Dissertation

zur Erlagung des Doktorgrades
der Naturwissenschaften
Dr. rer. nat.

Chemoenzymatic cascade reactions towards aliphatic nitriles and amines starting from biorenewable resources

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vorgelegt der Fakultät für Chemie
der Universität Bielefeld

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Alessa Hinzmann, M. Sc.

geboren am 08.10.1993 in Bielefeld

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Erstgutachter: Prof. Dr. Harald Gröger

Zweitgutachter: Jun.-Prof. Dr. Stephan Hammer

Erklärung

Die vorliegende Arbeit wurde am Lehrstuhl „Industrielle Organische Chemie und Biotechnologie“ der Universität Bielefeld unter Leitung von Prof. Dr. Harald Gröger und an der *Toyama Prefectural University* (TPU) (Oktober 2017 bis Dezember 2017) in der Zeit von Oktober 2017 bis Juli 2020 angefertigt. Die Finanzierung dieser Arbeit erfolgte durch die Fachagentur Nachwachsender Rohstoffe e.V. (FNR) und das Bundesministerium für Ernährung und Landwirtschaft (BMEL) im Rahmen des Projektes „Fettnitrile“ (Fördernummer: 22001716) sowie des Deutschen Akademischen Austauschdienstes (DAAD) und *Japan Society for Promotion of Science* (JSPS) im Rahmen des gemeinsam geförderten Projektes „DAAD PPP Japan 2017/2018“ (Fördernummer: 57345562).

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Alessa Hinzmann

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Publikationen

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“Would you tell me, please, which way I ought to go from here?”
“That depends a good deal on where you want to get to,” said the Cat.
“I don’t much care where—” said Alice.
“Then it doesn’t matter which way you go,” said the Cat.
“—so long as I get somewhere,” Alice added as an explanation.
“Oh, you’re sure to do that,” said the Cat, “if you only walk long enough.” —

Chapter 6, *Pig and Pepper* in *Alice’s Adventures in Wonderland* by Lewis Carroll

Let’s tesselate

Tesselate in *An Awesome Wave* by Alt-J

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Abbreviation

bww	Bio wet weight
Cy	Cyclohexane
dest.	Distilled
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
eq	equivalents
GC	Gas chromatography
IR	Infrared
Ni-NTA	Nickel nitrilotriacetic acid
NMR	Nuclear magnet resonance
Oxd	Aldoxime dehydratase
OxdA	Aldoxime dehydratase from *Pseudomonas chlororaphis* B23
OxdB	Aldoxime dehydratase from *Bacillus* sp. OxB-1
OxdFG	Aldoxime dehydratase from *Fusarium graminearum* MAFF305135
OxdK	Aldoxime dehydratase from *Pseudomonas* sp. K-9
OxdRE	Aldoxime dehydratase from *Rhodococcus erythropolis*
OxdRG	Aldoxime dehydratase from *Rhodococcus globerulus* A-4
PCR	Polymerase chain reaction
PAN	Phenylacetonitrile
PAOx	Phenylacetaldoxime
pH	*potentia Hydrogenii*
PIPO	Polyamine immobilised piperidinyl oxyl
PPB	Potassium phosphate buffer
syn gas	Synthetic gas (CO-H_2-mixture)
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TEOS	Tetraethyl orthosilicate
THF	Tetrahydrofurane
TLC	Thin layer chromatography
TPP	Triphenylphosphine
TPPS	Tetraphenylporphyrin Tetrasulfonic Acid
TPPTS	Triphenylphosphine-3,3',3"-trisulfonic acid trisodium salt

1 Background

1.1 Industrial importance of aliphatic nitriles and amines and their synthesis

Parts of this chapter have been published by the author of this thesis and her coauthors as a concept article in Chemistry – A European Journal *(Article 1).*[1]

Nitriles are substances which are found in a broad range of products from the *high-volume-low-price-* to the *high-price-low-volume*-segment (Figure 1). In case of *high-volume-low-price-*chemicals, especially solvents like acetonitrile or polymer-precursors like adiponitrile should be mentioned, while in the segment of *high-price-low-volume* chiral nitriles play a major role and are found in many pharmaceuticals, such as Vildagliptin and Saxagliptin, or in fine chemicals for different applications.[2–5]

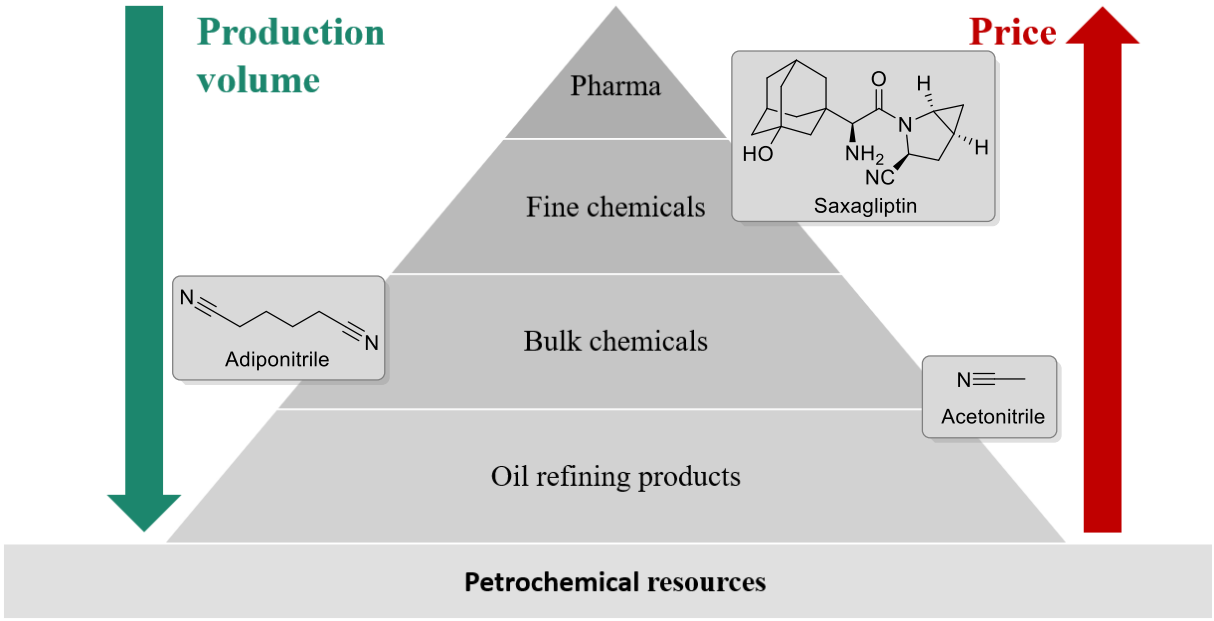
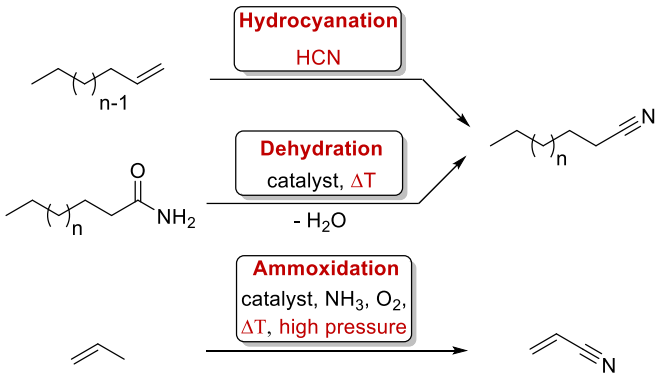


Figure 1. Product chain of chemical industry. Acetonitrile and adiponitrile are shown as examples for nitriles of the bulk-chemical segment, while Saxagliptin represents an example of a pharma product including a nitrile functionality.

Nitriles are nowadays usually synthesized by hydrocyanation, ammoxidation or dehydration of amides (Scheme 1).[2,4] One of the most important production process of nitriles by annual tonnage is the double *n*-terminal hydrocyanation of 1,3-butadiene to yield adiponitrile. Most of

the produced adiponitrile is hydrogenated to 1,6-hexanediamine, which is used for nylon production. Another frequently used process for nitrile synthesis is ammoxidation. This process is for example used to produce acrylonitrile starting from propylene, ammonia and oxygen by usage of harsh reaction conditions, especially in terms of temperature and pressure.[4,6] Starting from amides, nitriles can be obtained by dehydration under elevated temperatures and (usually) with heterogeneous catalysts. This process is mostly used for the synthesis of fatty nitriles due to the high accessibility of long chain, aliphatic fatty acids. These nitriles serve as precursor for aliphatic amines as surfactants or lubricants additives, for example against corrosion.[5]

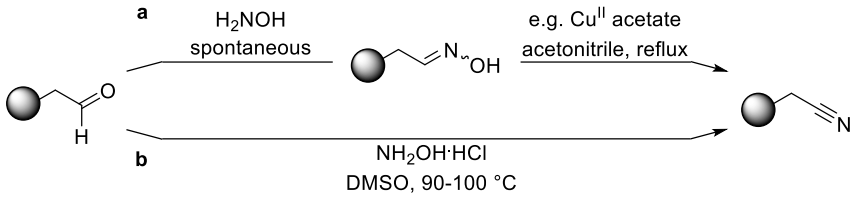


Scheme 1. Common methods for the synthesis of nitriles.

By careful evaluation of these existing processes several drawbacks become apparent. In case of the hydrocyanation reaction the use of toxic cyanide salts is inevitably, while in case of the dehydration of amides, the amide needs to be synthesized beforehand and high reaction temperatures of approximately 300 °C are required for the dehydration. High reaction temperatures and furthermore high pressures are also applied in the gas-phase ammoxidation process, which raises selectivity and side-product formation problems.

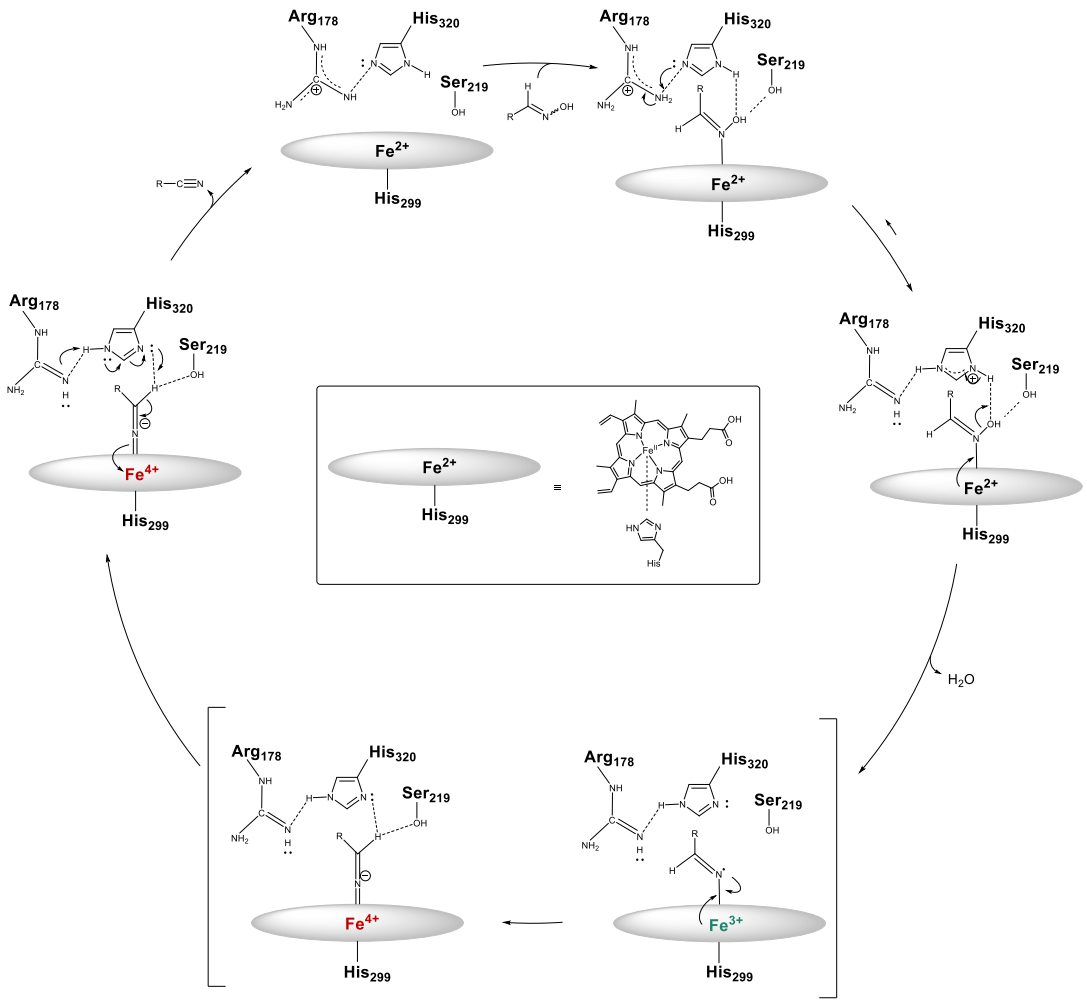
Since these drawbacks of nitrile synthesis are known, many investigations on alternative nitrile syntheses were performed by different groups in the last years. Aldoximes were found to be suitable starting materials for nitrile synthesis, enabling a potential process without need of cyanide salts, while aldoximes themselves are easily synthesized from aldehydes. A facile synthesis of nitriles is demonstrated by Lu *et al.* using transition metal salts as catalyst for the dehydration of aldoximes to nitriles using a nitrile as cosubstrate (Scheme 2, a).[7] The aldoximes themselves are easily accessible by condensation of the aldehyde with hydroxylamine, which is a multi-tons product in the chemical industry[8] and might also prepared by microorganisms in the future.[9] An even more elegant way to synthesize nitriles is a one-pot synthesis described by Mebane *et al.*[10] and Atta *et al.*[11] using the aldehyde as

starting material, hydroxylamine as cosubstrate and dimethyl sulfoxide (DMSO) as catalyst (Scheme 2, b).



Scheme 2. Alternative nitrile syntheses starting from aldehydes and aldoximes.[7,10,11]

Another alternative to conventional nitrile syntheses is based on a biocatalytical dehydration of aldoximes using aldoxime dehydratases (Oxds) as catalyst. Oxds are nowadays known to be involved in nitrile-degrading microorganisms, however, their natural function has not yet been fully clarified yet.[12–16] The first enzyme of its kind was found by the Asano group in 1998, namely OxdB (Oxd from *Bacillus* sp. OxB-1), which showed activity for the dehydration of phenylacetaldoxime (PAOx) to phenylacetonitrile (PAN).[13] Since the discovery of OxdB further enzymes were found and characterized.[17–22] These enzymes, for example found in *Pseudomonas chlororaphis* B23 (OxdA), *Fusarium graminearum* MAFF305135 (OxdFG), *Rhodococcus erythropolis* (OxdRE), *Rhodococcus globerulus* A-4 (OxdRG) and *Pseudomonas* sp. K-9 (OxdK), proved their catalytic efficiency for nitrile synthesis for many examples.[23–29] Oxds are heme-containing enzymes, carrying an Fe^{II} ion in their active center, which was found to be necessary for their catalytic activity and a loss of activity is observed by an oxidation of the ferrous iron to ferric iron.[14,15,17] The dehydration of an aldoxime in the active center of an Oxd is performed by a catalytic triade, consisting of a serine (or tyrosine in case of OxdB), a histidine and an arginine (Scheme 3). After coordination of the nitrogen of the aldoxime to the ferrous iron in the active center, the histidine is protonated by the arginine residue, which leads to an increased electrophilicity of the aldoximes OH-functionality. After the elimination of water and electron transfer from the ferrous iron to the aldoxime nitrogen an Fe^{IV}-species is formed and the aldoxime α-proton is then coordinated to the deprotonated histidine residue and the serine side chain. The nitrile precursor is deprotonated and electron transfer to the Fe^{IV} species proceeds, releasing the nitrile and recovering the ferrous iron. Subsequent proton shifts of the histidine and arginine residues complete the catalytic cycle.

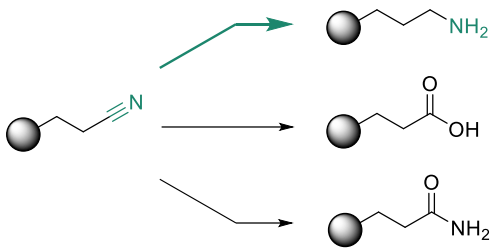


Scheme 3. Proposed mechanism of aldoxime dehydratases. *This figure was taken from Article 1.*[1]

Generally, Oxds show a broad substrate scope from aliphatic, to aromatic and chiral aldoximes, being usually active at moderate reaction temperatures (~30 °C) and neutral pH.[30] Oxds have proven their efficiency and selectivity for the biocatalytic synthesis of chiral nitriles.[23,25,30] In some cases, they can even produce both enantiomers of a nitrile using the separated *E*- or *Z*-aldoxime.[23,25] Not only for chiral nitriles, being important industrial chemicals used in pharma or as fine chemicals, but also for the synthesis of aliphatic *high-volume-low-price*-chemicals Oxds have been found to be applicable.[26] An efficient synthetic access to the multi-ton scale product adiponitrile has been discovered using Oxds as biocatalyst for the conversion of the dialdoxime, obtained from the dial.[26] Already these few examples from a broad synthetic spectrum[1,30] show the applicability of Oxds as catalyst for the synthesis of many different nitriles.

Not only as products itself, but also as precursor for other functional groups nitriles are interesting and often used compounds. For example, they can be transformed into amides, carboxylic acids or amines (Scheme 4), being valuable compounds of chemical industry.

Especially aliphatic primary amines are important products, which can be synthesized from nitriles and are frequently used, for example in the field of lubricants.[5]



Scheme 4. Examples of products, which are accessible from nitriles. The green arrow indicates the reaction studied in this thesis.

Nowadays aliphatic amines are mainly produced from the corresponding aldehydes via reductive amination,[5] however, this method using heterogeneous catalysts usually leads to selectivity problems and thus tedious isolation and purification of the primary amine products.[31–33] To overcome these selectivity problems, homogeneous catalysts can be used, but usually the synthesis of these catalysts is complex, the catalysts are often highly instable and usually cannot be reused.[33] An alternative access to primary amines is based on nitriles as precursor (see Scheme 4), which can be hydrogenated to the product of interest, but nevertheless, also the hydrogenation to nitriles often leads to selectivity problems when using heterogeneous catalysts.[34–36] However, in case of nitrile hydrogenation homogeneous catalysts can be used to overcome selectivity problems as well.[37–42] These catalysts are mainly based on ruthenium as metal,[41,43] although iron,[42] cobalt[37] and manganese[44] can also be used. Due to the higher stability of manganese catalysts, especially in comparison to iron and cobalt based catalysts, they are very interesting for the selective production of primary amines.[44]

For the alternative synthesis of aliphatic nitriles using for example Oxds or transition metal salts aldehydes are used as starting material. Therefore, it has to be evaluated carefully how aldehydes can be obtained and which resources (petrochemical-based or biorenewable resources) can be used.

1.2 Synthetic access to aliphatic aldehydes

The chemical industry's research is currently strongly focused on raw material change,[45] as the resources normally used for chemical products such as crude oil and natural gas are finite. With regard to the two classes of substances targeted by this theses, namely aliphatic nitriles and primary amines, crude oil after steam cracking of crude oil is often the basis of these products. Considering that the chemical industry has to decrease its dependency on fossil raw materials in the future, finding new synthesis routes based on renewable raw materials is a very current topic of research and development. Not only the raw materials used for a process, but also safety and sustainability in general are important topics of modern organic chemistry, due to global warming and other environmental issues. For this reason, Paul Anastas and John Warner developed the 12 principles of green chemistry (Figure 2) as a guideline for new sustainable processes which could replace the less sustainable existing processes in the future.[46]

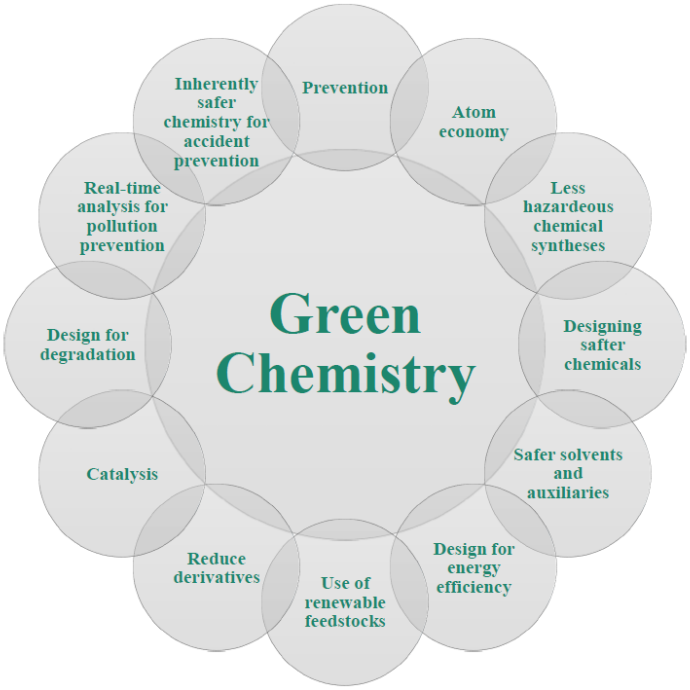
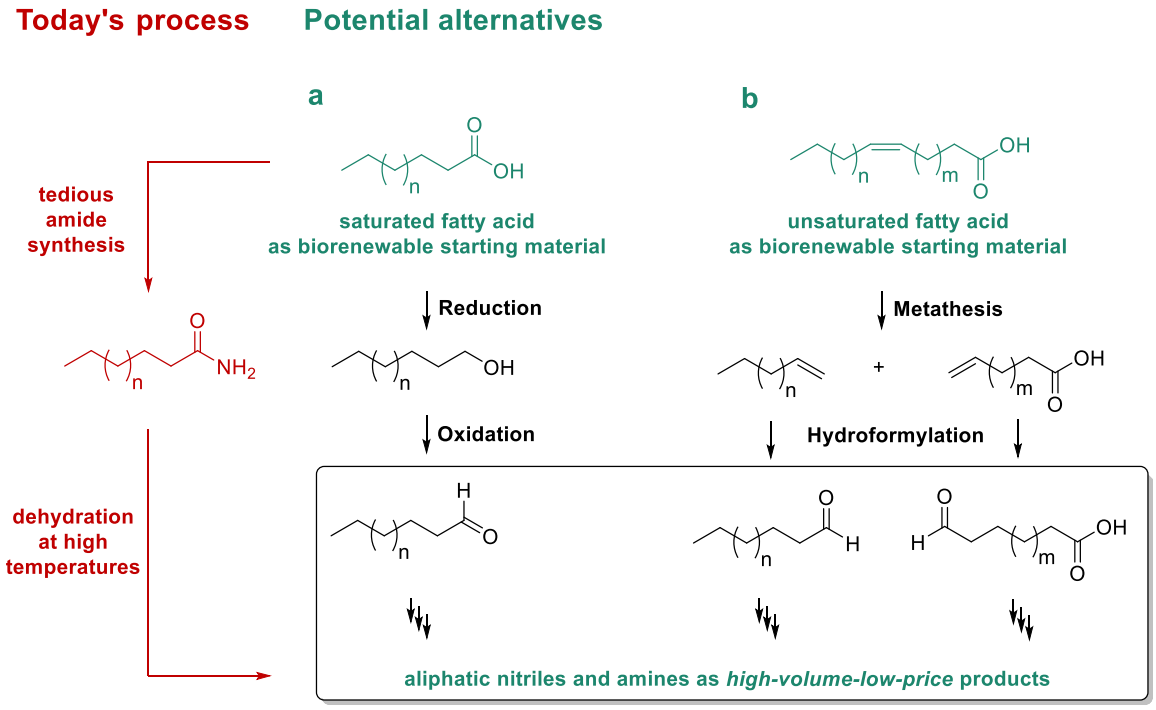


Figure 2. 12 Principles of green chemistry.[46]

In case of aliphatic nitriles and amines, usually unsaturated alkenes from steam cracking are used as precursor and are accessible via hydrocyanation (nitriles) or hydroformylation and reductive amination (amines). But especially for these products, a suitable alternative to crude-oil based starting material is available, since fatty acids are omnipresent in nature and range

from short to long alkyl chains, serving a large product range. Fatty acids have already proven to be an alternative raw material for the synthesis of aliphatic nitriles and amines,[5] however, in today's processes they are tediously converted to amides,[47] which can be dehydrated to nitriles under harsh reaction conditions with enormous energy consumption.[4] Therefore, an alternative synthesis nitriles and amines starting from fatty acids is desired. The idea of using fatty acids as biorenewable resource for the synthesis of aliphatic nitriles and amines and the design of a more sustainable synthesis is the basis of this thesis (Scheme 5).



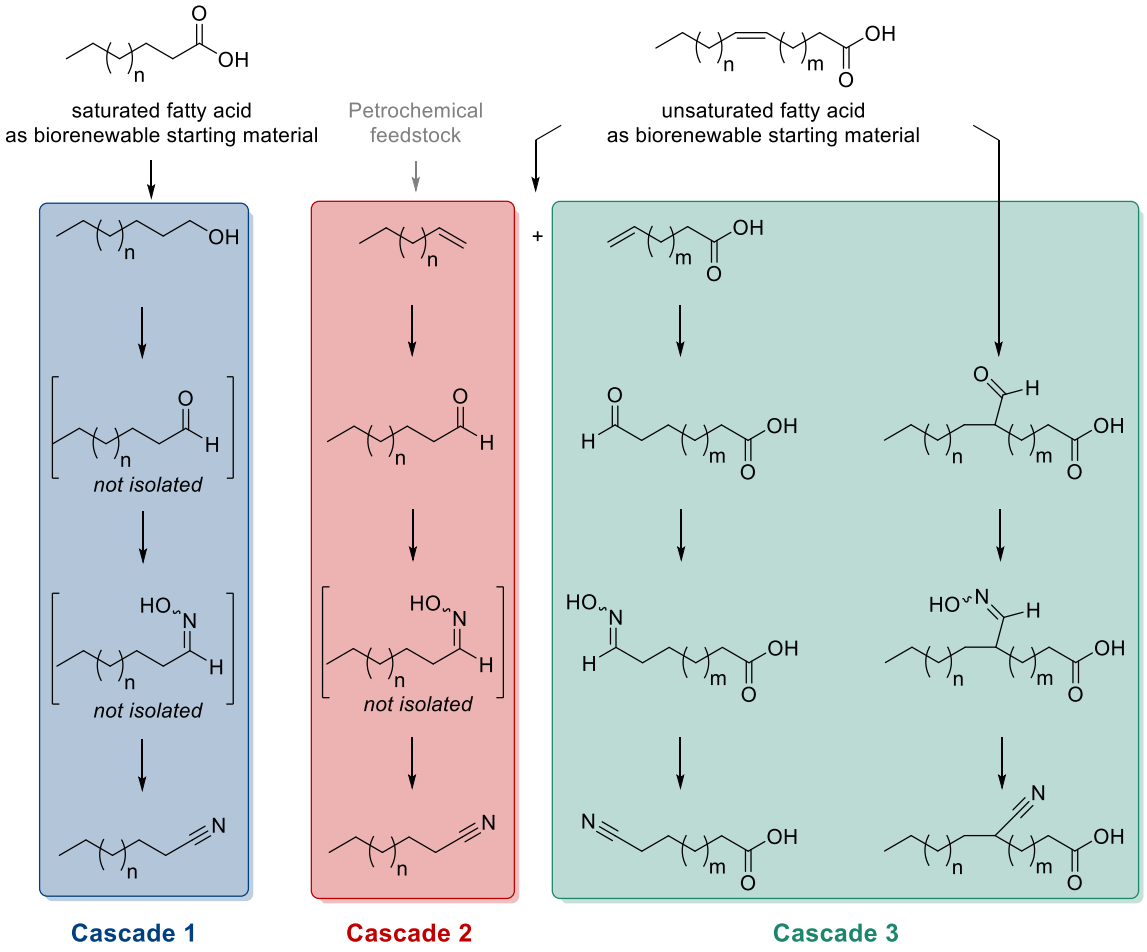
Scheme 5. Synthetic access to aldehydes, nitriles and amines starting from fatty acids.

To obtain aldehydes from fatty acids, they can initially be hydrogenated to the corresponding alcohols. The alcohols can be oxidized to the aldehydes in a subsequent step. One example of selective oxidation of primary alcohols to aldehyde is the Anelli-type TEMPO-oxidation ((2,2,6,6-Tetramethylpiperidin-1-yl)oxyl)) as a sustainable oxidation method in which TEMPO or TEMPO-derivatives are used as catalyst and usually hypochlorite as oxidation agent.[48,49] TEMPO is easily accessible from acetone and ammonia and hypochlorite is a component of house-hold bleach, being produced in large scale and is classified as green oxidation agents.[50] However, many investigations were performed to find an alternative solvent for the selective oxidation of primary alcohols to aldehydes using this method, since the classical solvent system for Anelli-type TEMPO-oxidation is a mixture of water and dichloromethane.[48,49] Dichloromethane is a chlorinated solvent and needs to be replaced for sustainability and safety

reasons. A general method in an alternative solvent was not found, being applicable to a variety of alcohol substrates, especially for aliphatic alcohols. Starting from unsaturated fatty acids a preceding cross-metathesis with ethylene can be performed to obtain unsaturated compounds, one aliphatic chain with terminal double bond and one carboxylic acid with a terminal double bond. The former can be used for the synthesis of aliphatic aldehydes via hydroformylation, the latter for the synthesis of a variety of bifunctional molecules, for example also by hydroformylation. Hydroformylation is a technically significant, usually homogeneously catalyzed reaction of alkenes with synthetic gas (syn gas) from carbon monoxide and hydrogen. The products of hydroformylation are aldehydes with one more carbon atom than the olefin substrate. As catalyst usually rhodium or cobalt catalysts are used. Hydroformylation is a highly productive and atom efficient process for the synthesis of a variety of aldehydes. Anelli-type TEMPO-oxidation and hydroformylation were both chosen as synthetic access to aldehydes potentially from biorenewables to produce aliphatic nitriles and amines.

2 Outline and scope

This thesis aims towards the design of chemoenzymatic cascade reactions for the synthesis of aliphatic nitriles and amines starting from biorenewable resources. In this project saturated and unsaturated fatty acids are chosen as renewable feedstock for nitrile and amine synthesis in chemoenzymatic cascades (Scheme 6), avoiding the use of toxic cyanide salts and harsh reaction conditions.



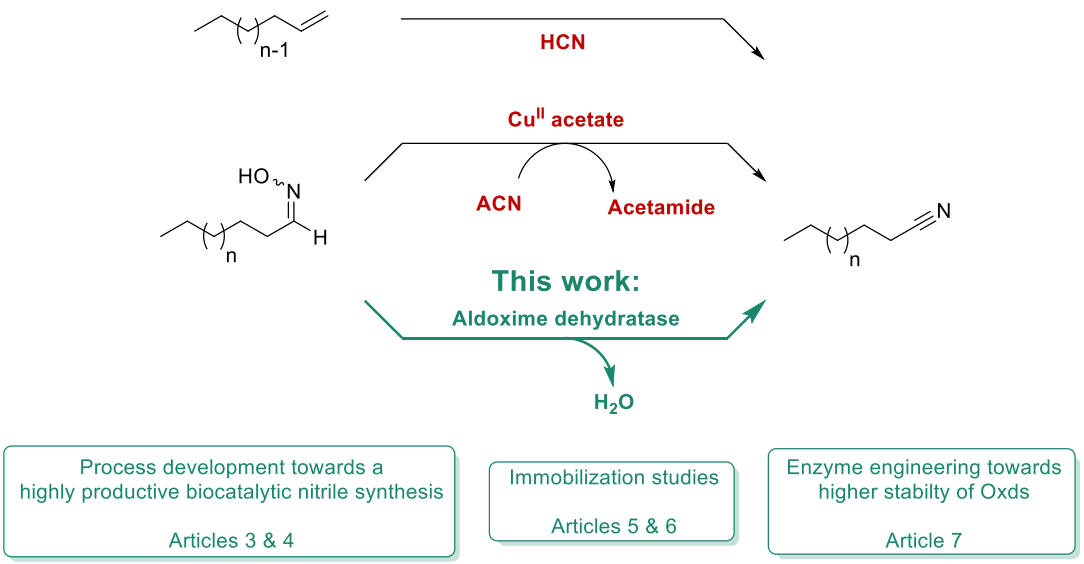
Scheme 6. Overview of chemoenzymatic cascades towards aliphatic nitriles starting from saturated or unsaturated fatty acids as starting materials investigated in this thesis.

Nowadays fatty acids can be already used as starting material for aliphatic nitrile and amine synthesis. However, amides are synthesized under harsh reaction conditions beforehand and dehydrated using high reaction temperatures.[4,5,47] Thus, the goal of this thesis is to develop alternative syntheses towards nitriles and amines starting from fatty acids, being less energy-consuming and more sustainable. Three different cascades are investigated, which are

highlighted in different colors in Scheme 6 (Cascade 1 (blue), Cascade 2 (red) and Cascade 3 (green)). Cascade 1 (Article 2-8) starts from saturated fatty acids by a hydrogenation to the corresponding alcohol. This step is very well known and is not investigated in this work. [51,52] Aliphatic alcohols are oxidized by a nitroxyl radical-catalyzed oxidation to the corresponding aldehydes in alternative solvents (Article 2), which are afterwards condensed with hydroxylamine hydrochloride in the presence of sodium carbonate to the corresponding aldoxime. In case of the nitroxyl radical-catalyzed oxidation of alcohols to aldehydes the major goal of this thesis is to investigate an alternative method, which do not require dichloromethane as solvent, which is nowadays state-of-the-art of this reaction.[48,49,53] The aldoximes are subsequently dehydrated by aldoxime dehydratases to the aliphatic nitriles by different methods. On the one hand, an aqueous approach is used (Article 3 & 4) and on the other hand, usage of these enzymes in pure organic medium by using an immobilized catalyst in superabsorber is investigated (Article 6). In a combination of all steps into one cascade the major goal is that the aldehydes and aldoximes, formed as intermediates, do not have to be isolated from the reaction medium and two work-up steps, isolation and purification are omitted (Article 8). In Cascade 2, unsaturated fatty acids can be used as starting material after initial cross-metathesis reaction with ethylene. This cross-metathesis process is already well known and an established process for many different unsaturated fatty acids,[54] wherefore this step is not investigated in this thesis. The alkenes can undergo hydroformylation reactions to form aldehydes, which basically can be used for the same cascade as described for Cascade 1 without the TEMPO-oxidation step. In this approach (Article 9) the goal is to establish a cascade without isolation of the aldoxime using a one-pot approach with removal of residual hydroxylamine of the condensation step by heat. Cascade 3 also starts from unsaturated fatty acids and goals the synthesis of bifunctional molecules as potential polymer building blocks. Within this cascade, two different approaches are used resulting in different products (Article 10). By a metathesis reaction with ethylene as described above for Cascade 2, the fatty acids would result in an aliphatic alkene, but also in an alkene with a terminal carboxylic acid functionality. This functionalized alkene can be utilized for hydroformylation reaction to obtain aldehydes, which can undergo the same cascade reaction towards carboxylic acid-functionalized nitriles. Secondly, the unsaturated fatty acids can directly be used for hydroformylation to give branched aldehydes with an alkyl chain. These aldehydes can be used for the same cascade to give branched and functionalized nitriles.

The key steps of all cascades presented in Scheme 6 is the biocatalytic dehydration of aldoximes to nitriles (Scheme 7). In comparison to established nitrile syntheses, for example

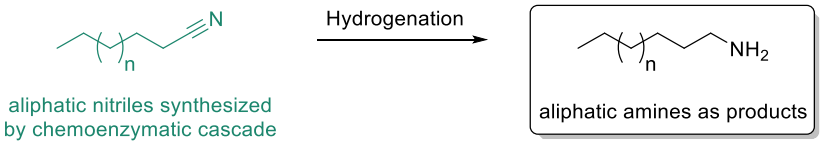
hydrocyanation and transition metal catalyzed dehydration, the biocatalytic dehydration does not require toxic cyanide salts, transition metals or a cosubstrate. Since the target products of this thesis, aliphatic nitriles and amines, are *high-volume-low-price* chemicals, a major topic of this thesis is the process development of the key step of enzymatic dehydration, as most enzymes today are not yet productive and stable enough to be used in bulk segment in the chemical industry.



