*)-5-NORBORNENE-2-CARBONITRILE

The following procedure was conducted according to SOP22 with *E/Z*-5-norbornene-2-carbaldoxime (3.43 mg, 0.025 mmol) in DMSO (125 µL).

Table 74. Time courses of aldoxime dehydration of *E/Z*-5-norbornene-2-carb-aldoxime



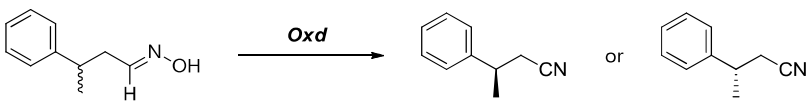
Entry	Substrate	Time [min]	ie[a] [%]	de[b] [%]	rel. Conv. (*R*) [%]	rel. Conv. (*S*) [%]	ee[c] [%]
1	*E/Z*-C	30	19.8 (E)	41.5 (*R*)	23.6	12.9	29.3 (*R*)
2	*E/Z*-C	60	19.8 (E)	41.5 (*R*)	46.0	28.3	23.8 (*R*)
3	*E/Z*-C	120	19.8 (E)	41.5 (*R*)	58.9	31.7	30.0 (*R*)
4	*E/Z*-C	240	19.8 (E)	41.5 (*R*)	64.9	35.1	29.8 (*R*)

(a) Isomeric excess (b) Diastereomeric excess was determined by ^{1}H-NMR integration (c) Determined by chiral HPLC (Daicel Chiralpak, 5 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 7.8 min (*S*), R_{t2}= 8.6 min (*R*)).

9.14.5 (*RS*)-3-PHENYLBUTYRONITRILE

The following procedure was conducted according to SOP22 with *E/Z*-3-phenyl-butyraldehyde oxime (4.08 mg, 0.025 mmol) in DMSO (125 µL).

Table 75. Time course of aldoxime dehydration of *E/Z*-3-phenylbutyraldoxime



Entry	Substrate	Time [min]	ie[a] [%]	Conv. [%]	ee[b] [%]
1	*E/Z*-E	30	0	29.0	60.6 (*S*)
2	*E/Z*-E	60	0	62.1	29.9 (*S*)
3	*E/Z*-E	120	0	97.3	2.3 (*S*)

4	*E/Z*-E	240	0	>99.5	0.3 (*S*)

(a) Isomeric excess (b) Determined by chiral HPLC (Daicel Chiralcel OJ-H, 2 % v/v 2-propanol in *n*-hexane, 0.7 mL/min, 210 nm, 20 °C, R_{t1} = 23.3 min (*major*), R_{t2}= 24.6 min (*minor*)).

9.15 STANDARD OPERATING PROCEDURE 26 (SOP26): SCALE-UP OF SUBSTRATE CONCENTRATION FOR THE BIOTRANSFORMATION OF *E/Z*-2 PHENYLPROPIONALDOXIME

A standard solution of *E/Z*-2-phenylpropionaldoxime in DMSO was added to the aqueous reaction mixture consisting of flavin mononucleotide (FMN, 1 M in H_2O, 1.25 mL), *OxdB* (resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*, 7 U/mL, 0.10 mL) and KPB (50 mM, pH 7.0, 3.525 mL) at room temperature. The reaction was reciprocally shaken at 180 min^{-1} and different temperatures. Aliquots of 0.50 mL were transferred to a 1.5-mL-microtube and diluted with dist. H_2O (0.50 mL). After centrifugation for five minutes at 15,000 rpm and 4 °C the supernatant (0.80 mL) was transferred to another microtube and centrifuged again (15,000 rpm, 4 °C, 5 min). The resulting supernatant (0.75 mL) was transferred to a HPLC vial and directly measured (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-1), R_{t2} = 12.4 min (*Z*-1), R_{t3} = 18.1 min (2)) for conversion.

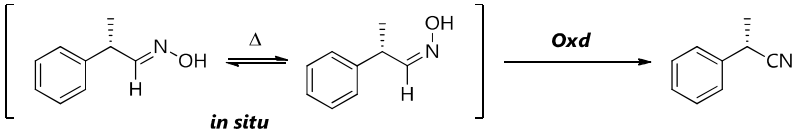
Table 76. Scale up of substrate concentrations for the biotransformation of *E/Z*-2-phenylpropionaldoxime

Entry	Substrate	Conc. [mM]	Temp. [C]	Time [min]	Spec. act. [U/$L_{culture}$]	Conv. [%]
1	*E/Z*-A	5	30	1	1240	n.d.
2	*E/Z*-A	10	30	1	1850	n.d.
3	*E/Z*-A	10	30	30	886.3	n.d.
4	*E/Z*-A	25	30	30	1205.7	3.4
5	*E/Z*-A	50	30	30	404.0	2.4
6	*E/Z*-A	100	30	30	31.3	0.1
7	*E/Z*-A	10	8	30	115.0	56.6
8	*E/Z*-A	20	8	30	174.0	5.2

9.16 (2*R*)-PHENYLPROPIONITRILE FROM INERT *E*-(2*R*)-PHENYLPROPIONALDEHYDE OXIME

A solution of *E*-(2*R*)-phenylpropionaldoxime (3.73 mg, 0.025 mmol) in DMSO (125 µL) was dissolved in a reaction mixture containing FMN (1.25 mL, 1 mM in dist. H_2O), *OxdB* (resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*, 100 µL, 0.7 U) and KPB (50 mM, pH 7.0) to a final volume of 5.0 mL at 5 mM substrate concentration. The resulting emulsion was mixed for three hours with reciprocal shaking at 180 min^{-1} and 30 °C. The progress of the reaction was monitored according to the procedures described in SOP23.

Table 77. Temperature-induced conversion of inert *E*-(2*R*)-phenylpropionaldoxime by *in situ*-isomerization to active *Z*-(2*R*)-phenylpropionaldoxime



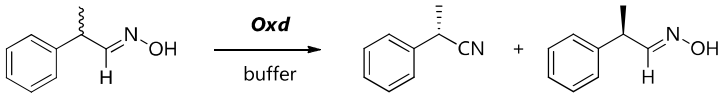
Entry	Substrate	Time	ie	[E]	[Z]	[CN]	ee
1	*E*-(2*R*)-A	60	>99.4 (E)	4.740	0.260	-	-
2	*E*-(2*R*)-A	173	>99.4 (E)	3.762	0.181	1.057	99.2 (*R*)

9.17 INVESTIGATION OF RACEMIZATION AND ISOMERIZATION IN AQUEOUS MEDIA

9.17.1 STANDARD OPERATING PROCEDURE 27 (SOP27): EFFECTS OF PH CHANGE IN AQUEOUS MEDIUM

E-2-Phenylpropionaldoxime (125 µL, 200 mM in DMSO) was dissolved in different buffer systems at various pH values and mixed with FMN (1250 µL, 1 mM in DIW). After addition of *OxdB* (resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*, 100 µL, 0.7 U) to a final volume of 5 mL at 5 mM substrate concentration the emulsion was mixed with reciprocal shaking (160 min^{-1}, 8 °C) for 240 minutes. The progress of the reaction was monitored according to the procedures described in SOP22.

Table 78. Biotransformation of *E*-2-phenylpropionaldoxime at different pH values



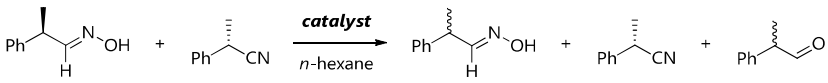
Entry	Buffer[a] (50 mM)	pH	Conversion [%]	ee [%]
1	NaOAc	5.0	<0.5[b]	n.d.
2	KPB	7.0	27.7[c]	98.1[d]
3	KPB	8.0	37.2[c]	97.9[d]
4	Ethanolamine	9.0	8.9	n.d.
5	Ethanolamine	10.0	7.3[b]	n.d.

(a) Sodium acetate (NaOAc), potassium phosphate buffer (KPB) (b) significant hydrolysis of the aldoxime (c) only product-related conversion, no hydrolysis observed (d) normal-phase HPLC showed no change in the content of *E*-(2*R*)-phenylpropionaldoxime even after 24 hours

9.17.2 STANDARD OPERATING PROCEDURE 28 (SOP28): RACEMIZATION AND ISOMERIZATION WITH ACID / BASE CATALYSTS IN *N*-HEXANE

The corresponding catalyst (0.0125 mmol, 50 mol-%), *E*-(2*R*)-phenyl-propionaldoxime (>99.8 % ee, 3.73 mg, 0.025 mmol, 25 mM) and (2*S*)-phenylpropionitrile (77.6 % ee, 3.28 mg, 0.025 mmol, 25 mM) were dissolved in *n*-hexane (1 mL) and stirred at 8° C for three hours. An aliquot of 100 μL was filtered over a small silica column (*tert*-butyl methylether, MTBE) and analyzed on a normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 5 % 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-**2**), R_{t2} = 14.4 min ((*S*)-**2**), R_{t3} = 20.3 min (*Z*-(*S*)-**1**), R_{t4} = 26.7 min (*E*-(*S*)-**1**), R_{t5} = 30.0 min (*Z*-(*R*)-**1**), R_{t6} = 31.5 min (*E*-(*R*)-**1**)) for racemization or isomerization effects.

Table 79. Racemization and Isomerization under acidic / basic catalysis in *n*-hexane



Entry	Catalyst[a]	*E/Z* (R-NOH)	ee (R-NOH) [%]	ee (R-CN) [%]	R-CHO [%]
1	none	99:1	>99.8	77.6	0.0
2	BzOH	71:29	>99.8	77.6	63.0
3	DBU	99:1	>99.8	63.6	0.0
4	DABCO	99:1	>99.8	77.6	0.0
5	DMAP	99:1	>99.8	77.6	0.0

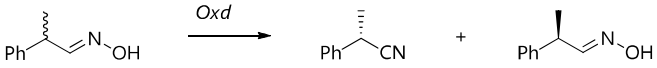
(a) Benzoic acid (BzOH), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), 1,4-diazabicyclo[2.2.2]octane (DABCO), 4-(dimethylamino)-pyridine (DMAP)

9.18 IDENTIFICATION OF PREFERABLE REACTION CONDITIONS FOR *OXD* BIOTRANSFORMATIONS IN A BINARY MEDIUM

9.18.1 STANDARD OPERATING PROCEDURE 29 (SOP29): EFFECT OF DMSO REMOVAL ON *OXDB* BIOTRANSFORMATIONS

To prevent the binary reaction media from mixing, DMSO was removed from the standard KPB medium. *E/Z*-2-phenylpropionaldoxime (37.3 mg, 0.25 mmol) was either directly emulsified in KPB (pH 7.0, 50 mM) or previously dissolved in DMSO (1.25 mL). After the addition of *OxdB* (1 mL resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*) and FMN (12.5 mL, 1 mM in DIW), KPB (50 mM, pH 7.0) was added to a final volume of 50 mL and mixed with reciprocal stirring (160 min^{-1}, 8 °C) for 22 hours. Aliquots of 500 µL were taken at different reaction times to investigate a changing time course of the biotransformations. The samples were analyzed as described in SOP22.