Scheme 7. Key step of Cascades 1-3: Biocatalytic dehydration of aldoximes to nitriles by usage of aldoxime dehydratases.

Especially high substrate loadings are necessary for a highly productive process and have not been evolved for aldoxime dehydratases for this substance class yet, except for very early works of the Asano group in the 1990th.[30] Besides the process development towards high substrate loadings for an efficient biocatalytic synthesis of nitriles and immobilization studies for an easy separation and recycling of the biocatalyst, a directed evolution study is performed to obtain Oxd-variants with higher stability (Article 7).

All these cascades can be completed by a hydrogenation of the nitrile functionality to the primary amine (Article 11).



Scheme 8. Nitrile hydrogenation towards primary amines.

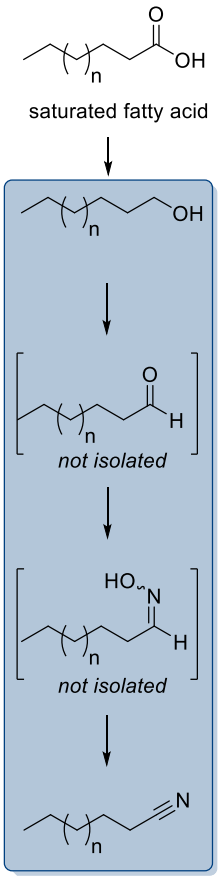
Within this work, different heterogeneous and homogeneous hydrogenation catalysts are screened. Lastly, a new manganese catalyst is developed, being highly suitable for the selective hydrogenation of nitriles to primary amines. Within this study, a heterogeneous palladium-substituted cerium-tin oxide catalyst is tested for hydrogenation reactions in general (Article 12).

3 Aliphatic nitrile and amine syntheses starting from biorenewables

3.1 Cascade 1 (Articles 2-8)

Towards aliphatic nitriles from saturated fatty acids

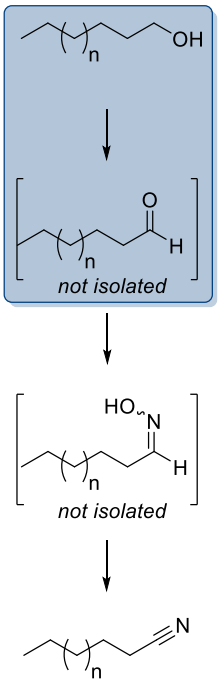
Existing processes for aliphatic nitrile syntheses are started from petrochemical feedstocks, which should be avoided in future processes. Starting from fatty acids as biobased material usually the fatty acid amides are synthesized initially by a tedious and energy intense process.[47] These amides are afterwards dehydrated to the corresponding nitriles using harsh reaction conditions.[4] For this reason, the development of an alternative access to aliphatic nitriles was goal of this thesis. A chemoenzymatic cascade was developed from fatty acids to aliphatic nitriles, in which the intermediates are not isolated or purified. Skipping isolation and purification steps in a cascade saves solvent, time and energy. In Cascade 1, saturated fatty acids were chosen as biorenewable material, which initially are hydrogenated to the aliphatic alcohols. This step of the cascade was not investigated, since this step is already well known.[51,52] The aliphatic alcohols are, in a first step, oxidized to the corresponding aldehydes, which are subsequently condensed with hydroxylamine to form aldoximes. Aldoximes serve as substrates for the biocatalytic dehydration towards nitriles using Oxds as catalysts. For the first step of the cascade, a nitroxyl radical-catalyzed oxidation method based on Anelli-type TEMPO-oxidation with hypochlorite as oxidation agent was developed. Considering that this cascade should be a more sustainable alternative to the existing nitrile production processes, it was decided to use this oxidation method. Details of this step were published by the author of this thesis and her coauthors in *European Journal of Organic Chemistry* (Article 2)[55] and are explained in Chapter 3.1.1. The second part of the cascade is a condensation step towards aldoximes and subsequent dehydration by Oxds. The biocatalytic dehydration was first investigated in aqueous reaction medium (Chapter 3.1.2), which is the standard for biotransformations since the native environment of enzymes is usually aqueous and the stability is higher compared to in the presence of organic media. These results were published



by the author of this thesis and her coauthors in *Journal of Organic Chemistry* (Article 3)[56] and afterwards also summarized in a book section in *Applied Biocatalysis: The Chemist's Enzyme Toolkit* (Article 4).[57] To facilitate separation and recycling of the catalyst, an immobilization study of purified Oxds and Oxds in whole cells was performed for the use in aqueous reaction media (Article 5).[58] In order to be able to incorporate the biocatalysts in the later combined process without isolation and purification of the intermediates, an immobilization method for Oxds in whole cells was also developed, making its use in a purely organic medium possible (Chapter 3.1.2.1). These results were published by the author of this thesis and her coauthors in *European Journal of Organic Chemistry* (Article 6)[59]. Finally, the steps were combined in a cascade without isolation and purification of the aldehyde and aldoxime intermediates by performing all steps in the product nitrile as solvent. Since the product nitrile was used as solvent and all transformations resulted in high conversions and selectivities even the step of isolation and purification of the product after the last step of the cascade was eliminated (Chapter 3.1.3). The results of this cascade were submitted to *ACS Sustainable Chemistry & Engineering* (Article 7).[60]

3.1.1 Nitroxyl radical-catalyzed oxidation of alcohols to aldehydes (Article 2)

The results of this chapter were published by the author of this thesis and her coauthors in European Journal of Organic Chemistry (Article 2).[55]



Cascade 1

In our study for a selective oxidation method from alcohols to aldehydes using the Anelli-type TEMPO-oxidation, an optimization of reaction conditions in terms of substrate concentration, phase transfer catalyst or additives and hypochlorite amount mentioned in the Supporting Information was performed by Jasmin Busch in her Master thesis.[61] An initial solvents study to overcome the limitation of classical TEMPO-oxidation using dichloromethane as solvent was performed by Jasmin Busch using the optimized conditions for *n*-octan-1-ol and *n*-decan-1-ol with dichloromethane, ethyl acetate, methyl-*tert*-butyl ether as solvent and under neat conditions. In all alternative solvents to dichloromethane conversions of >5% were found, wherefore, Sylvia Glinski performed an additional solvent screening based on these results using butyronitrile and *n*-octanenitrile in her bachelor thesis supervised by the author of this

thesis.[62] In this study nitriles were found to be as suitable as dichloromethane for the selective TEMPO-oxidation of *n*-octan-1-ol to *n*-octanal. Based on these initial experiments of Jasmin Busch[61] and Sylvia Glinski[62] a comparative solvent study of all tested solvents and additional nitrile solvents was performed showing that all nitrile solvents were found to be suitable for this type of oxidation with high selectivities. Especially *n*-octanenitrile was found to be suitable, which was later the basis of our concept to combine all steps on the way to nitriles into one cascade. Since TEMPO is a homogeneous catalyst, the catalyst was switched to PIPO,[63] a polymer-based TEMPO-derivative obtained from Chimassorb 944, which is a multi-ton scale product of chemical industry and is used for example as light stabilizer in plastics. PIPO is heterogeneous in a variety of solvents and may be recycled after the oxidation reaction. An additional solvent study on this catalyst was performed by using nitrile solvents in comparison to dichloromethane. High selectivities and conversions were obtained in butyronitrile or *n*-octanenitrile as solvent, being as high as in case of dichloromethane or even higher (Figure 3).

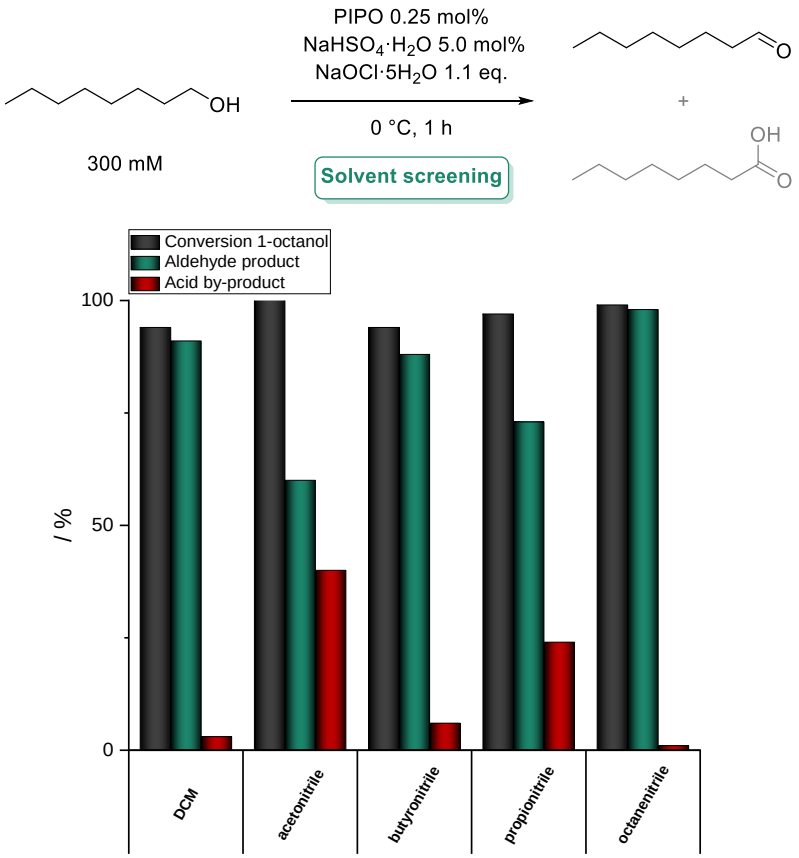


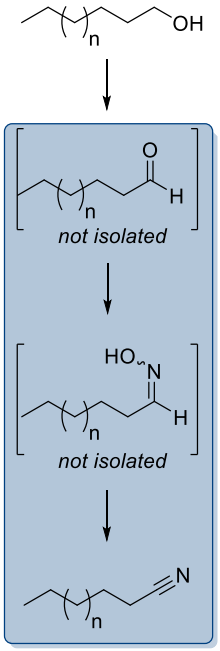
Figure 3. Solvent study for the PIPO-catalyzed oxidation of *n*-octan-1-ol to *n*-octanal. *This figure was taken from Article 2*.[55]

Based on this newly established method in butyronitrile as alternative solvent to dichloromethane was used for the oxidation of a variety of primary alcohols. Ten primary alcohols were successfully oxidized to the corresponding aldehydes using the PIPO-catalyzed oxidation method in butyronitrile with selectivities >91% in all cases. We broaden the substrate scope also to the oxidations of diols to dials. These experiments were performed by Michael Stricker in his doctoral thesis. To show that this method is also applicable for the oxidation of secondary alcohols to ketones four different secondary alcohols were successfully oxidized using our established method.

This work showed that the green oxidation method using nitroxyl radical-catalysts and hypochlorite as oxidation agent can be applied for the selective synthesis of aliphatic aldehydes in nitriles as solvents. Within this study, a general method for alcohol oxidation was developed being applicable to a variety of alcohols and in nitriles as solvents as an alternative to dichloromethane. These results are the basis of the Cascade 1, which starts with this oxidation to obtain the aldehyde as precursor for aldoximes.

3.1.2 Biocatalytical synthesis of aliphatic nitriles from aliphatic aldehydes via aldoximes (Article 3 & 4)

The results of this chapter were published by the author of this thesis and her coauthors in Journal of Organic Chemistry *(Article 3)*[64] *and were summarized in a book chapter in* Applied Biocatalysis: The Chemist's Enzyme Toolkit *(Article 4).*[57]



Cascade 1

The next important intermediates in the cascade to aliphatic nitriles are the aldoximes, which can be obtained very easily by condensation of the aldehydes obtained from alcohols by nitroxyl radical-catalyzed oxidation, and hydroxylamine. For safety reasons hydroxylamine is formed *in situ* from hydroxylamine hydrochloride in the presence of sodium carbonate. Using this method in aqueous reaction medium and subsequent filtration of the products, aliphatic aldoximes were obtained in a very pure form in isolated yields of 63 -96%. After successful synthesis of the aldoximes, the next step is the biocatalytic dehydration using Oxds to the nitriles. Aldoxime dehydratases were already found to be active for aliphatic aldoximes,[30] but high substrate loadings which are required in industrial processes for bulk chemical syntheses were not tested so far. The process optimization towards

a highly productive nitrile synthesis using Oxds were performed in this thesis. Initial studies showed that aldoximes of chain length >12 were not or only in small extent converted by aldoxime dehydratases to the corresponding nitriles. Furthermore, OxdB was found to be the most active enzyme for the conversion of aliphatic aldoximes, wherefore subsequent experiments were conducted exclusively with this enzyme. Since aldoxime dehydratases were found to be relatively unstable,[30] biotransformations were performed with OxdB in whole cells as catalyst.

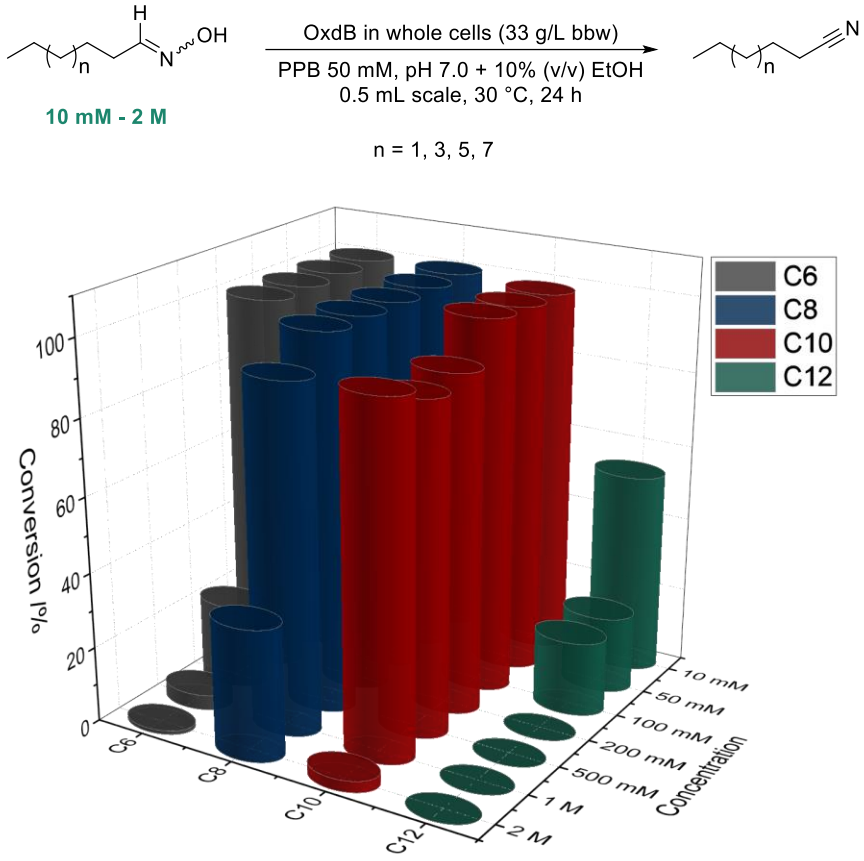
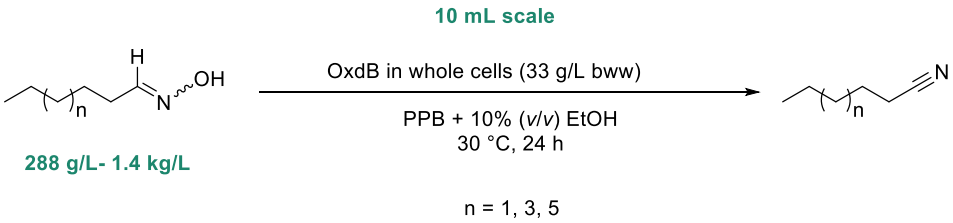


Figure 4. Screening of conversion of various aliphatic aldoximes into the corresponding nitriles using the enzyme OxdB in whole cells on a 0.5 mL scale. *This figure was taken from Article 3.*[64]

After an initial screening using different substrates and substrate loadings in small scale (Figure 4), 10 mL-scale experiments with isolation of the nitrile products were performed with C6-, C8- and C10-aldoximes. Very high conversions and yields were obtained with substrate loadings of up to 1.4 kg substrate per liter of aqueous reaction medium (Table 1). Besides the very high productivity of these biotransformations using OxdB in whole cells, also the product separation is very simple. After the reaction, an easy phase separation can be performed of the aqueous phase including OxdB whole cells and the nitrile product. Based on these results, an

up-scaling experiment for *n*-octanenitrile was performed by Sylvia Glinski, resulting in >182 g of pure *n*-octanenitrile from 250 mL aqueous reaction medium.[62]

Table 1. Summary of 10 mL-scale biotransformation experiments using C6-, C8-, and C10-aldoximes.



#	n	Final substrate loading	Conversion /%	Isolated yield /%
1	**1**	288 g/L	>99	81
2	**3**	665 g/L	>99	98
3	**3**	**1.4 kg/L**	**93**	--
4	**5**	342 g/L	>99	84
5	**5**	428 g/L	93	--

These results clearly show the potential of OxdB as whole cells catalyst for the dehydration of aliphatic aldoximes within in our planed cascade. In the following, the dehydration step was further optimized towards an easier implementation into Cascade 1 without the isolation of intermediates.

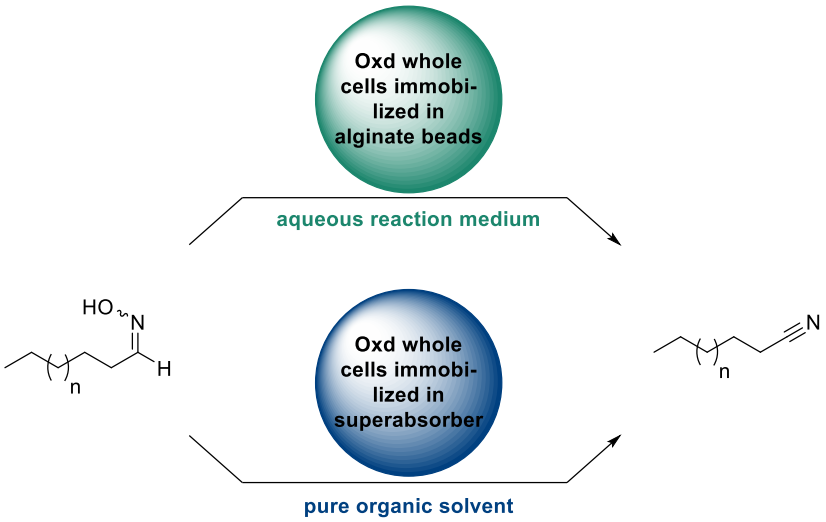
3.1.2.1 Immobilization of OxdB and OxdRE for the use in aqueous reaction media (Article 5) and in pure organic medium (Article 6)

The results of this chapter were submitted by the author of this thesis and her coauthors to Catalysts *(Article 5).*[58] *The results of the superabsorber immobilization was published by the author and her coauthors in* European Journal of Organic Chemistry *(Article 6).*[59]

In Chapter 3.1.2 described and reported in Articles 4 and 5 Oxds can be used for the efficient synthesis of aliphatic nitriles from aldoximes. For a potential recycling of the biocatalyst or even simpler separation of the biocatalyst from the product, heterogenization of the biocatalyst would be a favorable option. For this purpose, different immobilization techniques for the immobilization of OxdB and OxdRE were tested using purified enzymes and whole cell catalysts. First, different immobilization carriers were investigated, which immobilize free

enzymes by hydrophobic interactions (three different were tested) or covalently (two epoxy carrier and one amino carrier were tested). It was found that the residual activity after immobilization with high immobilization efficiencies (~80-90%) were <20% in all cases. Since the immobilization of purified enzymes showed low activity in all cases the focus was further on the immobilization of whole cells. For this purpose, the encapsulation of whole cells into calcium alginate beads[65–68] and absorbance into superabsorber[69,70] were tested. Using an immobilization strategy in calcium alginate beads compared to calcium alginate beads coated with tetraethyl orthosilicate (TEOS), OxdB and OxdRE in whole cells were successfully immobilized with residual activities of up to ~70%. TEOS-coating changes the polarity of the beads' surfaces from very polar into non-polar, facilitating diffusion of the non-polar substrate molecules into the beads.[65] Compared to the immobilization of purified enzymes, the residual activity of the whole cells entrapped in the alginate beads is remarkable. For both enzymes higher activities for the TEOS-coated beads in comparison to the uncoated beads were observed, which is probably due to a better diffusion of the substrate in the beads. It was found that the alginate beads, especially the TEOS-coated beads, are significantly more stable in ethanol-containing buffer with ~80% residual activity after 24 h incubation time in 10% ethanol of OxdB in TEOS-coated beads compared to ~20% for the free cells. Recycling of the beads led to decreased activity, however, the immobilizates can be used three times for 24 h reactions until a decrease of the activity to <85% is observed. An alternative immobilization-technique for OxdB in whole cells was published by the author in *European Journal of Organic Chemistry*.[59] Whole cells in buffer are absorbed into polyacrylate (superabsorber), resulting in an immobilized aqueous phase. These immobilizates can then be used in pure organic medium, preferably in very non-polar organic solvents like cyclohexane, for the conversion of aldoximes to nitriles. 500 mM of *n*-octanaloxime were quantitatively converted to the corresponding nitrile within 24 h in pure cyclohexane as reaction medium. Since Oxds in non-immobilized whole cells do not show any activity in pure organic medium and almost no conversion is found, when using whole cells in a classical biphasic medium, a stabilizing effect of the superabsorber is expected. These remarkable results show that very labile Oxds can also be used in pure organic medium by a simple immobilization in superabsorber, which facilitates the combination of all steps into a cascade starting from alcohols to nitriles. All immobilization methods presented and discussed in this chapter are the first examples for heterogenization of Oxds and the results, especially of whole cell immobilization (Scheme 9), show the applicability of Oxd immobilizates in aqueous and organic reaction media. The latter, made possible by

immobilizing whole cells in superabsorber, opens up completely new possibilities for this class of enzymes, which previously could only be used in aqueous media.



Scheme 9. Whole cell immobilization of Oxd-catalysts in calcium alginate beads (green) and in superabsorber (blue), being usable in aqueous reaction medium or pure organic solvent.

3.1.2.2 OxdRE-mutagenesis study (Article 6)

A manuscript about OxdRE mutagenesis study was submitted by the author of this thesis and her coauthors to ChemBioChem *(Article 7).*[71]

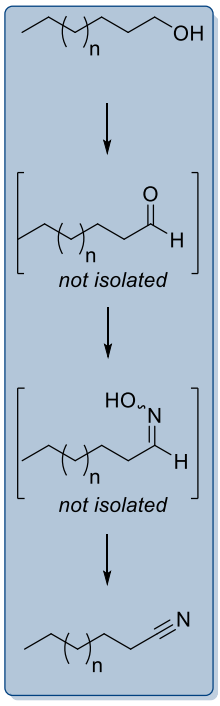
The low stability of aldoxime dehydratases, for example in the presence of high temperatures and organic solvents, is a well-known problem and is possibly a reason why this enzyme class is not used in industrial processes so far.[30,59] Besides immobilization (Chapter 3.1.2.1), which can stabilize biocatalysts and makes them easier recyclable, enzymes can also be engineered towards higher stability. Whether or not recycling is possible successfully without loss of catalyst activity is mainly influenced by the stability of the catalyst. A mutagenesis study was conducted to increase the stability. Here OxdRE was used, because the X-ray structure of this enzyme is solved and thus the changes by directed evolution are better understandable. After implementation of a color-assay for the screening of a mutant library, error-prone PCR was conducted. Indeed, an OxdRE-variant with 10 mutations was found, which showed increased stability by treatment with acetonitrile and at higher temperatures. This result shows, that the stability of Oxds can be engineered. Since for this mutagenesis study OxdRE was used, which is compared to OxdB even more instable in contact with solvents and at high temperatures, this

OxdRE-variant was not used for the further development of the cascade. Future enzyme engineering for stable Oxds might be performed with OxdB instead of OxdRE to obtain a very stable OxdB-variant.

3.1.3 Combination of steps into a chemoenzymatic cascade (Article 8)

The results of this chapter were submitted by the author of this thesis and her coauthors to ACS Sustainable Chemistry & Engineering *(Article 8).*[60]

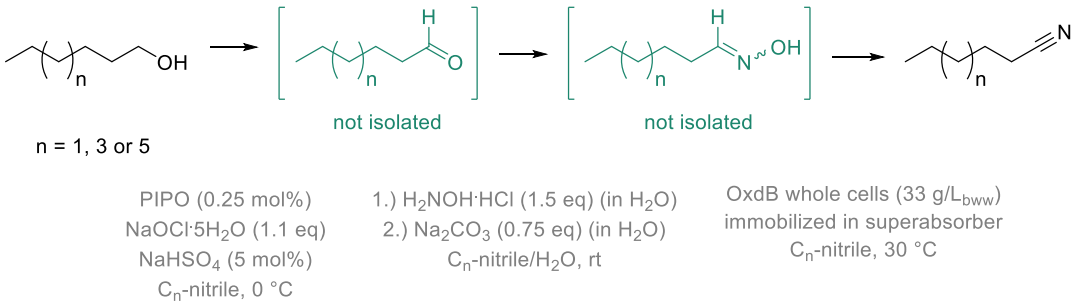
After the successful development of the single steps oxidation (Chapter 3.1.1), condensation and dehydration (Chapter 3.1.2), we had all in hand to develop a cascade which does not require isolation and purification of the intermediates. From the TEMPO-oxidation study it was known that this step can be performed in nitriles as solvent, however, the isolation of aldehydes is always a problem especially because of the oxidation sensitivity of these compounds. Therefore, a sequential one-pot two reaction process was developed in which first the oxidation step is performed by using PIPO as catalyst, hypochlorite as oxidation agent and butyronitrile as solvent. After full conversion, an aqueous phase is added, which contains hydroxylamine hydrochloride and sodium carbonate. In this two-phase system for condensation, the great advantage is that the product aldoxime is dissolved in butyronitrile and residues of hydroxylamine, which is toxic to the biocatalyst OxdB, remain in the aqueous phase. After phase separation and removal of the solvent isolated yields of 64-70% were achieved for C6-, C8- and C10-aldoximes. In the final step, after removal of the organic solvent, OxdB in whole cells converted the aldoximes in an aqueous reaction medium to the corresponding nitriles, with isolated yields over all three steps of 50-64%. This sequential one-pot two reaction and subsequent biotransformation process was a first big step on the way to a chemoenzymatic cascade without any isolations and purification. In the study of OxdB in whole cells immobilized in superabsorbent it was shown that these are also active in pure organic medium. These results indicate that the cascade can be performed without any isolation of the intermediates. Since it was found that *n*-octanenitrile is a suitable solvent for TEMPO oxidation our idea was that *n*-octanenitrile could be used as a solvent directly from the first step on. Indeed, when using the product nitrile directly for the oxidation step full conversion and high selectivities (99%) were



Cascade 1

achieved. In the further course of the cascade, the aqueous phase with hydroxylamine hydrochloride and sodium carbonate was then added for the aldoxime synthesis and the phases were subsequently separated from each other. This organic phase consisting of *n*-octanaloxime dissolved in *n*-octanenitrile, was then used directly for reaction with OxdB in whole cells immobilized in superabsorbers. This process was analogously performed for C6- and C10-nitrile in the product nitriles as solvent. Isolated yields of 63-67% were obtained over this three-step process. In this cascade no isolation of the aldehyde and aldoximes, formed as intermediates, is necessary, only a phase separation on aldoxime stage needs to be performed to separate excess hydroxylamine dissolved in the aqueous phase. Even a purification or solvent removal of the product is not required since the whole cascade is performed in the nitrile product as solvent. All three steps result in quantitative conversion and very high selectivities of ≥99%, wherefore a simple filtration step of the superabsorbed biocatalyst is sufficient to obtain the pure nitrile.

Table 2. Chemoenzymatic cascade reaction towards aliphatic nitriles without isolation of aldehyde and aldoxime intermediates using immobilized OxdB whole cells. *This Table was taken with minor changes from Article 8.*[60]



n =	Conversion of alcohol	Conversion aldehyde to aldoxime	Conversion aldoxime to nitrile	Isolated yield over 3 steps
1	97% Aldehyde (1% Acid)	>99%	>99%	63%
3	99% Aldehyde (1% Acid)	>99%	>99%	70%
5	99% Aldehyde (1% Acid)	>99%	>99%	67%

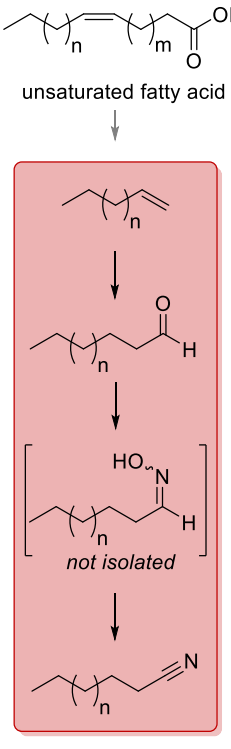
All in all, a very productive and easy three-step chemoenzymatic cascade was designed and implemented for aliphatic nitriles of chain length of C_6, C_8 and C_{10}. Overall yields of up to 70% could be obtained without isolation of the intermediates, with just one phase separation step and in the product as solvent. This cascade provides a new alternative access to aliphatic nitriles starting from fatty acids as bio-based material with high conversions, selectivities and yields.

3.2 Cascade 2 (Article 9)

Approaching Bulk Chemical Nitriles from Alkenes: A Hydrogen Cyanide-Free Approach through a Combination of Hydroformylation and Biocatalysis

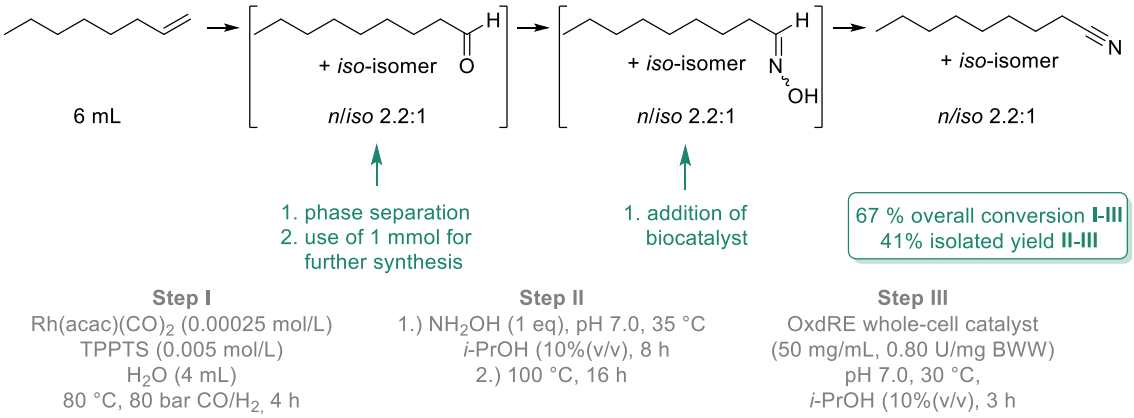
Results of this chapter are published by the author of this thesis and her coauthors in ACS Catalysis.[72]

Another example to synthesize aliphatic nitriles from biorenewable starting material, namely Cascade 2, was investigated in this thesis. Starting from unsaturated fatty acids by cross-metathesis with ethylene,[54] unsaturated aliphatic compounds can be obtained, which also serve as precursors for aldehydes and thus to produce aliphatic nitriles. A major advantage of unsaturated fatty acids as starting material is that not only unsaturated alkenes but also terminally unsaturated carboxylic acids are formed during cross-metathesis with ethylene, which can serve as a starting point for the synthesis of polymer building blocks. This was investigated within Cascade 3 (Chapter 3.3) of this thesis. Alkenes can easily be converted by the well-established process hydroformylation with syn-gas, resulting in aldehydes with one additional carbon atom. In this study the author of this thesis, her coworkers from the Gröger group and coworkers from the Vorholt group designed a chemoenzymatic cascade from alkenes to nitriles, exemplified for *n*-oct-1-ene to *n*-nonanenitrile including a hydroformylation step and Oxds. For this purpose, a screening was performed by the author of this thesis, which Oxds can convert the aldoxime mixture consisting of *n*-nonanaloxime and 2-methyl-octanaloxime, which is formed after condensation with hydroxylamine from the hydroformylation by-product (*branched* aldehyde). The screening showed that all Oxds (OxdA, OxdB, OxdFG, OxdRE and OxdRG) are active for the conversion of *n*-nonanaloxime, however, OxdFG showed the lowest conversion. 2-Methyl-octanaloxime was also converted by all Oxds, however, in this case the conversions were lower compared to *n*-nonanaloxime. Especially OxdB and OxdRG showed less conversion compared to the linear aldoxime, although OxdA and OxdRE showed full conversion of the branched aldoxime. In the cascade from alkenes to nitriles, the hydroformylation step was performed in a biphasic reaction medium consisting of water including rhodium salt



Cascade 2

(Rh(acac)(CO)$_2$) and triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt (TPPTS) as ligand and *n*-oct-1-ene. An optimization for this step was performed by Michael Terhorst of the Voholt group. After hydroformylation a phase separation was performed to obtain the product (organic phase) as well as the catalyst (aqueous phase). The aldehyde mixture is then used for the condensation with hydroxylamine hydrochloride in the presence of sodium carbonate. In this case, residual hydroxylamine in this case is removed by heating overnight before usage of the crude mixture for the biotransformation step (Scheme 10). The whole cascade presented in Article 9 was performed by the author of this thesis in cooperation with Carmen Plass.



Scheme 10. Chemoenzymatic cascade from *n*-oct-1-ene to *n*-nonanenitrile.

67% overall conversion over three steps and 41% isolated yield over the last two steps were achieved for this cascade.

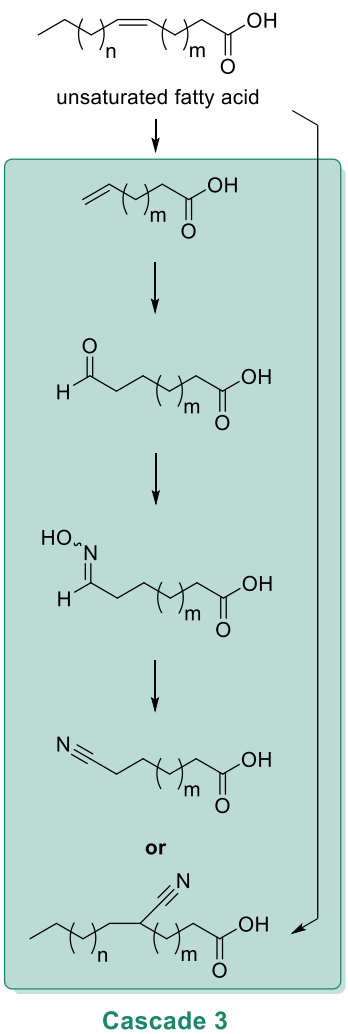
These results show that the “two worlds” of high-pressure reactions, namely hydroformylation and biocatalysis, can be merged into one cascade with good results. The cascade is very simple because of the easy separation of the hydroformylation catalyst by phase separation. Furthermore, the aldehydes and aldoximes, formed as intermediates, do not have to be purified during the cascade and a heating step is sufficient to remove residual hydroxylamine from the condensation step, which is also from a process point of view feasible. Starting from unsaturated fatty acids as biorenewable material only two isolation steps of intermediates are necessary, namely the isolation of the alkenes after metathesis of unsaturated acids with ethylene and the nitrile product after hydroformylation, condensation and dehydration. This concept principally can compete with the petrochemical-based nitrile synthesis starting from alkenes after steam-cracking. They are usually hydroformylated as well and afterwards a hydrocyanation is performed to produce the nitrile from the aldehydes. A major disadvantage of this process is the need of toxic cyanide salts, which is circumvented in the herein presented Cascade 2.

3.3 Cascade 3 (Article 10)

From biorenewables to industrial applicable bifunctional molecules using chemoenzymatic transformations of bioderived unsaturated fatty acids

The results of this chapter were submitted by the author of this thesis and her coauthors to Sustainable Chemistry *(Article 10).*[73] *Experiments were performed by the author of this thesis and Selina Sophie Druhmann*[74] *(Bachelor stundent supervised by the author of this thesis).*

When starting from unsaturated fatty acids a variety of different compounds can be synthesized. As described in Chapter 3.2 after cross-metathesis with ethylene alkenes are obtained, which can be hydroformylated to aldehydes for example and used in the presented cascade to nitriles. However, by doing cross-metathesis with unsaturated acids and ethylene also the unsaturated acid is produced, which can be used for the synthesis of polymer building blocks. Even without metathesis the unsaturated fatty acids directly can be hydroformylated to gain branched compounds as polymer building blocks. These two ways of synthesizing polymerizable compounds were studied and the chemoenzymatic cascade was transferred to these substrates.



Cascade 3

When starting from acids carrying a terminal double bond hydroformylation was performed to produce aldehydes. C6-, C8- and C10- unsaturated acids were used as model substrates and hydroformylation of these compounds using Rh-TTPTS as catalytic system was optimized for these substrates in terms of reaction time, pressure and temperature. Without isolation of the aldehydes, hydroxylamine hydrochloride and sodium carbonate were added to the crude biphasic hydroformylation mixture after completion. Fortunately, it was found that the linear aldoximes precipitated from the reaction medium, while the branched aldoxime remained in the filtrate when isolating the linear aldoximes. Therefore, pure linear aldoximes were used for a screening of Oxds converting them into nitriles. 50 mM of the aldoximes could be converted completely by OxdB, however, to have a productive process higher substrate loadings are needed. For this reason, Cu^{II} acetate catalyzed nitrile synthesis was

performed using acetonitrile as cosubstrate, resulting in the nitriles in good yields of 71-89% using 500 mM substrate concentration. These nitriles could subsequently be hydrogenated to the amines and the resulting amino acids represent polymerizable compounds, being suitable for a variety of polyamides. Oleic acid itself was hydroformylated, first with the Rh-TPPTS biphasic system, resulting in very low conversion to the aldehyde. Thus, a monophasic Rh-triphenylphosphine (TPP) approach was applied using oleic acid as solvent and substrate. Herein, full conversion was obtained and the aldehyde was converted directly to the aldoxime in a sequential one-pot reaction. Since Oxds do not accept such bulky substrates, the nitrile synthesis was solely performed using Cu^{II} acetate and acetonitrile with a substrate concentration of 500 mM. Also this nitrile was obtained in high yields of 91%, being a suitable substrate for hydrogenation to the amine.

Using a modification of our chemoenzymatic cascade, unsaturated fatty acids were turned into bifunctional molecules that can be used as polymer building blocks. This process is simple and achieves good yields with no by-products, which makes it an attractive alternative to existing routes.

3.4 Artificial hydroformylase

Usually used catalysts for hydroformylation are rhodium or cobalt metals in combination with phosphine or phosphite ligands. The ligands control the selectivity of the reaction in terms of the ratio of linear to branched and by-product amounts (especially of formed alcohols) during the hydroformylation process. Another way to achieve high selectivity would be, for example, the use of enzymes, as they can often generate high selectivities due to their overall structure. There are already some examples in which enzymes were used for hydroformylation, [75–78] however, in these cases only small turnover numbers were obtained. In this work, an artificial hydroformylation with high selectivities and turnover numbers should be developed. For this purpose, two different approaches were investigated in a collaboration project together with Michael Stricker, namely an artificial hydroformylase based on the streptavidin-biotin system (Chapter 3.4.1) and an artificial hydroformylase based on a cytochrome P450 monooxygenase from *Sulfolobus acidocaldarius* (strain ATCC 33909) (CYP119) (Chapter 3.4.2). Compared to literature known artificial hydroformylases both approaches are based on very different concepts. In case of the streptavidin-biotin approach the big advantage is, that a ligand system

for the catalytically active rhodium species, which is very close what typically is used for hydroformylation can be applied. In case of the metal-exchange approach of iron to rhodium in the CYP119 monooxygenase, the native enzyme is highly stable to pressure and high temperatures, which should enable the usage of this enzyme under hydroformylation conditions without inactivation of the hydroformylase. Compared to the known hydroformylases, for example published by the Kazlauskas group in 2010, the ligand system for the rhodium is very different to "classical" hydroformylation catalysts.[77] The rhodium is bound to a carbonyl anhydrase via three histidine residues in the protein and resulting in low turnover for hydroformylation reactions. Previous works on artificial hydroformylases are even based on non-specific binding of rhodium to human serum albumin (HSA), resulting in very unspecific catalysts for hydroformylation (high amounts of branched aldehyde product) and low turnover numbers.[78] The Kamer group developed an artificial hydroformylase based on a fatty acid transporter protein in which they metal-binding phosphine ligands are bound to the protein and enables hydroformylation with rhodium in a "classical"-like ligand sphere of the metal.[75,76] Since the transporter protein is not very stable and the activity needed to be evolved to obtain high turnover numbers, our approaches based on streptavidin and the temperature and pressure stable CYP119 monooxygenase seemed to be promising concepts to develop artificial hydroformylases with high turnover numbers and selectivities.

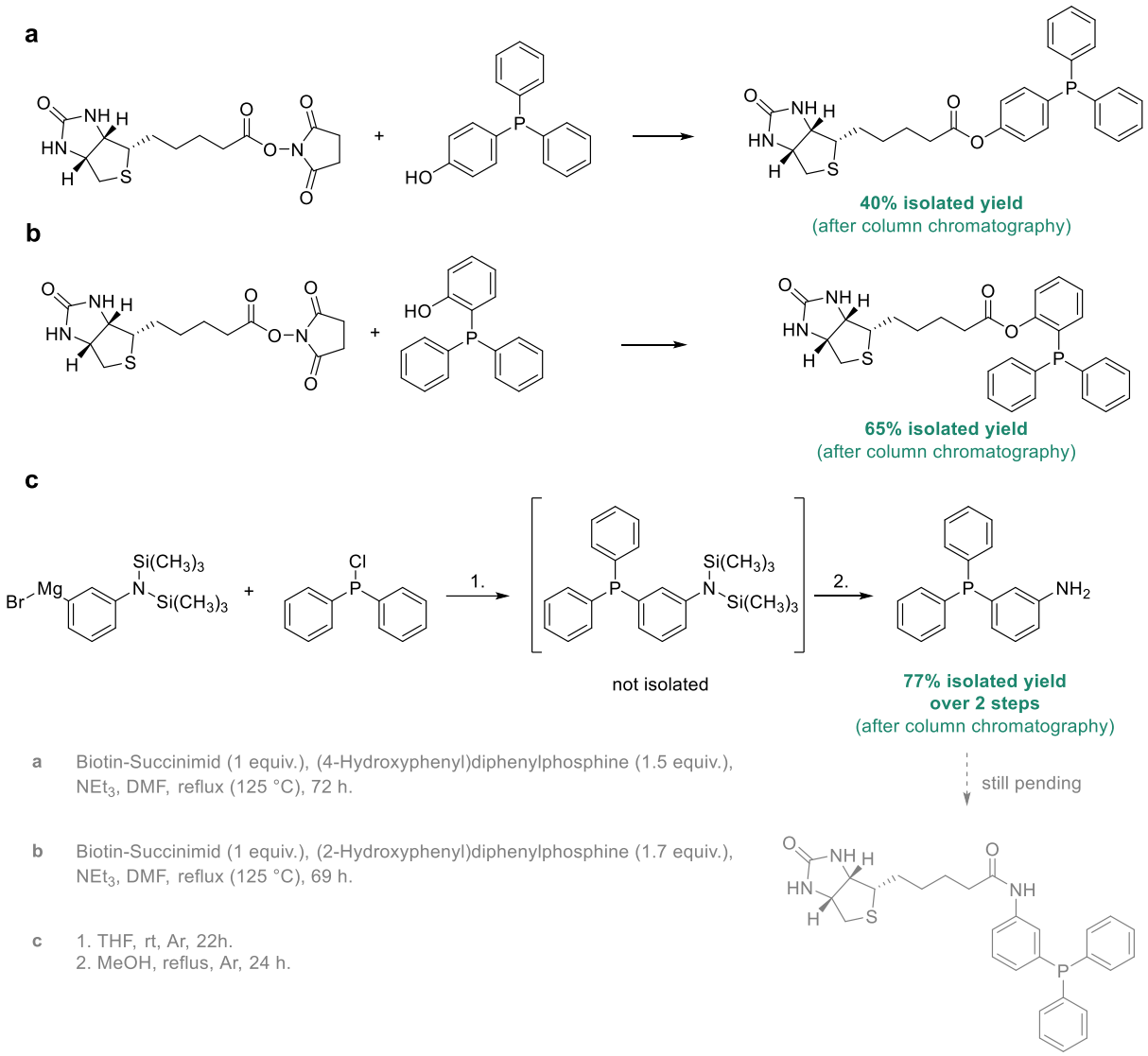
3.4.1 Streptavidin-biotin approach

The experimental methods and data of these unpublished results are presented in Chapter 6.9.

Artificial metalloenzymes based on the streptavidin-biotin system are frequently used because they are conceptionally easy to prepare due to the strong binding of biotin to streptavidin.[79–81] Biotin can be derivatized with different residues, which can act as ligand for a metal, being essential for catalytic activity. Metalloenzymes based on streptavidin-biotin were established in the 80th and used for many different new enzymatic reactions.[81] In this thesis, biotin was coupled to different phosphines, which should act in the complex of phosphine-substituted biotin with streptavidin as ligand for Rh^{I} having the catalytic activity for hydroformylation. A coupling of biotin to phosphine ligands was already shown to be successful for the design of an "artificial suzukiase" based on the streptavidin-biotin system,[82] which shows that this concept seems to be possible in principle. Furthermore, goal of this thesis was to find hydroformylation

conditions for *n*-oct-1-ene with rhodium in presence of phosphine ligands, which could be suitable for a streptavidin-biotin-based hydroformylase in terms of temperature, pressure and reaction time. These results should serve as basis for further investigations on this hydroformylase approach by Michael Stricker in his doctoral thesis.

In a first step, biotin-derivatives were synthesized to obtain phosphine-substituted biotins (Scheme 11).

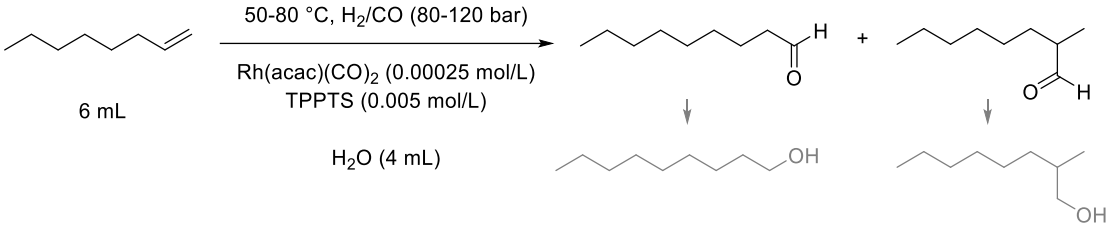


Scheme 11. Ligand synthesis for streptavidin-biotin approach.

Coupling of (4-hydroxyphenyl)diphenylphosphine and (2-hydroxyphenyl)diphenylphosphine with biotin was already successful with isolated yields of 40 and 65% after column chromatography. (3-Aminophenyl)diphenylphosphine was not coupled to biotin yet. The biotin-coupled phosphine ligands can now be bound to streptavidin and screened for hydroformylation activity by complexing rhodium as a metal.

Since streptavidin has a melting point of ~75-83 °C[83] the conditions used for *n*-oct-1-ene hydroformylation using Rh-TPPTS cannot be applied (80 °C, 80 bar). For this reason, a screening for alternative conditions was conducted (Table 3).

Table 3. Hydroformylation conditions of *n*-oct-1-ene hydroformylation at various reaction temperatures, pressure and time.



#	*T* /°C	*p* /bar	*t* /h	Conv. /%[a]	Not-defined impurities /%[b]	*n*-Aldehyde /%[c]	*iso*-Aldehyde /%[c]	*n*-Alcohol /%[b]	*iso*-Alcohol /%[b]
1	80	80	4	67	1	45	21	<1	<1
2	80	100	4	85	6	46	27	4	3
3	70	80	4	38	<1	25	13	<1	<1
4	70	100	4	65	2	39	20	2	1
5	60	80	4	23	<1	14	8	<1	<1
6	60	100	4	39	1	24	13	<1	<1
7	60	100	4	6	<1	4	2	<1	<1
8	60	120	4	29	<1	18	11	<1	<1
9	60	110	20	93	6	42	28	10	7
10	60	120	20	96	6	44	31	9	6
11	50	120	4	9	<1	5	3	<1	<1
12	50	120	20	57	3	27	18	5	4

[a] *Conversions were determined by GC-analysis. GC-areas of octene were compared to all other areas of substances.*
[b] *Amounts were determined by comparison of the GC-areas of this compound to all other areas of substances in the chromatogram.*
[c] *Amounts of aldehyde were determined by comparison to standard-curves and to all other areas of substances in the chromatogram.*

The temperature can be lowered to 50 °C by increased pressure up to 120 bar resulting in 57% conversion and comparably low by-product formation. These conditions might be used for hydroformylation reactions using an artificial hydroformylase based on streptavidin-biotin. Since for hydroformylation only very low catalyst-loadings per substrate amount is needed, this hydroformylation reaction is a very promising reaction for the design of a catalytically active metalloenzyme. Usually very high amounts of metalloenzymes need to be used for various

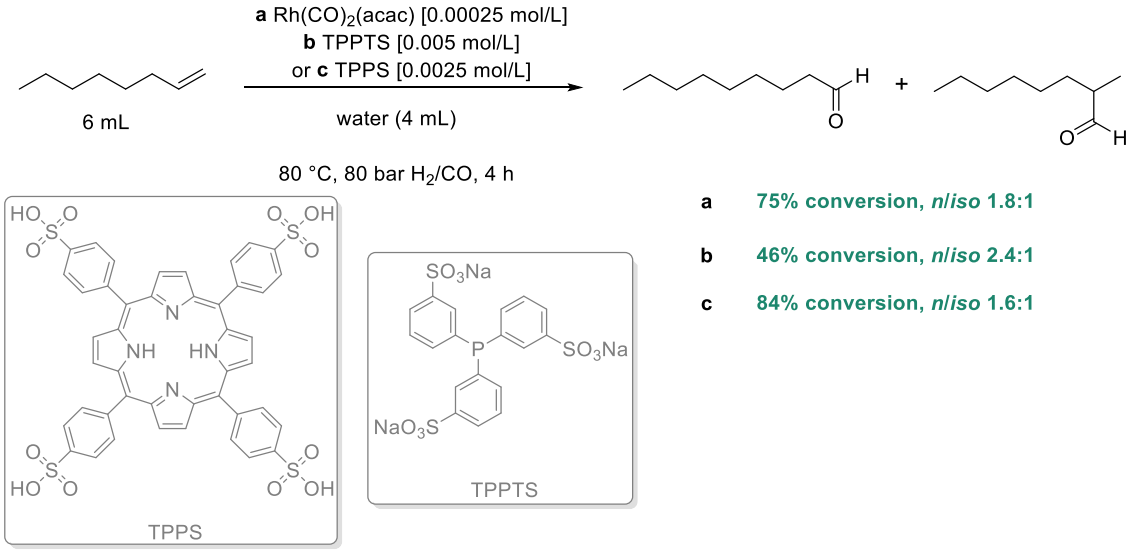
reactions, for example in case of the artificial suzukiase 1 mol% catalyst is needed for the cross-coupling reaction catalyzed by the metalloenzyme.[82] In hydroformylation reactions, catalysts are typically used in ppm-scale based on the amount of substrate, which enables usage of very low amounts of metalloenzyme.

The biotinylated phosphine ligands and hydroformylation conditions serve as a basis for subsequent experiments of Michael Stricker during his doctoral thesis.

3.4.2 Metal-exchange approach of CYP119 monooxygenase

The experimental methods and data of these unpublished results are presented in Chapter6.10.

Another approach towards an artificial hydroformylase is based on a CYP119 monooxygenase, which is a temperature and pressure stable P450 monooxygenase. Because the stability for both, temperature and pressure is very high,[84,85] this enzyme has best starting conditions to produce a hydroformylase, which must be stable and active at high temperatures and high pressures. This project is investigated in a collaboration project with Michael Stricker, who is focusing on the development of the metalloenzyme, while goal of this thesis was the investigation of rhodium porphyrin complexes for hydroformylation and characterization of these complexes.



Scheme 12. Hydroformylation of *n*-oct-1-ene using Rh with TPPTS as phosphine ligand or with TPPS as porphyrin ligand.

The CYP119 monooxygenase was already used for the design of an artificial metalloenzyme by an exchange of the iron heme center by an iridium heme complex, resulting in a metalloenzyme being active for insertions of carbenes into C–H bonds with high activities.[85] Since CYP119 monooxygenase is a heme enzyme, naturally harboring an Fe^{II}-heme b complex in their active site, the easiest way to implement a rhodium ion into this enzyme is by exchanging the metal or the full complex to Rh-heme b. However, hydroformylation is usually performed using phosphine ligands rather than porphyrin ligands, whereas the activity of this complex is needed to be evaluated first (Scheme 12). It was found that the activity of Rh in the coordination with tetraphenylporphyrin tetrasulfonic acid (TPPS) is lower (46% conversion vs. 75%) compared to Rh-TPPTS, however, it is still in the same range and the selectivity towards the linear aldehyde is higher (2.4:1 vs 1.8:1 (*n:iso*)). Thus, Rh-porphyrins generally can be used for hydroformylation reactions and a Fe-heme b exchange by Rh-heme b in the CYP119 monooxygenase seems to be a valid option. A Rh heme b complex was synthesized in dichloromethane in the presence of triethylamine and comparative absorbance spectra were recorded of the substituted porphyrin and the ligand (protoporphyrin IX) (Figure 5).

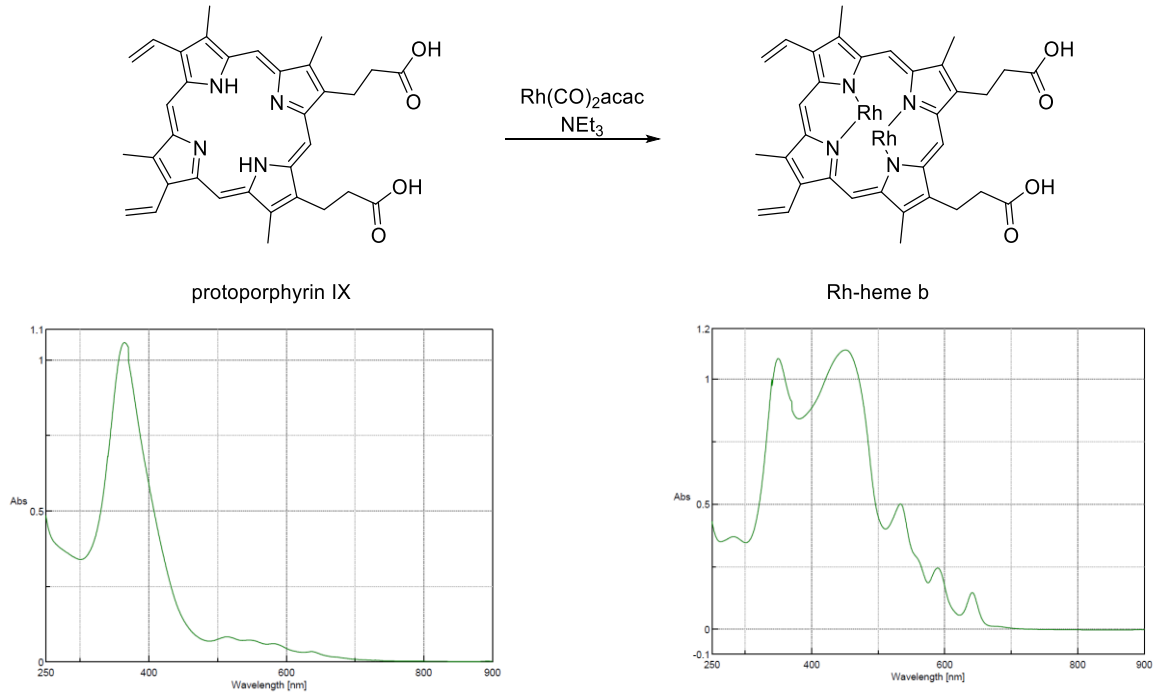
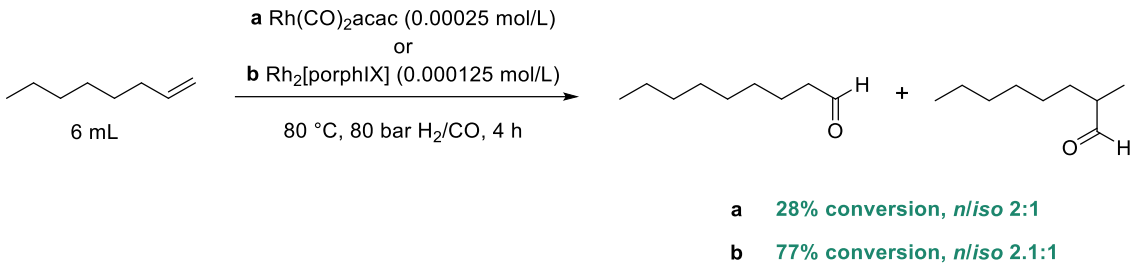


Figure 5. Absorbance spectra of protoporphyrin IX and protoporphyrin IX substituted with Rh in water with 5% DMSO.

It is shown that the absorbance spectrum is different between the Rh-substituted and the unsubstituted protoporphyrin IX, showing another maximum at ~450 nm besides the maximum at ~380 nm. These differences in absorbance spectra are in accordance to the literature.[86]the f

Further characterization of the rhodium heme complex has turned out to be very difficult. A similar complex has been published in earlier work and a crystal structure of which has been elucidated.[86] Therefore, it can be assumed that the herein reported complex is also a dinuclear rhodium porphyrin complex. This complex was then used for the hydroformylation of *n*-oct-1-ene (Scheme 13), however, in this case without an aqueous phase since the complex is insoluble in water.

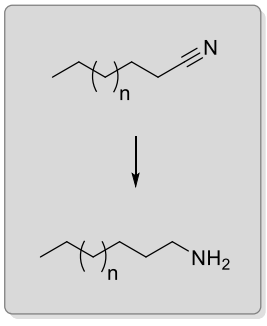


Scheme 13. Hydroformylation of *n*-oct-1-ene using Rh bound to protoporphyrin IX in comparison to free Rh.

The activity of the Rh_2[porphIX] was found to be even higher as for the Rh_2[tpps] in a biphasic reaction medium. These are promising results for the design of an artificial hydroformylation based on the CYP119 monooxygenase and serve as basis for further experiments performed by Michael Stricker in his doctoral thesis.

3.5 Hydrogenation of nitriles to amines (Article 11)

The results of this chapter were published by the author of this thesis and Harald Gröger in European Journal of Lipid Science and Technology (Article 11).[87]

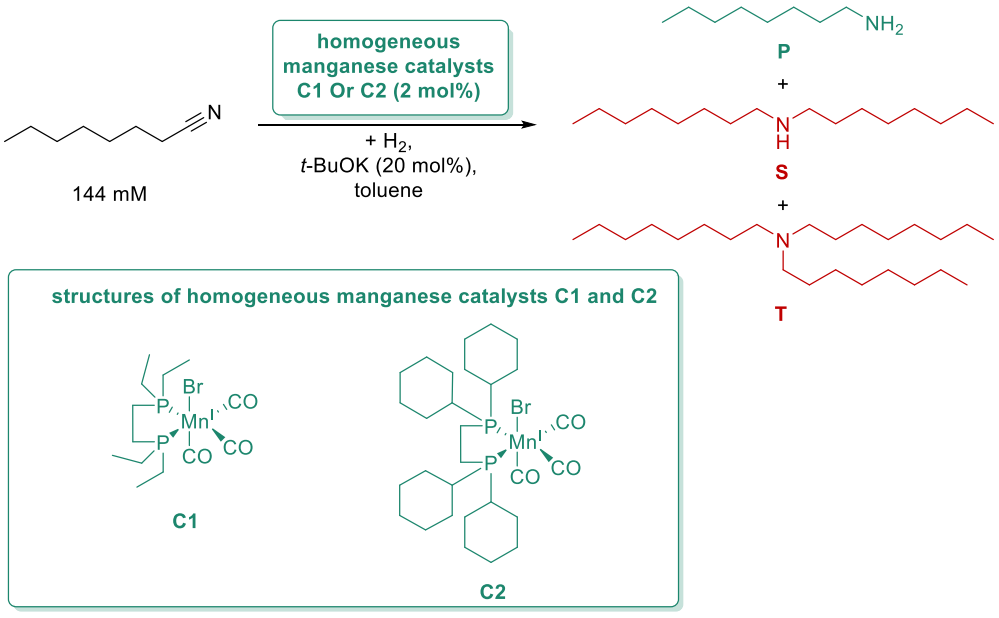


Nitrile hydrogenation

As a part of all cascades towards aliphatic nitriles and primary amines, the last step is a hydrogenation reaction of the nitriles to the amines. Since aliphatic amines are usually *high-volume-low-price*-chemicals their synthesis needs to be highly efficient and inexpensive. Thus, heterogeneous catalysts are usually the catalysts of choice, as they can be easily separated and reused. In our study on finding a suitable catalyst

for the selective hydrogenation of aliphatic nitriles to primary amines, classical heterogeneous catalysts like Pd/C, Ru/C, Pt/Al_2O_3 and others showed very low conversions to the product of interest and high amounts of secondary and tertiary amines are formed. Therefore, the study was expanded to homogeneous catalysts. Homogeneous and commercially available catalysts based on ruthenium (for example Ru-MACHO-BH, Ru-Gusev and Ru-PNNP) were found to be highly selective, however, ruthenium is very expensive and homogeneous catalysts are difficult to recycle. Hence, novel homogeneous manganese catalysts (**C1** and **C2**, Table 4) were developed based on the work of the Kirchner group,[44] which showed excellent selectivity and conversions for the hydrogenation of aliphatic nitriles to primary amines. They are easily prepared from commercially available phosphine ligands and Mn^I-salts. Manganese has the advantage over ruthenium that it is much cheaper and larger quantities are available, however, also this catalyst is homogeneous and is hard to recycle. This hurdle is currently under investigation together with the Vogt group. They are trying to use membrane systems for separating the catalyst from the reaction medium Afterwards the catalyst might be reusable for another hydrogenation reaction.

Table 4. Hydrogenation experiments using novel homogeneous manganese catalysts.



#	Catalyst	T /°C	p /bar	t /h	Conversion /%[a]	Isolated yield /%	P/S/T[a]
1	**C1**	100	50	19	>99	68	>99/0/0
2	**C2**	100	50	19	>99	96	>99/0/0

[a] *Conversions and ratios (P/S/T) were determined by ^{1}H-NMR analysis.*

New hydrogenation catalysts were developed for the hydrogenation of aliphatic nitriles to primary amines based on manganese, which is a cheap transition metal being a reasonable alternative to ruthenium. Potentially even a recycling could be realized by membranes to separate and reuse the catalyst. Both catalysts convert 144 mM octanenitrile within 19 h quantitively and with perfect selectivities towards the primary amine. Catalyst **C1** and also **C2** to certain extend furthermore seem to be very stable by storage and in contact with air and water, which is another remarkable benefit of these hydrogenation catalysts over ruthenium-, iron- or cobalt-based hydrogenation catalysts.[35,37,42,88]

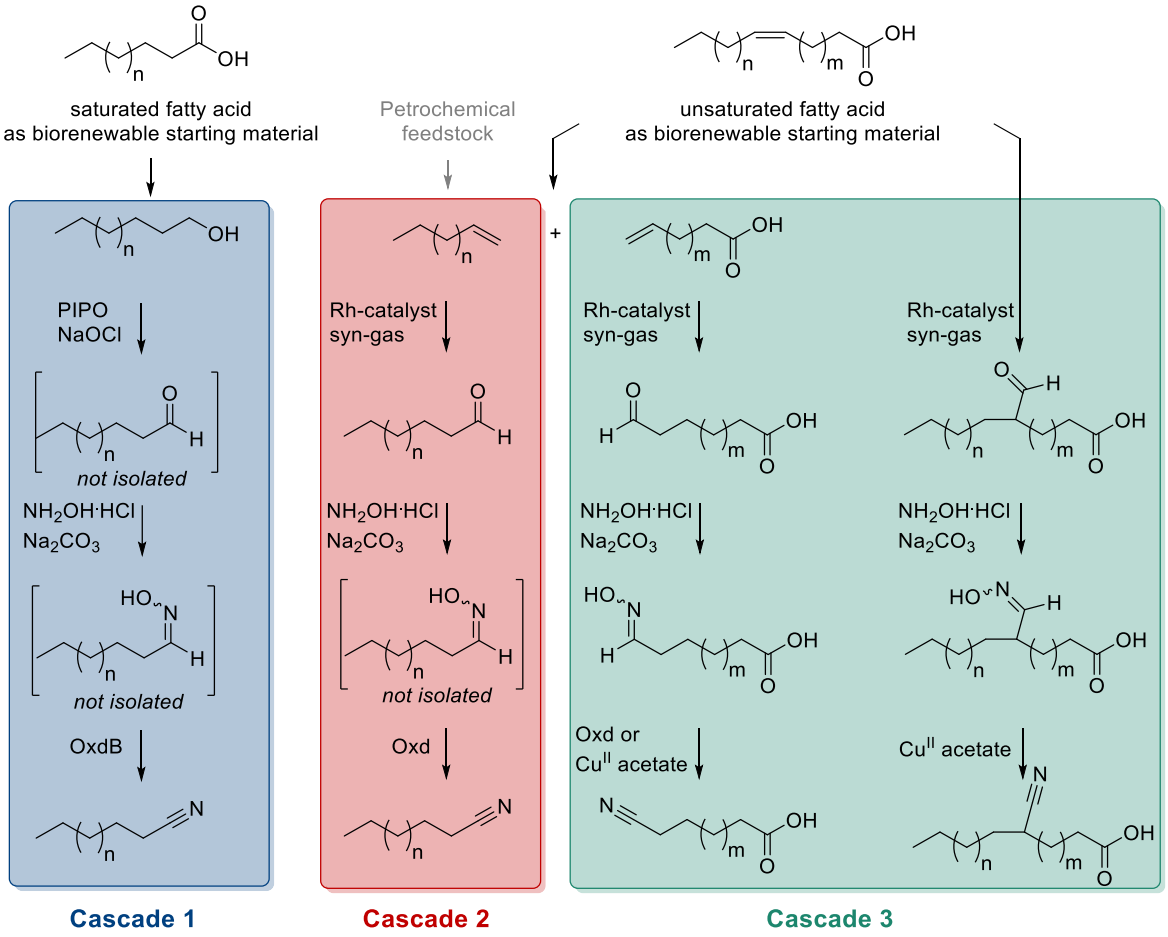
3.6 Evaluation of the catalytic activities of $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$

The results of this chapter are submitted by the author of this theses and her coauthors to Chemistry (Article 12).[89]

Within the framework of nitrile hydrogenation, a palladium-substituted mixed cerium-tin oxide catalyst from the Gruber-Wölfler group of Graz University of Technology was also used in addition to the classic hydrogenation catalysts. This catalyst ($Ce_{0.20}Sn_{0.79}Pd_{0.01}O_2$) has only been used for cross-coupling reactions so far and its activity for hydrogenation has been evaluated in this thesis. Since $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_2$ has not been tested for hydrogenations reactions before this study, it was interesting to show the catalytic activity of this catalyst for double-bond hydrogenations. Cinnamic acid and derivatives were hydrogenated with full conversion using $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_2$ in the presence of a base and hydrogen pressure. Esters and halogen substituents aromatic rings remain stable under the hydrogenation conditions in the presence of this catalyst, which enables a chemoselective hydrogenation of double bonds using this catalyst. Since $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_2$ shows very low leaching of palladium[90] compared to for example Pd/C,[91,92] an often used hydrogenation catalyst, this new catalyst might be a useful alternative.

4 Conclusion

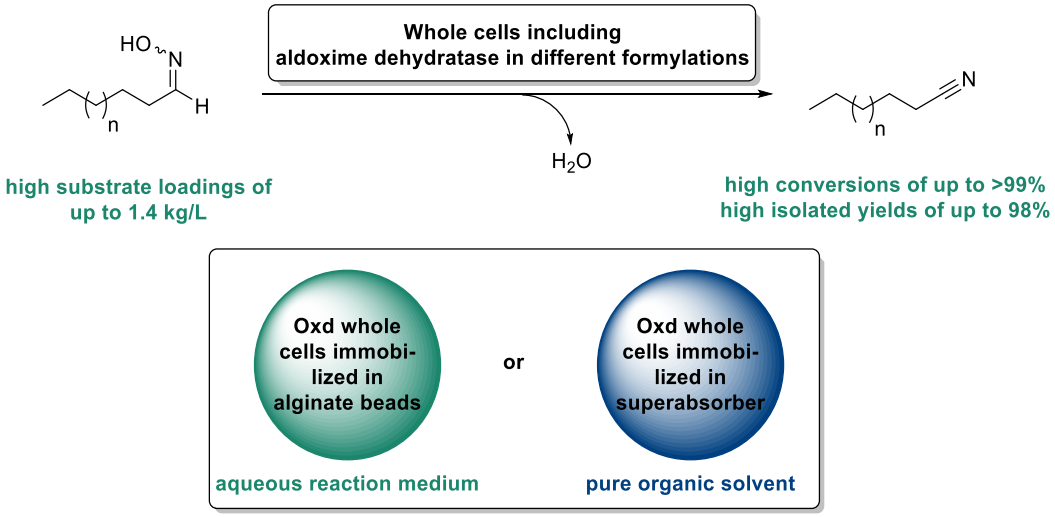
In summary, three different chemoenzymatic cascade reactions were developed starting from saturated or unsaturated fatty acids as biorenewable resources towards aliphatic nitriles and amines as potential alternative processes without need of cyanide salts and high energy consumption. Within all three cascades high conversions, selectivities and isolated yields were achieved.



Scheme 14. Established cascades towards nitriles and amines starting from fatty acids.

In Cascade 1 (Article 8) a cascade towards aliphatic nitriles via aliphatic alcohols, aldehydes and aldoximes was established using nitroxyl-radical catalyzed oxidation (Article 2), condensation of the aldehydes with hydroxylamine and biocatalytic dehydration using OxdB (Articles 3-7). In this cascade the aldehyde and aldoxime intermediates neither have to be isolated or purified and since the whole cascade is performed in the product nitrile as solvent,

nor an isolation or purification of the product is needed. High overall isolated yield for C6-, C8-, and C10-aldoximes were achieved with up to 70%. Within Cascade 1 the selective TEMPO-oxidation of alcohols to aldehydes was investigated in detail and it was found that aliphatic nitriles can be used as organic solvents as an alternative to dichloromethane, which was up to now a strong limitation of this method. An alternative cascade (Article 9) starting from alkenes (obtainable from unsaturated fatty acids) was developed including hydroformylation using a rhodium catalyst, condensation with hydroxylamine and dehydration using OxdRE without isolation of the aldoxime intermediate. In Cascade 3 (Article 10) bifunctional molecules from unsaturated fatty acids were synthesized by hydroformylation, condensation of the aldehydes with hydroxylamine and dehydration by Oxds or Cu^{II} acetate with acetonitrile to nitrile-functionalized carboxylic acids. In this cascade the aldehyde intermediate is not isolated or purified and linear aldoxime acids were isolated by simple filtration. In addition, no purification of the product is needed. The key step of all presented cascade is the aldoxime dehydratase catalyzed dehydration of aldoximes to nitriles, which was intensively studied in this thesis (Scheme 15).



Scheme 15. Achievements of aldoxime dehydratase catalyzed dehydration of aliphatic aldoximes to nitriles.

Great achievements towards a potential industrial application of aldoxime dehydratases were accomplished. High productivity of aliphatic nitrile synthesis with up to 1.4 kg/L substrate loading were achieved and immobilization methods for these label enzymes were found, making them usable not only in aqueous medium (immobilized in alginate beads), but also in pure organic medium by immobilization in superabsorber. The nitriles of all cascades can be hydrogenated to the corresponding amines by a novel manganese catalyst (exemplified for

linear nitriles in Article 11). These amines can for example be used in lubricant industry (especially primary aliphatic amines) or as precursor for polyamides. A new hydrogenation catalyst was found in $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (Article 12), which selectively hydrogenates double bonds while other functional groups remain stable. In the future, hydroformylation could be carried out by means of an artificial hydroformylase. In this work, initial experiments on the production of artificial hydroformylases based on streptavidin and a cytochrome P450 monooxygenase (CYP119) were carried out, which will be further developed by Michael Stricker in his doctoral thesis.

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6 Articles

6.1 Article 1 *accepted*

Synthetic processes towards nitriles without cyanide: A biocatalytic concept based on dehydration of aldoximes in water

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Author contribution

AH and TB wrote the manuscript. AH, TB, YA and HG read and edited the manuscript.