Table 80. Biotransformation of *E*/*Z*-2-phenylpropionaldoxime in the presence or absence of DMSO as cosolvent



Entry	Cosolvent	Time [min]	2PPN [mM]	Spec. Activity [U/L_{cult}][b]
1	DMSO	18	0.323	897.2
2	none	19	0.377	992.1
3	DMSO	69	0.511	-[a]
4	none	70	0.677	-[a]
5	DMSO	170	0.886	-[a]
6	none	171	1.086	-[a]
7	DMSO	285	1.230	-[a]
8	none	286	1.387	-[a]
9	DMSO	1296	2.464	-[a]
10	none	1297	2.030	-[a]

(a) The rapid decrease in activity due to partial product inhibition makes these values obsolete

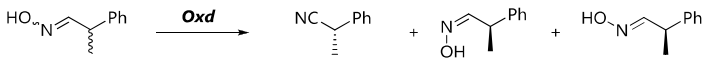
(b) Units per liter of cell culture

9.18.2 STANDARD OPERATING PROCEDURE 30 (SOP30): COMPATIBILITY OF *OXDB* WITH BINARY REACTION MEDIA

The stability of *OxdB* in binary mixtures was tested according to the following procedure: *E*-2-phenylpropionaldoxime (98 µL, 255 mM in DMSO) was dissolved in KPB (2.55 mL, pH 7.0, 50 mM) and mixed with FMN (1.250 mL, 1mM in DIW) and organic solvent (1.00 mL). After addition of *OxdB* (resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*, 100 µL, 0.7 U) to a final volume of 5 mL at 5 mM substrate concentration the emulsion was mixed with reciprocal shaking (160 min^{-1}, 8 °C) for 24 hours. After centrifugation at 15,000 rpm and 4 °C for 10 minutes, an aliquot of 0.50 mL of the aqueous phase was transferred to a 1.5-mL-microtube and diluted with dist. H_2O (0.50 mL). The solution was centrifuged (15,000 rpm, 4 °C, 5 min) again and the supernatant (0.80 mL) was transferred to another microtube before being centrifuged a third time (15,000 rpm, 4 °C, 5 min). The resulting supernatant (0.75 mL) was transferred to a HPLC vial and directly measured on a reversed-phase column (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-1), R_{t2} = 12.4 min (*Z*-1), R_{t3} = 18.1 min (2)) for conversion. Subsequently, the sample was again transferred to a 1.5-mL-

microtube and overlaid with *tert*-butyl methylether (MTBE, 0.50 mL). After mixing on a vortexer, the phases were separated by centrifugation (15,000 rpm, 4 °C, 5 min) and the organic phase (0.40 mL) was transferred to another microtube. Brine was added (0.40 mL) and the mixture was again mixed by vortexing and centrifuged. The organic phase (0.30 mL) was transferred to a 1.0-mL-HPLC vial and diluted with additional MTBE (0.45 mL). The sample was measured on normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 5% 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-2), R_{t2} = 14.4 min ((*S*)-2), R_{t3} = 20.3 min (*Z*-(*S*)-1), R_{t4} = 26.7 min (*E*-(*S*)-1), R_{t5} = 30.0 min (*Z*-(*R*)-1), R_{t6} = 31.5 min (*E*-(*R*)-1)) for the enantiomeric excess of both product and remaining substrate.

Table 81. Biotransformation of *E/Z*-2-phenylpropionaldoxime in binary reaction media



E/Z-**1** **2** *Z*-**1** *E*-**1**

Entry	Solvent	Homogenity	Conv. [%]	ee (2) [%]	ee (*Z*-**1**) [%]	ee (*E*-**1**) [%]
1	MTBE	biphasic	33.8	29.3 (*S*)	85.8 (*S*)	27.7 (*R*)
2	EtOAc	biphasic	20.0	17.2 (*R*)	79.3 (*S*)	6.8 (*R*)
3	*n*-Hexane	biphasic	62.5	60.5 (*S*)	-	>99.8 (*R*)
4	DCM[a]	biphasic	21.6	50.4 (*R*)	97.0 (*S*)	6.70 (*R*)
5	*n*-BuOAc	biphasic	20.4	44.8 (*S*)	30.6 (*R*)	29.4 (*R*)
6	$CHCl_3$[a]	biphasic	7.40	56.3 (*R*)	5.50 (*S*)	0.50 (*R*)
7	THF	heterogenous	22.8	13.0 (*S*)	38.3 (*S*)	13.7 (*R*)
8	2-PrOH	homogenous	33.3	39.6 (*S*)	0.20 (*R*)	37.2 (*R*)

(a) Cell denaturation was observed

9.18.3 STANDARD OPERATING PROCEDURE 31 (SOP31): SOLUBILITY OF *E/Z*-2-PHENYLPROPIONALDOXIME AND 2-PHENYLPROPIONITRILE IN AQUEOUS BUFFER

E/Z-rac-2-phenylpropionaldoxime (2-PPAOx, 200 mg) or *rac*-2-phenylpropionitrile (2-PPN, 200 mg), respectively, were transferred to 1.5-mL microtubes and overlaid with 500 µL of KPB (50 mM, pH 8.0). The phases were mixed on a vortexer at full speed for 10 minutes and rested for additional two

hours. After centrifugation at 15,000 rpm and 4 °C for 10 minutes an aliquot of 100 µL KPB was analyzed by reversed-phase HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-2-PPAOx), R_{t2} = 12.4 min (*Z*-2-PPAOx), R_{t3} = 18.1 min (2-PPN)).

Table 82. Solubility of *E/Z*-2-phenylpropionaldoxime and 2-phenylpropionitrile in aqueous buffer (pH 8.0)

Entry	Compound	Concentration [mM][a]	Solubility [g/L]
1	2-PPAOx	20.79 ± 0.13	
2	2-PPAOx	20.79 ± 0.32	3.10 ± 0.03
3	2-PPAOx	20.78 ± 0.20	
4	2-PPN	13.69 ± 1.84	1.80 ± 0.24

(a) interpolated from a standard curve (2.50 – 50.00 mM), each value is the average of three injections

9.18.4 Standard operating procedure 32 (SOP32): Distribution coefficients for *E/Z*-2-phenylpropionaldoxime in binary reaction media

The distribution coefficients were determined by quantitative measurement of remaining *E/Z*-2-phenylpropionaldoxime in the aqueous phase. *E/Z-rac*-2-phenyl-propionaldoxime (3.73 mg, 0.025 *mmol*) was overlaid with 1.00 mL of KPB (50 mM, pH 7.0) and the desired volume of organic solvent. The phases were mixed on a vortexer at full speed for 10 minutes and rested for additional two hours. After centrifugation at 15,000 rpm and 4 °C for 10 minutes an aliquot of 500 µL of the aqueous phase was analyzed by reversed-phase HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-2-PPAOx), R_{t2} = 12.4 min (*Z*-2-PPAOx)) for remaining aldoxime concentration.

Table 83. Distribution coefficients for various organic solvents at 5 mM substrate concentration

Entry	Solvent	Vol. ratio [V_{org}/V_{aq}]	2-PPAOx (aq.) [mM]	log D (pH 7.0)
1	MTBE	1:5	< 0.05	n.d.
2	MTBE	2:5	< 0.05	
3	DCM	1:5	< 0.05	n.d.
4	DCM	2:5	< 0.05	
5	*n*-hexane	1:5	0.50	
6	*n*-hexane	2:5	0.74	
7	*n*-hexane	3:5	1.18	0.34 ±0.07
8	*n*-hexane	4:5	1.26	
9	*n*-hexane	5:5	1.33	
10	EtOAc	1:5	< 0.05	n.d.
11	EtOAc	2:5	< 0.05	

9.18.5 STANDARD OPERATING PROCEDURE 33 (SOP33): ACTIVE AQUEOUS CONCENTRATIONS FOR *E/Z*-2-PHENYLPROPIONALDOXIME IN A BINARY REACTION MIXTURE OF KPB AND *N*-HEXANE

The active concentrations were determined by quantitative measurement of remaining *E/Z*-2-phenylpropionaldoxime in the aqueous phase. An appropriate amount of *E/Z-rac*-2-phenylpropionaldoxime was dissolved in *n*-hexane (2.50 mL) to set the desired concentration (10 – 50 mM). KPB (50 mM, pH 7.0, 2.50 mL) was added and the phases were mixed on a vortex at full speed for 10 minutes and rested for additional two hours. After centrifugation at 15,000 rpm and 4 °C for 10 minutes, an aliquot of 500 µL of the aqueous phase was analyzed by reversed-phase HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-2-PPAOx), R_{t2} = 12.4 min (*Z*-2-PPAOx)) for the remaining aldoxime concentration.

Table 84. Distribution of *E*/*Z*-2-phenylpropionaldoxime in *n*-hexane/KPB mixtures (1:1 v/v) at various starting concentrations

Entry	2-PPAOx (org.) [mM][a]	2-PPAOx (aq.) [mM][b]
1	10.0	1.45
2	20.0	2.49
3	30.0	3.23
4	40.0	3.81
5	50.0	4.13

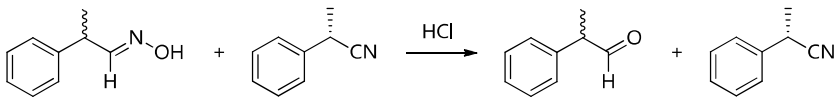
(a) Determined by weigh-in (b) Determined via reversed-phase HPLC

9.19 PURIFICATION OF CHIRAL NITRILES FROM AQUEOUS PRODUCT MIXTURES

9.19.1 STANDARD OPERATING PROCEDURE 34 (SOP34): HYDROLYSIS OF REMAINING ALDOXIME IMPURITIES FROM CRUDE MIXTURES

A solution of *E/Z*-2-phenylpropionaldoxime (2.50 mM) and *rac*-2-phenylpropio-nitrile (2.50 mM) in KPB (50 mM, pH 7.0, 10 mL), mimicking a 50% conversion, was stirred at 20 °C or 80 °C and subsequently acidified by addition of aqueous hydrochloric acid. Aliquots of 500 µL were taken after 60 minutes and analyzed by HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-2-PPAOx), R_{t2} = 12.4 min (*Z*-2-PPAOx), R_{t3} = 18.1 min (2-PPN)).

Table 85. Acid-catalyzed hydrolysis of remaining aldoxime in non-quantitative conversions



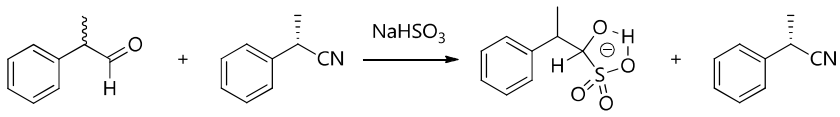
Entry	2PPAOx [mM]	2PPN [mM]	HCl [mM]	Temp. [°C]	Conv.[a] [%]
1	2.49	2.47	30	20	2.0
2	1.51	2.49	90	20	39.6
3	0.89	2.49	200	20	64.4
4	2.39	2.40[b]	30	80	4.4
5	1.10	2.39[b]	90	80	56.0
6	0.64	2.29[b]	200	80	74.4

(a) The conversion was calculated from the decrease of educt since the hydrolysis product was distributed between the free aldehyde and its hydrate (b) The whereabouts of decreasing nitrile concentration remain unclear.

9.19.2 STANDARD OPERATING PROCEDURE 35 (SOP35): EXTRACTIVE SEPARATION OF ALDEHYDE IMPURITIES AS BISULFITE ADDUCTS

A mixture of 2-phenylpropionaldehyde (2PPA, 2.5 mM) and 2-phenylpropionitrile (2PPN, 2.5 mM) was dissolved in KPB (10 mL, 50 mM, pH 7.0, containing 2.5 vol-% DMSO) and stirred at room temperature. Solid sodium bisulfite was added to the mixtures to obtain different bisulfite concentrations. The solutions were stirred for 30 minutes before aliquots of 500 µL were overlaid with 500 µL MTBE and extracted using a vortex mixer at full speed for 20 seconds. After centrifugation at 15,000 rpm and 4 °C for 5 minutes, 300 µL of the organic phase were transferred to an HPLC vial and diluted with 700 µL MTBE. The solution was filtrated over a short silica column to remove insoluble contaminants and analyzed by normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 5% 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-2), R_{t2} = 14.4 min ((*S*)-2), R_{t3} = 20.3 min (*Z*-(*S*)-1), R_{t4} = 26.7 min (*E*-(*S*)-1), R_{t5} = 30.0 min (*Z*-(*R*)-1), R_{t6} = 31.5 min (*E*-(*R*)-1)).