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CONCEPT

Synthetic processes towards nitriles without cyanide: A biocatalytic concept based on dehydration of aldoximes in water

Alessa Hinzmann[a], Tobias Betke[a], Yasuhisa Asano[b] and Harald Gröger*[a]

Abstract: While belonging to the most fundamental functional groups, nitriles represent a class of compound still raising challenges in terms of an efficient, cost-effective and at the same time sustainable way for their synthesis. Complementing existing chemical routes recently a cyanide-free enzymatic process technology based on the use of an aldoxime dehydratase (Oxd) as biocatalyst component has been developed and successfully applied for a range of nitrile products. In these biotransformations, these Oxd enzymes catalyze the dehydration of aldoximes as readily available substrates to the desired nitrile products. Within this concept article these developments with such enzymes are summarized with a strong focus on the synthetic application side. By means of the achieved synthetic examples, it will be demonstrated that this biocatalytic technology has the potential to "cross the bridge" between the production of fine chemicals and pharmaceuticals on the one hand and bulk and commodity chemicals on the other hand. For example, Oxd enzymes were found to give high enantioselectivities and can give access to both enantiomers when starting either from the (*E*)- or (*Z*)-aldoxime racemate. However, besides their high potential towards stereoselective synthesis making the attractive for the area of fine chemicals and pharmaceuticals, Oxd enzymes also turned out to suitable for the dehydration of a range of aldoximes derived from aliphatic achiral commodity chemicals, leading to the corresponding nitriles, e.g., adiponitrile and fatty nitriles, with high substrate loading up to the Kg per L range as a required criteria for the field of bulk chemicals production.

Introduction

The expeditious depletion of our world's resources, which is especially true for noble metals, prompts us to rethink the production methods for many of today's chemical compounds. The increasing product demand in all segments of the chemical industry forces us to develop reliable (and at the same time sustainable) production processes which can meet our needs now and in the future. Biocatalysis is considered to represent one of the key technologies for enabling these processes.[1–6] Not only do biocatalytic processes run under milder conditions than most of conventional chemical processes and excel in selectivity, they also are not affected by limited raw material sources as in case of a range of previous metals used in metal catalysis. While such metal catalyzed processes always depend on the current price and availability of the corresponding metal, biocatalysts can be simply produced by fermentation. Additionally, the precious metals need to be efficiently recycled and have to be restricted in their exposition towards animals, humans and environment due to their, at least in part, high toxicity. Biocatalysts on the other hand are completely biodegradable and, under optimized cultivation procedures, easily produced. However, the successive implementation of biocatalytic processes into the chemical industry should always be regarded and used as an additional alternative to other catalytic processes,[7] This additional alternative should be viewed as a broadening of the chemical repertoire and not as the all-promising solution to every synthetic problem. By abiding these standpoints, new and fascinating possibilities open up.

As for nowadays nitrile synthesis, mainly four approaches are used in the industry, dependent on the structure and application of the produced nitrile.[8,9] The most important production process of nitriles by annual tonnage is the double *n*-terminal hydrocyanation of 1,3-butadiene to yield adiponitrile. Adiponitrile is a key intermediate in nylon production and is almost exclusively hydrogenated to hexamethylenediamine. In addition, adiponitrile is synthesized by electro-hydrodimerization of acrylonitrile. Acrylonitrile, an unsaturated nitrile, exemplifies another industrial process for nitrile synthesis: Ammoxidation. This process utilizes unsaturated hydrocarbons, ammonia and air to yield the corresponding nitrile. Since ammoxidation benefits from the easy modification of C-H bonds next to a C=C-moiety, aromatic nitriles (starting from toluene derivatives) are also accessible. Lastly, amides can be dehydrated towards their corresponding nitriles under elevated temperatures and presence of heterogeneous catalysts. This process is mostly used for the synthesis of fatty nitriles due to the high accessibility of long chain, aliphatic fatty acids. The formed fatty nitriles are hydrogenated (mainly heterogeneously) towards fatty amines, which are used as surfactants or lubricant additives against corrosion. If one evaluates these processes, in spite of their impressive utilization on large scale also several drawbacks are apparent. For example, highly toxic cyanide has to be used for the hydrocyanation approaches. Moreover, amide dehydration requires tedious

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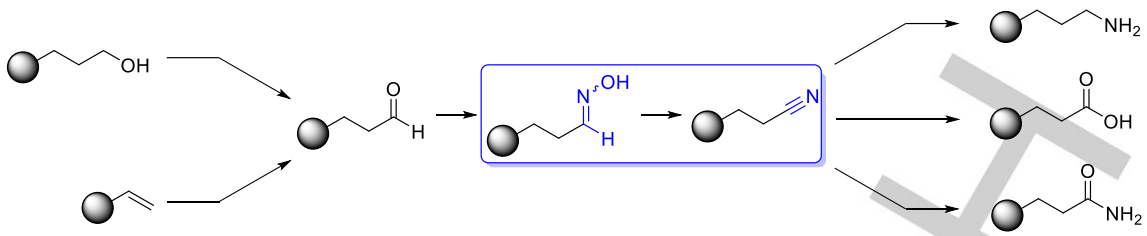


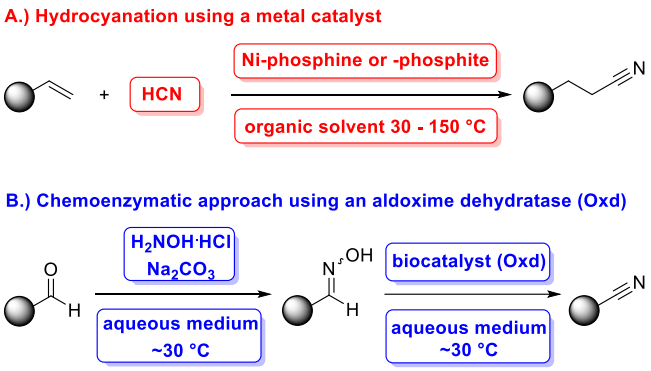
Scheme 1. Alternative synthetic route towards nitriles using aldehydes accessed by hydroformylation of alkenes or oxidation of alcohols as starting material. Aldehydes are condensed with hydroxylamine to give aldoximes which subsequently can serve as substrates for aldoxime dehydratase-catalyzed nitrile synthesis. Nitriles can afterwards be used for several transformations, resulting in valuable chemical products.

amide formation and excessive temperatures around 300 °C to proceed efficiently. These harsh conditions are also applied in the gas-phase ammoxidation process, which raises selectivity and side-product formation concerns. For ammoxidation, acetonitrile and hydrogen cyanide are formed as side products, while the double hydrocyanation of butadiene also leads to regioisomers of adiponitrile. In case of the amide dehydration, purification by distillation is paramount to obtain the nitrile in high purity.

Avoiding the above-mentioned drawbacks, biocatalytic concepts based on the dehydration of aldoximes in water, yielding the nitriles highly selectively, has just recently presented itself as a promising approach for a mild, sustainable nitrile synthesis. The required aldoximes are easily accessible by condensation of the bulk chemical hydroxylamine with aldehydes (Scheme 1). The required aldehydes are themselves broadly accessible by homogeneous catalyzed hydroformylation of alkenes with syngas or by oxidation of alcohols by usage of, for example, a piperidin-1-yl)oxyl radical as catalyst and sodium hypochlorite as oxidation agent.[10,11] The utilized enzymes for this biotransformation are so-called aldoxime dehydratases (Oxds). Aldoxime dehydratases, belonging to the enzyme class of lyases (EC 4.99.1.5-4.99.1.7), have firstly been described in 1998 by *Asano et al*. and their high potential for organic synthesis has just recently been started to unravel.[12–19] Their independence of cofactors and already high specific activity of the wild-type enzymes for many substrates (including aryl-aliphatic, aliphatic, aromatic and chiral aldoximes) may allow this enzyme class to be a “bridge builder” in the future between the fine chemical and bulk chemical industry, where many other enzyme classes are still struggling due to their high cost and low productivity that makes them, while highly valuable e.g. in the pharmaceutical industry, unprofitable for bulk chemistry.[20–22] The key advances in aldoxime dehydratase catalysis of the last years with respect to synthetic applications are summarized in this concept article.

Discovery of aldoxime dehydratases

Aldoximes were found to be intermediates in the biosynthesis of certain biological active compounds,[23–28] but it took a long time before aldoxime degrading enzymes have been isolated, purified and characterized. The first aldoxime dehydratase (Oxd), namely OxdB from *Bacillus* sp. OxB-1, was discovered in 1998 by screening microorganisms from soil using (*Z*)-phenylacetaldoxime (PAOx) as substrate.[24] These enzymes are nowadays known to be involved in nitrile-degrading microorganisms and plants.[23–28] Other proteins found in this pathway are nitrilases and nitrile hydratases. Since the discovery of the first Oxd another five enzymes of this enzyme class were found and characterized.[29–34] These five enzymes were found in *Pseudomonas chlororaphis* B23 (OxdA), *Fusarium graminearum* MAFF305135 (OxdFG), *Rhodococcus erythropolis* (OxdRE), *Rhodococcus globerulus* A-4 (OxdRG) and *Pseudomonas* sp. K-9 (OxdK), are also nitrile-degraders. The Oxd enzymes carry a heme b group in their active center, in which a ferrous iron as center ion is crucial for their catalytic activity.[25,26,29] Generally, PAOx was chosen as standard substrate for the determination of the catalytic activity of Oxds for the dehydration of aldoximes to nitriles because this substrate was identified as natural substrate being derived from phenylalanine. However, the Oxd enzymes not only accept PAOx as substrate but show a broad substrate spectrum. With their catalytic activity as well as high robustness in synthetic processes, Oxds enables an attractive access to nitriles without using toxic cyanide as reagent. The aldoxime substrates can be easily prepared starting from the corresponding aldehydes and hydroxylamine as cheap bulk chemical. In many cases, aldehydes are commercially available or easily accessible from cheap raw materials. For example, aldehydes can be prepared in an elegant fashion through hydroformylation of alkenes or oxidation of alcohols.[10,11] A comparison of the chemoenzymatic synthesis of nitriles from aldehydes using Oxds for the dehydration of aldoximes and the hydrocyanation as a well-known example for “classical” nitrile synthesis is shown in Scheme 2.

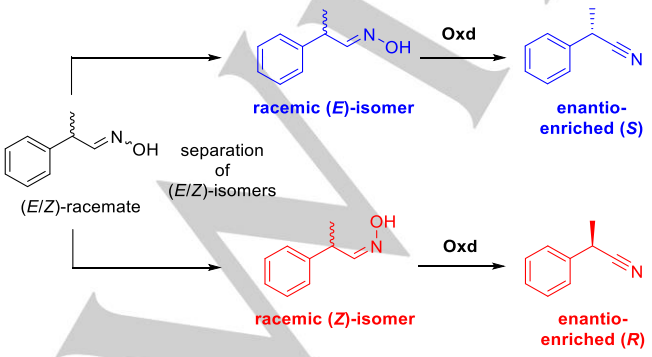


Scheme 2. Comparison of hydrocyanation as a common reaction for nitrile synthesis with the chemoenzymatic approach from aldehydes to nitriles using aldoxime dehydratases (Oxds) as biocatalysts.

In the following chapters we describe the capability of Oxd enzymes as catalysts in synthetic processes for a cyanide-free synthesis of nitriles at moderate reaction temperatures in water as an alternative synthesis of nitriles to hydrocyanation, ammoxidation and amide dehydration.

Enantioselective synthesis of chiral nitriles

Aldoxime dehydratases have proven themselves as versatile biocatalysts for the chiral nitrile synthesis. Initial studies in the early 2000s proved that aldoximes bearing a chiral center in the α-position of the oxime moiety are accepted as substrates by many Oxds, however the stereochemical course of these reactions had not been investigated.[25,30,31,33–36] In this initial work, (*E*/*Z*)-2-phenylpropionaldoxime and (*E*/*Z*)-mandelaldoxime were found to be accepted by up to five different Oxds and showed K_m-values ranging from 1.70-11.9 mM as well as specific activities of 0.57-18.1 U/mg. The stereoselective synthesis of nitriles starting from racemic aldoximes was investigated in detail jointly by the *Asano* and *Gröger* groups, who discovered in their preliminary studies that the aldoxime dehydratase from *Bacillus sp*. OxB-1 (OxdB) was able to convert racemic 2-phenylpropionaldoxime with highly enantioselectively towards (*S*)-2-phenylpropionitrile if solely the (*E*)-isomer of the aldoxime was used as racemic substrate.[18] Furthermore, it was found paramount to conduct the biotransformations at 8 °C to suppress the thermal (*E*) to (*Z*)-isomerization of the aldoxime since the inversion barrier of the nitrogen's lone pair is rather low. Based on this initial discovery, we then expanded the substrate scope for this type of chiral nitrile synthesis and the biocatalyst toolbox broadly, demonstrating that Oxds do accept a broad range of racemic aldoxime for the chiral nitrile synthesis.[12] In particular substrates with their stereogenic center in α-position as well as a strong steric differentiation of the substituents at the chiral center showed high enantioselectivity in the reaction course, yielding the corresponding nitriles with up to 99% *ee* in these kinetic resolutions. Furthermore it is noteworthy that Oxds can change their enantiopreference when changing the substrate from the *E*- to *Z*-racemate. This unique stereochemical behaviour has also a valuable synthetic consequence as with the same enzyme and based on the same racemic aldehyde both enantiomers are enantioselectively accessible by using either the *E*- or *Z*-isomer of the racemic aldoxime (Scheme 3).

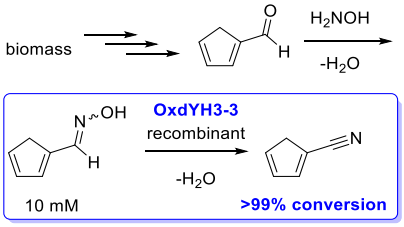


Scheme 3. Access to different enantiomers of the same nitrile with the same enzyme dependent on the *E*- or *Z*-configuration of an aldoxime.

For example, when separating the (*E*)- and (*Z*)-isomers of 2-(3-bromophenyl)propanal oxime and using them separately in a biotransformation with the Oxd from *Fusarium graminearum* (OxdFG), the (*S*)-nitrile is obtained 87% *ee* at 37% conversion, when starting from the (*E*)-isomer of the aldoxime. However, if the (*Z*)-isomer of the aldoxime is used, the (*R*)-nitrile is obtained with 88% *ee* at 51% conversion. This stereochemical phenomenon is in general quite unusual in catalysis, and from a synthetic perspective advantageous as it avoids the necessity to screen for further enzymes with the opposite enantiopreference if a proper separation of the aldoxime isomers can be conducted.

Synthesis of aromatic nitriles and utilization of biorenewable feedstocks as raw materials

A further class of nitriles being of importance for various industrial segments are aromatic nitriles. Among them, a focus in recent years has been on those being accessible from biorenewable feedstocks. An example is 2-furonitrile being an intermediate in the field of fine chemicals and pharmaceuticals as well as a potential sweetener.[37] 2-Furonitrile can be synthesized, e.g., by ammoxidation of furfural in a gas phase process at temperatures >400 °C.[37] As alternative approach starting from furfural, which is available from pentoses as a biorenewable source, a synthesis of 2-furonitrile utilizing Oxd enzymes was reported.[35,38,39] The chemoenzymatic synthetic concept and a preparative result are shown in Scheme 4.



Scheme 4. Biocatalytic synthesis of 2-furonitrile starting from furfural, which can be obtained from biomass.[38]

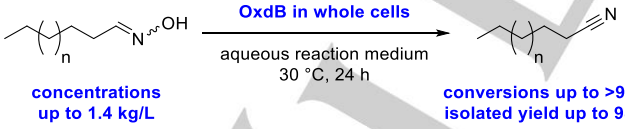
A task of this study was to get access to a recombinant form of the aldoxime dehydratase from *Rhodococcus* sp. strain YH3-3 (OxdYH3-3). This Oxd was found to be able for the conversion of some aromatic aldoximes and also proved to be suitable to convert furfural oxime.[35] Thus, the gene of OxdYH3-3 was cloned into expression vectors and expressed in *E. coli*.[38] With this whole cell-catalyst, 2-furonitrile was successfully synthesized *via* this biocatalytic dehydration of 2-furfuraldoxime (Scheme 4). Since usually Oxds were found to be (mostly) inactive for the conversion of aromatic aldoximes to nitriles,[19] this recombinant OxdYH3-3 provides now a practical access towards aromatic nitriles using this route.[38] Furthermore, mutants of OxdYH3-3 were generated by directed evolution, which showed increased activity for the synthesis of 2-furonitrile and 3-cyanopyridine of up to six fold compared to the wild-type enzyme.[39]

Synthesis of aliphatic nitriles with utilization as bulk and commodity chemicals

Aliphatic nitriles are a further class of nitriles being widely used in industry.[9,40] Direct applications of such nitriles are particularly known for the short-chain nitriles such as acetonitrile, whereas a large portion of the longer-chain aliphatic nitriles serves as intermediates for hydrogenation to the corresponding amines. For example, the resulting fatty amines can be found in many home products as well as industrial products with a worldwide demand being reported to be 800.000 tons already in 2011.[41] Furthermore, here fats and oils as renewable building blocks can serve as raw material source and, thus, as an alternative to petrochemicals.

Already in early work with Oxds, such enzymes were shown to catalyze the dehydration of aliphatic aldoximes to give aliphatic nitriles by the *Asano* group.[36] For example, it was demonstrated that Oxds form acetonitrile when starting from acetaldoxime as the smallest substrate for Oxds. When using whole cells with OxdB, a nearly quantitative conversion of 97% to acetonitrile was observed at a substrate concentration of 0.1 M (~18 g·L^{-1}) of acetaldoxime. In the same work also linear aliphatic aldoximes with a chain length between three and six were found to be converted by different Oxds. At a substrate concentration of 0.3 M (~35 g·L^{-1}) of hexanal oxime, a quantitative conversion of OxdB as a biocatalyst was achieved within 3 h reaction time at 30 °C. However, besides linear aliphatic aldoximes also branched ones such as isobutyraldoxime and isovaleraldoxime were found to be converted by Oxds. Based on these initial studies, a detailed process development of aliphatic nitrile synthesis using OxdB in whole cells as a catalyst was conducted very recently by the *Gröger* group utilizing *n*-hexanaloxime, *n*-octanaloxime and *n*-decanaloxime as model substrates.[14] Selected results are shown in Table 1, demonstrating the high productivity of OxdB as catalyst for aliphatic nitrile synthesis. It is noteworthy that these biotransformations can be conducted at a substrate loading of up to 1.4 kg of aldoxime per liter of aqueous reaction medium, leading to the desired aliphatic nitriles with conversions exceeding 90% and being even quantitative in some cases.

Table 1. Preparative biotransformation of aliphatic aldoximes to nitrile using OxdB in whole cells.[14]

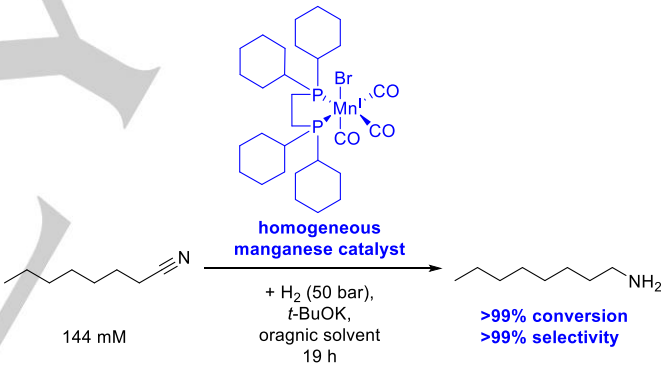


Entry	n =	Substrate concentration	Conversion /%	Yield /%
1	1	288 g/L	>99	81
2	2	342 g/L	>99	84
3	2	428 g/L	93	n.d.
4	3	665 g/L	>99	98
5	3	1430 g/L	93	n.d.

These substrate loadings belong to the highest ever reported in biocatalysis, in particular for converting a nearly water-insoluble substrate in aqueous medium. Since also the productivity of OxdB for the synthesis of aliphatic nitriles is very high, this biocatalytic reaction represents a process which has a promising potential for being transferred into an industrial process in the future.

Furthermore, Oxds were found to be active in pure organic solvent when using a superabsorber-based immobilization technique.[15] Since many aldoximes, especially long-chain aliphatic aldoximes are hardly soluble in aqueous reaction medium this possibility enables the use of a biocatalyst in organic medium, in which the aldoxime is better soluble. This superabsorber-immobilized Oxd-catalyst was also applied in a flow-reactor showing stable activity for at least 3 h run-time.

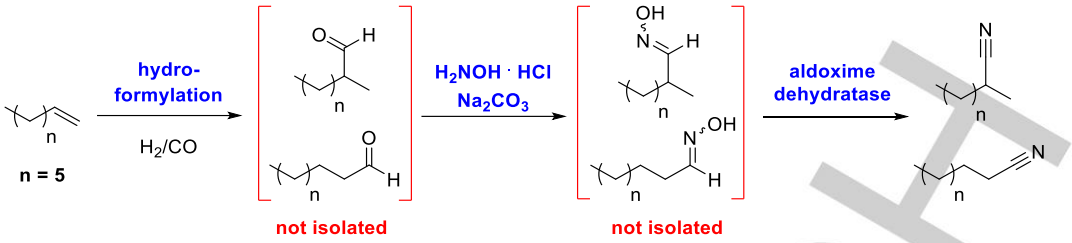
As in industry most of the aliphatic nitriles, in particular fatty nitriles, are utilized as intermediates for production of the corresponding amines,[40] the development of novel hydrogenation methods for their synthesis is also a field of current interest. Industrial valuable primary aliphatic amines can be also synthesized starting from these aliphatic nitriles by hydrogenation. However, hydrogenation of nitriles in a very selective manner is still a challenge, especially by usage of heterogeneous catalysts.[42] The Kirchner group[43] and based on their work recently also our group[42] investigated homogeneous manganese catalysts for such transformations with high selectivities. For example, *n*-octanenitrile can be selectively hydrogenated to *n*-octan-1-amine with >99% conversion and >99% selectivity using such a manganese catalyst (Scheme 5).



Scheme 5. Hydrogenation of *n*-octanenitrile using a Mn^{I}-based catalyst yielding in high selectivity and conversion towards *n*-octan-1-amine.[39]

In addition, researchers from BASF reported the application of Oxds for the synthesis of citronellyl nitrile, which is used as a fragrance compound.[44] In these reactions running under neat conditions directly in citronellaloxime, the Oxds were also used as whole cell-catalysts, leading to quantitative conversion after 90 h at 30 °C. This example underlines the high industrial potential of Oxds since it shows the opportunity to use Oxds in a pure organic system (neat), thus achieving high space-time yields.

Besides process development of individual reaction steps, also process integration of various reaction steps towards cascades without the need of intermediate purification represents a versatile concept for industrial production. Addressing the combination of enzymatic and “classic” chemical as well as chemocatalytic reactions,[45] a chemoenzymatic cascade with Oxds for aliphatic nonane nitrile synthesis starting from 1-octene as readily available raw material was developed.[16] This chemoenzymatic

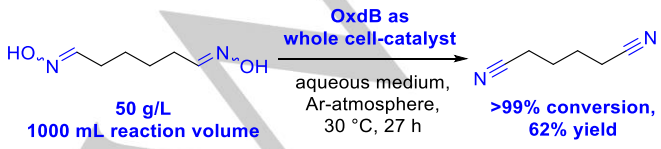


Scheme 6. Chemoenzymatic cascade from 1-octene towards nonane nitrile and 1-methyl-octanenitrile without intermediate isolation and purification.[16]

cascade combines a hydroformylation of 1-octene to *n*-/*iso*-C_9-aldehydes with subsequent condensation of these aldehydes with hydroxylamine under formation of the aldoxime, followed by the biocatalytic dehydration of the aldoximes to the nitriles (Scheme 6). The initial hydroformylation step was conducted in a biphasic system consisting of water and 1-octene using a rhodium complex with the commercial TPPTS ligand as a catalyst. The catalyst is water-soluble, thus enabling a simple separation of the organic product-substrate-mixture from the catalyst. After this phase separation, the organic phase mainly consists of *n*-nonanal and 2-methyl-octanal as products from hydroformylation of 1-octene and an isomer of 1-octene. This mixture was then treated in aqueous reaction medium with hydroxylamine before heating overnight to remove residual traces of hydroxylamine, which causes deactivation of the aldoxime dehydratase. Subsequently, the aldoxime mixture was converted by an Oxd using an aqueous reaction medium. Using this chemoenzymatic cascade reaction an overall conversion of 67% and a yield of the desired *n*-/*iso*-C_9-nitriles of 41% was obtained, thus demonstrating that the Oxd-based biotransformation is also compatible with chemical reaction steps for aldoxime substrate synthesis after optimization.

Synthesis of aliphatic dinitriles

Another potential application area for the industrial use of Oxds in the field of commodity chemicals is the biocatalytic synthesis of aliphatic dinitriles.[13] Among them adiponitrile is the one with the largest production volume being in the range of ca. 1 million tons annually.[9] Adiponitrile is utilized mainly for the manufacture of 1,6-hexanediamine as monomer for the polymerization with adipic acid to nylon-6,6 which is produced on multimillion-ton scale per year. As an alternative to today's existing methods such as the hydrocyanation of butadiene as mainly applied process in terms of production volume, recently the capability of Oxd enzymes for transforming the *bis*-aldoxime of adipaldehyde into adiponitrile was successfully demonstrated (Scheme 7).



Scheme 7. Adiponitrile synthesis in water at 30 °C using OxdB as biocatalyst on liter scale.[13]

This cyanide-free approach towards adiponitrile is conducted in water as a solvent and at a low reaction temperature (30 °C). The process has been already demonstrated at a 1 L reaction scale with 50 $g \cdot L^{-1}$ substrate loading, leading to full conversion within 27 h in the presence of a recombinant whole cell-catalyst bearing the OxdB as biocatalyst.

Furthermore, other linear dinitriles (C_4-C_{10}) were shown to be converted by Oxds under similar reaction conditions. The *bis*-aldoxime substrates can also be synthesized starting from the corresponding *bis*-aldehydes or their acetal-protected derivatives. Recently, not only oxidation of monoalcohols to aldehydes but also the oxidation of diols to dialdehydes was successfully performed by usage of TEMPO-oxidation in nitriles as solvent.[11] These examples underline that Oxds also accept *bis*-aldoxime as substrates very well as exemplified for linear aliphatic representatives of this compound class.

Summary

While their synthetic potential has just started to unravel, aldoxime dehydratases (Oxds) have already proven to be highly capable for the synthesis of chiral nitriles, aromatic nitriles, aliphatic nitriles and aliphatic dinitriles. In case of the chiral nitriles, such enzymes are able to even yield both enantiomers of a nitrile in spite of using the same enzyme due to the dependency of the enantio-preference on the *E*- and *Z*-configuration of the utilized aldoxime, thus generating the possibility to gain access to both enantiomers of a high-value chiral nitrile building blocks with the same enzyme. Furthermore, the potential of Oxd enzymes for the synthesis of bulk chemicals has been demonstrated by the liter scale synthesis of the polyamide intermediate adiponitrile with up to 50 g/L substrate loading. In addition, Oxds have been implemented in a chemoenzymatic reaction cascade to obtain fatty nitriles starting from alkenes in combination with hydroformylation. Fatty nitriles as a further nitrile product class of industrial interest can be prepared by Oxds in a very productive manner at substrate loadings of up to 1.4 kg per liter of reaction medium. For the future, we expect that the utilization of Oxds in cyanide-free synthesis of nitriles under mild reaction conditions will gain increasing interest for various segments of the chemical industry such as, for example, commodity chemicals, bulk and fine chemicals, and pharmaceuticals.

MANUSCRIPT

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Article 2

Selective TEMPO-Oxidation of Alcohols to Aldehydes in Alternative Solvents

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Author contribution

HG initiated the project. Initial optimization experiments, mentioned in the Supporting Information, were designed by HG and performed by JB. Based on the solvent study of JB, SG has conducted a solvent study. Optimization study, final solvent studies using TEMPO and PIPO as catalyst and substrate scope in terms of primary and secondary monoalcohols published were designed and performed by AH. MS performed an optimization study based on the method for the oxidation of monoalcohols to transfer the method to the oxidation of diols and tested different diols for the substrate scope. SG (bachelor student supervised by AH) performed initial TEMPO-oxidations in different solvents including nitriles. KO performed initial experiments of Anelli-type TEMPO-oxidation (not mentioned in the article). AH wrote the manuscript with the help of MS. AH, MS and HG read and edited the manuscript.

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Oxidation Reactions

Selective TEMPO-Oxidation of Alcohols to Aldehydes in Alternative Organic Solvents

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Abstract: The TEMPO-catalyzed oxidation of alcohols to aldehydes has emerged to one of the most widely applied methodologies for such transformations. Advantages are the utilization of sodium hypochlorite, a component of household bleach, as an oxidation agent and the use of water as a co-solvent. However, a major drawback of this method is the often occurring strict limitation to use dichloromethane as an organic solvent in a biphasic reaction medium with water. Previous studies show that dichloromethane cannot easily be substituted because a decrease of selectivity or inhibition of the reaction is observed by using alternative organic solvents. Thus, up to now, only a few examples are known in which after a tedious optimization of the reaction dichloromethane could be replaced. In order to overcome the current limitations, we were interested in finding a TEMPO-oxidation method in alternative organic solvents, which is applicable for various alcohol oxidations. As a result, we found a method for N-oxyl radical-catalyzed oxidation using sodium hypochlorite as an oxidation agent in nitriles as an organic solvent component instead of dichloromethane. Besides the oxidation of aromatic primary alcohols also aliphatic primary alcohols, secondary alcohols as well as dialcohols were successfully converted when using this method, showing high selectivity towards the carbonyl compound and low amounts of the acid side-product.

Introduction

Selective oxidation of alcohols to aldehydes is still a major challenge in organic chemistry.[1] Many methods are known, which are applicable in such transformations. Among them, chromium-based oxidations, oxidations using activated dimethyl sulfoxides, oxidations with hypervalent iodine species, ruthenium-based oxidations or 2,2,6,6-tetramethylpiperidin-1-yl)-oxyl (TEMPO)-catalyzed oxidations are prominent and widely applied methods.[1] However, chromium-based oxidation methods are problematic to use, especially in industrial applications due to the high toxicity of chromium salts.[2] Swern-oxidation of primary alcohols to aldehydes is usually performed at very low temperatures of approx. –80 °C, which makes this method also less favored.[3] Oxidation methods with hypervalent iodine species such as the Dess–Martin oxidation is usually performed in halogenated solvents and the Dess–Martin periodinane used as oxidation agent is an expensive and explosive compound, which needs to be synthesized beforehand.[4] Anelli-type TEMPO-catalysed oxidation is a mild method performed at 0–15 °C using TEMPO as a catalyst,[5,6] which is a stable nitroxyl radical and readily accessible starting from acetone and ammonia.[7] As an oxidation agent, hypochlorite is used in a biphasic reaction medium consisting of water and dichloromethane. Besides the Anelli-type TEMPO oxidation, many other types of this oxidation method were developed. TEMPO as a catalyst for oxidation of alcohols to carbonyl compound was investigated using metal salt additives or different oxidation agents, different TEMPO-derivatives or immobilized TEMPO as a catalyst or different solvent systems.[8–12] Nevertheless, a major disadvantage of the "classical" TEMPO-oxidation using sodium hypochlorite as an oxidation agent and TEMPO as a catalyst is the strong limitation of the solvent system for selective oxidation of primary alcohols to aldehydes, which consists of a biphasic system of water and dichloromethane (DCM).[5,6,13,14] Many studies were performed to find alternative solvents, but despite some examples for replacement no general solvent or solvent type was found to be suitable for the selective TEMPO-catalysed oxidation of primary alcohols to aldehydes.[15,16,8,17,18,19] The Sheldon group, for example, investigated different systems for the selective oxidation of alcohols with aromatic residues to the corresponding aldehydes using alternative solvents like ethyl acetate (EtOAc) or methyl-*tert*-butyl ether (MTBE).[17] They could show in principle that TEMPO-oxidation can be performed selectively in alternative organic solvents compared to dichloromethane, but no general procedure was found, which was suitable for all substrates. As the authors mentioned, especially the oxidation of aliphatic alcohols such as citronellol to citronellal was challenging in the alternative organic solvents to DCM and led to selectivity problems. However, TEMPO is not the only nitroxyl radical which is active for oxidation reactions, but also

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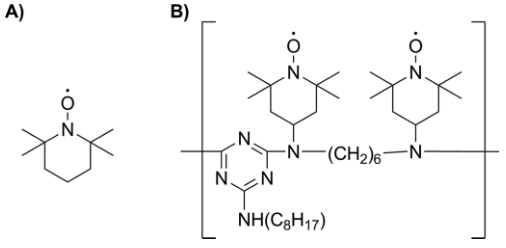
for example PIPO as a polymer-immobilised TEMPO-derivative (Scheme 1),[20,12] which was found to be suitable for catalysing the oxidation of alcohols to aldehydes and/or ketones.[20] PIPO is synthesised from the antioxidant and light-stabiliser Chimassorb 944, which is used as ingredient in different plastics.



Scheme 1. Structures of **A)** 2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and **B)** PIPO.

Since Chimassorb 944 is commercially available and manufactured at large amounts, the use of PIPO as catalyst is very attractive. In their first report in 2000 about PIPO-catalysed oxidation of alcohols, the Sheldon group studied the reactivity and suitability of this catalyst for different alcohol oxidations.[20] Within this study, they found that a solvent-free approach can be used for aromatic primary alcohols with high selectivity to the aldehydes. They also discovered an approach using *n*-hexane as solvent for the selective oxidation of *n*-octan-1-ol and *n*-hexan-1-ol to the corresponding aldehydes with high selectivity. This study principally shows that PIPO seems to be a more convenient catalyst for nitroxyl radical-catalyzed oxidation of primary alcohols in which also other solvents can easier be used. In our study, we were interested in finding a general protocol for the selective nitroxyl radical-catalyzed oxidation of primary and secondary alcohols and dialcohols to the corresponding aldehydes, ketones or dialdehydes, based on the use of alternative organic solvents than DCM. Such a method would remove the limitation of the solvent system for TEMPO oxidation. In the following, we report a general working procedure for the synthesis of aldehydes or ketones in alternative organic solvents without the need for optimization for each alcohol substrate. We present the utilization of aliphatic water-immiscible nitriles as preferred and generally applicable solvent components for the TEMPO as well as PIPO-oxidation. To the best of our knowledge, these solvents have not been tested before for this oxidation method.

Results and Discussion

Optimisation Study of TEMPO-Catalysed Oxidation of *n*-Octan-1-ol and *n*-Decan-1-ol

To investigate other solvents being suitable as alternative reaction medium for a selective TEMPO-catalyzed oxidation of alcohols to aldehydes, we decided to first optimize the TEMPO-catalysed oxidation of *n*-octan-1-ol (**1**) and *n*-decan-1-ol (**4**) in DCM to obtain a benchmark system which we can use for a solvent screening. As a starting point, we chose the TEMPO-catalysed oxidation system described by Kimura et al.[21] In this process, sodium hypochlorite pentahydrate is used as oxidation agent instead of an aqueous bleach solution. A major advantage of the use of this pentahydrate is the easy dosage of the oxidation agent. In the case of the 13 % aqueous bleach as an oxidation agent the amount of hypochlorite usually has to be determined before usage for the oxidation reaction by titration. Kimura et al. found that this method based on the use of hypochlorite pentahydrate can be used for the selective oxidation of a variety of primary and secondary alcohols.[21] Usually, for TEMPO-catalysed oxidation bromide ions were used as a co-catalyst, since the in situ-formed hypobromite is even more reactive than hypochlorite and was reported to represent the actual oxidation agent in this process.[5,6] The Kimura group found, however, that bromide is not necessary when using the pentahydrate as oxidation agent as long as a phase-transfer catalyst or additives like $NaHSO_4$ are used.[21,22] To get a deeper insight into the reaction system of Kimura et al., we first investigated the influence of different phase-transfer catalysts and additives. The oxidation of *n*-octan-1-ol (**1**) to *n*-octanal (**2**) was performed with 0.3 M *n*-octan-1-ol (**1**), 1 mol-% TEMPO, 1.1 equiv. $NaOCl{\cdot}5H_2O$ and 5 mol-% phase-transfer catalyst or $NaHSO_4$ in DCM at 0 °C for 1 h. As phase-transfer catalyst tetrabutylammonium hydrogensulfate (Bu_4NHSO_4), tetrabutylammonium chloride (Bu_4NCl) and acetylcholine hydrochloride were tested. As an additive, sodium hydrogensulfate ($NaHSO_4$) was tested and additionally, one experiment without phase-transfer catalyst or $NaHSO_4$ was conducted (see Supporting Information). With acetylcholine hydrochloride, Bu_4NCl and without any phase-transfer catalyst the reaction did not proceed. In contrast, when using Bu_4NHSO_4 and $NaHSO_4$ nearly the same conversion of 85 % and acid formation of 4 % was observed. Overoxidation through initial oxidation of primary alcohols to aldehydes and subsequent further oxidation to the acids is an often-reported problem in TEMPO-catalysed oxidations.[5,6,8,17,20,23] In particular aliphatic aldehydes tend to overoxidize to the acid, which was one major reason for us to first focus on the investigation of the oxidation of the aliphatic alcohols *n*-octan-1-ol (**1**) and *n*-decan-1-ol (**4**) to find a generally applicable system for also challenging substrates. Both Bu_4NHSO_4 and $NaHSO_4$ then were used again under the same reaction conditions but with a prolonged reaction time of 2 h. With both phase-transfer catalysts and additive, a high conversion exceeding 95 % was achieved, but with $NaHSO_4$ less acid formation of 6 % was observed in contrast to an acid formation of 20 % with Bu_4NHSO_4. In further experiments, the amount of $NaHSO_4$, the amount of TEMPO and the amount of $NaOCl{\cdot}5H_2O$ was varied to find optimal reaction conditions (see Supporting Information). The results show that a change in the amount of $NaHSO_4$ in the range of 1 mol-% to 10 mol-% has no relevant effect on the reaction. Thus, we chose 5 mol-% as the "standard" amount for further experiments. The optimal catalyst loading was found to be 0.25 mol-% and the optimal amount of $NaOCl{\cdot}5H_2O$ turned out to be 1.1 equiv. (both related to the amount of substrate). Using higher amounts of oxidation agent leads to an increased acid formation. As a last parameter the substrate loading was investigated. We increased the substrate concentration from 0.3 M to 1 M and performed the oxidation of *n*-octan-1-ol (**1**) to *n*-octanal (**2**) under the optimised reactions

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conditions consisting of 0.25 mol-% TEMPO, 1.1 equiv. $NaOCl{\cdot}5H_2O$ and 5 mol-% $NaHSO_4$ in DCM at 0 °C. The increase in the substrate concentration led to a significant increase in the reaction speed (Supporting Information). Full conversion was obtained after 15 min with 98 % selectivity, whereas at a lower substrate concentration of 0.3 M full conversion was reached after 45 min with a selectivity of 96 %. We defined selectivity as the ratio of aldehyde concentration to aldehyde and side-products concentrations in the reaction mixture. All optimisation experiments were also carried out for the oxidation of *n*-decan-1-ol (**4**) to *n*-decanal (**5**) (Supporting Information). The optimised reaction conditions for both reactions are summarized in Table 1.

Table 1. Optimized reaction parameters for the oxidation of *n*-octan-1-ol (**1**) and *n*-decan-1-ol (**4**).

	***n*-Octan-1-ol (1)**	***n*-Decan-1-ol (4)**
Substrate concentration	1 M	1 M
TEMPO	0.25 mol-%	0.25 mol-%
$NaHSO_4{\cdot}H_2O$	5 mol-%	5 mol-%
$NaOCl{\cdot}5H_2O$	1.1 eq	1.0 eq

Optimization of the solvent component. The usual solvent system for TEMPO-catalysed oxidations consists of aqueous hypochlorite solution and DCM. Although some examples are known in which DCM could be replaced by other solvents,[15,16,8,17,18,19] there is still a lack of universal generally applicable oxidation method in solvents other than DCM using nitroxyl radicals as catalyst and hypochlorite as oxidation agent without using metal salts as co-catalyst. To identify such a desired alternative reaction medium, we screened several organic solvents for the oxidation of *n*-octan-1-ol (**1**) using the optimized reaction conditions (Figure 1). It was found, that when using ethyl acetate (EtOAc), methyl *tert*-butyl ether (MTBE) and 2-methyl-tetrahydrofuran (2-Me-THF) as organic solvents nearly no conversion of *n*-octan-1-ol (**1**) was observed. However, we were pleased to find that the oxidation reaction proceeded smoothly when using different types of aliphatic nitriles as an organic solvent. In particular, *n*-octanenitrile and isobutyronitrile turned out to be highly suitable as (nearly) no side-product formation was found in the presence of these solvents. By decreasing the reaction time to 30 min also in *n*-butyronitrile, formation of less than 1 % acid **3** was detected (results not shown). To the best of our knowledge, this is the first solvent study for TEMPO-catalysed oxidation of alcohols in which nitriles except for acetonitrile were tested as alternative solvents to DCM, revealing that such solvents are highly suitable for this type of transformations. Recently our group provided protocols for the biocatalytic access to nitriles using aldoxime dehydratases as biocatalyst,[24–26] enabling an alternative access to these nitrile solvents without the need for toxic cyanide and harsh reaction conditions.

It is noteworthy that aliphatic nitriles with short and long chain length are suitable solvents for TEMPO-oxidation because as shown in Figure 2 the solvent parameters and properties of long- and short-chain aliphatic nitriles are very different and, thus, complementary to each other.[27,28]

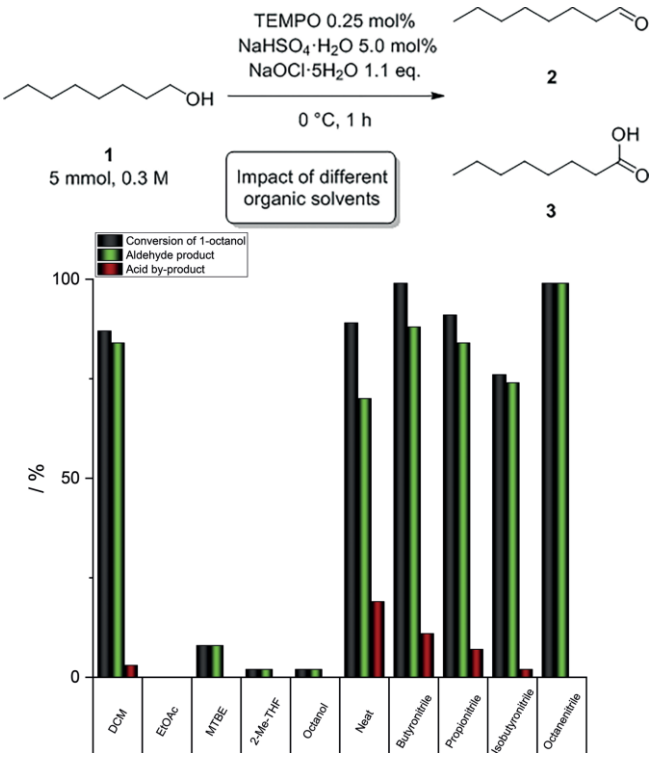

Figure 1. Solvent study of TEMPO-catalyzed oxidation of *n*-octan-1-ol (1) to *n*-octanal (2).

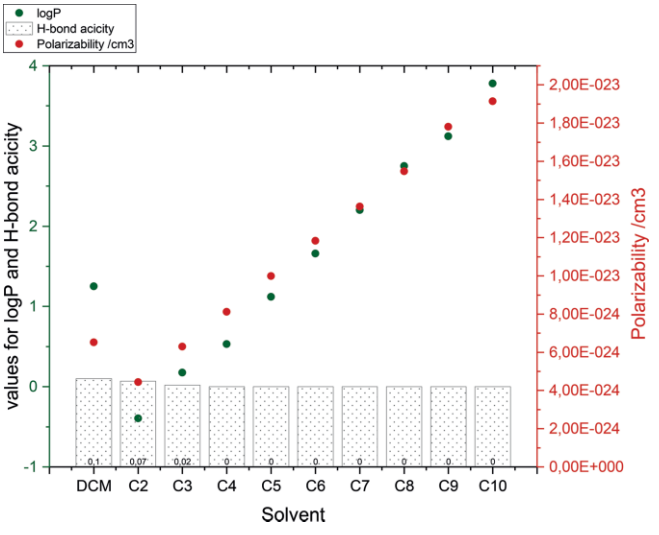

Figure 2. Partition coefficient (log P) (green dots),[28] summation of H-bond-acidity (bars, values are given as numbers in the bars) and polarizability in cm^3 (red dots) of from left to right: DCM, acetonitrile (C2), propionitrile (C3), butyronitrile (C4), pentanenitrile (C5), hexanenitrile (C6), heptanenitrile (C7), octanenitrile (C8), nonanenitrile (C9) and decanenitrile (C10). Y-axis on the left side presents values of log P and H-bond acidity and y-axis on the right represents polarizability.[27]

As the solvent properties of dichloromethane and the different nitrile solvents differ, at least in part, strongly from each other it is difficult to explain on this basis the phenomenon that DCM and nitriles are suitable solvents and other solvents usually not. Thus, rationalizing the effect of the solvent needs

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further investigations. From a synthetic perspective, a replacement of dichloromethane as a solvent for TEMPO-oxidation is of major interest due to the chronical toxicity and the fact that chlorinated solvents should be substituted in industrial processes.[29] However, the potential substitution of dichloromethane with aliphatic nitriles needs to be carefully considered. Short-chain aliphatic nitriles, e.g. *n*-butyronitrile,[30] are acute toxic by exposure, whereas longer-chained aliphatic nitriles, such as *n*-octanenitrile,[31] are harmful to health. However, short-chain and especially long-chain aliphatic nitriles are not fully characterized in terms of toxicity and environmental impact, making it difficult to evaluate the benefits of replacement of dichloromethane with aliphatic nitriles at the current stage.

Since we could find these promising results for all tested aliphatic nitriles as solvents in these oxidation reactions, we expanded our study to other nitroxyl radicals as catalysts in the oxidation of alcohols to aldehydes and ketones using sodium hypochlorite as oxidation agent and nitriles as solvents.

Transfer of the oxidation conditions for TEMPO to PIPO as a catalyst. As mentioned above, PIPO is a polymeric nitroxyl radical, which is based on Chimassorb 944 that is a stabilizer for plastics and therefore a tons-product and commercially readily available. Since the polymer is insoluble in many solvents, the catalyst can potentially be recycled and easily separated from the reaction mixture.[20] As described above, for the TEMPO-catalysed oxidation of *n*-octan-1-ol (**1**) nitriles turned out to represent a suitable organic solvent as an alternative for DCM enabling the reaction at a high substrate concentration of up to 1 M. These conditions were then transferred to the use of PIPO instead of TEMPO to improve the system further. Initially, a solvent screening (Figure 3) was performed using PIPO and a substrate concentration of 0.3 M of *n*-octan-1-ol (**1**) for a better comparison with the solvent screening which we conducted with TEMPO as a catalyst. These experiments showed that in particular *n*-butyronitrile and *n*-octanenitrile are also suitable solvents for the oxidation of *n*-octan-1-ol (**1**) to *n*-octanal (**2**) when using PIPO as a catalyst. In these oxidation reactions, only small amounts of acid (<5 %) were observed and conversions of >90 % of *n*-octan-1-ol (**1**) were reached within a reaction time of 1 h. The same reaction was performed then at 1 M substrate concentration since this concentration was found to be suitable for the oxidation using TEMPO as catalyst. As an organic solvent *n*-butyronitrile was used because this nitrile solvent can be easily removed in vacuo, which simplifies the isolation of the formed aldehyde. In this reaction full conversion of *n*-octan-1-ol (**1**) was reached and a selectivity of 93 % towards

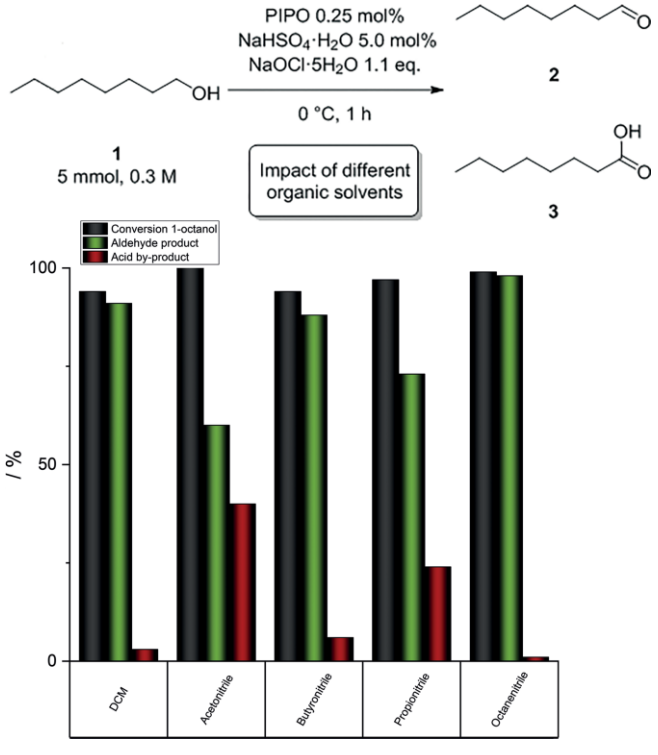
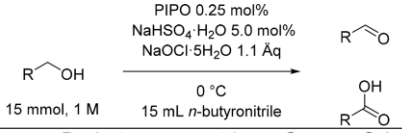


Figure 3. Solvent study for the PIPO-catalyzed oxidation of *n*-octan-1-ol (1) to *n*-octanal (2).

Table 2. Substrate scope of PIPO-catalyzed oxidation of primary alcohols to aldehydes.



Entry	Product	t / min	Conv. /%[a,b]	Sel. /%[c]	Yield /%[d]
1	**2**	15	99	93	90
2	**5**	45	94	96	89
3	**8**	1290[e]	>99	91	95[f]
4	**11**	75[g]	>99	95	58[f]
5	**14**	75	98	>99	95
6	**17**	420	>99	91	75[h]
7	**20**	75	>99	>99	94
8	**23**	25	>99	>99	92
9	**26**	150[i]	95	>99	91
10	**29**	105[i]	95	>99	88

[a] The reactions were quenched by the addition of 2 M HCl (15 mL). [b] Conversion of alcohol to the corresponding aldehyde or side-products (e.g. acid) was determined by GC-analysis in comparison to standard-curves. [c] Selectivities are defined as: $c_{aldehyde}/(c_{aldehyde} + c_{side\text{-}products})$. [d] Yields were calculated by isolation of the aldehydes from the reaction mixture by phase-separation after quenching with acid and extraction of the organic phase. [e] Reaction was performed in *n*-octanenitrile as solvent. After 12 h reaction time, further reaction at room temperature instead of 0 °C. [f] Isolated yield after distillation. [g] Reaction was performed in *n*-decanenitrile as solvent. [h] Isolated yield after column chromatography. [i] Reaction was performed at room temperature.

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the aldehyde **2** was detected (Table 2, entry 1). In addition, *n*-octanal (**2**) was isolated in 90 % yield from the reaction mixture.

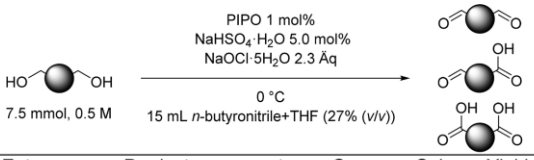
Substrate Scope of PIPO-Catalysed Oxidation in *n*-Butyronitrile as a Solvent

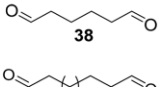
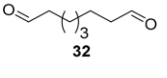
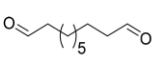
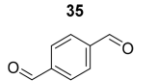
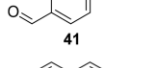
Since this oxidation method using *n*-butyronitrile as solvent, PIPO as catalyst and sodium hypochlorite pentahydrate as an oxidation agent is very simple and as the reaction progress can be easily tracked by GC-analysis, this method was applied for further oxidation experiments using primary and secondary alcohols. In this study, the oxidation of different substrates with different electronic properties was tested to demonstrate the applicability of this method for the oxidation of a broad range of substrates. We started with the substrate scope of "mono" alcohols since the reaction conditions described above were optimized for a mono-alcohol and we expected that our method would be easily applicable also for the preparation of other mono-aldehydes. Taking into account that the synthesis of dialdehydes is much more complicated because many more side-products can occur, further optimization of our method was conducted later for the oxidation of dialcohols to dialdehydes. The substrate scope was also expanded to secondary alcohols to synthesize ketones, but in this case, no side-products due to overoxidation were expected. The results of the substrate scope in terms of mono-primary alcohols, secondary alcohols and dialcohols are shown and discussed in the following sub-chapters.

Substrate scope study, part 1: Oxidation of primary alcohols to aldehydes. Since we performed detailed studies about the oxidation of *n*-octan-1-ol (**1**) to *n*-octanal (**2**), we became interested in the performance of the PIPO-oxidation in *n*-butyronitrile as an alternative solvent to DCM when utilizing electronically different alcohols as substrates such as, e.g., alcohols with aromatic residues (Table 2, entry 5–10). It was found that aromatic primary alcohols with electron-withdrawing groups such as a nitro-group (Table 2, entry 7,8), are very rapidly converted into the corresponding aldehydes with high selectivity at 0 °C reaction temperature. In contrast, when utilizing alcohols substituted with an electron-donating group such as a methoxy-group (Table 2, entry 9,10), the oxidation is much slower even when being performed at room temperature instead of 0 °C. Nevertheless, also in these cases, high conversions of 95 % were reached and a selectivity of >99 % towards the aldehyde was achieved in both cases. When synthesizing cinnamaldehyde **17** (Table 2, entry 6), this product needed to be purified by column-chromatography due to undefined side-products found in the GC-chromatogram. However, also for this oxidation, a selectivity of 91 % was found (calculated by comparison of the aldehyde peak with those of all product peaks detected in the GC-chromatogram). All aliphatic aldehydes were synthesized with high conversions of >90 % and selectivities of >90 % (Table 2, entry 1–4). In case of the synthesis of cyclohexanecarbaldehyde **11** (Table 2, entry 4), *n*-decanenitrile was used as a solvent instead of *n*-butyronitrile and in case of the preparation of *n*-hexanal (**8**), *n*-octanenitrile was chosen as a solvent component because these products are very volatile and *n*-butyronitrile could not be removed from the product. When using *n*-decanenitrile or *n*-octanenitrile as solvent, however, the products cyclohexanecarbaldehyde **11** and *n*-hexanal (**8**) could easily be distilled from the solvent after quenching of the reaction with HCl, phase separation and extraction. These results show that this oxidation method proceeds with a variety of primary alcohols as substrates to synthesize the corresponding aldehydes with high selectivities.

Substrate scope study, part 2: Oxidation of primary diols to dialdehydes. We were further interested in the challenging oxidation of diols bearing two primary alcohol groups to the corresponding dialdehydes utilizing the same solvent system. Therefore, dialcohols such as various α,ω-*n*-alkanediols (Table 3, entry 1–3) and phenylenedimethanols (Table 3, entry 4, 5) were oxidized using PIPO as a catalyst, sodium hypochlorite pentahydrate as oxidation agent and *n*-butyronitrile as organic solvent. Initially, we tried to use the same reaction conditions as for the mono-alcohols, but we could not achieve full conversion and only moderate selectivities were obtained for the oxidation of *n*-octan-1,8-diol (**31**). A major problem was the low solubility of the substrate *n*-octan-1,8-diol (**31)** in *n*-butyronitrile. We could overcome this problem by adding THF (27 % *v*/*v*) to the reaction solution. This led to full conversion but the selectivity was only in a moderate range (see Supporting Information). Thus, we decided to conduct a reaction optimization addressing substrate loading and the amount of $NaHSO_4$ and sodium hypochlorite, respectively. We were pleased to find such desired re-

Table 3. Substrate scope of PIPO-catalyzed oxidation of diols to dialdehydes.



Entry	Product	t / min	Conv. /%[a,b]	Sel. /%[c]	Yield /%[d]
1	 **38**	45	96	91	37
2	 **32**	40	>99	90	69
3	 **35**	10	>99	93	56
4	 **41**	45	>99	94	87[e]
5	 **44**	45	>99	91	71

[a] The reactions were quenched by addition of 1 M HCl (15 mL). [b] Conversions are defined as consumption of substrate. [c] Selectivities are defined as: GC-Area$_{dialdehyde}$/(GC-Area$_{dialdehyde}$ + GC-Area$_{side\text{-}products}$). [d] Yields were calculated by isolation of the aldehydes from the crude reaction mixture by phase-separation after quenching with acid, extraction of the organic phase and purification via automated column chromatography. [e] Yields were calculated by isolation of the aldehydes from the reaction mixture by phase-separation after quenching with acid and extraction of the organic phase.

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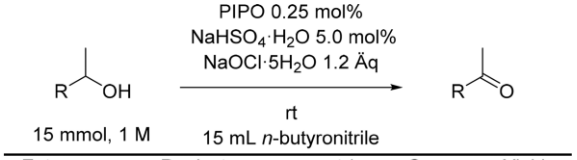
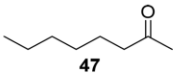
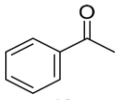
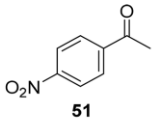
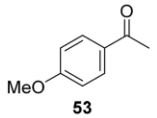
action conditions, leading to the oxidation of *n*-octan-1,8-diol (**31**) to *n*-octanedial (**32**) with >99 % conversion and 90 % selectivity. After purification by automated column chromatography the desired product *n*-octanedial (**32**) was obtained in 69 % isolated yield (Table 3, entry 2). We also applied these optimized reaction conditions for the oxidation of *n*-hexan-1,6-diol (**37**) and *n*-decan-1,10-diol (**34**) as two related aliphatic diols, reaching over 90 % selectivity and moderate yields (Table 3, entries 1,3). The relatively low yields are due to the instability of the dialdehydes. During the workup oxidation to various acids was observed. Furthermore, this method was successfully applied towards the oxidation of two phenylenedimethanols leading to high selectivity of 94 % and high yields (Table 3, entries 4,5), which underlines the generality of *n*-butyronitrile as a suitable solvent system for TEMPO-oxidation even for the challenging dialcohols. However, the oxidation of diols to dialdehydes is rather sensitive in comparison to the oxidation of monoalcohols. Slightly modified conditions in the oxidation of diols using our method can lead to significant changes in conversion and selectivity.

Substrate scope study, part 3: Oxidation of secondary alcohols to ketones. Since we could show that primary mono- as well as dialcohols can be oxidized by our TEMPO-oxidation method, we were further interested in applying this technique for the oxidation of secondary alcohols to the relating ketones (Table 4). We used the standard oxidation conditions as described for the oxidation for the mono-alcohols with slight modifications. Since we did not expect any side-product formation due to the formation of ketones instead of aldehydes as reaction products, we increased the reaction temperature to room temperature. The results of the four oxidation experiments using secondary alcohols as substrates are shown in Table 4. We were pleased to find that also secondary alcohols were converted into the corresponding ketones with quantitative conversions. Also in the case of the secondary alcohols, we found a significantly higher reaction velocity for the nitro-substituted phenylethanol compared to the methoxy-substituted one (Table 4, entry 3, 4). This is in accordance with literature results. Kimura et al. also found that nitro-substituted benzyl alcohol is faster converted than methoxy-substituted benzyl alcohol.[21] However, an explanation for this effect is still missing. *n*-Octan-2-ol (**46**) was converted slower than the primary alcohol *n*-octan-1-ol (**1**) with a reaction time of 40 min compared to 15 min for the primary alcohol, although higher reaction temperature was used for the oxidation of the secondary alcohol **46**. This result indicates that the oxidation of primary alcohols is favored.

Table 4. Substrate scope of PIPO-catalyzed oxidation of secondary alcohols to ketones.



Entry	Product	t / min	Conv. /%[a,b]	Yield /%[b]
1	 **47**	40	>99	22
2	 **49**	70	>99	49
3	 **51**	5	>99	90
4	 **53**	90	>99	65

[a] The reactions were quenched by addition of 2 M HCl (15 mL). [b] Conversions are defined as consumption of substrate. [c] Yields were calculated by isolation of the ketones from the crude reaction mixture by phase-separation after quenching with acid and extraction of the organic phase.

Oxidation of a Mixture of *n*-Octan-1-ol and *n*-Octan-2-ol

In many TEMPO-oxidation studies, the chemoselectivity of this oxidation method is addressed.[20,21] In the case of the "classical" TEMPO-catalysed oxidation using bleach as oxidation agent the oxidation of primary alcohols to the aldehyde was found to proceed much faster than the oxidation of secondary alcohols as shown for the oxidation of a mixture of *n*-nonan-1-ol and *n*-nonan-2-ol.[5] Generally, TEMPO-catalyzed oxidations can proceed in two different ways, following different mechanisms.[32–34] Under acidic conditions, a hydride or proton transfer between catalyst and alcohol substrate can occur, while under basic conditions a pre-oxidation complex is formed via an alkoxide attack on the electrophilic nitrogen of the oxammonium cation. The formation of the pre-oxidation intermediate is slower for secondary alcohols due to sterically hindrance, which leads to a faster conversion of primary alcohols compared to secondary alcohols. However, Kimura et al. found that the primary and secondary alcohol are oxidized with equal conversions when using sodium hypochlorite pentahydrate as oxidation agent instead of an aqueous solution of hypochlorite.[21] In detail, the same conversion of *n*-octan-1-ol (**1**) and *n*-octan-2-ol (**46**) in the oxidation reaction of this mixture was observed. Thus, we were interested to gain an insight into the chemoselectivity of our approach for the oxidation of *n*-octan-1-ol (**1**) and *n*-octan-2-ol (**46**). Therefore, we performed the oxidation of *n*-octan-2-ol (**46**) at 0 °C, since this temperature was also used for the oxidation of *n*-octan-1-ol (**1**). We found 40 % conversion of *n*-octan-2-ol (**46**) to *n*-octan-2-one (**47**) after 1 min. The reaction proceeds further with a very low reaction velocity leading to 42 % conversion of the alcohol **46** after 1 h reaction time. The reason for the high reaction rate at the beginning is not clear yet, but since we could obtain only 42 % conversion after 1 h reaction time in case of the secondary alcohol **46**, the overall process efficiency is lower in comparison to the one for the primary alcohol **1** (for which a full conversion after 15 min

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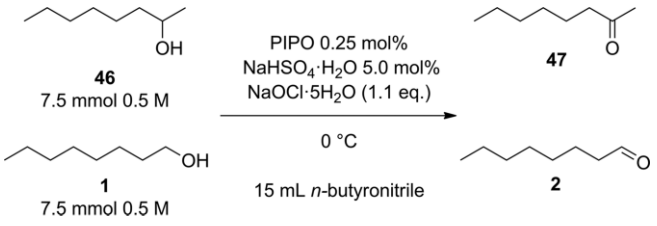
reaction time was observed; Table 2 entry 1). Afterward, we conducted another oxidation reaction starting from a 1:1-mixture of *n*-octan-1-ol (**1**) and *n*-octan-2-ol (**46**) at 0 °C in *n*-butyronitrile and utilizing sodium hypochlorite pentahydrate as an oxidation agent (Scheme 2).

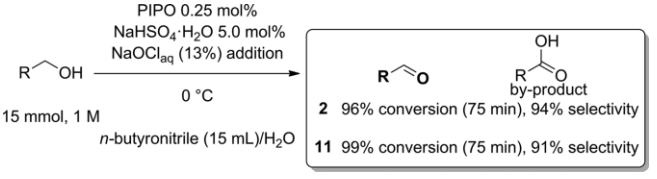


Scheme 2. Oxidation of a mixture of *n*-octan-1-ol (**1**) and *n*-octan-2-ol (**46**).

For this process, we found a complete conversion of *n*-octan-1-ol (**1**) after 2 min reaction time and a conversion of 61 % of *n*-octan-2-ol (**46**) within the same reaction time. When prolonging the reaction time to 1 h, we observed still the same conversion of *n*-octan-2-ol (**46**) with 62 %. Thus, this experiment shows that the oxidation of secondary alcohols is slower than the oxidation of primary alcohols, indicating that in our method the oxidation follows a basic reaction mechanism. To proof this hypothesis, in principle, a simple pH-measurement could be conducted. However, using our method without having an aqueous phase, the pH-value cannot be determined directly. To overcome this problem, we performed one experiment in which we added water to our system, thus making a pH-measurement possible. The pH measurement was performed after 5 min reaction time and after completion of the reaction. While the reaction was proceeding, we could find a basic pH of 8. After completion of the reaction, however, the pH turned out to be neutral. These results support the hypothesis that our system follows a basic reaction mechanism. Interestingly, the group of Kimura et al. could show that primary and secondary alcohols were converted equally in their reaction system, although they applied very similar reaction conditions.[21] The major differences between our system and the system of Kimura et al. are the choice of organic solvent (*n*-butyronitrile vs. DCM) and nitroxyl radical-derivative as catalyst (PIPO vs. TEMPO).

Oxidation of Alcohols to Aldehydes Using Aqueous Bleach Solution as Oxidation Agent

As in the "classical" TEMPO-oxidation method typically a 13 % aqueous bleach solution is used as oxidation agent,[5] we also modified our system by using this oxidation agent. Thus, we used the same conditions as described above with 1 M substrate concentration, an amount of sodium hydrogen sulfate of 5 mol-%, 0.25 mol-% PIPO, *n*-butyronitrile as an organic solvent and 1.1 equiv. hypochlorite in aqueous solution. In this experiment, however, we could not obtain full conversion of the alcohol and additionally, we found significant amounts of acid as side-product. By increasing the amount of hypochlorite solution full consumption of the alcohol was detected, but also high amounts of acid were formed. Therefore, we investigated a dosage approach of the hypochlorite solution when conducting the oxidation reaction. We started with 3 mL of 13 % sodium hypochlorite solution using 15 mL *n*-butyronitrile phase and added 3 mL after every 15 min reaction time with an overall reaction time 75 min. We tested this method using two aliphatic primary alcohols, namely *n*-octan-1-ol (**1**) and cyclohexylmethanol **16** (Scheme 3).



Scheme 3. Oxidation of *n*-octan-1-ol (**1**) and cyclohexylmethanol **10** to the corresponding aldehydes using 13 % bleach solution and *n*-butyronitrile as an organic solvent.

We were pleased to find that the resulting products *n*-octanal (**2**) and cyclohexanecarbaldehyde **11** were formed with conversions of >95 % and selectivities of >90 %. These results show that this system using *n*-butyronitrile as an organic solvent component is also suitable for the use of bleach solution instead of hypochlorite pentahydrate as an oxidation agent.

Conclusions

In conclusion, we reported a nitroxyl radical-catalysed oxidation method running in nitriles as an organic solvent component, being suitable for the selective synthesis of a broad range of aldehydes, dialdehydes and ketones. Thus, a convenient and easy-to-use method has been developed for the selective oxidation of alcohols and diols to the corresponding aldehydes or ketones, avoiding the need for chlorinated solvents used in the "classical" Anelli-type oxidation.[5,6] We also could show that solid sodium hypochlorite pentahydrate as well as bleach solution can be used as oxidation agent in this system. In this study we investigated the oxidation of 19 different mono- and dialcohols, which were successfully converted into the corresponding carbonyl compounds with high conversion and selectivities at moderate reaction conditions.

Experimental Section

Chemicals were purchased by Sigma Aldrich, VWR Chemicals, Fluka Chemicals, TCI Chemicals, Fluorochem, Alfa Aesar, and Carl Roth and were used without further purification. The oxidant $NaOCl{\cdot}5H_2O$ were purchased by TCI Chemicals (Germany) and used without further purification. NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^1H). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$ or $(CD_3)_2SO$). Reaction progress was monitored by GC analytics. Further information may be found in the Supporting Information. Optimization studies of oxidation of alcohols were performed in analogy to Kimura et al. using TEMPO or PIPO as catalyst.[21]

Optimization of the TEMPO-Catalyzed Oxidation of *n*-Octan-1-ol and *n*-Decan-1-ol: Phase-transfer catalyst or $NaHSO_4$ as additive (0.1–1 mmol, 1–10 mol-%) was suspended in dichloromethane (15 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (4.5–7.5 mmol, 0.74–1.23 g, 0.9–1.5 equiv.) and TEMPO (0.005–

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0.05 mmol, 0.78–7.81 mg, 0.1–1 mol-%) were added. The substrate (5 mmol, 0.3 M) was added under vigorous stirring and the reaction mixture was stirred at 0 °C until completion (GC-control). The reaction was quenched by addition of aqueous HCl solution (15 mL of a 1.2 M solution), the phases were separated. The organic phase was analyzed by GC chromatography. Further work-up and isolation of the product(s) were not performed. For the substrate *n*-octan-1-ol (**1**) a reaction optimization was carried out in terms of phase-transfer catalyst or additive amount, sodium hypochlorite amount, catalyst amount and substrate loading. The results of the optimization studies are shown in the Supporting Information (Tables S3–S4).

Optimization in Terms of Solvent: $NaHSO_4 \cdot H_2O$ (0.22 mmol, 31.1 mg, 5 mol-%) was suspended in organic solvent (15 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (0.81 g, 5 mmol, 1.1 equiv.) and TEMPO (0.0075 mmol, 1.2 mg, 0.25 mol-%) were added. *n*-Octan-1-ol (**1**) (0.80 mL, 5 mmol, 0.3 M) was added under vigorous stirring and the reaction mixture was stirred at 0 °C for 1 h. Reaction progress was monitored by GC. The results of the optimization study are shown in the Supporting Information (Table S5) and in the publication (Figure 1).

Synthesis of Polyamine-Immobilized Piperidinyloxyl Radical (PIPO): PIPO radical was prepared in analogy to Dijksman et al.[20] from Chimassorb 944 (M_n ca. 3,000 g/mol). Chimassorb 944 (10.12 g, 3.5 mmol) was suspended in 15 % aqueous H_2O_2 solution (120 mL) and the reaction mixture was stirred at room temperature for 5 days. The solid was filtered, washed with H_2O (500 mL) and dried in high vacuum. PIPO (9.78 g, 3.3 mmol, 94 %) was obtained as slightly orange solid.

Transfer of Optimized Conditions to PIPO as Catalyst: $NaHSO_4 \cdot H_2O$ (0.22 mmol, 31.1 mg, 5 mol-%) was suspended in organic solvent (15 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (0.81 g, 5 mmol, 1.1 equiv.) and PIPO (0.005–0.01125 mmol, 33.7 mg, 0.25 mol-%) were added. The *n*-octan-1-ol (**1**) (0.80 mL, 5 mmol, 0.3 M) was added under vigorous stirring and the reaction mixture was stirred at 0 °C for 1 h. Reaction progress was monitored by GC. The results of the optimization study are shown in the Supporting Information (Table S6) and in the publication (Figure 2).

Optimization of the PIPO-Catalyzed Oxidation Reaction of *n*-Octan-1,8-diol: $NaHSO_4 \cdot H_2O$ (0.5–1 mmol, 69.04–138 mg, 5–10 mol-%) was suspended in *n*-butyronitrile (16–20 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (22 – 35 mmol, 3.62–5.73 g, 2.2–3.5 equiv.) and PIPO (0.025–0.1 mmol, 75–300 mg, 0.25–1 mol-%) were added. The *n*-octan-1,8-diol (**32**) (10–20 mmol, 1.46–2.92 g, 0.5 – 1 M) was dissolved in THF (0–4 mL, 0–27 % *v*/*v*) by gentle warming and added to the reaction mixture. The reaction mixture was stirred at 0 °C until a color change from red to colorless occurred. The reaction was quenched by addition of aqueous HCl solution (50 mL of a 1 M solution), the phases were separated and the organic phase was dried with $MgSO_4$. The organic phase was analyzed by GC chromatography. Further work-up and isolation of the product(s) were not performed. For *n*-octan-1,8-diol (**32**) an optimization study was carried out. The results of the optimization study are shown in the Supporting Information (Table S7).

General Protocol for PIPO-Oxidation of Primary Alcohols to Aldehydes: $NaHSO_4 \cdot H_2O$ (0.75 mmol, 103.6 mg, 5 mol-%) was suspended in *n*-butyronitrile (15 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (16.5–22.5 mmol, 2.71–3.70 g, 1.1–1.5 equiv.) and PIPO (0.0375 mmol, 112.5 mg, 0.25 mol-%) were added. The primary alcohol (15 mmol) was added under vigorous stirring and the reaction mixture was stirred at 0 °C until completion (GC-control). The reaction was quenched by addition of aqueous HCl solution (15 mL of a 2 M solution), the phases were separated and the aqueous phase extracted with *n*-butyronitrile (2 × 5 mL). Organic phases were combined, dried with $MgSO_4$ and the solvent removed in vacuo. The product was analyzed using ^{1}H-NMR spectroscopy in [D_6]DMSO.