Table 86. Extractive separation of remaining aldehyde from product mixtures



Entry	HSO_3^- [mM]	2PPN [rel. %]	2PPA [rel. %]
1	0.00	100	100
2	5.04	98.9	82.7
3	54.7	99.1	21.6
4	81.0	99.5	5.80
5	99.9	98.1	1.10

9.20 RECYCLING OF WHOLE-CELL BIOCATALYSTS

9.20.1 STANDARD OPERATING PROCEDURE 36 (SOP36): SUBSEQUENT ADDITION OF NEW SUBSTRATE AFTER FIRST REACTION CYCLE

E/Z-2-Phenylpropionaldoxime (25.0 µmol, 200 mM in DMSO, 125 µL) was added to the aqueous reaction mixture consisting of flavin mononucleotide

(FMN, 1 M in H_2O, 1.25 mL), *OxdB* (resting cell suspension of recombinant *E. coli HB101/pOxD-9OF*, 7 U/mL, 0.10 mL) and KPB (50 mM, pH 7.0, 3.525 mL) at room temperature. The reaction was reciprocally shaken for 24 hours at 180 min^{-1} and 8.0 °C. Aliquots of 500 µL were analysed by reversed-phase HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-1), R_{t2} = 12.4 min (*Z*-1), R_{t3} = 18.1 min (2)) for conversion and normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 5% 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-2), R_{t2} = 14.4 min ((*S*)-2), R_{t3} = 20.3 min (*Z*-(*S*)-1), R_{t4} = 26.7 min (*E*-(*S*)-1), R_{t5} = 30.0 min (*Z*-(*R*)-1), R_{t6} = 31.5 min (*E*-(*R*)-1)) for the enantiomeric excess of both product and remaining substrate. New *E/Z*-2-Phenylpropionaldoxime (0.025 mmol, 200 mM in DMSO, 125 µL) was added and the reaction was reciprocally shaken for another 24 hours at 180 min^{-1} and 8.0 °C.

Table 87. Subsequent addition of new substrate after the first reaction cycle

Entry	Time	ie	[E]	[Z]	[CN]	ee
1	82	98.9 (E)	5.838	-	4.162	95.6 (*S*)
2	270	98.9 (E)	5.194	-	4.808	96.1 (*S*)
3	1395	98.9 (E)	4.527	-	5.470	n.d.
4	82	90.2 (Z)	0.453	3.852	5.695	n.d.
(5)[a]	270	90.2 (Z)	(0.860)	(6.936)	(2.415)	n.d.
6	1395	90.2 (Z)	0.422	1.757	7.822	n.d.

(a) artifact

9.20.2 STANDARD OPERATING PROCEDURE 37 (SOP37): EXCHANGE OF AQUEOUS REACTION MIXTURE AFTER FIRST REACTION CYCLE

E/Z-2-Phenylpropionaldoxime (0.025 mmol, 200mM in DMSO, 125 µL) was added to the aqueous reaction mixture consisting of flavin mononucleotide (FMN, 1M in H_2O, 1.25 mL), *OxdB* (resting cell suspension of recombinant *E.coli* HB101/p*Oxd*-90F, 7 U/mL, 0.10 mL) and KPB (50 mM, pH 7.0, 3.525 mL) at room temperature. The reaction was reciprocally shaken for 24 hours at 180 min^{-1} and 8.0 °C. An aliquot of 0.50 mL was analysed by reversed-phase HPLC (COSMOSIL C18-MS-II, 30% CH_3CN in H_2O, 1.0 mL/min, 40 °C, 254 nm, R_{t1} = 11.0 min (*E*-1), R_{t2} = 12.4 min (*Z*-1), R_{t3} = 18.1 min (2)) for conversion and normal-phase chiral HPLC (Daicel Chiralcel OJ-H, 5% 2-propanol in *n*-hexane, 0.7 mL/min, 20 °C, 210 nm, R_{t1} = 13.8 min ((*R*)-2), R_{t2} = 14.4 min ((*S*)-2), R_{t3} = 20.3 min (*Z*-(*S*)-1), R_{t4} = 26.7 min (*E*-(*S*)-1), R_{t5} = 30.0 min (*Z*-(*R*)-1), R_{t6} = 31.5 min (*E*-(*R*)-1)) for the enantiomeric excess of both product and

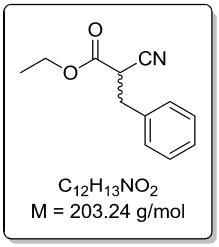
remaining substrate. The mixture was centrifuged for 5 minutes at 15,000 rpm and 4 °C. The supernatant was removed and the cells were resuspended in the new reaction mixture. The reaction was reciprocally shaken for 24 hours at 180 min^{-1} and 8.0 °C and analysed like the preceding reaction.

Table 88. Recycling of whole-cell biocatalyst by centrifugation and resuspending

Entry	Spec. activity [$U/L_{culture}$]	Nitrile conc. (5min) [mM]	Nitrile conc. (24h) [mM]
1	705	0.705	2.428
2	700	0.700	2.637
3	12	0.012	0.230

10 EXPERIMENTAL PROCEDURES – BETA-HYDROXYCARBONYL DERIVATIVES

10.1 ETHYL *RAC*-2-CYANO-3-PHENYLPROPIONATE



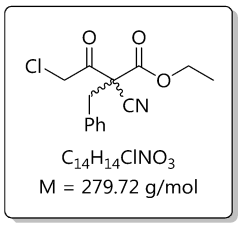
Benzyl bromide (1.78 mL, 15.0 mmol), ethyl cyanoacetate (4.76 mL, 45.0 mmol) and potassium carbonate (4.15 g, 30.0 mmol) were mixed without solvent and stirred for 12 hours at room temperature. The mixture was partitioned between MTBE (100 mL) and dist. H_2O (100 mL). The aqueous phase was brought to pH 1 by titration with hydrochloric acid (12 N) and extracted twice with MTBE (2 x 100mL). The combined organic phase was washed once with brine (100 mL). After drying over $MgSO_4$, all volatile compounds were removed *in vacuo*. NMR analysis shows quantitative conversion of benzyl bromide and a bis-alkylated side-product which could be identified by mass spectrometry. The main impurity of the crude product was remaining ethyl cyanoacetate. The product was isolated by column chromatography on silica (10% v/v ethyl acetate in cyclohexane, d=6cm, h=15cm) to obtain ethyl *rac*-2-cyano-3-phenylpropionate (2.46 g, 12.1 mmol, 80.7%) as a colorless oil. The racemic compound can be separated on chiral HPLC (Daicel Chiralpak AD-H, 80:18:2 SC-CO_2/*n*-hexane/*i*PrOH, 0.8 mL/min, 216 nm, R_{t1} = 12.8 min, R_{t2} = 18.3 min).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.42 – 7.26 (m, 5H, Ar-*H*), 4.26 (q, *J* = 7.1 Hz, 2H, O-C*H*$_2$), 3.74 (dd, *J* = 8.4, 5.8 Hz, 1H, C*H*-CN), 3.26 (qd, *J* = 13.8, 7.1 Hz, 2H, C*H*$_2$-Ph), 1.29 (t, *J* = 7.1 Hz, 3H, CH_2-C*H*$_3$).

^{13}C-NMR (75 MHz, $CDCl_3$): δ [ppm] = 165.52, 135.30, 129.04, 128.88, 127.79, 116.17, 62.94, 39.70, 35.77, 13.95.

The data corresponds to literature-known values.[148]

10.2 ETHYL *RAC*-2-BENZYL-4-CHLORO-2-CYANO-3-OXOBUTANOATE



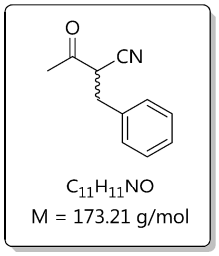
A suspension of sodium hydride (50 mg, 1.25 mmol, 60 % in mineral oil) in dry THF (10 mL) was stirred at room temperature. A solution of ethyl *rac*-2-benzyl-2-cyano-acetate (203 mg, 1.0 mmol) in dry THF (10 mL) was added dropwise at room temperature. Subsequently, the mixture was heated to reflux and stirred for additional two hours. A solution of chloroacetyl chloride (200 µL, 2.5 mmol) in dry THF (10 mL) was added dropwise to the enolate and the resulting mixture was stirred for 30 minutes at reflux temperature and additional three hours while cooling to room temperature. The reaction was quenched by the addition of brine (20 mL), the phases were

separated, the organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. Analysis by ^{1}H-NMR spectroscopy revealed a product-related conversion of 40.6%. No side-products except chloroacetic acid and starting material were observed. The racemic compound can be partially separated on chiral HPLC (Daicel ChiralPak OJ-H, 80:18:2 SC-CO_2/*n*-hexane/*i*PrOH, 0.8 mL/min, 220 nm, R_{t1} = 12.8 min, R_{t2} = 18.3 min).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.40 – 7.25 (m, 5H, Ar-*H*), 4.33 (s, 2H, Cl-C*H*$_2$), 4.24 (q, *J* = 7.2 Hz, 2H, O-C*H*$_2$-CH_3), 3.40 (d, *J* = 2.7 Hz, 2H, C*H*$_2$-Ph), 1.28 (t, *J* = 7.1 Hz, 3H, CH_2-C*H*$_3$).

The compound is unknown to literature.

10.3 RAC-3-CYANO-4-PHENYL-2-BUTANONE



$C_{11}H_{11}NO$
M = 173.21 g/mol

The synthesis is based on a condensation procedure from JI et al..[149] Potassium *tert*-butoxide (1.68 g, 15.0 mmol, 3.0 eq.) is added to a stirring solution of 3-phenylpropionitrile (655 mg, 5.0 mmol) in anhydrous THF (20 mL). After addition of ethyl acetate (1.97 mL, 20.0 mmol, 4.0 eq.) the mixture is stirred for 18 hours at room temperature. The reaction is quenched by addition of hydrochloric acid (1 N, 25 mL), water (75 mL) and ethyl acetate (100 mL). The organic layer is separated, washed with water (2 x 50 mL), brine (2 x 25 mL), dried over $MgSO_4$ and concentrated in vacuo. The crude product is purified via column chromatography on silica (6:1 v/v ethyl acetate in cyclohexane) to obtain a pale yellow oil.

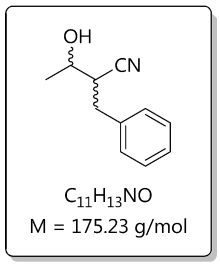
Isolated yield: 742 mg (4.28 mmol, 85.7 %).

^{1}H NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.42 – 7.23 (m, 5H, Ar-*H*), 3.66 (dd, J = 8.6, 5.5 Hz, 1H, C*H*-CN), 3.30 – 3.05 (m, 2H, C*H*$_2$-Ph), 2.35 (s, 3H, C*H*$_3$).

^{13}C NMR (75 MHz, $CDCl_3$): δ [ppm] = 198.15, 135.56, 128.99, 128.95, 127.71, 117.18, 46.42, 34.74, 28.91.

The compound is known to literature.

10.4 RAC-3-CYANO-4-PHENYL-2-BUTANOL



$C_{11}H_{13}NO$
M = 175.23 g/mol

Sodium borohydride (75.7 mg, 2.0 mmol) is added to a stirring solution of rac-3-cyano-4-phenyl-2-butanone (346 mg, 2.0 mmol) in THF (25 mL) at 0-4°C. The mixture is stirred for 1.5 hours and quenched by the addition of saturated sodium bicarbonate solution (15 mL). The solution is extracted with MTBE (3x 50 mL) and the combined organic phase is washed with brine (1x 50 mL). After drying over $MgSO_4$ all volatile compounds are removed in vacuo and the crude product is analyzed by ^{1}H-NMR spectroscopy.

Isolated yield: 278 mg (1.59 mmol, 79.3%).