***n*-Octanal (2)**: Yield: 90 % (1.728 g, 13.5 mmol). (**2**) was obtained from *n*-octan-1-ol (**1**) (2.35 mL, 15 mmol) as colorless oil using sodium hypochlorite pentahydrate (2.70 g, 16.42 mmol, 1 equiv.) as oxidation agent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (t, *J* = 1.6 Hz, 1 H), 2.41 (td, *J* = 1.6, 7.3 Hz, 2 H), 1.51 (p, *J* = 7.2 Hz, 2 H), 1.25 (m, 6 H), 0.86 (t, *J* = 6.9 Hz, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

***n*-Decanal (5)**: Yield: 89 % (2.09 g, 13.4 mmol). (**5**) was obtained from *n*-decan-1-ol (**4**) (2.86 mL, 15 mmol) as colorless oil using sodium hypochlorite pentahydrate (2.70 g, 16.42 mmol, 1.1 equiv.) as oxidation agent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (t, *J* = 1.5 Hz, 1 H), 2.41 (td, *J* = 1.5, 7.3 Hz, 2 H), 1.51 (p, *J* = 7.1 Hz, 2 H), 1.25 (m, 6 H), 0.86 (t, *J* = 6.9 Hz, 3 H). The ^{1}H-NMR spectrum is in accordance to the literature.[35]

***n*-Hexanal (8)**: Yield: 85 % (1.275 g, 12.75 mmol). Synthesis of *n*-hexanal (**8**) from *n*-hexan-1-ol (**7**) (1.88 mL, 15 mmol) was performed in *n*-octanenitrile as solvent instead of *n*-butyronitrile using sodium hypochlorite pentahydrate as oxidation agent (2.70 g, 16.42 mmol, 1.1 equiv.). *n*-Hexanal (**8**) (85 %) was obtained as colorless oil. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (t, *J* = 1.6 Hz, 1 H), 2.41 (td, *J* = 1.6, 2.4 Hz, 2 H), 1.52 (p, *J* = 7.3 Hz, 2 H), 1.26 (m, 4H), 0.86 (t, *J* = 7.1 Hz, 3 H). The ^{1}H-NMR spectrum is in accordance to the literature.[35]

Cyclohexancarbaldehyde (11): Yield: 58 % (0.98 g, 8.7 mmol). Synthesis of cyclohexancarbaldehyde **11** from cyclohexanemethanol **10** (1.84 mL, 15 mmol) was performed in *n*-decanenitrile as solvent instead of *n*-butyronitrile using sodium hypochlorite pentahydrate as oxidation agent (2.70 g, 16.42 mmol, 1.1 equiv.). Cyclohexancarbaldehyde **11** (0.98 g, 8.7 mmol, 58 %) was obtained as colorless oil. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.55 (s, 1 H), 2.27 (m, 1 H), 1.81 (m, 2 H), 1.65–1.55 (m, 3 H), 1.33–1.18 (m, 5 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

Benzaldehyde 14. Yield: 95 % (1.51 g, 14.23 mmol). **14** was obtained from benzyl alcohol **13** (1.62 g, 15 mmol) as colorless oil using sodium hypochlorite pentahydrate (2.96 g, 18.0 mmol, 1.2 equiv.) as oxidation agent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 10.02 (s, 1H), 7.92 (dd, *J* = 1.4, 8.2 Hz, 2H), 7.71 (m, 1H), 7.61 (t, *J* = 7.6 Hz, 2H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

Cinnamaldehyde (16). Yield: 75 % (1.49 g, 11.25 mmol). The oxidation of cinnamyl alcohol **16** (2.01 g, 15 mmol) was performed using sodium hypochlorite pentahydrate (2.96 g, 18.0 mmol, 1.2 equiv.) as oxidation agent. Cinnamaldehyde **17** (1.49 g, 11.25 mmol, 75 %) was obtained as yellowish oil after automated column chromatography using cyclohexane and ethyl acetate as solvent (gradient from 10 to 40 % ethyl acetate in cyclohexane), a flow of 75 mL/min on a Biotage® SNAP Ultra 50 g column. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.69 (d, *J* = 7.6 Hz, 1 H), 7.75 (m, 4 H), 7.47 (m, 2 H), 6.87 (dd, *J* = 7.8, 16.0 Hz, 1 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

4-Nitrobenzaldehyde 20. Yield: 94 % (2.12 g, 14.0 mmol). **20** was obtained from 4-nitrobenzyl alcohol **19** (2.30 g, 15 mmol) as a slightly yellowish solid using sodium hypochlorite pentahydrate as oxidation agent (2.96 g, 18.0 mmol, 1.2 equiv.). ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 10.17 (s, 1 H), 8.42 (d, *J* = 8.5 Hz, 2 H), 8.17 (d, *J* = 8.5 Hz, 2 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

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3-Nitrobenzaldehyde 23. Yield: 92 % (2.09 g, 13.8 mmol) **23** was obtained from 3-nitrobenzyl alcohol **22** (2.30 g, 15 mmol) as yellowish solid using sodium hypochlorite pentahydrate (2.96 g, 18.0 mmol, 1.2 equiv.) as oxidation agent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 10.14 (s, 1 H), 8.67 (m, 1 H), 8.52 (ddd, *J* = 1.1, 2.4, 8.3 Hz 1 H), 8.33 (d, *J* = 7.7 Hz, 1 H), 7.89 (t, *J* = 7.9 Hz, 1 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

4-Methoxybenzaldehyde 26. Yield: 88 % (1.79 g, 13.15 mmol). **26** was obtained from 4-methoxybenzyl alcohol **25** (2.07 g, 15 mmol) as slightly yellowish liquid using sodium hypochlorite pentahydrate as oxidation agent (2.96 g, 18.0 mmol, 1.2 equiv.) at room temperature instead of 0 °C. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.87 (s, 1 H), 7.86 (d, *J* = 8.8 Hz, 2 H), 7.11 (d, *J* = 8.7 Hz, 2 H), 3.33 (s, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

3-Methoxybenzaldehyde 29. Yield: 88 % (1.79 g, 13.2 mmol). **29** was obtained from 3-methoxybenzyl alcohol **28** (2.07 g, 15 mmol) as slightly yellowish liquid using sodium hypochlorite pentahydrate (2.96 g, 18.0 mmol, 1.2 equiv.) as oxidation agent at room temperature instead of 0 °C. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.98 (s, 1 H), 7.52 (m, 2 H), 7.42 (m, 1 H), 7.28 (m, 1 H), 3.83 (s, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

General Protocol for PIPO-Oxidation of Dialcohols to Dialdehydes. $NaHSO_4{\cdot}H_2O$ (0.5 mmol, 51.8 mg, 5 mol-%) was suspended in *n*-butyronitrile (11 mL) and cooled to 0 °C. Sodium hypochlorite pentahydrate (17.3 mmol, 2.84 g, 2.3 equiv.) and PIPO (0.38 mmol, 225 mg, 1 mol-%) were added. The primary dialcohol (7.5 mmol, 1 M) was dissolved in THF (4 mL, 27 % *v*/*v*) by gentle warming and added to the reaction mixture after cooling to r.t. The reaction mixture was stirred at 0 °C until a color change from red to colorless occurred. The reaction was quenched by addition of aqueous HCl solution (50 mL of a 1 M solution), the phases were separated and the aqueous phase extracted with ethyl acetate (3 × 30 mL). Organic phases were combined, dried with $MgSO_4$ and the solvent removed in vacuo. The crude product was analyzed using GC analysis and further purified using automated column chromatography. The product was then analyzed using ^{1}H-NMR spectroscopy in $[D_6]$DMSO.

***n*-Hexanedial (38).** Yield: 37 % (316 mg, 2.8 mmol). **38** was obtained from *n*-hexan-1,6-diol (**37**) (886 mg, 7.5 mmol) as colorless liquid using sodium hypochlorite pentahydrate as oxidation agent (2.84 g, 17.3 mmol, 2.2 equiv.) after automated column chromatography using cyclohexane and ethyl acetate as solvent (gradient from 5 to 50 % ethyl acetate in cyclohexane), a flow of 75 mL/min on a Biotage® SNAP Ultra 25 g column. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (t, *J* = 1.4 Hz, 2 H), 2.47–2.40 (m, 4 H), 1.55–1.47 (m, 4 H). The ^{1}H-NMR spectrum is in accordance with the literature.[36]

***n*-Octanedial (32).** Yield: 69 % (740 mg, 5.2 mmol). **32** was obtained from *n*-octan-1,8-diol (**37**) (1.1 g, 7.5 mmol) as colorless liquid using sodium hypochlorite pentahydrate as oxidation agent (2.84 g, 17.3 mmol, 2.2 equiv.) after automated column chromatography using cyclohexane and ethyl acetate as solvent (gradient from 10 to 50 % ethyl acetate in cyclohexane), a flow of 75 mL/min on a Biotage® SNAP Ultra 25 g column. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (s, 2 H), 2.41 (td, *J* = 7.3, 1.6 Hz, 4 H), 1.56–1.43 (m, 4 H), 1.31–1.19 (m, 4 H). The ^{1}H-NMR spectrum is in accordance with the literature.[37]

***n*-Decanedial (35).** Yield: 56 % (710 mg, 4.17 mmol). **35** was obtained from *n*-decan-1,10-diol (**34**) (1.3 g, 7.5 mmol) as colorless liquid using sodium hypochlorite pentahydrate as oxidation agent (2.84 g, 17.3 mmol, 2.2 equiv.) after automated column chromatography using cyclohexane and ethyl acetate as solvent (gradient from 5 to 50 % ethyl acetate in cyclohexane), a flow of 75 mL/min on a Biotage® SNAP Ultra 25 g column. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 9.66 (t, *J* = 1.7 Hz, 2 H), 2.42 (td, *J* = 7.2, 1.7 Hz, 4 H), 1.56–1.47 (m, 4 H), 1.26 (s, 8 H). The ^{1}H-NMR spectrum is in accordance with the literature.[38]

Benzene-1,4-dicarboxaldehyde 42. Yield: 87 % (876 mg, 6.5 mmol). **42** was obtained from 1,4-benzendimethanol **41** (1.0 g, 7.5 mmol) as colorless solid using sodium hypochlorite pentahydrate as oxidation agent (2.84 g, 17.3 mmol, 2.2 equiv.). The substrate was added neat to a reaction already containing the THF cosolvent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 10.14 (s, 2 H), 8.12 (s, 4 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

Benzene-1,3-dicarboxaldehyde 45. Yield: 71 % (719 mg, 5.4 mmol) was obtained from 1,4-benzendimethanol **44** (1.0 g, 7.5 mmol) as colorless solid using sodium hypochlorite pentahydrate as oxidation agent (2.84 g, 17.3 mmol, 2.2 equiv.) after automated column chromatography using cyclohexane and ethyl acetate as solvent (gradient from 12 to 86 % ethyl acetate in cyclohexane), a flow of 75 mL/min on a Biotage® SNAP Ultra 25 g column The substrate was added neat to a reaction already containing the THF solvent. ^{1}H NMR (500 MHz; $(CD_3)_2SO$): 10.13 (s, 2 H), 8.43 (d, *J* = 1.9 Hz, 1 H), 8.22 (d, *J* = 7.6 Hz, 2 H), 7.84 (t, *J* = 7.6 Hz, 1 H). The ^{1}H-NMR spectrum is in accordance to the literature.[35]

General Protocol for PIPO-Oxidation of Secondary Alcohols to Ketones. $NaHSO_4{\cdot}H_2O$ (0.75 mmol, 103.6 mg, 5 mol-%) was suspended in *n*-butyronitrile (15 mL) at room temperature. Sodium hypochlorite pentahydrate (22.5 mmol, 3.70 g, 1.5 equiv.) and PIPO (0.0375 mmol, 112.5 mg, 0.25 mol-%) were added. The secondary alcohol (15 mmol) was added under vigorous stirring and the reaction mixture was stirred at room temperature until completion (GC-control). The reaction was quenched by addition of aqueous HCl solution (15 mL of a 2 M solution), the phases were separated and the aqueous phase extracted with *n*-butyronitrile (2 × 5 mL). Organic phases were combined, dried with $MgSO_4$ and the solvent removed in vacuo. The product was analyzed by ^{1}H-NMR spectroscopy in $CDCl_3$.

2-Octanone (47). Yield: 22 % (0.415 g, 3.24 mmol). **47** was obtained from *n*-octan-2-ol (**46**) (2.38 mL, 15 mmol) as colorless liquid. ^{1}H NMR (500 MHz; $CDCl_3$): 2.39 (t, *J* = 7.5 Hz, 2 H), 2.10 (s, 3 H), 1.54 (p, *J* = 7.4 Hz, 2 H), 1.26 (m, 6 H), 0.85 (t, *J* = 6.8 Hz, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

Acetophenone 49. Yield: 49 % (0.899 g, 7.48 mmol) was obtained from 1-phenylethanol (**48**) (1.81 g, 15 mmol) as colorless liquid. ^{1}H NMR (500 MHz; $CDCl_3$): 7.97 (d, *J* = 1.4, 8. Hz, 2 H), 7.55 (t, *J* = 7.4 Hz, 1 H), 7.45 (t, *J* = 7.7 Hz, 2 H), 2.59 (s, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

4-Nitroacetophenone 51. Yield: 90 % (2.23 g, 14 mmol). **51** was obtained from 4-nitro-α-methylbenzyl alcohol (**50**) (2.51 g, 15 mmol) as colorless solid. ^{1}H NMR (500 MHz; $CDCl_3$): 8.32 (d, *J* = 8.5 Hz, 2 H), 8.12 (d, *J* = 8.5 Hz, 2 H), 2.68 (s, 3 H). The ^{1}H-NMR spectrum is in accordance to the literature.[35]

4-Methoxyacetophenone 53. Yield: 65 % (1.46 g, 9.73 mmol). **53** was obtained from 4-methoxy-α-methylbenzyl alcohol (**52**) (2.28 g, 15 mmol) as colorless solid. ^{1}H NMR (500 MHz; $CDCl_3$): 7.93 (d, *J* = 8.8 Hz, 2 H), 7.45 (d, *J* = 8.8 Hz, 2 H), 3.84 (s, 3 H). The ^{1}H-NMR spectrum is in accordance with the literature.[35]

Oxidation of *n*-Octan-2-ol at 0 °C and 1.1 eq. Sodium Hypochlorite Pentahydrate. $NaHSO_4{\cdot}H_2O$ (0.75 mmol, 103.6 mg, 5 mol-%) was suspended in *n*-butyronitrile (15 mL) at 0 °C. Sodium hypochlorite pentahydrate (16.5 mmol, 2.71 g, 1.1 equiv.) and

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PIPO (0.0375 mmol, 112.5 mg, 0.25 mol-%) were added. The secondary alcohol (15 mmol) was added under vigorous stirring and the reaction mixture was stirred at 0 °C. The reaction progress was monitored by GC. The results are shown in the Supporting information (Table S8).

Oxidation of a Mixture of *n*-Octan-1-ol and *n*-Octan-2-ol. $NaHSO_4$ H_2O (0.75 mmol, 103.6 mg, 5 mol-%) was suspended in *n*-butyronitrile (15 mL) at 0 °C. Sodium hypochlorite pentahydrate (16.5 mmol, 2.71 g, 1.1 equiv.) and PIPO (0.0375 mmol, 112.5 mg, 0.25 mol-%) were added. The alcohol mixture consisting of *n*-octan-1-ol (**1**) (1.18 mL, 7.5 mmol) and *n*-octan-2-ol (**46**) (1.19 mL, 7.5 mmol) (in total 15 mmol) was added under vigorous stirring and the reaction mixture was stirred at 0 °C. The reaction progress was monitored by GC. The results are shown in the Supporting Information (Table S9) and in Scheme 2.

Oxidation of Alcohols to Aldehydes Using Aqueous Bleach Solution as Oxidation Agent. $NaHSO_4$•H_2O (0.75 mmol, 103.6 mg, 5 mol-%) was suspended in *n*-butyronitrile (15 mL) at 0 °C. 13 % aqueous sodium hypochlorite solution (3 mL, 5 mmol, 0.33 equiv.) and PIPO (0.0375 mmol, 112.5 mg, 0.25 mol-%) were added. The alcohol (15 mmol) was added under vigorous stirring and the reaction mixture was stirred at 0 °C. Each 15 min reaction time 13 % aqueous sodium hypochlorite solution (3 mL, 5 mmol, 0.2 equiv.) was added. The reaction progress was monitored by GC. The results are shown in the Supporting Information (Table S10) and the publication (Scheme 3).

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Keywords: Aldehydes · Dialdehydes · Nitriles · Oxidation · Synthetic methods

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Supporting Information

Selective TEMPO-Oxidation of Alcohols to Aldehydes in Alternative Organic Solvents

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1 Experimental information

Chemicals were purchased by Sigma Aldrich, VWR Chemicals, Fluka Chemicals, TCI Chemicals, Fluorochem, Alfa Aesar and Carl Roth and were used without further purification. The oxidant $NaOCl \cdot 5H_2O$ were purchased by TCI Chemicals (Germany) and used without further purification

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^1H). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$ or $(CD_3)_2SO$).

Reaction progess was monitored and conversions determined by GC measurements (Shimadzu GC-2010 Plus) in comparison to calibration curves (mono-alcohols) or by comparison of GC-areas (dialcohols). Selectivities of the oxidations reactions are defined as: $amount_{aldehyde}/(amount_{aldehyde}+amount_{by\text{-}products})$. Measurements were conducted on a chiral SGE Analytik B6B-174 column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) or an achiral Zebron Phenomenex ZB-5MSi column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with nitrogen as carrier gas. In following table, temperature gradients of the GC-methods are shown.

Table S1. GC-methods and temperature profiles used in this project.

Method no.	GC-column	Rate /°C·min^{-1}	End /°C	Hold /min
1	SGE Analytik B6B-174	--	140	1
		20	190	0.5
		50	200	0
2	SGE Analytik B6B-174	--	60	0.0
		20	150	0.5
3	SGE Analytik B6B-174	--	60	0
		20	150	0.5
		100	199	1
4	Zebron Phenomenex ZB-5MSi	--	40	0
		20	250	0.5
		100	300	0
5	Zebron Phenomenex ZB-5MSi	--	90	2
		30	260	0
6	Zebron Phenomenex ZB-5MSi	--	130	2
		30	230	0.5
7	Zebron Phenomenex ZB-5MSi	--	130	2
		30	300	0
8	SGE Analytik B6B-174	--	50	0
		1	60	0
		20	100	0
		5	135	0
		40	200	2.38
9	SGE Analytik B6B-174	--	100	0
		1	110	0
10	Zebron Phenomenex ZB-5MSi	--	40	0
		20	250	0.5
		100	300	5

The retention times for the compounds of interest are as follows:

Table S2. Retention times and methods used for the detection of compounds of interest.

Compound	GC-method	Retention time /min	Calibration curve
1	1	2.0	Yes
2	1	1.8	Yes
3	1	3.0	Yes
4	1	3.0	Yes
5	1	2.5	Yes
6	1	3.4	Yes
7	2	2.0	Yes
8	2	1.8	Yes
9	2	2.5	Yes
10	3	3.5	Yes
11	3	4.0	Yes
12	3	5.1	Yes
13	4	4.7	Yes
14	4	4.1	Yes
15	4	6.4	Yes
16	4	6.9	Yes
17	4	6.6	Yes
18	4	8.0	Yes
19	4	8.6	Yes
20	4	7.2	Yes
21	4	9.2	Yes
22	4	8.6	Yes
23	4	7.3	Yes
24	4	9.2	Yes
25	4	6.8	Yes
26	4	6.5	Yes
27	4	8.3	Yes
28	4	6.8	Yes
29	4	6.4	Yes
30	4	7.9	Yes
31	6	2.8	No
32	6	2.1	No
33a	6	3.1	No
33b	6	4.1	No
34	7	4.1	No
35	7	3.3	No
36b	7	5.1	No
37	8	3.4	No
38	8	2.4	No
39b	8	5.4	No
41	10	7.9	No
42b	10	6.2	No
43b	10	11.5	No
44	10	8.2	No
45	10	6.4	No
47	10	10	No
48	8	16.1	Yes
49	8	15.6	Yes
50	9	7.9; 8.2	Yes
51	9	6.2	Yes
52	4	8.6	Yes

2 Oxidation methods

2.1 Optimization studies

2.1.1 Optimisation of the TEMPO-catalyzed oxidation of *n*-octan-1-ol and *n*-decan-1-ol

For the substrate *n*-octan-1-ol (**1**) a reaction optimization was carried out in terms of phase-transfer catalyst and additive, sodium hypochlorite amount, catalyst amount and substrate loading. All conducted experiments are listed in Table S3.

Table S3: List of all experiments carried out for the reaction optimization of the oxidation of *n*-octan-1-ol (**1**).

Entry	Octanol 1 / M	Phase transfer catalyst or additive (%mol)	$NaOCl \cdot 5H_2O$ / eq.	TEMPO / %mol	t / min	Conv. / %	Sel. / %
1	0.3	Bu_4NHSO_4 (5)	1.1	1	60	84	80
2	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	1	60	87	84
3	0.3	Bu_4NCl (5)	1.1	1	60	1	1
4	0.3	Acetylcholin-hydrochlorid (5)	1.1	1	60	0	0
5	0.3	-	1.1	1	60	0	0
6	0.3	Bu_4NHSO_4 (5)	1.1	1	110	>99	78
7	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	1	110	98	91
8	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.1	90	>99	93
9	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	90	>99	96
10	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.5	90	>99	95
11	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.75	90	>99	93
12	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	1	90	>99	92
13	0.3	$NaHSO_4 \cdot H_2O$ (5)	0.9	0.25	120	85	83
14	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	120	98	94
15	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.5	0.25	120	>99	90
16	0.3	$NaHSO_4 \cdot H_2O$ (1)	1.1	0.25	60	>99	96
17	0.3	$NaHSO_4 \cdot H_2O$ (2.5)	1.1	0.25	60	92	90
18	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	60	>99	94
19	0.3	$NaHSO_4 \cdot H_2O$ (7.5)	1.1	0.25	60	>99	94
20	0.3	$NaHSO_4 \cdot H_2O$ (10)	1.1	0.25	60	98	94
21	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	45	>99	97
22	1	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	45	>99	95

For the substrate *n*-decan-1-ol (**4**) a reaction optimization was carried out in terms of phase-transfer catalyst and additive, sodium hypochlorite amount, catalyst amount and substrate loading. All conducted experiments are listed in Table S4.

Table S4: List of all experiments carried out for the reaction optimization of the oxidation of *n*-decan-1-ol (**1**).

Entry	Decanol 4 / M	Phase transfer catalyst or additive (%mol)	$NaOCl \cdot 5H_2O$ / eq.	TEMPO / %mol	t / min	Conv. / %	Sel. / %
1	0.3	Bu_4NHSO_4 (5)	1.1	1	120	>99	81
2	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	1	120	>99	95
3	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.1	60	69	68
4	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	60	99	93
5	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.5	60	96	87
6	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.75	60	97	88
7	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	1	60	96	87
8	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	120	>99	95
9	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.5	0.25	120	>99	87
10	0.3	$NaHSO_4 \cdot H_2O$ (1)	1.1	0.25	60	44	44
11	0.3	$NaHSO_4 \cdot H_2O$ (2.5)	1.1	0.25	60	94	94
12	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	60	85	85
13	0.3	$NaHSO_4 \cdot H_2O$ (7.5)	1.1	0.25	60	91	90
14	0.3	$NaHSO_4 \cdot H_2O$ (10)	1.1	0.25	60	93	91
15	0.3	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	55	71	71
16	1	$NaHSO_4 \cdot H_2O$ (5)	1.1	0.25	55	98	97

2.1.2 Optimization in terms of solvent

Table S5. Results after 1 h reaction time of TEMPO-catalyzed oxidation of *n*-octan-1-ol (**1**) in different organic solvents. Selectivity is defined as follows: $c_{n\text{-octanal}}/(c_{n\text{-octanal}}+c_{\text{octanoic acid}})$.

Entry	Organic solvent	Conversion /%	Selectivity /%
1	Dichloromethane	87	96
2	Ethyl acetate	0	--
3	Methyl-*tert*-butylether	8	--
4	2-Methyl tetrahydrofuran	2	--
5	Solvent-free	89	79
4	Acetonitrile	70	83
7	*n*-Butyronitrile	99	89
8	Propionitrile	91	92
9	Isobutyronitrile	76	97
10	*n*-Octanenitrile	99	>99

2.2 Transfer of optimized conditions to PIPO as catalyst

2.2.1 PIPO-catalyzed oxidation of *n*-octan-1-ol in different organic solvents

Table S6. Results after 1 h reaction time of PIPO-catalyzed oxidation of *n*-octan-1-ol (**1**) in different organic solvents. Selectivity is defined as follows: $c_{n\text{-octanal}}/(c_{n\text{-octanal}}+c_{\text{octanoic acid}})$.

Entry	Organic solvent	Conversion /%	Selectivity /%
1	Dichloromethane	94	97
2	Acetonitrile	>99	60
3	*n*-Butyronitrile	94	94
4	Propionitrile	97	75
5	*n*-Octanenitrile	99	99

2.3 Optimisation of the PIPO-catalyzed oxidation reaction of *n*-octan-1,8-diol

For *n*-octan-1,8-diol (**32**) a optimization study was carried out. The conducted experiments are listed in Table S6.

Table S7: List of all experiments carried out for the reaction optimization of the oxidation of *n*-octan-1,8-diol (**32**).

Entry	35 / M	$NaHSO_4 \cdot H_2O$ / %mol	$NaOCl \cdot 5H_2O$ / eq.	PIPO / %mol	Amount THF / % (*v*/*v*)	T / °C	t / min	Conv. / %	Sel.: / %
1	1	5	2.2	0.25	-	0	5		
2	1	5	2.2	0.25	27	0	10		
3	0.5	5	2.45	0.5	20	0	60	100	75
4	0.5	5	2.45	0.75	20	0	50	100	76
5	0.5	5	2.45	1	20	0	48	100	80
6	0.5	5	2.2	1	20	0	45	100	81
7	0.5	5	3.5	1	20	0	50	100	65
8	0.5	5	2.45	1	-	0	240	100	65
9	0.5	10	2.45	1	20	0	20	100	74
10	0.5	5	2.3	1	27	0	40	100	90

2.4 Oxidation of a mixture of *n*-octan-1-ol and *n*-octan-2-ol

2.4.1 Oxidation of 2-octanol at 0 °C and 1.1 eq. sodium hypochlorite pentahydrate

Table S8. Conversion of 2-octanol (**46**) to 2-octanone (**47**) determined by GC after different reaction times.

Entry	Reaction time /min	Conversion /%
1	1	40
2	3	41
3	5	41
4	10	41
5	20	41
6	40	42
7	60	42

2.4.2 Oxidation of a 1:1-mixture of *n*-octan-1-ol and 2-octanol

Table S9. Conversion of *n*-octan-1-ol (**1**) to *n*-octanal (**2**) (and octanoic acid **3** as by-product) and 2-octanol (**46**) to 2-octanone (**49**) determined by GC after different reaction times.

Entry	Reaction time /min	Conversion *n*-octan-1-ol (**1**) /%	Ratio *n*-octanal (**2**) to octanoic acid **3**	Conversion 2-octanol (**46**) to 2-octanone (**47**) /%
1	2	>99	95/5	61
2	3	>99	94/6	62
3	5	>99	94/6	62
4	10	>99	94/6	62
5	20	>99	94/6	62
6	40	>99	94/6	62
7	60	>99	94/6	62

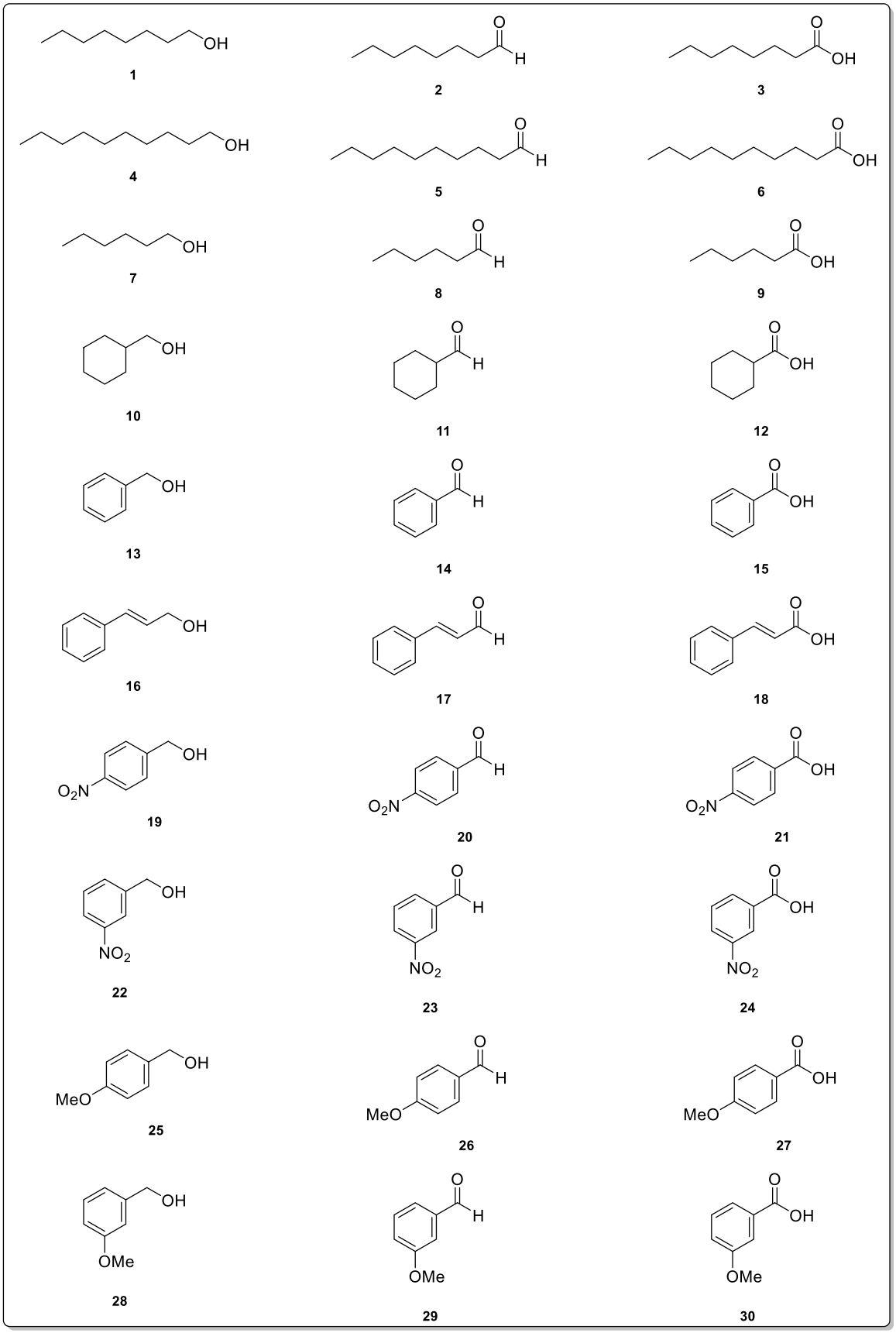
2.5 Oxidation of alcohols to aldehydes using aqueous bleach solution as oxidation agent

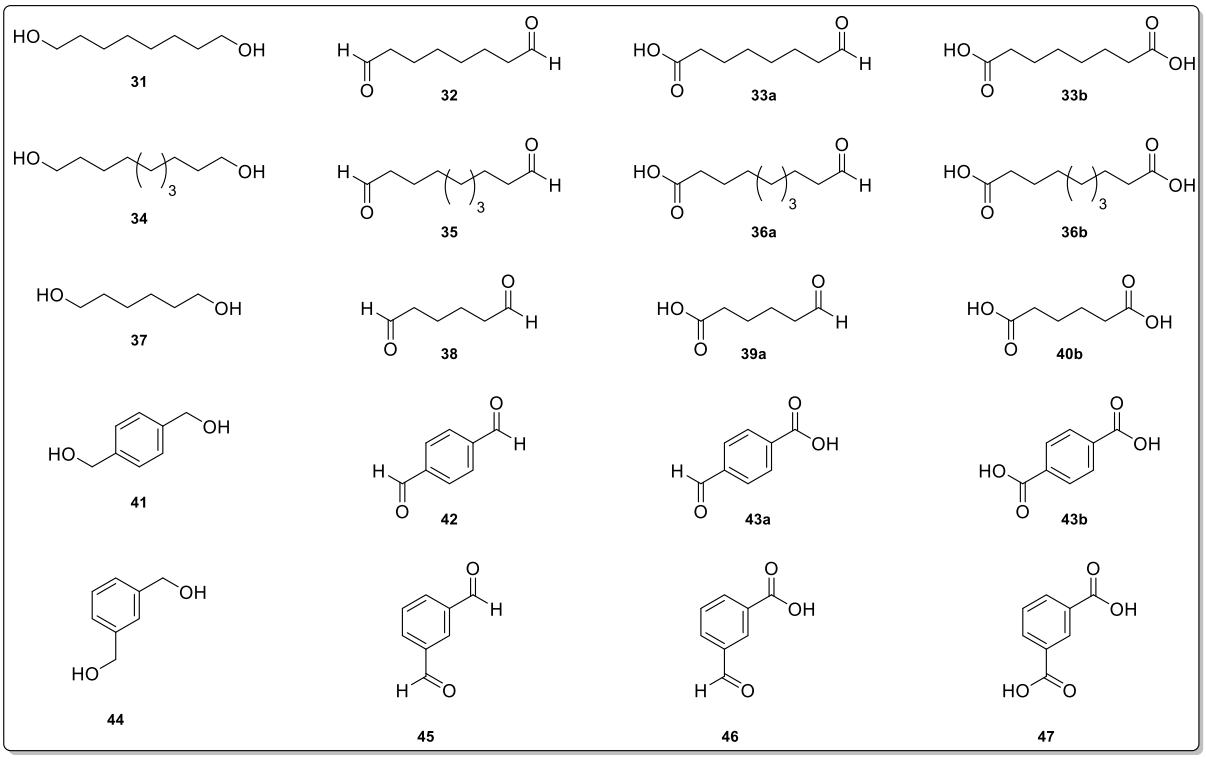
Table S10. Conversion of *n*-octan-1-ol (**1**) to *n*-octanal (**2**) (and octanoic acid **3** as by-product) and 2-octanol (**46**) to 2-octanone (**47**) determined by GC after different reaction times. Selectivity is defined as follows: $c_{aldehyde}/(c_{aldehyde}+c_{acid})$.