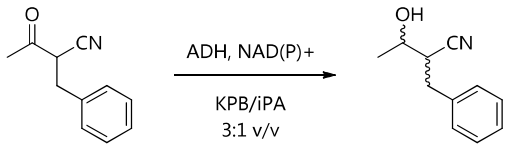
^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.42 – 7.23 (m, 5H, Ar-*H*), 3.99 – 3.86 (m, 1H, C*H*-OH), 3.09 – 2.73 (m, 1H, C*H*-CN), 1.43 (dd, J = 10.3, 6.3 Hz, 1H, CH_3).

The compound is known to literature.

10.5 STANDARD OPERATING PROCEDURE 38 (SOP38): BIOCATALYTIC REDUCTION OF BETA-OXONITRILES WITH ALCOHOL DEHYDROGENASES

A solution of NAD(P)+ (2.5 mol%) and the respective alcohol dehydrogenase in KPB (pH 8.0, 50 mM, 7.5 mL) is stirred at room temperature. The corresponding ・-oxonitrile (0.5 mmol) is dissolved in 2-propanol (2.5 mL) and added to the stirring biocatalyst solution. The resulting mixture is stirred for 24 hours at room temperature. The reaction is quenched by addition of neat sodium chloride to saturation and extraction with MTBE (3x 25 mL). The combined organic phase is washed with brine (25 mL) and dried over $MgSO_4$. All volatile compounds are removed *in vacuo* and the crude product is analyzed by ^{1}H-NMR spectroscopy. The products are not purified yet.

Table 89. Enantio- and diastereoselective reduction of 3-cyano-4-phenyl-2-butan-one with alcohol dehydrogenases



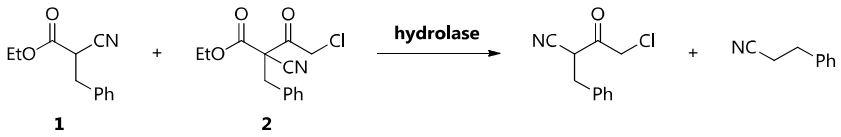
Entry	ADH (amount)	Cofactor	Conversion [%]	dr[a] (major/minor)
1	*Rhodococcus* sp. (50 uL)	NAD^+	>95.0	92:8
2	evocatal 1.1.200 (5.0 mg)	NAD^+	>95.0	70:30

[a] Identification of syn- and anti- diastereomers not yet established

10.6 STANDARD OPERATING PROCEDURE 39 (SOP39): PHOTOMETRIC SCREENING ASSAY OF HYDROLASES FOR THE BIOCATALYTIC DECARBOXYLATION OF BETA-OXOESTERS

A solution of the corresponding substrate or mixtures of substrates (20 µL, 0.2 M in DMSO), containing bromothymol blue (75 nM), was added to a solution of hydrolase (1 mg/mL, 180 µL) in aqueous buffer (10 mM, pH 7). The solutions were mixed on a 96-well plate and reference mixtures of the corresponding hydrolase with bromothymol blue were added. A color change from blue to yellow due to formation of carboxylic acids indicates activity for the given substrate. The well plate was immediately controlled for active enzymes at room temperature and was further mixed by shaking at 40 °C for 4-24 hours.

Table 90. Hydrolase-Screening for ethyl *rac*-2-benzyl-2-cyanoacetate and the mixture after chloroacetylation



1 **2**

	1	1	1+2[a]	1+2[a]	BL[b]
A	0.65	0.64	0.88	0.89	0.61
B	0.67	0.67	0.89	0.90	0.60
C	0.66	0.66	0.88	0.87	0.60
D	0.70	0.69	0.89	0.84	0.64
E	0.76	0.79	0.74	0.75	0.63
F	0.62	0.61	0.87	0.88	0.56
G	0.76	0.77	0.79	0.76	0.56
H	0.57	0.59	0.88	0.88	0.53

	1	1	1+2[a]	1+2[a]	BL[b]
I	0.63	0.61	0.84	0.81	0.56
J	0.64	0.67	0.89	0.89	0.61
K	0.80	0.77	0.80	0.89	0.60
L	0.59	0.59	0.84	0.83	0.55
M	0.64	0.62	0.87	0.88	0.57
N	0.55	0.54	0.81	0.79	0.52
O	0.71	0.69	0.97	0.92	0.67
P	0.62	0.61	0.70	0.70	0.51

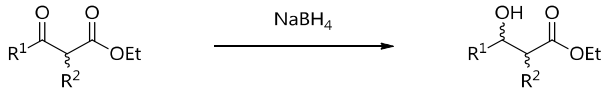
(a) mixture incl. remaining chloroacetic acid, not neutralized (b) blank; hydrolase with indicator, no substrate present

A	Protease from *Rhizopus* sp.	**I**	Pronase from *Streptomyces griseus*
B	Alcalase ® CLEA	**J**	Protease from *Aspergillus saitoi*
C	Protease from *Aspergillus* sp. (ABCR)	**K**	Protease from bovine pancreas
D	Amano Acylase (*Aspergillus melleus*)	**L**	Protease S, *Pyrococcus furiosus*
E	Porcine liver esterase (PLE, crude)	**M**	Amano Lipase M, *Mucor javanicus*
F	Protease from *Bacillus amilolyquefaciens*	**N**	Protease from *Bacillus* sp. (Sigma)
G	α-Chymotrypsin (Biozym CHY-03)	**O**	Protease from *Bacillus licheniformis*
H	Amano Lipase PS (*Pseudomonas cepacia*)	**P**	Protease from *Aspergillus oryzae*

10.7 STANDARD OPERATING PROCEDURE 40 (SOP40): SYNTHESIS OF RACEMIC BETA-HYDROXY ESTERS BY REDUCTION OF RACEMIC BETA-OXO ESTERS WITH SODIUM BOROHYDRIDE

A solution of the corresponding β-oxo ester (5.0 – 17.2 mmol) in THF (10-35 mL) was cooled to 0-4 °C. Sodium borohydride (1.0 eq.) was added in portions and the resulting suspension was stirred for 2-3.5 hours at the same temperature. The progress of the reaction was monitored *via* TLC. The reaction was quenched by the addition of hydrochloric acid (0.5-1.0 M, 1:1 v/v) and MTBE (2:1 v/v). The phases were separated and the aqueous phase was extracted with MTBE (2 x 2:1 v/v). The combined organic phase was washed with brine (25 mL), filtered over a small silica column and dried over $MgSO_4$. After removal of all volatile compounds *in vacuo* the product was obtained as colorless oil and was used without further purification.

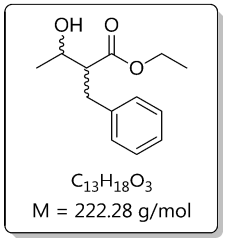
Table 91. Unselective reduction of racemic β-oxo esters with $NaBH_4$



Entry	R^1	R^2	Conv. [%]	Yield[a] [g (%)]
1	Me	Bn	>95.0	1.02 (91.8)
2	Ph	Me	>95.0	3.15 (87.9)

[a] β-Oxo esters were readily reduced to their corresponding 1,3-diols by $NaBH_4$; the crude product was always a mixture of β-hydroxy ester and 1,3-diol which can be easily purified by recrystallization in the following saponification

10.7.1 ETHYL *RAC*-2-BENZYL-3-HYDROXYBUTANOATE



$C_{13}H_{18}O_3$
M = 222.28 g/mol

The following synthesis was conducted according to SOP35. A solution of ethyl *rac*-2-benzylacetoacetate (1.07 mL, 5.0 mmol) in THF (10 mL) was cooled to 0-4 °C. Sodium borohydride (187 mg, 5.0 mmol) was added in portions and the suspension was stirred for two hours at 0-4 °C. The work-up was done as described in SOP35. The enantio- and diastereomers can be separated on normal-phase chiral HPLC (Daicel Chiralpak OJ-H, 2 % v/v 2-propanol in *n*-hexane, 0.8 mL/min, 220 nm): 23.4 min (2*S*,3*S*), 23.5 min (2*R*,3*R*), 33.2 min (2*S*,3*R*), 39.8 min (2*R*,3*S*).

Yield: 1.02 g (4.58 mmol, 91.8 %)

Ethyl syn-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.30 - 7.16 (m, 5H, Ar-*H*), 4.03 (q, *J* = 7.2 Hz, 2H, O-C*H*$_2$-CH$_3$), 4.04 (m, 1H, C*H*-OH), 2.97 (d, *J* = 7.8 Hz, 2H, C*H*$_2$-Ph), 2.74 (m, 1H, C*H*-COOEt), 1.26 (d, 3H, *J* = 6.3 Hz, *H*$_3$C-CH-OH), 1.09 (t, *J* = 7.1 Hz, 3H, CH$_2$-C*H*$_3$).

^{13}C-NMR (100 MHz, DMSO-d_6): δ [ppm] = 173.08, 139.67, 128.57, 128.20, 126.01, 67.18, 59.47, 55.93, 34.78, 21.44, 13.95.

Ethyl anti-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (400 MHz, $CDCl_3$): δ [ppm] = 7.30 - 7.16 (m, 5H, Ar-*H*), 4.08 (q, *J* = 7.2 Hz, 2H, O-C*H*$_2$-CH$_3$), 3.87 (m, 1H, C*H*-OH), 2.97 (d, *J* = 7.8 Hz, 2H,

C*H*$_2$-Ph), 2.66 (m, 1H, C*H*-COOEt), 1.25 (d, *J* = 6.3 Hz, 3H, *H*$_3$C-CH-OH), 1.13 (t, *J* = 7.2 Hz, 3H, CH$_2$-C*H*$_3$).

^{13}C-NMR (100 MHz, DMSO-d$_6$): δ [ppm] = 173.01, 139.57, 128.68, 128.15, 126.01, 67.29, 59.32, 55.56, 33.65, 20.89, 13.97.

IR: ṽ [cm^{-1}] = 3442 υ(O-H), 2977 υ(C-H), 1720 υ(C=O), 735 δ(C-H$_{Ar}$), 699 δ(C=C$_{Ar}$).

MS (EI): [m/z] = 204 (M$^+$-H$_2$O), 132 (M$^+$-C$_3$H$_5$O-OH), 131 (M$^+$-C$_7$H$_7$), 104 (M$^+$-C$_7$H$_7$-C$_2$H$_4$), 91 (C$_7$H$_7^+$), 72 (C$_3$H$_5$O$^+$).

EA: C$_{13}$H$_{18}$O$_3$

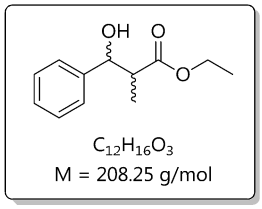
Calculated: 70.24 % C, 8.16 % H, 21.59 % O.

Measured: 69.93 % C, 8.34 % H.

The compound is literature-known. However, no NMR data is available.

10.7.2 Ethyl rac-3-hydroxy-2-methyl-3-phenylpropionate

The following synthesis was conducted according to SOP35. A solution of ethyl 2-methyl-3-oxo-3-phenylpropionate (3.54 g, 17.2 mmol) in dry THF (35 mL) was cooled to 0-4 °C. Sodium borohydride (649.4 mg, 17.2 mmol, 1.0 eq.) was added and the mixture was stirred for 3.5 hours at the same temperature. The work-up was done as described in SOP35.



C$_{12}$H$_{16}$O$_3$
M = 208.25 g/mol

Yield: 3.15 g (15.1 mmol, 87.9 %)

Ethyl syn-3-hydroxy-2-methyl-3-phenylpropionate

^{1}H-NMR (400 MHz, CDCl$_3$): δ [ppm] = 7.40-7.25 (m, 5H, Ar-*H*), 5.09 (d, 1H, *J* = 4.0 Hz, C*H*-OH), 4.13 (q, 2H, *J* = 7.1 Hz, O-C*H*$_2$-CH$_3$), 2.74 (m, 1H, C*H*-COOEt), 1.21 (t, 3H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$), 1.13 (d, 3H, *J* = 7.1 Hz, CH-C*H*$_3$).

^{13}C-NMR (100 MHz, CDCl$_3$): δ [ppm] = 175.50, 141.53, 128.05, 127.30, 125.94, 73.73, 60.52, 46.52, 13.92, 10.95.

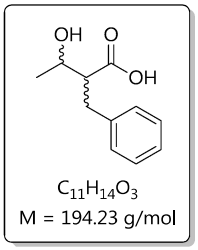
Ethyl anti-3-hydroxy-2-methyl-3-phenylpropionate

^{1}H-NMR (400 MHz, CDCl$_3$): δ [ppm] = 7.40 - 7.25 (m, 5H, Ar-*H*), 4.75 (d, 1H, *J* = 8.3 Hz, C*H*-OH), 4.19 (q, 2H, *J* = 7.1 Hz, O-C*H*$_2$-CH$_3$), 2.80 (m, 1H, C*H*-COOEt), 1.26 (t, 3H, *J* = 7.1 Hz, CH$_2$-C*H*$_3$), 1.02 (d, 3H, *J* = 7.1 Hz, CH-C*H*$_3$).

^{13}C-NMR (100 MHz, $CDCl_3$): δ [ppm] = 175.67, 141.58, 128.24, 127.77, 126.53, 76.13, 60.56, 47.08, 14.27, 13.98.

The data corresponds to literature-known values.[150]

10.8 *RAC*-2-BENZYL-3-HYDROXYBUTANOIC ACID



$C_{11}H_{14}O_3$
M = 194.23 g/mol

The crude product of ethyl *rac*-2-benzyl-3-hydroxybutanoate (max. 5.0 mmol) was dissolved in ethanol (5 mL). After addition of aqueous sodium hydroxide solution (2.5 M, 5 mL) the mixture was stirred for four hours at 60 °C. The solution was diluted with dist. H_2O (20 mL), cooled to room temperature and saturated with neat sodium chloride. After extraction with MTBE (30 mL) at pH 12, the solution was acidified (pH 1-2) with hydrochloric acid (12 M) and extracted with MTBE (3 x 30 mL). The combined organic phases of the acidic extraction were washed with brine (30 mL), dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was taken up in 2-propanol (1.5 mL) at reflux temperature. Light petroleum ether (10 mL) was added and the solution was slowly cooled to room temperature and further to 0-4 °C with an ice bath. The precipitate was filtered off and washed with cold ether (0-4 °C). After drying in high vacuum the product was obtained as colorless solid.

Isolated yield: 201 mg (1.03 mmol, 20.7 %).

syn-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (500 MHz, Acetone-d_6): δ [ppm] = 7.28 - 7.14 (m, 5H, Ar-*H*), 4.30 – 3.95 (m, 1H, C*H*-OH), 2.92 (m, 2H, CH_2-Ph), 2.74 – 2.69 (m, 1H, C*H*-COOH), 1.25 (d, 3H, *J* = 6.3 Hz, H_3C-CH-OH).

^{13}C-NMR (125 MHz, Acetone-d_6): δ [ppm] = 175.10, 141.00, 129.81, 129.08, 126.90, 68.41, 55.92, 34.78, 21.33.

anti-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (400 MHz, Acetone-d_6): δ [ppm] = 7.28 - 7.14 (m, 5H, Ar-*H*), 3.95 – 3.89 (m, 1H, C*H*-OH), 2.92 (m, 2H, CH_2-Ph), 2.69 – 2.64 (m, 1H, C*H*-COOH), 1.25 (d, 3H, *J* = 6.3 Hz, H_3C-CH-OH).

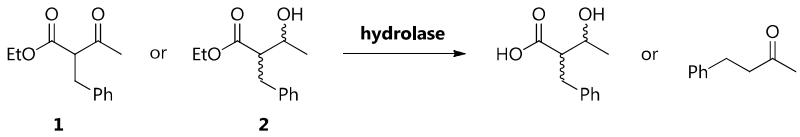
^{13}C-NMR (125 MHz, Acetone-d_6): δ [ppm] = 175.10, 141.00, 129.71, 129.08, 126.86, 68.64, 56.57, 55.56, 35.51, 21.67.

The compound is literature-known. However, NMR data is only available for D_2O.[151]

10.9 STANDARD OPERATING PROCEDURE 41 (SOP41): PHOTOMETRIC SCREENING ASSAY OF HYDROLASES FOR THE BIOCATALYTIC SYNTHESIS OF BETA-HYDROXYCARBOXYLIC ACIDS

A solution of the corresponding substrate or mixtures of substrates (20 µL, 0.2 M in DMSO), containing bromothymol blue (75 nM), was added to a solution of hydrolase (1 mg/mL, 180 µL) in KPB (10 mM, pH 7). The solutions were mixed on a 96-well plate and reference mixtures of the corresponding hydrolase with bromothymol blue were added. A color change from blue to yellow due to formation of carboxylic acids indicates activity for the given substrate. The well plate was immediately controlled for active enzymes at room temperature and was further mixed by shaking at 40 °C for 4-24 hours.

Table 92. Hydrolase-screening for ethyl *rac*-2-benzyl-3-oxobutanoate and the racemic β-hydroxy ester.



1 **2**

	1	1	2	2	BL
A	0.83	0.83	0.94	0.91	0.90
B	1.60	1.23	1.29	1.48	1.37
C	0.88	0.91	1.00	1.03	1.00
D	0.89	0.94	1.06	1.01	0.99
E	0.44	0.45	0.60	0.60	0.88
F	0.90	0.93	1.03	1.03	0.95
G	0.83	0.74	1.01	0.94	0.91
H	1.39	1.30	1.49	1.07	1.58

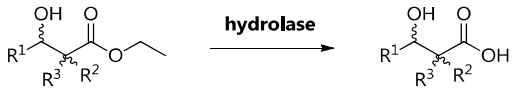
	1	1	2	2	BL
I	0.83	0.84	0.95	0.96	0.92
J	0.93	0.89	1.01	1.02	1.05
K	0.71	0.72	0.86	0.85	0.85
L	0.97	0.97	1.08	1.13	0.99
M	0.87	0.89	1.05	1.02	0.94
N	0.89	0.92	1.02	0.94	0.91
O	0.87	0.90	1.04	1.03	0.98
P	0.93	0.94	1.04	1.08	0.99

A	Protease from *Rhizopus* sp.	**I**	Pronase from *Streptomyces griseus*
B	Alcalase ® CLEA	**J**	Protease from *Aspergillus saitoi*
C	Protease from *Aspergillus* sp. (ABCR)	**K**	Protease from bovine pancreas
D	Amano Acylase (*Aspergillus melleus*)	**L**	Protease S, *Pyrococcus furiosus*
E	Porcine liver esterase (PLE, crude)	**M**	Amano Lipase M, *Mucor javanicus*
F	Protease from *Bacillus amilolyquefaciens*	**N**	Protease from *Bacillus* sp. (Sigma)
G	α-Chymotrypsin (Biozym CHY-03)	**O**	Protease from *Bacillus licheniformis*
H	Amano Lipase PS (*Pseudomonas cepacia*)	**P**	Protease from *Aspergillus oryzae*

10.10 STANDARD OPERATING PROCEDURE 42 (SOP42): PURIFICATION OF MIXTURES OF BETA-HYDROXY ESTERS BY BIOCATALYTIC DECARBOXYLATION *VIA* HYDROLASES

The corresponding racemic or enriched β-hydroxy or β-oxo ester was emulsified in KPB or mixtures of KPB with appropriate cosolvents, *e.g.* 2-propanol. A solution of hydrolase in buffer was added and the resulting mixture was stirred for 24 hours at room temperature. The pH was maintained by titration with aqueous sodium hydroxide solution (0.2 M) by a titrino apparatus. The progress of the reaction was monitored by the consumption of titer which was equivalent to the amount of product formed during hydrolysis. The solution was subsequently saturated with neat sodium chloride and extracted with *tert*-butyl methyl ether (1:1 v/v) at pH 8.0. If necessary, the pH of the solution was basified by addition of neat sodium bicarbonate. If the desired product was the corresponding decarboxylated ketone, the aqueous phase was extracted with additional MTBE (2 x 1:1 v/v) before the combined organic phase was washed with brine (1:1 v/v), dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. In the case of the β-hydroxycarboxylic acid as the desired product, the first organic phase was discarded and the aqueous phase was brought to pH 1 by the addition of hydrochloric acid (12 M). After extraction with MTBE (3x 1:1 v/v) the combined organic phase was treated as described before. The crude product was analyzed with ^{1}H-NMR spectroscopy and was purified by recrystallization or column chromatography on silica, if necessary.

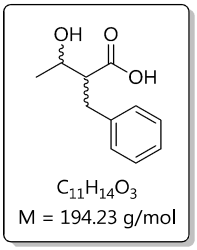
Table 93. Biocatalytic synthesis of β-hydroxycarboxylic acids *via* hydrolase.



Entry	R^1	R^2	R^3	Hydrolase	Conversion [%]	Isol. Yield [g (%)]
1	Me	Bn	H	PLE	40.2[a]	0.19 (39.4)
2	Cl-Me	Bn	CN	PLE	>95.0[b]	n.d. (< 1.0)[c]

(a) determined by consumption of titer (b) nearly quantitative conversion to hydrolyzed by-product (c) not determined

10.10.1 (2*RS*,3*RS*)-2-BENZYL-3-HYDROXYBUTANOIC ACID



The synthesis was conducted according to SOP29. To an emulsion of ethyl *rac*-2-benzyl-3-hydroxybutanoate (556 mg, 2.5 mmol) in KPB (pH 7.0, 50 mM, 25 mL) was added porcine liver esterase (52.4 mg). The mixture was stirred for 24 hours at room temperature. The pH was maintained by titration with aqueous sodium hydroxide solution (0.2M) by a titrino apparatus. The work-up was done as described in SOP29.

Isolated yield: 191 mg (0.98 mmol, 39.4%).

syn-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (500 MHz, Acetone-d_6): δ [ppm] = 7.28 - 7.14 (m, 5H, Ar-*H*), 4.30 – 3.95 (m, 1H, C*H*-OH), 2.92 (m, 2H, CH_2-Ph), 2.74 – 2.69 (m, 1H, C*H*-COOH), 1.25 (d, 3H, *J* = 6.3 Hz, H_3C-CH-OH).

^{13}C-NMR (125 MHz, Acetone-d_6): δ [ppm] = 175.10, 141.00, 129.81, 129.08, 126.90, 68.41, 55.92, 34.78, 21.33.

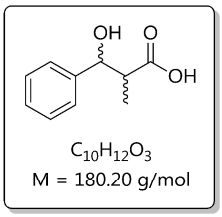
anti-2-benzyl-3-hydroxybutanoate

^{1}H-NMR (400 MHz, Acetone-d_6): δ [ppm] = 7.28 - 7.14 (m, 5H, Ar-*H*), 3.95 – 3.89 (m, 1H, C*H*-OH), 2.92 (m, 2H, CH_2-Ph), 2.69 – 2.64 (m, 1H, C*H*-COOH), 1.25 (d, 3H, *J* = 6.3 Hz, H_3C-CH-OH).

^{13}C-NMR (125 MHz, Acetone-d_6): δ [ppm] = 175.10, 141.00, 129.71, 129.08, 126.86, 68.64, 56.57, 55.56, 35.51, 21.67.

The data corresponds to literature-known values.