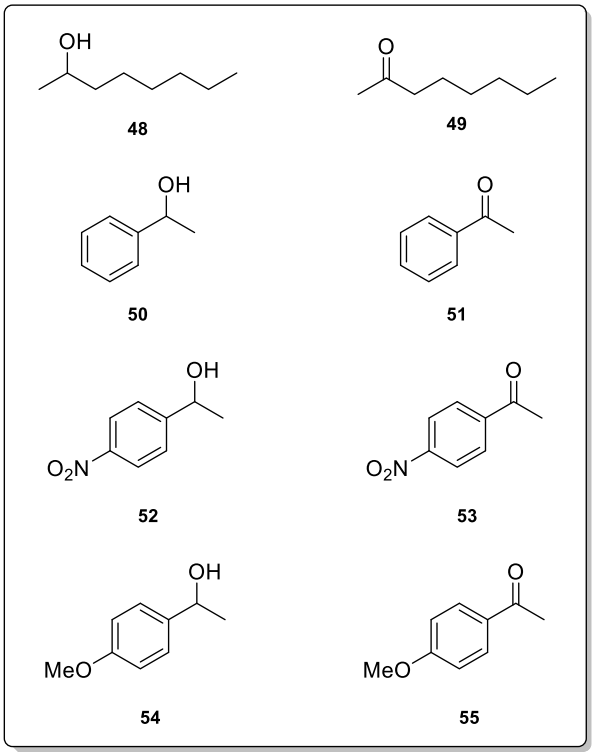
Entry	Alcohol	Reaction time /min	Added portions of hypochlorite solution	Conversion /%	Selectivity /%
1	**1**	75	5 (1.7 eq. in total)	96	94
2	**16**	75	5 (1.7 eq. in total)	99	91

3 Attachment

3.1 List of molecules

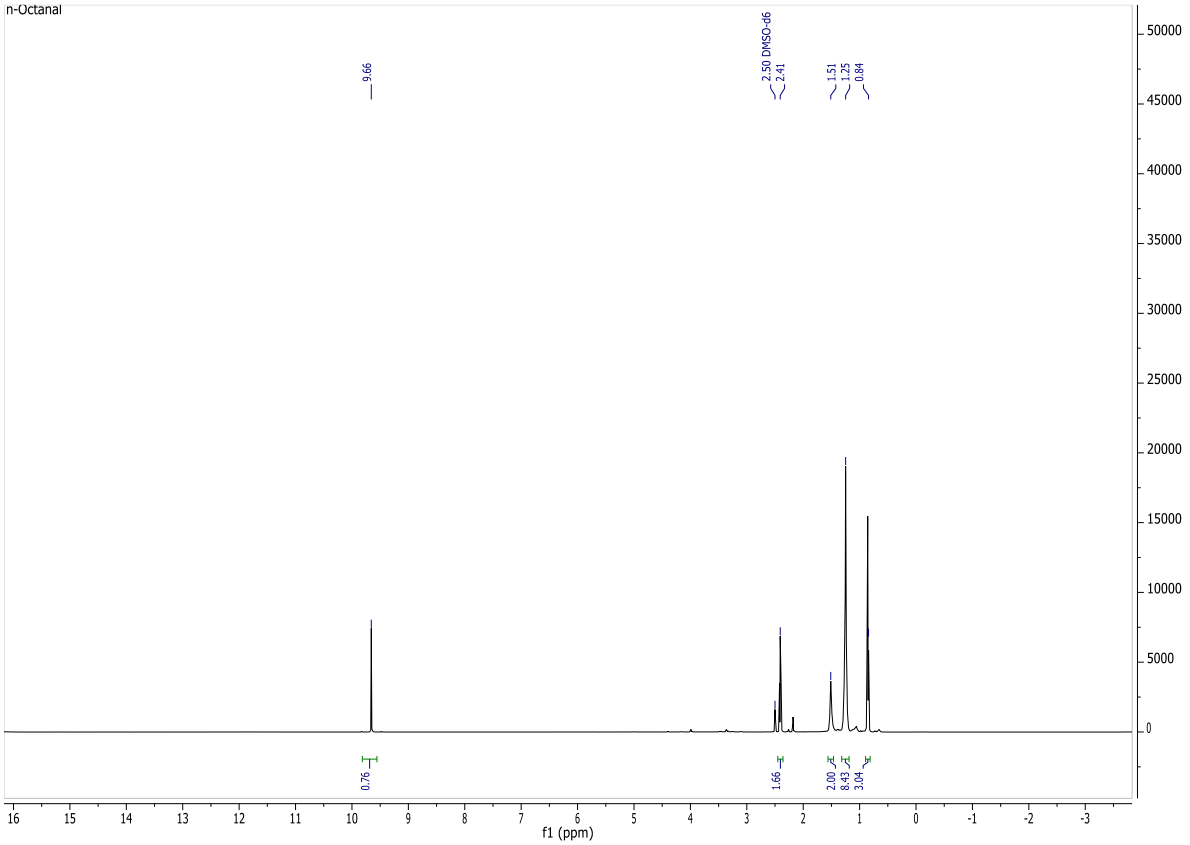


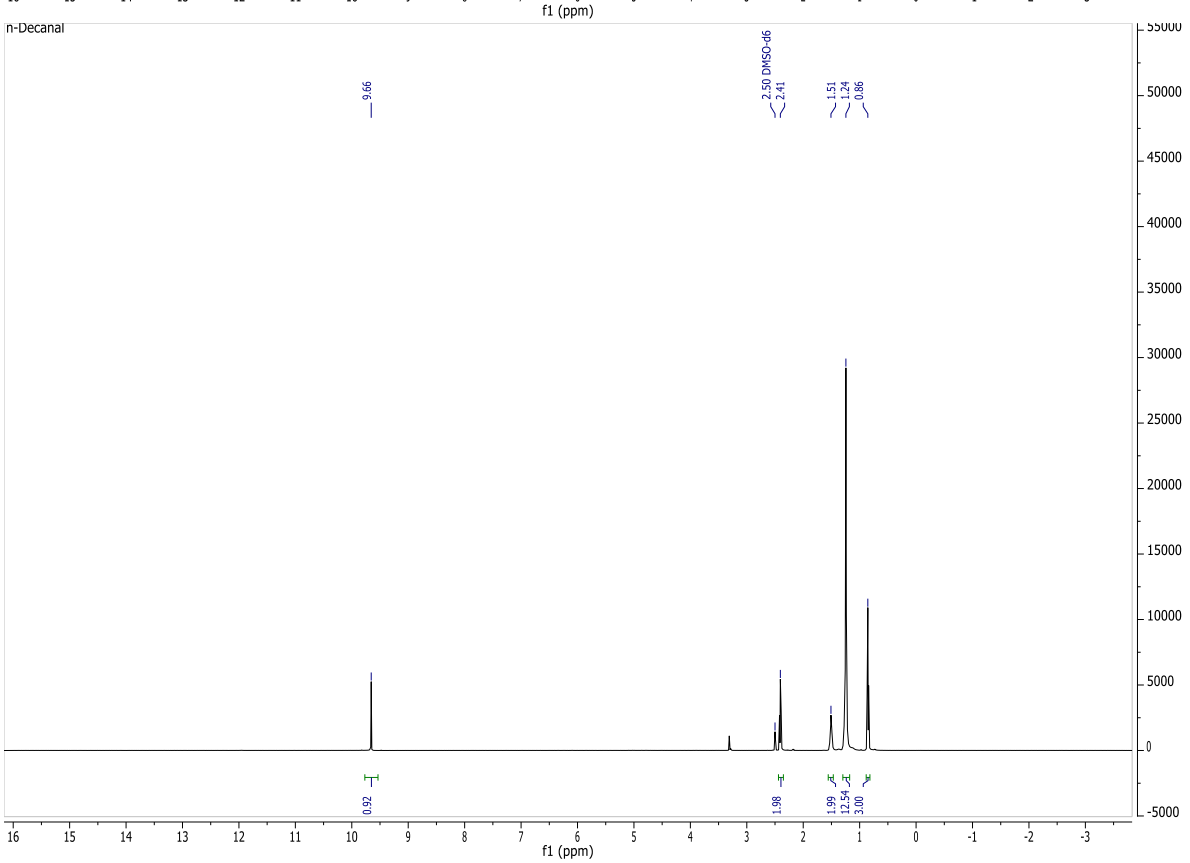

31
32
33a
33b
34
35
36a
36b
37
38
39a
40b
41
42
43a
43b
44
45
46
47

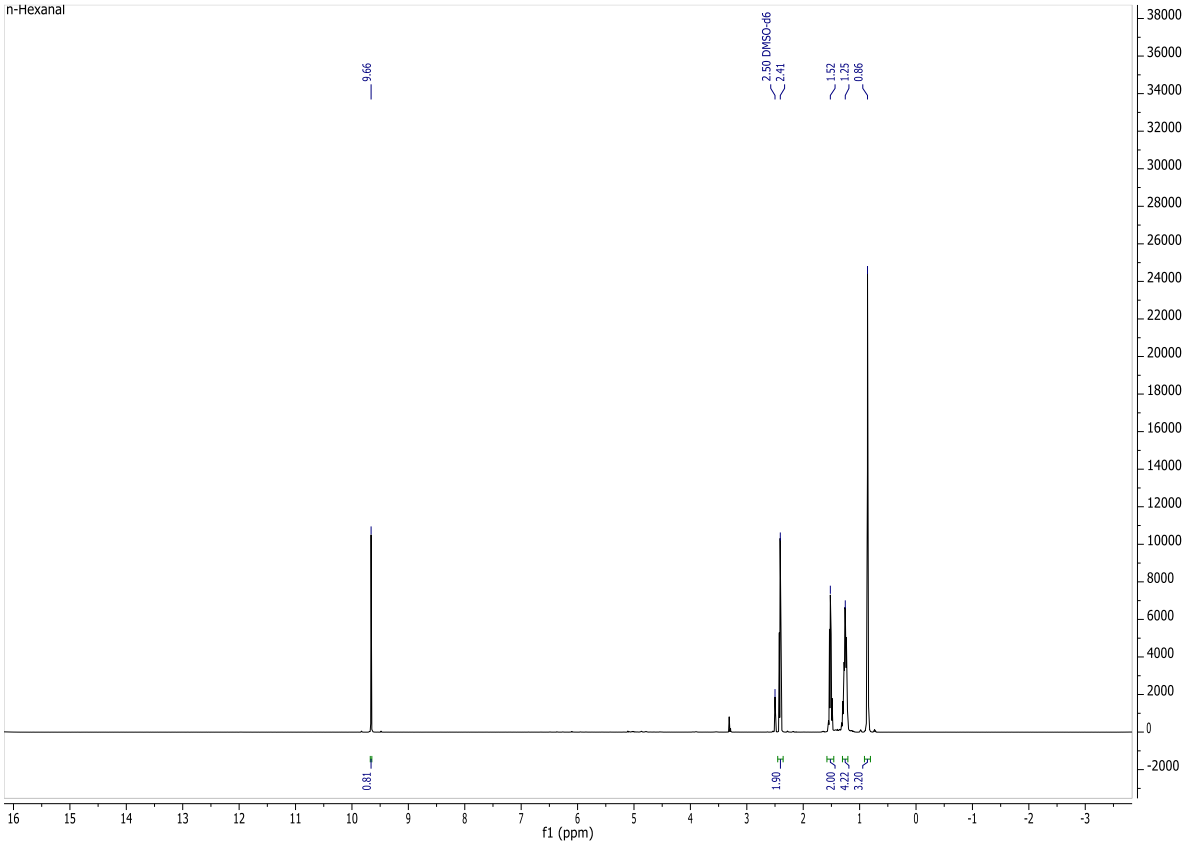

48
49
50
51
52
53
54
55

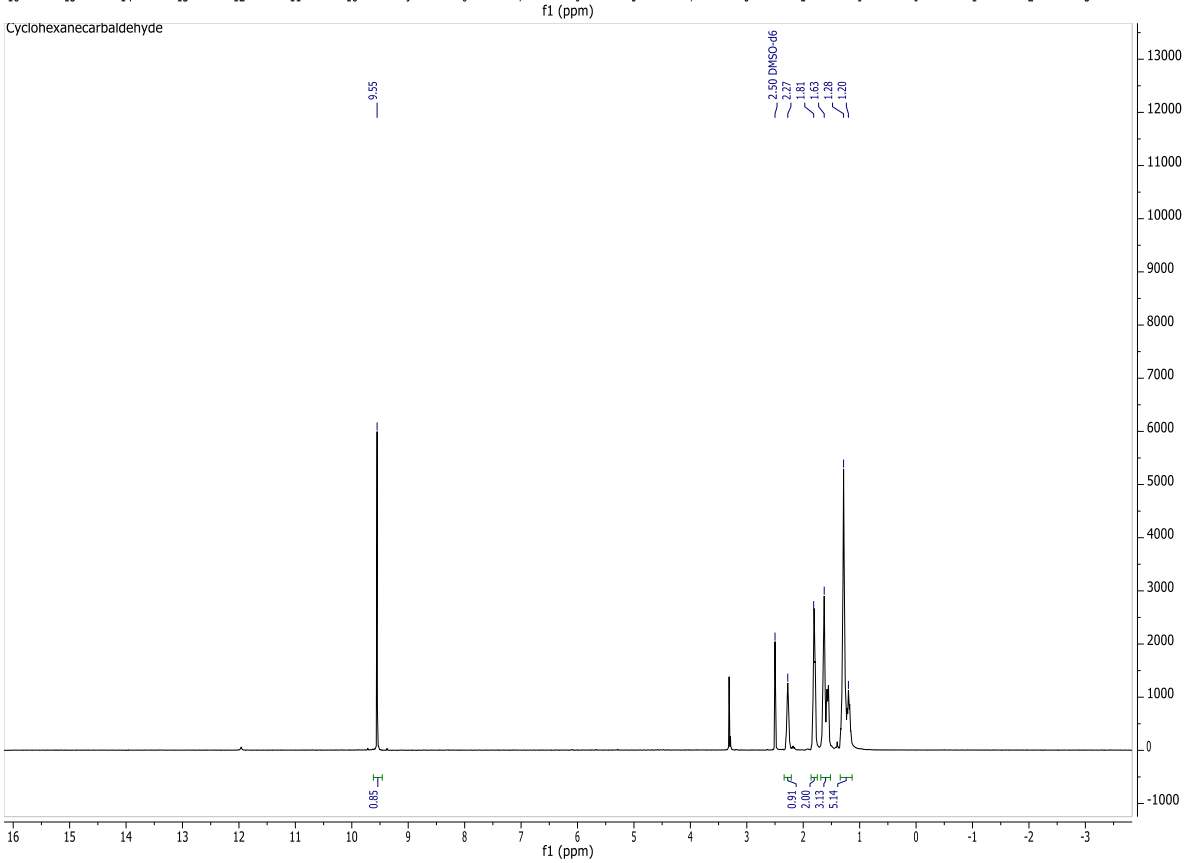
3.2 NMR-spectra

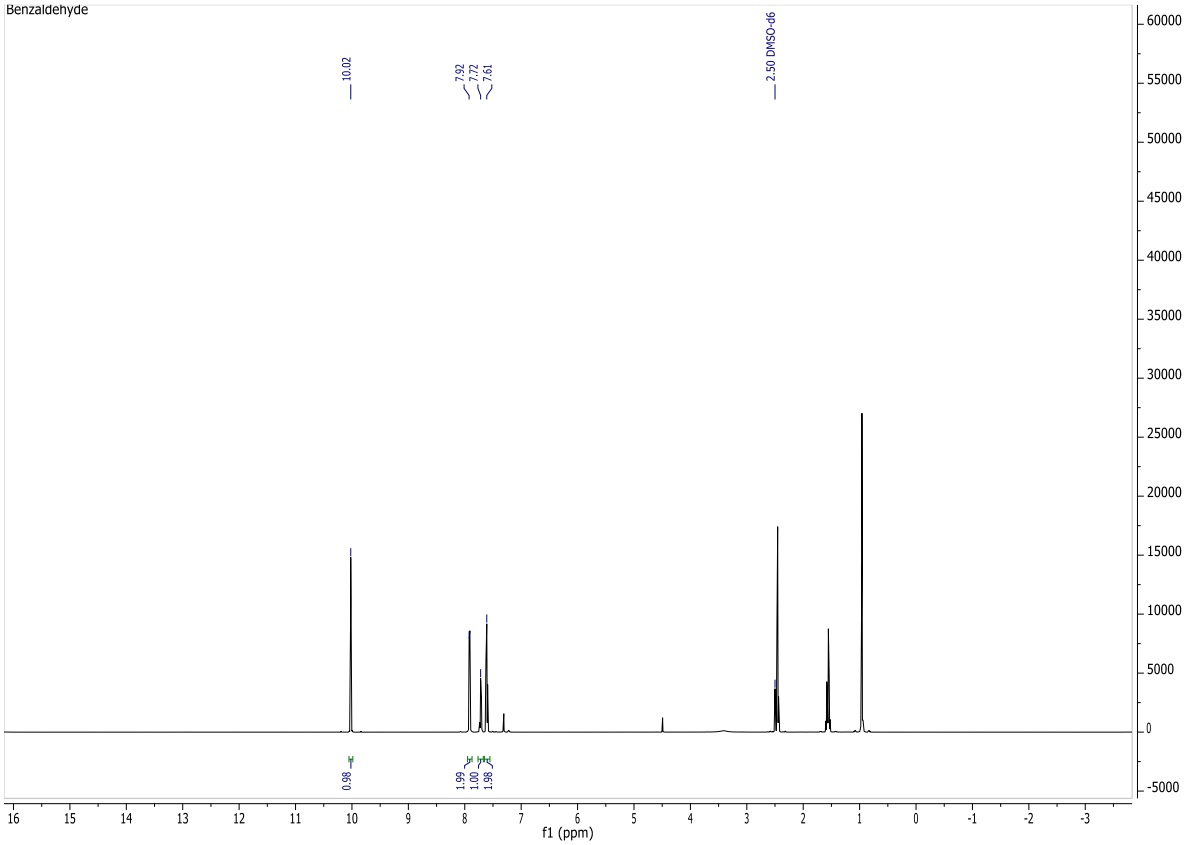
3.2.1 “Mono”-aldehydes






n-Hexanal
9.66
2.50 DMSO-d6
2.41
1.52
1.25
0.86
0.81
1.90
2.00
4.22
3.20
f1 (ppm)


Cyclohexanecarbaldehyde
9.55
2.50 DMSO-d6
2.27
1.81
1.63
1.28
1.20
0.85
0.91
2.00
3.13
5.14
f1 (ppm)


Benzaldehyde
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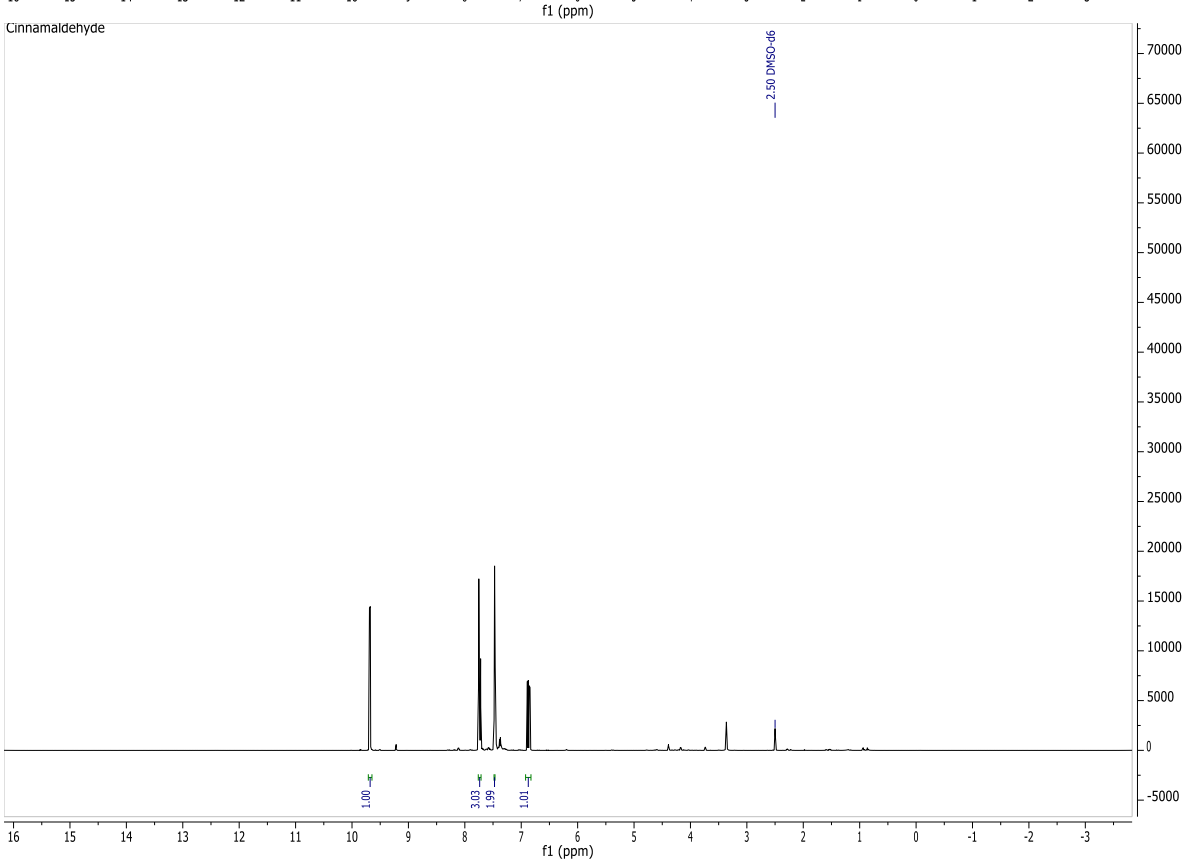
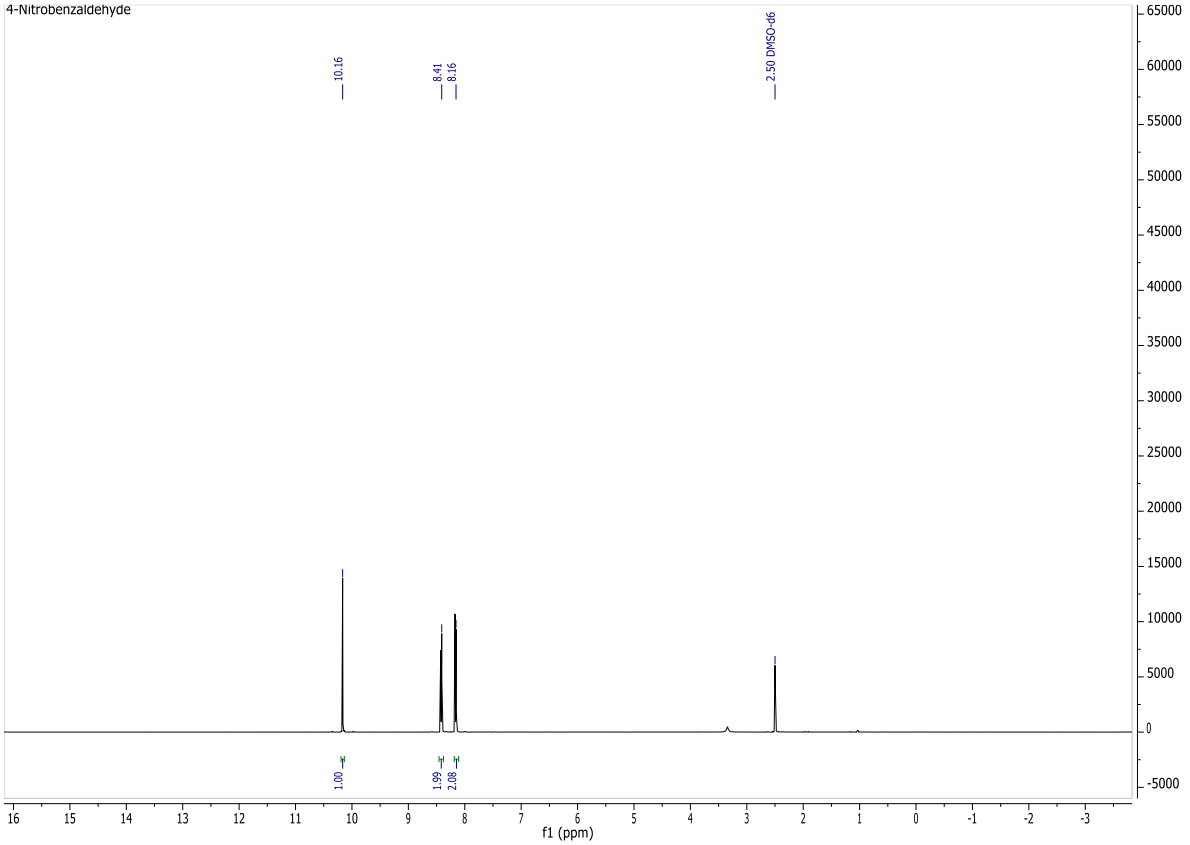
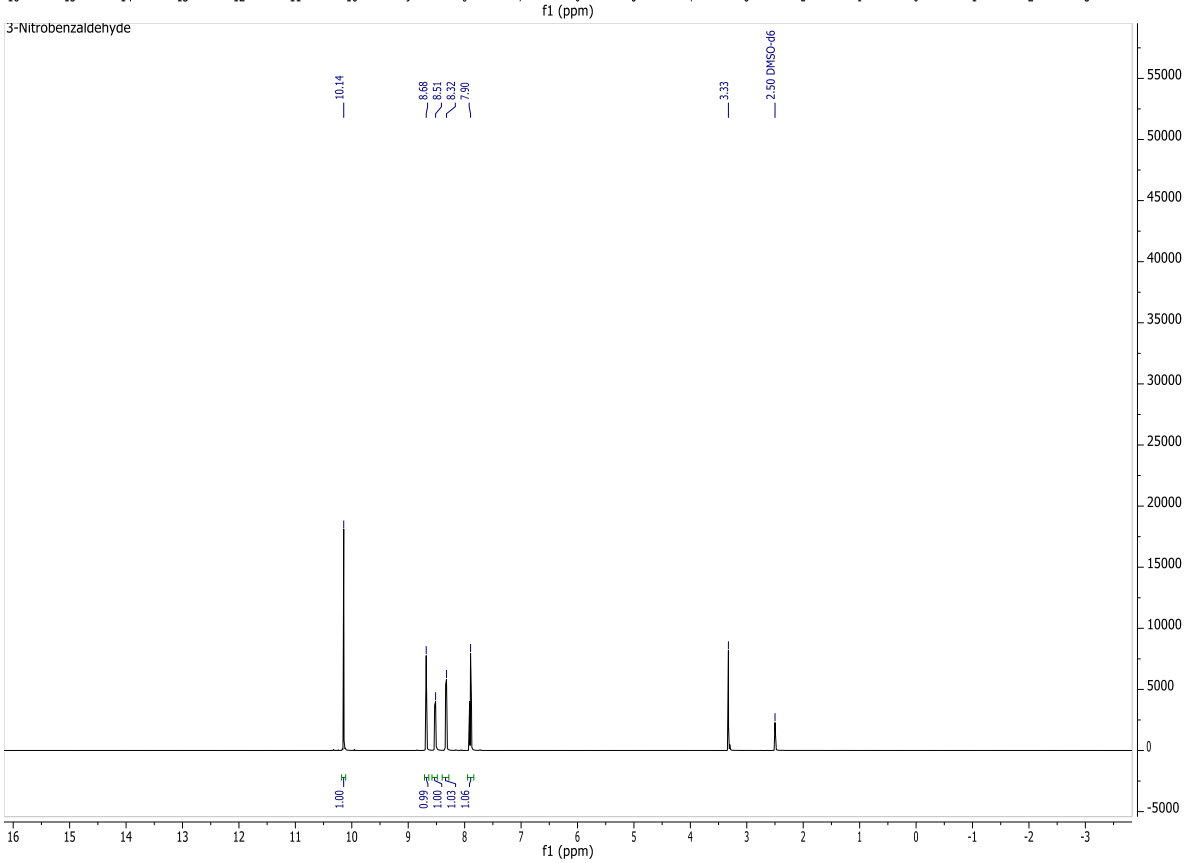
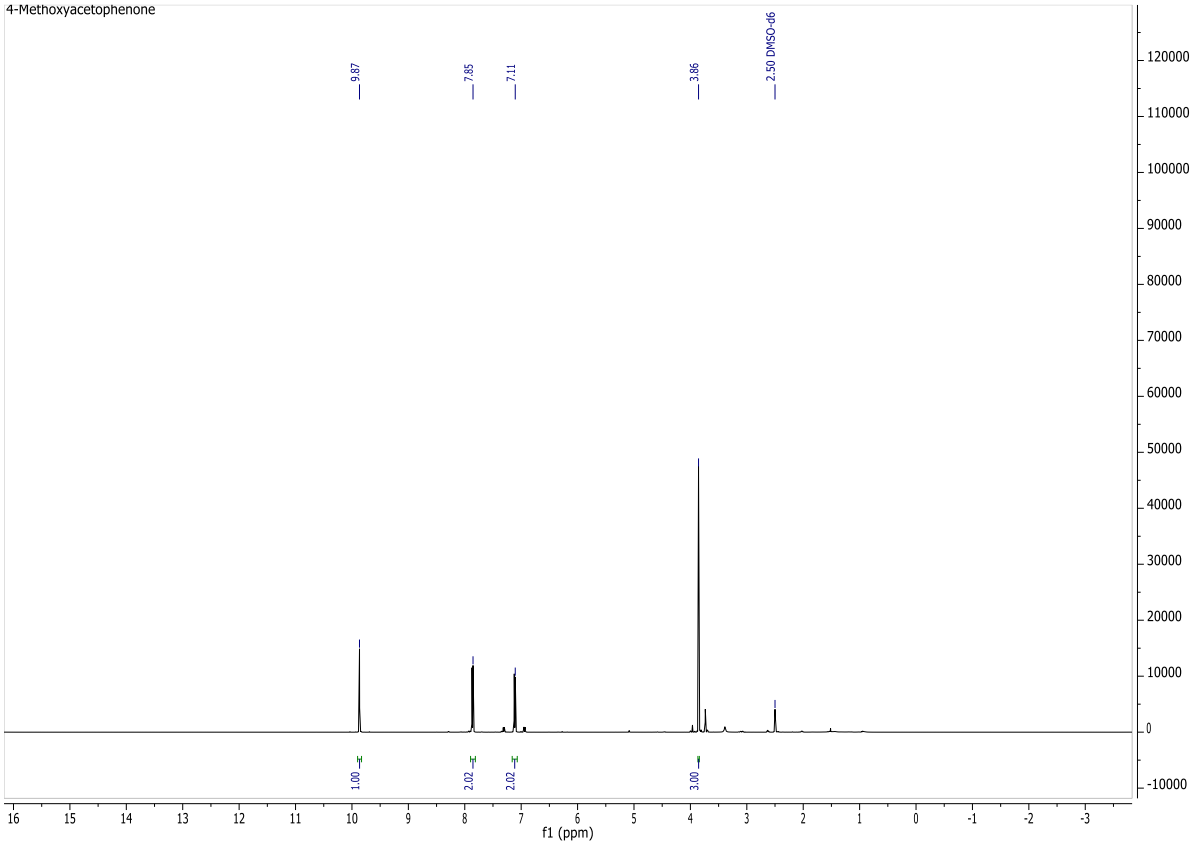
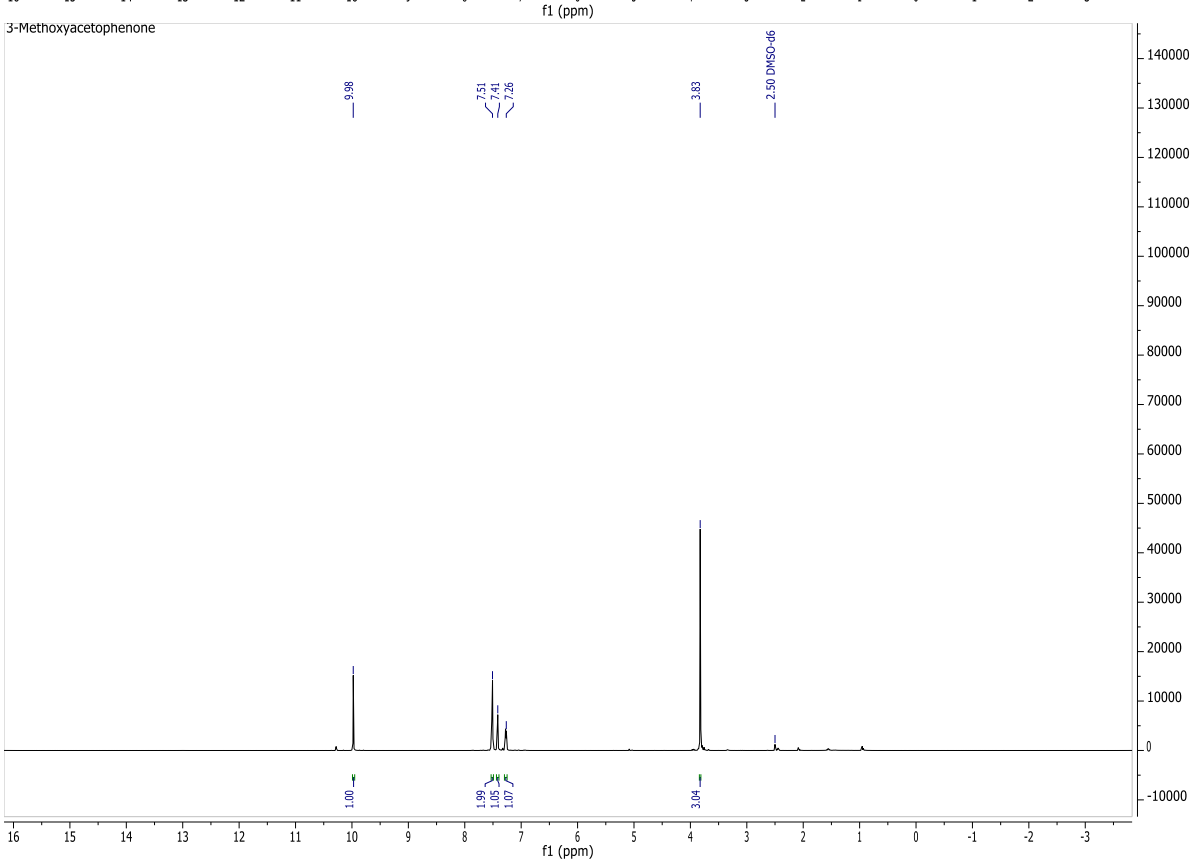
7.92
7.72
7.61
2.50 DMSO-d6
0.98
1.99
1.00
1.98
f1 (ppm)


Cinnamaldehyde
2.50 DMSO-d6
1.00
3.03
1.99
1.01
f1 (ppm)


4-Nitrobenzaldehyde
10.16
8.41
8.16
2.50 DMSO-d6
1.00
1.99
2.08
f1 (ppm)


3-Nitrobenzaldehyde
10.14
8.68
8.51
8.32
7.90
3.33
2.50 DMSO-d6
1.00
0.99
1.00
1.03
1.06
f1 (ppm)


4-Methoxyacetophenone
9.87
7.85
7.11
3.86
2.50 DMSO-d6
1.00
2.02
2.02
3.00
f1 (ppm)