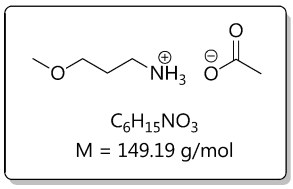
10.10.2 *RAC*-3-HYDROXY-2-METHYL-3-PHENYLPROPANOIC ACID



Ethyl rac-2-benzyl-3-hydroxybutanoate (1.04 g, 5.0 mmol) was dissolved in ethanol (5 mL). After addition of aqueous sodium hydroxide solution (2.5M, 5mL) the mixture was stirred for four hours at 60°C. The solution was diluted with dist. H_2O (20 mL), cooled to room temperature and saturated with neat sodium chloride. After extraction with MTBE (2x 30 mL) at pH 12, the solution was acidified (pH 1-2) with hydrochloric acid (12M) and extracted with MTBE (3x 30 mL). The combined organic phases of the acidic extraction were washed with brine (30 mL), dried over $MgSO_4$ and all volatile compounds were

removed *in vacuo*. The crude product was a colorless oil which crystallized while standing at room temperature. The purification is not finished yet.

10.11 3-Methoxypropylamine acetate



$C_6H_{15}NO_3$
M = 149.19 g/mol

A mixture of acetic acid (57.2 mL, 1.00 mol) and 3-methoxypropylamine (81.6 mL, 0.80 mol) was stirred for six hours at 60 °C. The excess of acetic acid was removed in high vacuum (60 °C, 0.02 mbar) to obtain the ionic liquid as yellow oil.

Isolated yield: 119.2 g (0.80 mmol, 99.8%).

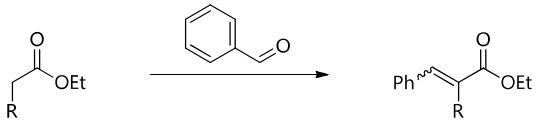
^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 3.56 – 3.48 (m, 2H, C*H*$_2$-NH_3), 3.36 – 3.30 (s, 3H, O-C*H*$_3$), 3.04 – 2.94 (t, *J* = 6.6 Hz, 2H, O-C*H*$_2$), 2.02 – 1.93 (s, 3H, (O=)C-C*H*$_3$), 1.94 – 1.84 (m, 2H, CH_2-C*H*$_2$-CH_2).

The data corresponds to literature-known values.

10.12 Standard operating procedure 43 (SOP43): Knoevenagel condensation of benzaldehyde with alpha-C-H acidic esters in an ionic liquid

A mixture of the corresponding *α*-C-H acidic ester (45.0 – 100 mmol), benzaldehyde (1.0 - 2.0 eq.) and 3-methoxypropylamine acetate (0.08 - 0.17 eq.) was stirred at room temperature. If the viscosity was too high for appropriate stirring, additional ethyl acetate (10 mL) was added for dilution. The solution was stirred for 3-18 hours at room temperature. The progress of the reaction was monitored *via* TLC. After complete consumption of starting material, ethyl acetate (200 mL) was added and the organic phase was washed with hydrochloric acid (6 M, 100 mL) and sodium bisulfite solution (40 %, 100 mL). The organic phase was dried over $MgSO_4$ and all volatile compounds were removed *in vacuo*. The crude product was purified *via* column chromatography or recrystallization, if necessary.

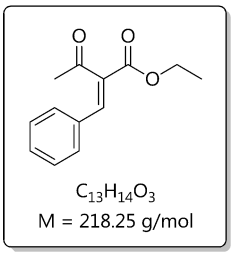
Table 94. Knoevenagel condensation for the synthesis of 2-substituted ethyl cinnamates



Entry	R	Time [h]	Conv. [%]	Yield [g(%)]
1	Ac	18.0	68.3	12.2 (56.1)
2	Cl-Ac	4.5	72.4	3.89 (51.3)[a]
3	CN	3.0	>95.0	6.51 (71.9)

[a] an aliquot of 6.0 g crude product was purified

10.12.1 ETHYL *E/Z*-2-BENZYLIDENE-3-OXOBUTANOATE



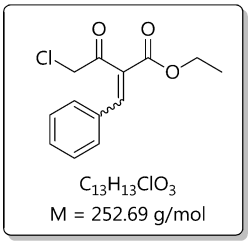
The following synthesis was conducted according to SOP38. A highly viscous mixture of ethyl acetoacetate (12.7 mL, 100 mmol), benzaldehyde (10.1 mL, 100 mmol) and 3-methoxypropylamine acetate (1.2 g, 8.0 mmol, 0.08 eq.) was vigorously stirred for 18 hours at room temperature. The work-up was done as described in SOP38. The crude product shows a product content of >95% and was not further purified. The product was obtained as yellow oil.

Isolated yield: 12.2 g (56.1 mmol, 56.1 %).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.86 (s, 1H, Ar-*H*C=C), 7.45 – 7.35 (m, 5H, Ar-*H*), 4.33 (q, *J* = 7.1 Hz, 2H, O-C*H*$_2$-CH$_3$), 4.31 (s, 3H, *H*$_3$C-C=O), 1.35 (t, *J* = 7.1 Hz, 3H, C*H*$_3$).

The data corresponds to literature values.[152]

10.12.2 ETHYL *E/Z*-2-BENZYLIDENE-4-CHLORO-3-OXOBUTANOATE



The following synthesis was conducted according to SOP38. A highly viscous mixture of ethyl 4-chloro-3-oxobutanoate (13.6 mL, 100 mmol), benzaldehyde (10.1 mL, 100 mmol) and 3-methoxypropylamine acetate (2.4 g, 16.0 mmol, 0.16 eq.) was diluted with ethyl acetate (10 mL) and vigorously stirred for 4.5 hours at room temperature. The work-up was done as described in SOP38. An aliquot of the crude product (6 g, 30 wt-%) was purified *via* column chromatography on silica (d=6 cm, h=20 cm, 1-10% v/v ethyl acetate in

cyclohexane) to yield the product as pure *Z*-isomer (0.65 g, 2.6 mmol) and an *E/Z*-mixture (3.24 g, 12.8 mmol, *E/Z* = 78:22).

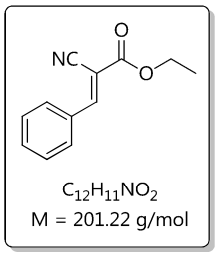
Isolated yield: 3.89 g (15.4 mmol, 51.3%).

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.87 (s, 1H, Ar-*H*C=C), 7.54 – 7.33 (m, 5H, Ar-*H*), 4.34 (q, *J* = 7.2 Hz, 2H, O-C*H*$_2$-CH$_3$), 4.31 (s, 3H, *H*$_3$C-C=O), 1.35 (t, *J* = 7.2 Hz, 3H, C*H*$_3$).

ESI-MS: m/z [fragment] = 275 [M+Na]+(3^5Cl, 100%), 277 [M+Na] (3^7Cl, 35%).

The data corresponds to literature-known values.[153]

10.12.3 ETHYL *E*-2-CYANOCINNAMATE



$C_{12}H_{11}NO_2$
M = 201.22 g/mol

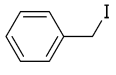
The following synthesis was conducted according to SOP38. A highly viscous mixture of ethyl cyanoacetate (4.76 mL, 45.0 mmol), benzaldehyde (9.10 mL, 90 mmol) and 3-methoxypropylamine acetate (2.5 g, 16.7 mmol, 0.17 eq.) was vigorously stirred for three hours at room temperature. The work-up was done as described in SOP38. The crude product was purified by recrystallization in light petroleum ether to obtain the product as colorless solid.

Isolated yield: 6.51 g (32.3 mmol, 71.9%).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 8.27 (s, 1H, C=CH), 8.04 – 7.98 (m, 2H, *o*-Ar-*H*), 7.62 – 7.48 (m, 3H, *m,p*-Ar-*H*), 4.41 (q, *J* = 7.1 Hz, 2H, O-C*H*$_2$-CH$_3$), 1.42 (t, *J* = 7.1 Hz, 3H, O-CH$_2$-C*H*$_3$).

The data corresponds to literature-known values.[154]

10.13 BENZYL IODIDE



C_7H_7I
M = 218.03 g/mol

Sodium iodide (7.79 g, 52.0 mmol) was suspended in acetone (40 mL) at room temperature and under exlusion of light. Benzyl bromide (5.95 mL, 50.0 mmol) was added dropwise and the resulting suspension was stirred for 60 minutes at room temperature. GC analysis reveals a product-related conversion of >98.5%. The suspension was filtered and the filtrate was concentrated *in vacuo* to obtain the product as pale yellow oil which was stored under exclusion of light.

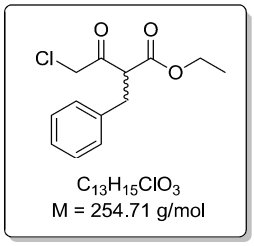
Isolated yield: 10.9 g (50.0 mmol, 100%).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.43 – 7.24 (m, 5H, Ar-*H*), 4.49 (s, 2H, C*H*$_2$-I).

EI-MS (m/z) = 91 $[Trp]^+$, 127 $[I]^+$.

The data corresponds to literature-known values.

10.14 Ethyl *rac*-2-benzyl-4-chloro-3-oxobutanoate



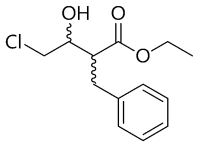
$C_{13}H_{15}ClO_3$
M = 254.71 g/mol

The crude product of synthesis **10.8.2** was diluted with ethyl acetate (50 mL). Palladium on charcoal (Pd/C, 100 mg) was added to the solution and stirred under hydrogen atmosphere at room temperature. Additional hydrogen was bubbled through the solution *via* syringe. Celite (1 g) was added after 12 hours and the suspension was filtered. The solvent and all volatile compounds were removed *in vacuo*. The crude product was analyzed by NMR spectroscopy. It shows a >95.0% conversion with minor impurities from the previous step. NMR analysis reveals dehalogenation of 58.8% under these conditions.

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.32 – 7.15 (m, 5H, Ar-*H*), 4.23 (s, 2H, C*H*$_2$-Cl), 4.22 (q, *J* = 7.2 Hz, 2H, O-C*H*$_2$-CH$_3$), 4.05 (t, *J* = 7.0 Hz, 3H, C*H*-COOEt), 3.22 (d, *J* = 7.1 Hz, 2H, C*H*$_2$-Ph), 1.22 (t, *J* = 7.2 Hz, 3H, O-CH$_2$-C*H*$_3$).

The compound is unknown to literature.

10.15 Ethyl *rac*-2-benzyl-4-chloro-3-hydroxybutanoate



$C_{13}H_{17}ClO_3$
M = 256.73 g/mol

Lithium *N,N*-diisopropylamide (2.0 M in THF/*n*-heptane/ethylbenzene, 20.0 mL, 40.0 mmol) was dissolved in abs. THF (40 mL) and cooled to -78 °C (acetone/dry ice). A solution of ethyl 3-phenylpropionate (7.03 mL, 40.0 mmol) in abs. THF (10 mL) was added dropwise under temperature control. The resulting mixture was stirred for 60 minutes at -78 °C before a solution of chloroacetaldehyde (59% in DCM, 5.32 mL, 40.0 mmol) in abs. THF (10 mL) was added dropwise under temperature control. The solution was stirred for additional two hours at -78 °C and was subsequently quenched by the addition of saturated ammonium chloride solution (20 mL). The phases were separated and the organic phase was directly filtrated over silica (20 % v/v ethyl acetate in cyclohexane). The filtrate was concentrated *in vacuo* and the residue was

purified *via* column chromatography on silica (1-10 % v/v ethyl acetate in cyclohexane) to obtain the product as colorless oil.

Isolated yield: 1.95 g (7.60 mmol, 19.0 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.42 – 7.07 (m, 5H, Ar-*H*), 4.03 – 3.90 (m, 2H, O-C*H*$_2$-CH_3), 3.32 – 2.72 (m, 5H, Cl-C*H*$_2$, C*H*-OH, C*H*$_2$-Ph), 2.29 – 2.00 (m, 1H, C*H*-COOEt), 1.06 – 0.94 (m, 3H, CH_2-C*H*$_3$).

EI-MS (m/z) = 178 $[M\text{-}C_2H_2ClO]^+$, 104 $[PhC_2H_4]^+$, 91 $[Trp]^+$.

The compound is unknown to literature.

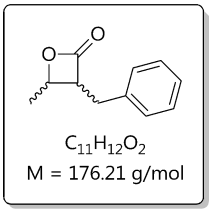
10.16 STANDARD OPERATING PROCEDURE 44 (SOP44): LACTONIZATION OF BETA-HYDROXYCARBOXYLIC ACIDS UNDER RETENTION OF THE ABSOLUTE CONFIGURATION

The corresponding β-hydroxy carbonacid (1.0 – 5.0 mmol) was dissolved in pyridine (5 – 25 mL) and cooled to 0-4 °C. After the addition of *p*-toluenesulfonyl chloride (2.0 eq.) the reaction was stirred for 4-24 hours at 0-4 °C. The reaction was quenched by the addition of dist H_2O (1:1 v/v) and ethyl acetate (2:1 v/v). The phases were separated and the aqueous phase was extracted with ethyl acetate (2x 2:1 v/v). The combined organic phase was washed with brine (1:1 v/v) and dried over $MgSO_4$ before all volatile compounds were removed *in vacuo*. The crude product was analyzed by NMR spectroscopy and purified *via* column chromatography, if necessary.

Table 95. Lactonization of β-hydroxy carbonacids

Entry	R^1	R^2	Conv. [%]	Isol. Yield [g(%)]
1	Me	Bn	>95.0	n.d.
2	Ph	Me	>95.0	n.d.

10.16.1 *RAC*-3-BENZYL-4-METHYLOXETAN-2-ONE



The following synthesis was conducted according to SOP39. A solution of *rac*-2-benzyl-3-hydroxybutanoic acid (194 mg, 1.0 mmol) in pyridine (5 mL) was cooled to 0-4 °C. After the addition of *p*-toluenesulfonyl chloride (381.3 mg, 2.0 mmol) the reaction was stirred for 24 hours at 0-4 °C. The work-up was done as described in SOP39. NMR analysis of the crude product reveals a quantitative conversion of starting material and a change in diastereomeric excess, most likely due to racemization by pyridine over the prolonged reaction time. The crude product was not further purified.

Yield: 206 mg

<u>*syn*-3-Benzyl-4-methyl-oxetan-2-one:</u>

^{1}H-NMR (400 MHz, $CDCl_3$)**:** δ [ppm] = 7.32-7.26 (m, 5H, Ar-*H*), 4.46 (m, 1H, H_3C-C*H*-O), 3.46 (m, 1H, C*H*-Bn), 3.20 – 3.00 (m, 2H, CH_2-Ph), 1.45 (d, *J* = 6.1 Hz, 3H, CH_3).

<u>*anti*-3-Benzyl-4-methyl-oxetan-2-one:</u>

^{1}H-NMR (400 MHz, $CDCl_3$)**:** δ [ppm] = 7.32-7.26 (m, 5H, Ar-*H*), 4.81 (m, 1H, H_3C-C*H*-O), 4.02 (m, 1H, C*H*-Bn), 3.20 – 3.00 (m, 2H, CH_2-Ph), 1.49 (d, *J* = 6.4 Hz, 3H, CH_3).

The compound is literature-known. However, no NMR data is available.

10.17 (2*RS*,3*RS*)-2-BENZYL-3-HYDROXYBUTANOIC ACID *VIA* BIENZYMATIC ONE-POT PROCEDURE COMBINING AN ALCOHOL DEHYDROGENASE AND A HYDROLASE

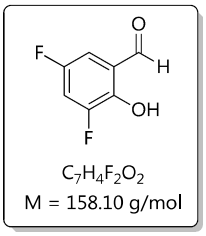
An aqueous reaction mixture containing NAD^+ (68.2 mg, 0.1 mmol), an alcohol dehydrogenase from *Rhodococcus* sp. (Rsp-ADH, cell-free extract, 1:1 v/v in glycerin, 1.00 mL) and porcine liver esterase (crude, 100 mg) in KPB (50 mM, pH 8.0, 75 mL) was stirred at room temperature. After the addition of a substrate solution of ethyl *rac*-2-benzyl-3-oxobutanoate (551 mg, 2.5 mmol) in 2-propanol (25 mL) the resulting mixture was stirred for 16 hours at pH 8.0 and room temperature. The pH was maintained by titration with sodium hydroxide solution (0.2 M) to neutralize the carboxylic acid formed during the reaction. The consumption of titer stopped at 50 % conversion after 60 minutes. To

exclude inactivation of the biocatalysts, additional ethyl *rac*-2-benzyl-3-oxobutanoate (551 mg, 2.5 mmol) was added and the mixture was stirred for 16 hours. The consumption of titer immediately increased up to a conversion of 86.5% in relation to 2.5 mmol substrate, corresponding to a total conversion of 43.3 %. The solution was brought to saturation with neat sodium chloride and acidified to pH 1 with hydrochloric acid (12 M). After extraction with MTBE (3x 75 mL) the combined organic phase was washed with brine (100 mL) and dried over $MgSO_4$. All volatile compounds were removed *in vacuo*. The crude product was analyzed by ^{1}H-NMR spectroscopy for conversion, diastereoselectivity and formation of side-products. It shows 15.2 % ethyl *rac*-2-benzyl-3-oxobutanoate, 44.5 % ethyl *rac*-2-benzyl-3-hydroxybutanoate, 11.7 % *rac*-2-benzyl-3-hydroxybutanoic acid, 8.4 % 4-phenyl-2-butanone and 20.2 % 4-phenyl-2-butanol. The crude product was not further purified.

Crude yield: 890 mg

11 EXPERIMENTAL PROCEDURES – CHROMENES

11.1 3,5-DIFLUORSALICYLALDEHYDE



$C_7H_4F_2O_2$
M = 158.10 g/mol

To a stirring solution of 2,4-difluorphenol (7.64 mL, 10.41 g, 80 mmol) in trifluoroacetic acid (65 mL) was added hexamethylentetramine (22.43 g, 160 mmol) in portions. The mixture was stirred for 18 hours at 75 °C. After addition of sulfuric acid (40%, 80 mL) and stirring for additional 2.5 hours at room temperature the mixture was poured into ice water (mixture of crushed ice and dist. H_2O, 0-4 °C). The precipitate was filtered off and dried in high vacuum to obtain the product as pale yellow solid.

Isolated yield: 6.85 g (43.3 mmol, 54.2%).

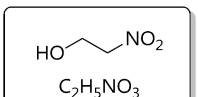
^{1}H-NMR (500 MHz, DMSO) δ [ppm] = 10.90 (s, 1H, C*H*O), 10.26 (d, *J* = 2.9 Hz, 1H, O*H*), 7.63 (ddd, *J* = 11.3, 8.4, 3.1 Hz, 1H, CF-C*H*-CF), 7.25 (ddd, *J* = 8.4, 3.0, 1.7 Hz, 1H, CF-C*H*-C).

^{13}C-NMR (126 MHz, $CDCl_3$) δ [ppm] = 195.2, 155.4, 153.5, 151.9, 149.9, 146.6, 121.0, 113.2, 112.0.

^{19}F-NMR (470 MHz, $CDCl_3$) δ [ppm] = -120.41 (s, CH-C*F*-COH), -131.17 (s, CH-C*F*-CH).

The compound was literature-known. However, no NMR data is available.

11.2 2-NITROETHANOL



$C_2H_5NO_3$
M = 91.07 g/mol

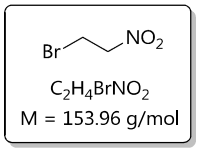
Paraformaldehyde (5.04 g, 168 mmol) and potassium carbonate (73 mg, 0.53 mmol, 0.3 mol%) were suspended in nitromethane (75 mL, 66.37 g, 1.70 mol) and stirred for three hours at reflux temperature. The mixture was cooled to room temperature, acidified with hydrochloric acid (1 M) and extracted with MTBE (2x 30 mL). The combined organic phase was washed with saline (2x 50 mL) and dried over $MgSO_4$. After removal of all volatile compounds the crude product was purified *via* fractional distillation (80 °C, 8 mbar) to obtain the product as colorless oil.

Isolated yield: 2.20 g (24.0 mmol, 15.0%).

^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm] = 4.54 (t, 2H, CH_2-OH), 4.15 (t, 2H, CH_2-NO_2).

The compound is literature-known. The data corresponds to a commercial reference.

11.3 2-BROMO-1-NITROETHANE



$C_2H_4BrNO_2$
M = 153.96 g/mol

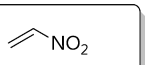
A solution of phosphor tribromide (2.14 mL, 22.8 mmol) in dichloromethane (5 mL) was cooled to 0-4 °C. A solution of 2-nitroethanol (5 mL, 70.9 mmol) in dichloromethane (10 mL) was added dropwise. The mixture was stirred for 60 minutes at 0-4 °C. Dist. H_2O (10 mL, 0-4 °C) was added and the phases were separated. The aqueous phase was extracted with dichloromethane (2x 15 mL) and the combined organic phase was washed with saturated sodium bicarbonate solution (20 mL). After drying over $MgSO_4$ all volatile compounds were removed *in vacuo*. The crude product was purified by fractional distillation (75 °C, 10 mbar) to obtain the product as colorless oil in a mixture with starting material.

Isolated yield: 1.25 g (84 % product content, 6.82 mmol, 9.6 %).

^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm] = 4.77 (t, *J* = 6.4 Hz, 2H, C*H*$_2$-NO_2), 3.82 (t, *J* = 6.3 Hz, 2H, C*H*$_2$-Br).

The compound is literature-known. However, no NMR data is available.

11.4 NITROETHYLENE



$C_2H_3NO_2$
M = 73.05 g/mol

Phthalic anhydride (9.63 g, 65 mmol) was mixed with 2-nitroethanol (3.5 mL, 49.6 mmol) and stirred at 150 °C. The product was directly distilled from the reaction mixture (30-45 °C, 11 mbar) and condensed at -78 °C (acetone/dry ice). The distillate was brought to room temperature and consists of two phases, one of them being H_2O. Dichloromethane (10 mL) was added and the phases were separated. After drying the organic phase over $MgSO_4$ all volatile compounds were removed under reduced pressure (>150 mbar, 40 °C).[i] The crude product was analyzed by ^{1}H-NMR spectroscopy to be a mixture of nitroethylene (40.0 %), nitromethane (44.3 %) and dichloromethane (15.7 %).

Isolated yield: 2.79 g (40 % product content, 15.3 mmol, 30.8 %).

[i] Due to the low boiling point of the product the pressure was not further decreased.

^{1}H-NMR (500 MHz, $CDCl_3$) δ [ppm] = 7.14 (dd, *J* = 14.9, 7.4 Hz, 1H, C*H*-NO_2), 6.65 (dd, *J* = 14.9, 2.0 Hz, 1H, H_EC=CH-NO_2), 5.91 (d, *J* = 5.9 Hz, 1H, H_ZC=CH-NO_2).

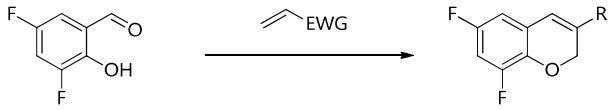
The data corresponds to literature-known values.[155]

11.5 STANDARD OPERATING PROCEDURE 45 (SOP45): CYCLOCONDENSATION OF 3,5-DIFLUOROSALICYLIC ALDEHYDE WITH VARIOUS ALPHA,BETA-UNSATURATED CARBONYL SYNTHONS

The following syntheses were conducted according to different literature procedures which will be given in parenthesis. In general, 3,5-difluorosalicylic aldehyde (5.0 mmol) and the corresponding Michael acceptor (1.5 – 5.0 eq.) were dissolved in an adequate organic solvent (10 mL). Different additives were added for stabilization of the monomer reagents, *in situ* formation of the monomers or acceleration of the cyclocondensation reaction. The mixtures were heated to reflux temperature of the solvent and under conditions for the irreversible elimination of H_2O from the reaction (e.g. azeotrope distillation).

After stirring for 4-24 hours the mixtures were cooled to room temperature and the solution was directly chromatographed on silica (dichloromethane / light petroleum ether 1:1 v/v) to remove the majority of impurities and to obtain the desired product in pure form or in a mixture with the starting aldehyde. If starting material was present, extraction with sodium hydroxide solution (1 M, 2 x 1:1 v/v), separation of the phases, drying over $MgSO_4$ and removal of all volatile compounds *in vacuo* will give the pure 2*H*-chromene derivative. The starting material can be recycled by acidification and extraction with MTBE (3 x 1:1 v/v). In the case of dioxane the solvent was first removed *in vacuo* before extraction or purification of the crude product. In the case of PEG400 the solvent was removed by additional washing with dist. H_2O (2 x 1:1 v/v) of the combined organic phase after extractive work-up.

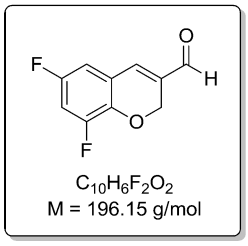
Table 96. Substrate synthesis of 3-substituted 6,8-difluoro-2*H*-chromenes



Entry	EWG[a]	Solvent	Base	Isolated yield
1	CHO	Dioxane	K_2CO_3	0.15 (15.5)
2	CN	-	DABCO	0.38 (39.3)
3	NO_2	Toluene	Bu_2NH	0.87 (40.9)
4	$CONH_2$	Toluene	Bu_2NH	n.d. (-)[a]

(a) Electron-withdrawing group (EWG): aldehyde (CHO), nitrile (CN), nitro (NO2), carbamide ($CONH_2$) (b) No product was isolated, probably acrylamide polymerization under the given conditions.

11.5.1 6,8-Difluoro-2*H*-chromene-3-carbaldehyde



The following synthesis was conducted according to a literature procedure from Conti and Desideri.[156] Acrolein (420 µL, 7.50 mmol) was added dropwise to a suspension of 3,5-difluorosalicylaldehyde (791 mg, 5.0 mmol) and K_2CO_3 (1.04 g, 7.5 mmol) in dry dioxane (20 mL). The mixture was refluxed for four hours. After cooling, the solvent was removed under reduced pressure and the residue was distributed between dichloromethane and 2 M NaOH. The organic phase was washed with brine and dried over $MgSO_4$ before all volatile compounds were removed *in vacuo*. The residue was purified by column chromatography on silica (1:1 v/v dichloromethane in light petroleum ether).

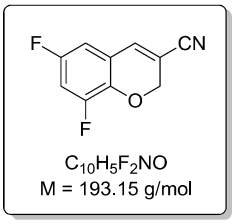
Isolated yield: 152 mg (0.77 mmol, 15.5 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 9.64 (s, 1H, C*H*O), 7.22 (m, 1H, Ar-C*H*-C), 6.91 (ddd, *J* = 10.3, 8.4, 2.9 Hz, 1H, FC-C*H*-CF), 6.79 (ddd, *J* = 7.7, 2.9, 1.8 Hz, 1H, FC-C*H*-C), 5.09 (d, *J* = 1.4 Hz, 2H, O-CH_2).

^{19}F NMR (282 MHz, $CDCl_3$): δ [ppm] = -118.88 (t, *J* = 8.0 Hz, 1F, *p*-C*F*), -131.15 (d, *J* = 10.0 Hz, 1F, *o*-C*F*).

The compound is literature-known.[157] However, no NMR data is available.

11.5.2 6,8-Difluoro-2*H*-chromene-3-carbonitrile



The following synthesis was conducted according to a patent procedure from Taugerbeck *et al.*.[158] A solution of 3,5-difluorosalicylaldehyde (791 mg, 5.0 mmol), 1,4-diazabicyclo[2.2.2]octane (DABCO, 169 mg, 1.5 mmol) and *p*-methoxyphenol (9.3 mg, 0.08 mmol) in acrylonitrile (8.0 mL) was stirred for 15 hours at 75 °C. The solvent was evaporated and the residue was taken up in dichloromethane (25 mL) and purified by flash chromatography on silica (dichloromethane) to obtain spectroscopically pure product.

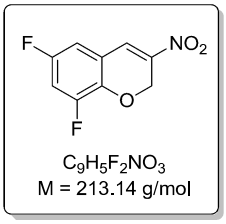
Isolated yield: 380 mg (1.97 mmol, 39.3 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.15 (d, *J* = 1.5 Hz, 1H, Ar-*H*C=C), 6.90 (ddd, *J* = 10.2, 8.4, 2.9 Hz, 1H, FC-C*H*-CF), 6.70 (m, 1H, FC-C*H*-C), 4.89 (d, *J* = 1.5 Hz, 2H, O-CH_2).

^{19}F NMR (282 MHz, $CDCl_3$): δ [ppm] = -117.70 (t, *J* = 8.1 Hz, 1F, *p*-C*F*), -130.88 (d, *J* = 10.9 Hz, 1F, *o*-C*F*).

The compound was literature-known.[158] *However, no NMR data was available.*

11.5.3 6,8-Difluoro-3-nitro-2*H*-chromene



The synthesis was conducted based on the literature procedure from Neirabeyeh *et al.*[159] A mixture of 3,5-difluorosalicylaldehyde (1.58 g, 10.0 mmol), phthalic anhydride (2.96 g, 20.0 mmol), 2-nitroethanol (1.41 mL, 20.0 mmol), *p*-methoxyphenol (1.24 mg, 0.01 mmol) and di-*n*-butylamine (843 µL, 5.0 mmol) was dissolved in toluene (100 mL). The solution was stirred for 24 hours at reflux temperature in a Dean-Stark-apparatus. After cooling to room temperature the solution was purified directly by column chromatography on silica (1:1 v/v dichloromethane in light petroleum ether) to obtain the product as orange solid.

Isolated yield: 872 mg (4.09 mmol, 40.9 %).

^{1}H-NMR (300 MHz, $CDCl_3$): δ [ppm] = 7.74 (d, *J* = 1.4 Hz, 1H, Ar-C*H*=C), 6.97 (ddd, *J* = 10.2, 8.3, 2.9 Hz, 1H, FC-C*H*-CF), 6.85 (m, 1H, FC-C*H*-C), 5.32 (d, *J* = 1.3 Hz, 2H, O-CH_2).

^{19}F NMR (282 MHz, $CDCl_3$): δ [ppm] = -117.36 (t, *J* = 7.8 Hz, 1F, *p*-C*F*), -129.91 (d, *J* = 10.3 Hz, 1F, *o*-C*F*).

FT-IR (neat): v [cm-1] = 3080, 2925, 2873, 1655, 1586, 1516, 1322, 1218.

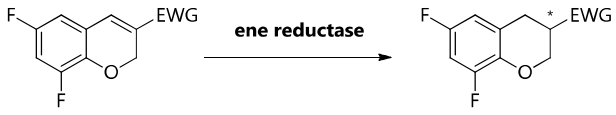
Mp (uncorrected): 114-116 °C.

The compound is unknown to literature.

11.6 STANDARD OPERATING PROCEDURE 46 (SOP46): BIOCATALYTIC *2H*-CHROMENE REDUCTION WITH AN ENOATE REDUCTASE FROM *GLUCONOBACTER OXYDANS* (*GOX-ER*)

The following synthesis was conducted with enzyme-coupled cofactor regeneration with an alcohol dehydrogenase from *Lactobacillus kefir* and 2-propanol as cosubstrate and cosolvent. This way the substrate was also distributed homogenously in the reaction mixture. A solution of the corresponding 3-substituted 6,8-difluoro-2*H*-chromene (0.1 mmol) in 2-propanol (2.5 mL) was added to an enzyme solution of Gox-ER (10 mg, 20 U, Batch-No. 361), $NADP^+$ (10 mg, 0.014 mmol) and LK-ADH (50 µL, 1:1 v/v in glycerin, 3.75 U) in KPB (7.5 mL, 50 mM, pH 6.0). The resulting emulsion was stirred for 24 hours at room temperature. The mixture was diluted with brine (10 mL) and brought to saturation with neat sodium chloride. After extraction with MTBE (3x 25 mL) the combined organic phase was washed with brine (20 mL) and dried over $MgSO_4$. All volatile compounds were removed *in vacuo* and the crude product was analyzed by NMR spectroscopy for conversion and HPLC for enantiomeric excess.

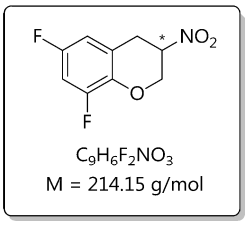
Table 97. Biocatalytic reduction of various 3-substituted 2*H*-chromenes with Gox-ER



Entry	EWG[a]	Conversion [%]	Yield [mg(%)]	ee [%]
1	CHO	>95.0[a]	n.d.	n.d.
2	CN	<1.0	-	-
3	NO_2	>95.0	14.1 (n.d.)	< 1.0 (6.7)[b]

(a) Electron-withdrawing group (EWG): aldehyde (CHO), nitrile (CN), nitro (NO_2) (b) LK-ADH-catalyzed reduction of carbaldehyde; 2*H*-chromene-3-carbinol and 3-hydroxymethylchroman as byproducts (c) racemic product after 24 hours, however, 6.7 % ee after 6 hours reaction time.

11.6.1 (3*RS*)-6,8-DIFLUORO-3-NITROCHROMAN



$C_9H_6F_2NO_3$
M = 214.15 g/mol

The following synthesis was conducted according to SOP41. A solution of 6,8-difluoro-3-nitro-2*H*-chromene (21.2 mg, 0.1 mmol) in 2-propanol (2.5 mL) was added to an enzyme solution of Gox-ER (10 mg, 20 U, Batch-No. 361), $NADP^+$ (10 mg, 0.014 mmol) and LK-ADH (50 µL, 1:1 v/v in glycerin, 3.75 U) in KPB (7.5 mL, 50 mM, pH 6.0). The resulting emulsion was stirred for 24 hours at room temperature. The work-up was done as described in SOP41. Analysis with ^{1}H-NMR shows quantitative conversion to the desired product. Chiral HPLC (Daicel Chiralcel OJ-H, 95:5 SC-CO_2/iPA, 0.8 mL/min, 212 nm, R_{t1} = 19.8 min, R_{t2} = 21.0 min) shows a poor enantioselectivity of 6.7 % ee (6 h) to a racemic product (24 h).

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12 LIST OF ABBREVIATIONS

3-DHG	diethyl 3-hydroxyglutarate
Ac	acetyl
ADH	alcohol dehydrogenase
API	active pharmaceutical ingredient
CAE	cephalosporin C acetylesterase
CHY	a-chymotrypsin
DABCO	1,4-diazabicyclo[2.2.2]octane
DCM	dichloromethane
DMAP	*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
EHG	monoethyl 3-hydroxyglutarate
ER	enoate reductase
FMN	flavin mononucleotide
HPLC	high-performance liquid chromatography
HWE	HORNER-WADSWORTH-EMMONS
IPA	*iso*-propanol
KPB	potassium phosphate buffer
LK	*Lactobacillus kefir*
MTBE	*tert*-butyl methyl ether
$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance
NOE	Nuclear-OVERHAUSER-Effect
Rsp	*Rhodococcus* sp.
T3P	*Tris*-*n*-propyltriphosphonic acid anhydride
TBAF	*tetra*-*n*-butylammonium fluoride
TBS	*tert*-butyldimethylsilyl
TEA	triethylamine
THF	tetrahydrofurane
THP	tetrahydropyran-2-yl
TMEDA	tetramethylethylendiamine
TPPO	triphenylphosphine oxide