3-Methoxyacetophenone
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7.51
7.41
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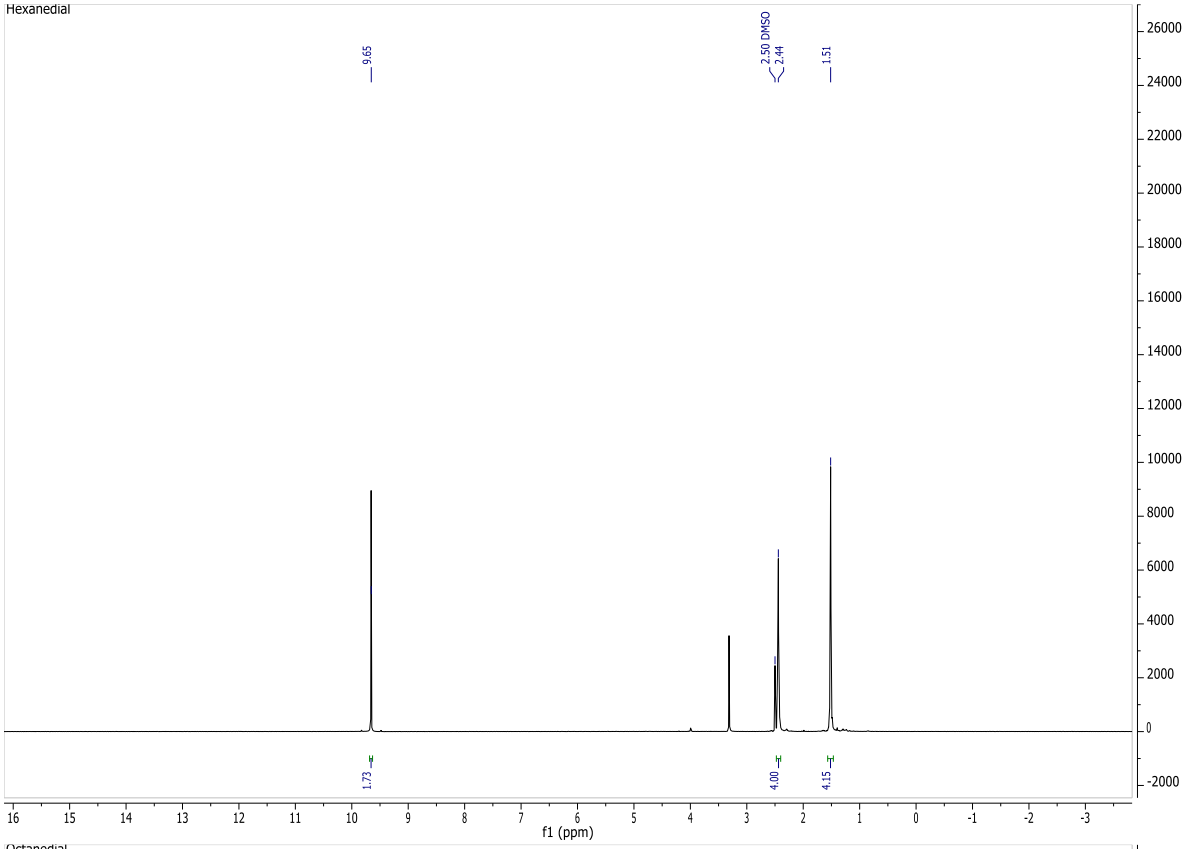
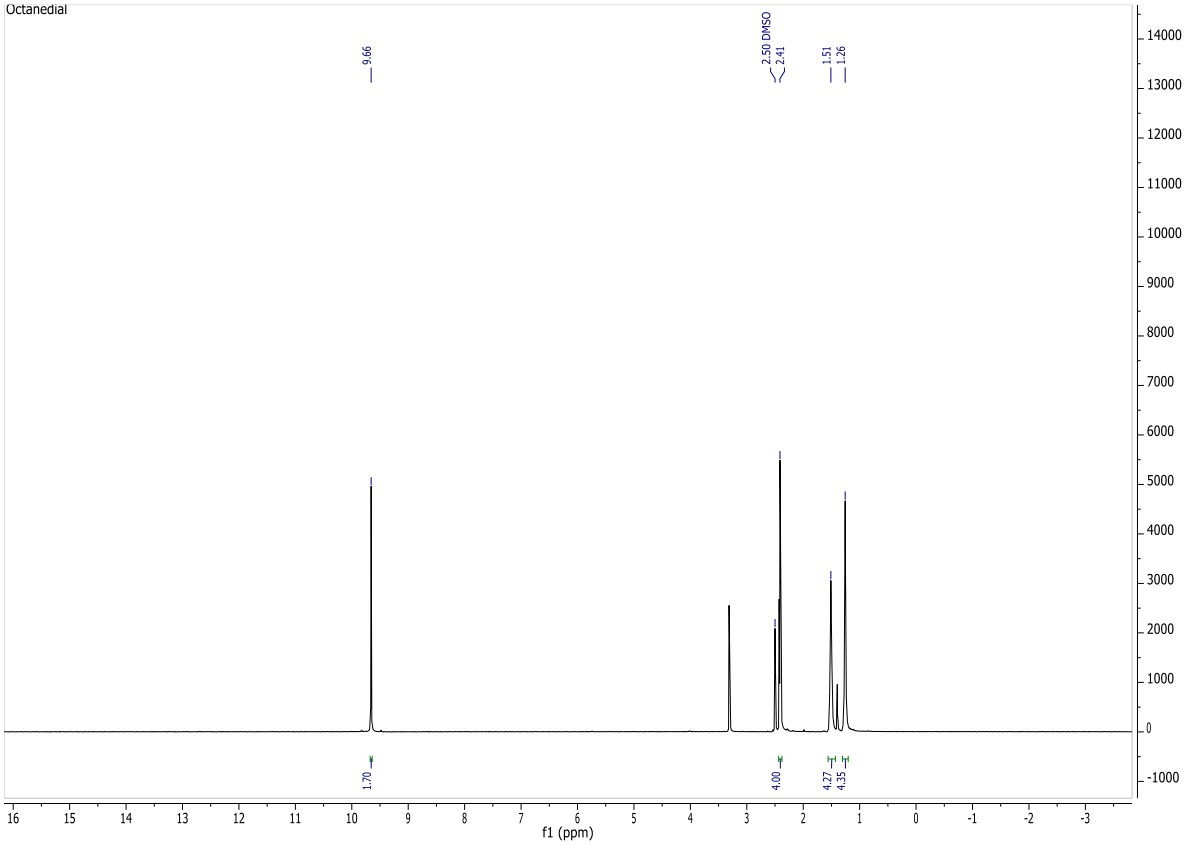
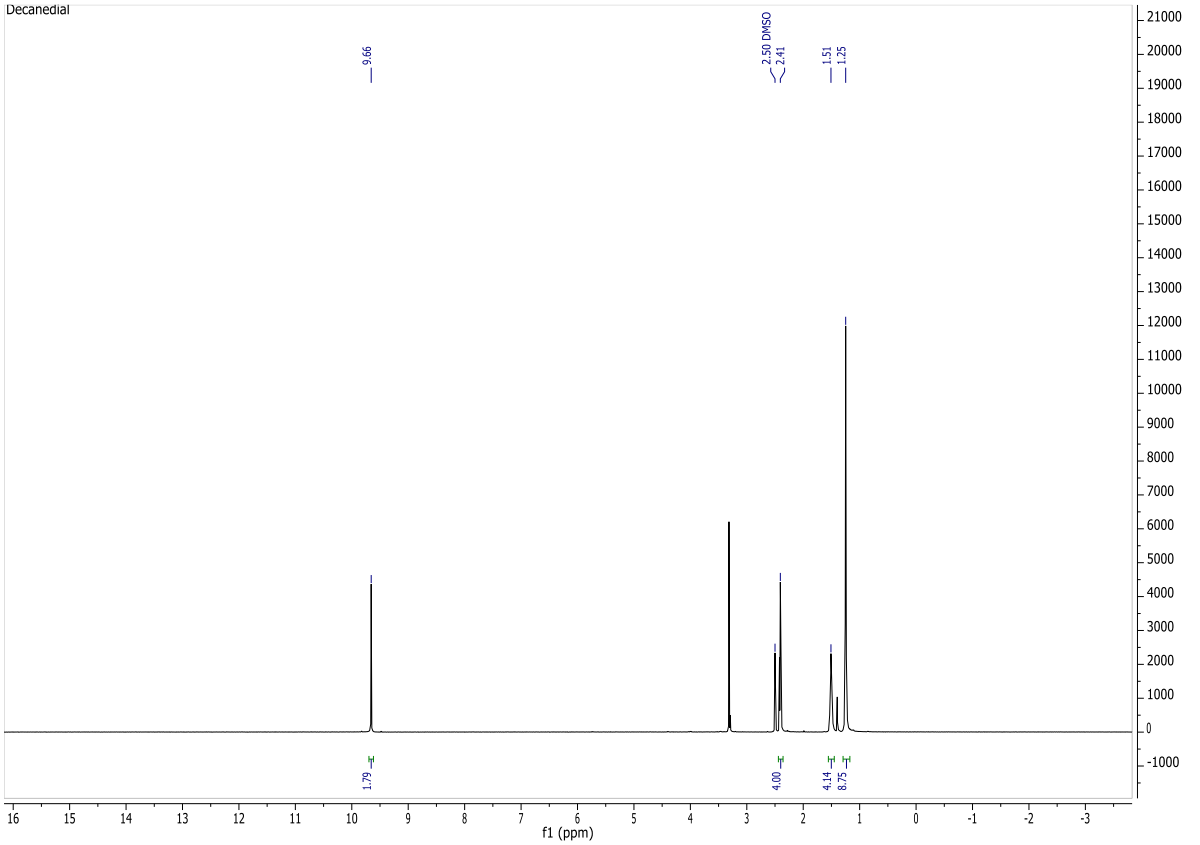
1.00
1.99
1.05
1.07
3.04
f1 (ppm)

3.2.2 Dialdehydes






Decanedial
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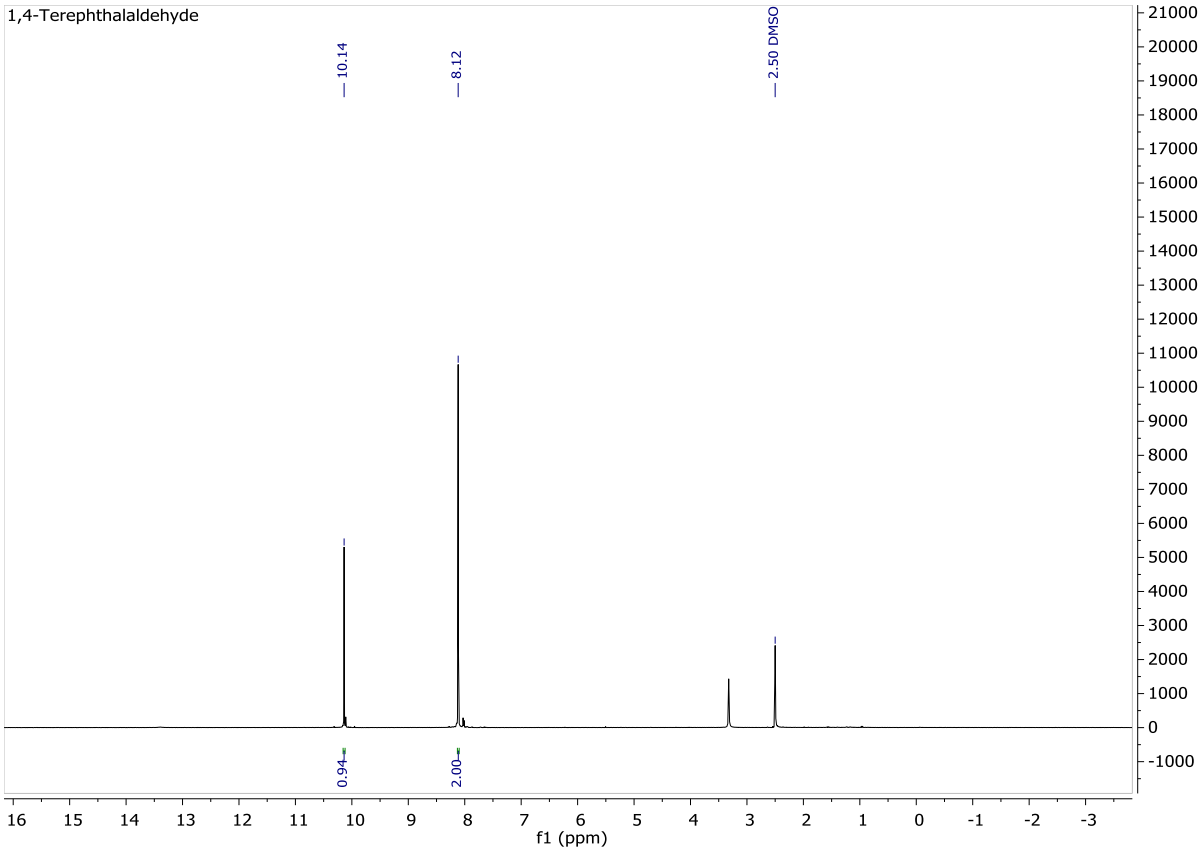
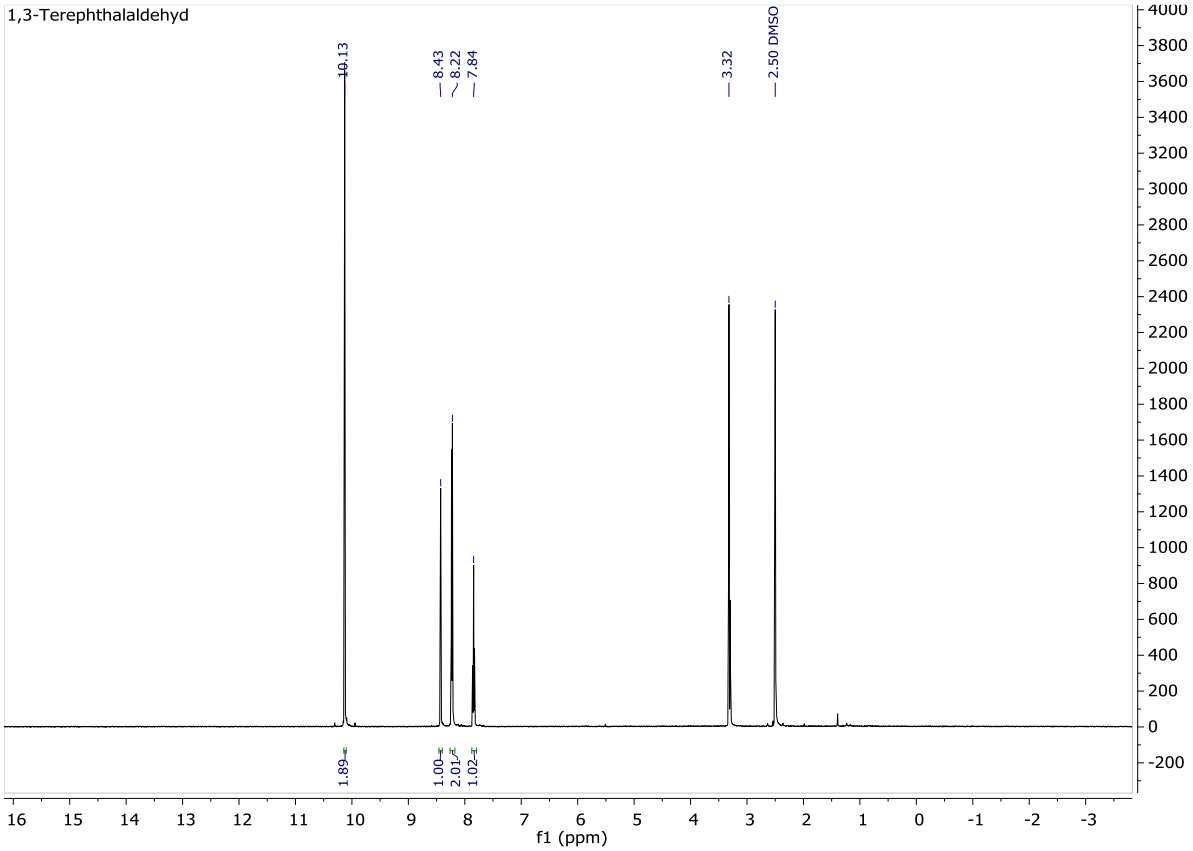
2.41
1.51
1.25
1.79
4.00
4.14
8.75
f1 (ppm)


1,4-Terephthalaldehyde
10.14
8.12
2.50 DMSO
0.94
2.00
f1 (ppm)


1,3-Terephthalaldehyd
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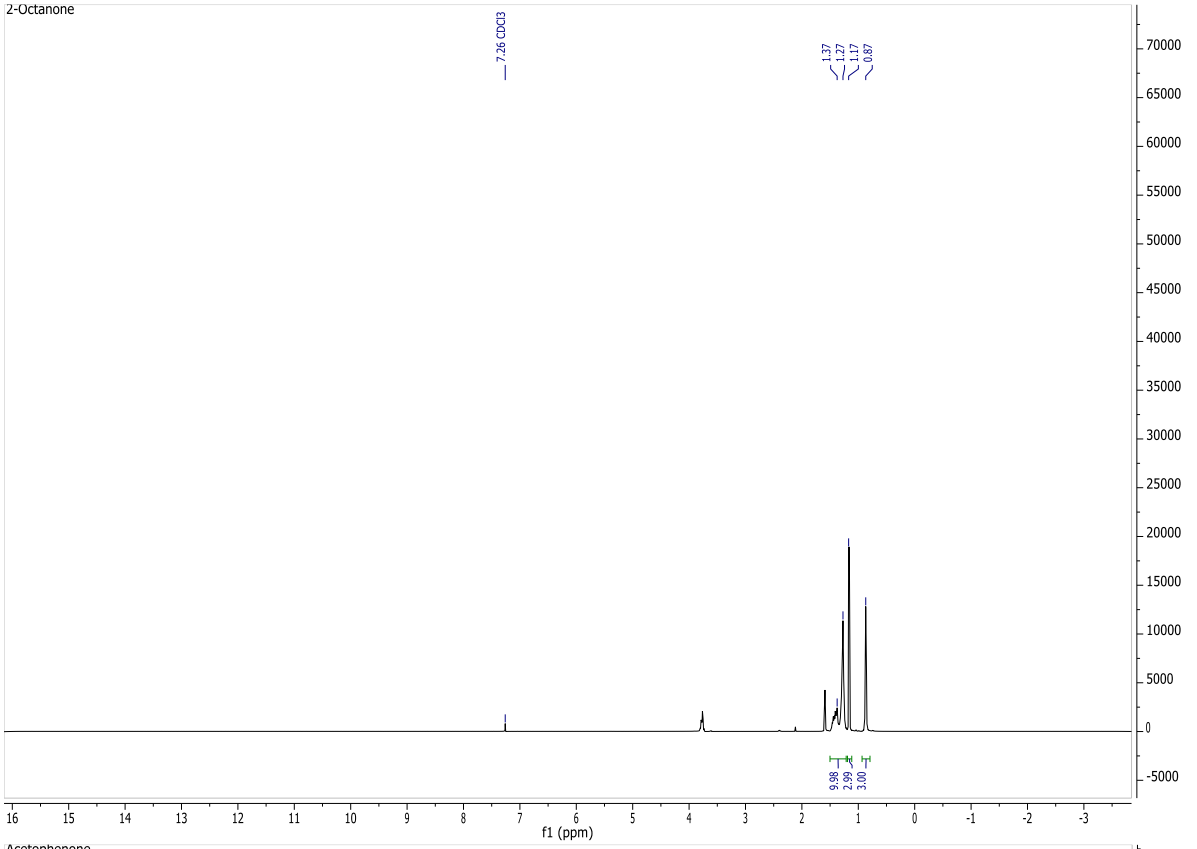
8.22
7.84
3.32
2.50 DMSO
1.89
1.00
2.01
1.02
f1 (ppm)

3.2.3 Ketones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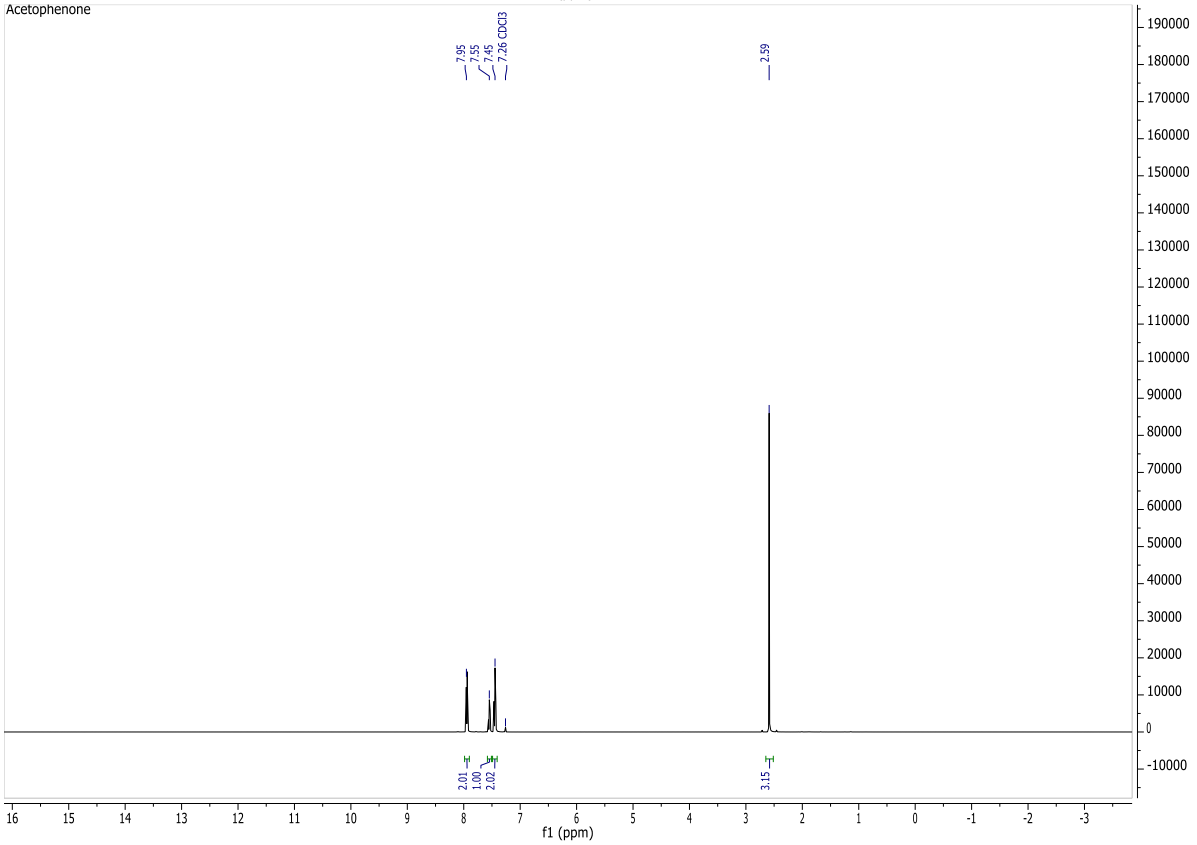
1.37
1.27
1.17
0.87
9.98
2.99
3.00
f1 (ppm)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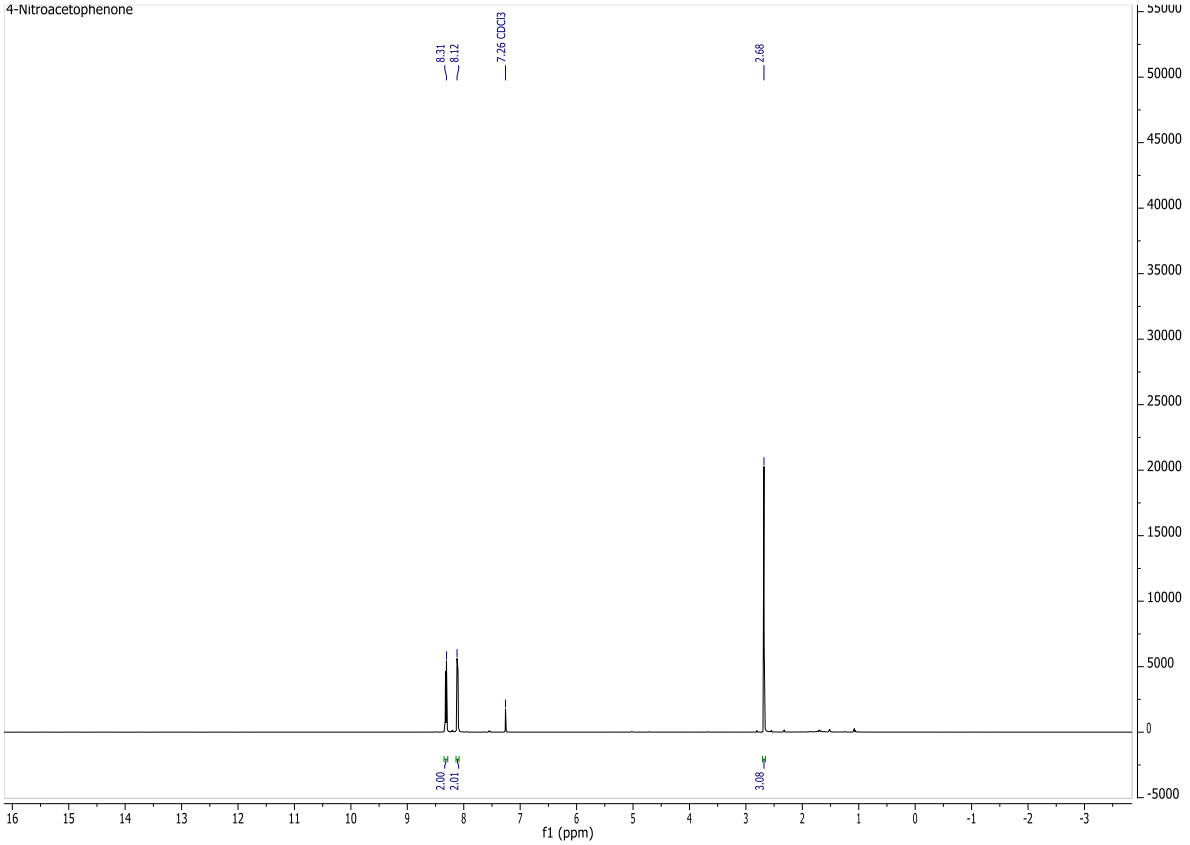
7.45
7.26 CDCl3
2.59
2.01
1.00
2.02
3.15
f1 (ppm)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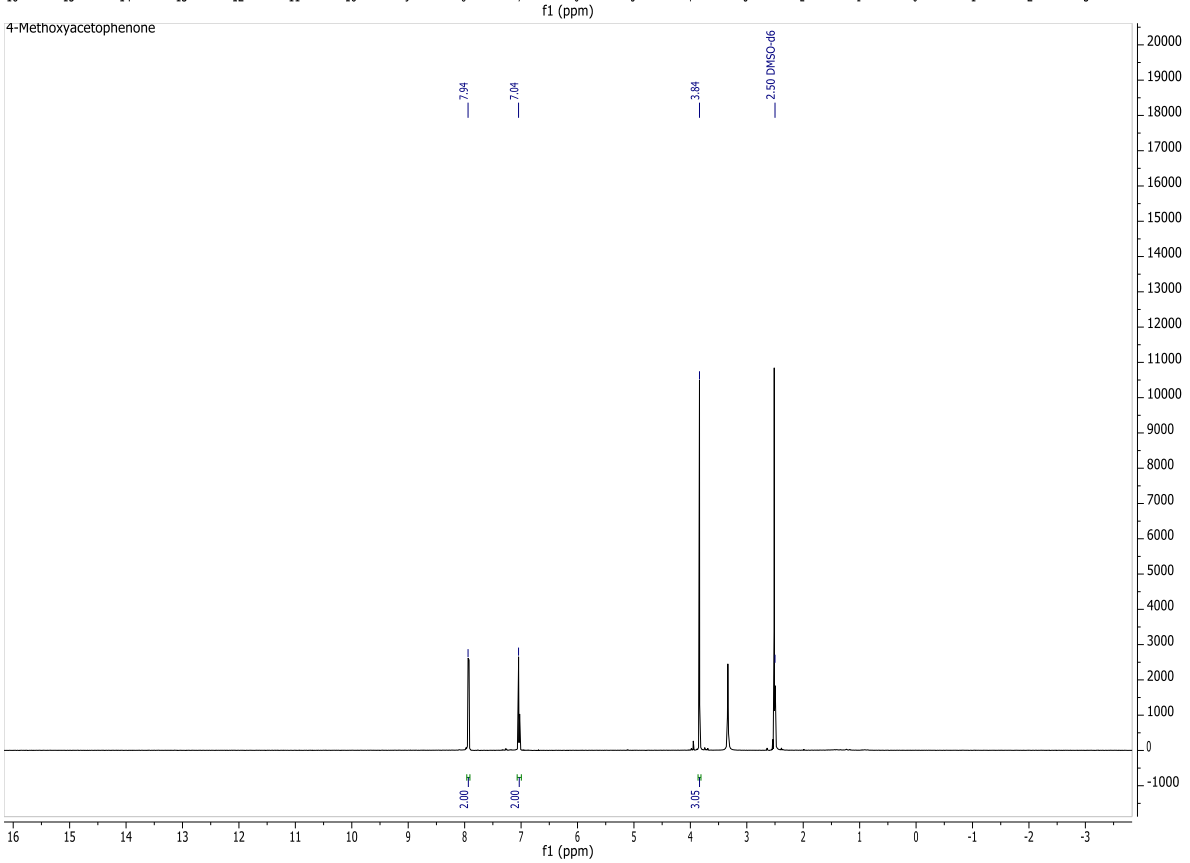
8.12
7.26 CDCl3
2.68
2.00
2.01
3.08
f1 (ppm)


4-Methoxyacetophenone
7.94
7.04
3.84
2.50 DMSO-d6
2.00
2.00
3.05
f1 (ppm)

Article 3

Enzymatic Synthesis of Aliphatic Nitriles at a Substrate Loading of up to 1.4 Kg/L: A Biocatalytic Record Achieved with a Heme Protein

A. Hinzmann, S. Glinski, M. Worm, H. Gröger

Journal of Organic Chemistry **2019**, 84, 8, 4867

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Author contribution

HG initiated the project. Experiments were planned and performed by AH. SG (bachelor student, supervised by AH) performed the 250 mL-scale experiment. MW (bachelor student, supervised by AH) performed the cosolvent-study, mentioned in the Supporting Information. AH performed all other experiments and wrote the manuscript. AH and HG read and edited the manuscript.

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Enzymatic Synthesis of Aliphatic Nitriles at a Substrate Loading of up to 1.4 kg/L: A Biocatalytic Record Achieved with a Heme Protein

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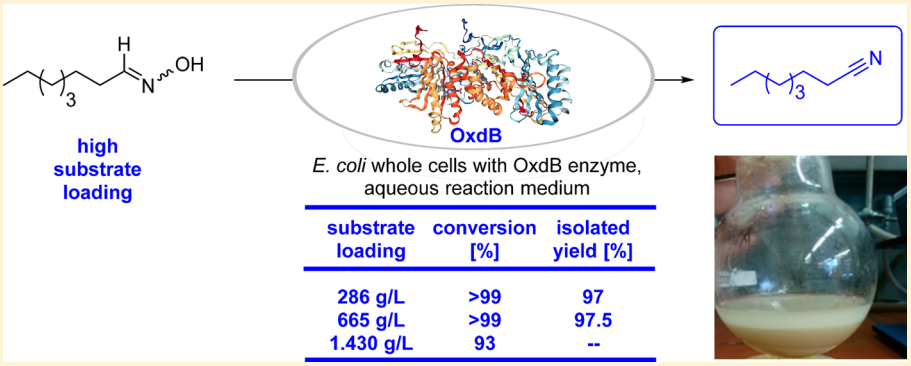
Supporting Information



ABSTRACT: A biocatalytic approach toward linear aliphatic nitriles being widely used as industrial bulk chemicals has been developed that runs at high substrate loadings of up to 1.4 kg/L as demonstrated for the synthesis of *n*-octanenitrile. This substrate loading is one of the highest ever reported in biocatalysis and to best of our knowledge the highest obtained for a water-immiscible product in aqueous medium. It is noteworthy that the biotransformation at such a high substrate loading was achieved by means of a metalloprotein bearing an iron-containing heme subunit in the active site. In detail, an aldoxime dehydratase from *Bacillus* sp. OxB-1 was used as a biocatalyst for a dehydration of aldoximes as readily available starting materials due to their easy preparation from aliphatic aldehydes through spontaneous condensation with hydroxylamine as bulk chemical. Excellent conversions toward the nitriles in the two-phase system were achieved and the products are easily separated from the reaction mixture without the need for further purification. Aliphatic nitriles are used in industry as solvents and intermediates for the production of surfactants and life sciences products.

INTRODUCTION

Although biocatalysis is recognized as a highly valuable tool in the fine and pharmaceutical industry with a proven track record for numerous processes on large scale,[1] analogous examples in the field of bulk chemicals[2,3] are still rare in spite of decade-long attempts addressing this challenge. The probably most prominent exception is the conversion of acrylonitrile into acrylamide utilizing a nitrile hydratase.[4,5] Having been developed by Yamada and Asano in the 1970s,[4,5] this process has been established on an industrial scale by Nitto Chemicals (today: Mitsubishi Rayon), running today on an annual multi-10000 ton scale with a substrate loading exceeding 500 g/L. At the same time, this process illustrates a major task in this field of applying enzymes for bulk chemical production, that is, to reach extremely high substrate loadings of several 100 g per L of reaction medium. Such high substrate loadings of hydrophobic chemicals (as a key property of most bulk chemicals known in our today's petrochemically dominated "product tree") in aqueous medium raises solubility issues as well as enzyme stability concerns. Thus, a particular challenge represents the development of processes for commodity chemicals which overcome such limitations and enable access to even hydrophobic bulk chemicals when operating at multi-100 g/L and starting from hardly water-soluble substrates. In the following we present the first example of a biocatalytic process for such a hydrophobic bulk chemical running in aqueous medium at a loading of >1 kg/L of a hardly water-soluble substrate with a water-solubility being below 1 g/L. Such a process has been exemplified for the synthesis of fatty nitriles in the presence of an aldoxime dehydratase[6−22] (Oxd) starting from the corresponding aldoxime substrates (that are easily accessible from readily available fatty aldehydes by spontaneous condensation with hydroxylamine). Aliphatic nitriles are widely used, for example, as solvents for many applications in industry and as key intermediates for the production of surfactants, agrochemicals, and pharmaceuticals.

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RESULTS AND DISCUSSION

At first, for this study, a range of aliphatic aldoxime substrates were prepared starting from the corresponding aldehydes and hydroxylamine as a further bulk chemical used in the Beckmann rearrangement for ε-caprolactam production. In detail, we synthesized the aldoximes derived from *n*-hexanal, *n*-octanal, *n*-decanal, and *n*-dodecanal (see the Experimental Section), and subsequently, a screening was conducted on a 0.5 mL scale to determine the conversion of these substrates to the corresponding nitriles at different concentrations (10 mM to 2 M) in an aqueous reaction system consisting of potassium phosphate buffer (PPB). In addition, as a result of a cosolvent screening carried out beforehand with *n*-octanaloxime (see the Supporting Information), 10% (v/v) EtOH was added as cosolvent to increase the solubility of the substrates. As a biocatalyst, a recombinant aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) was utilized as wet whole cells in a concentration of 33 g/L (bio wet weight (bww)). OxdB was prepared by expression in *E. coli* cells, which were then used as whole cell catalyst in biotransformations. From 1 L *E. coli* culture approximately 19 g wet cells active as biocatalyst can be obtained.

The reaction was performed at 30 °C for 24 h, and the conversion was determined after extraction with ethyl acetate (EtOAc) using GC. In general, all four aldoxime substrates were converted to the corresponding nitriles; however, the conversion of the C_{12}-aldoxime to the related nitrile was lower in comparison to C_6-, C_8-, and C_{10}-aldoximes (Figure 1). In

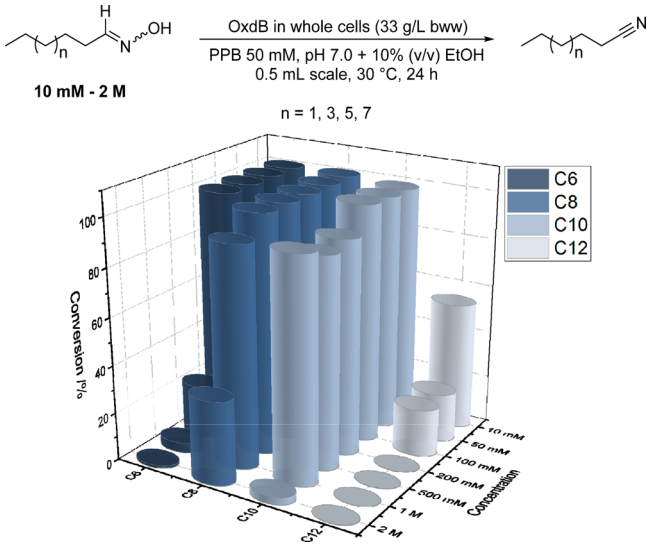
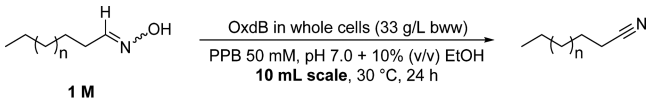


Figure 1. Screening of conversion of various aliphatic aldoximes into the corresponding nitriles using the enzyme OxdB in whole cells on a 0.5 mL scale. Reactions were performed in triplicate, and mean values of conversions, determined by GC after extraction using ethyl acetate (EtOAc), are shown. Conversion values within triplicates vary ±3%.

the case of the C_6-aldoxime, a full conversion was obtained for concentrations of up to 200 mM, and nearly full conversion of C_8- and C_{10}-aldoximes was reached with substrate concentrations of up to 1 M, which corresponds to ∼140 g/L (C_8) and ∼170 g/L (C_{10}). In this work the substrate concentration of 1 M is defined as 1 mol of substrate per liter of aqueous reaction medium (and, thus, does not refer to the total volume of the resulting mixture). Since all of these substrates have a very low solubility in water and although 10% ethanol was used as a cosolvent, a large amount of the substrates was not dissolved, thus being present as solid crystals in the reaction mixture. Hence, mass transfer and, thus, mixing could be a limitation of these screening experiments carried out on a 0.5 mL scale.

In the next step, this issue was addressed by conducting the reactions on a 10 mL scale in round-bottom flasks and with a magnetic stirrer. These 10 mL scale biotransformations were performed using C_6-, C_8-, and C_{10}-aldoximes in the presence of the enzyme OxdB as a biocatalyst. Due to the low conversion of C_{12}-aldoxime in the 0.5 mL scale experiments, this substrate was not used for further investigations. The results of the 10 mL scale experiments using 1 M substrate concentration of C_6-, C_8-, and C_{10}-aldoximes are shown in Table 1.

Table 1. Biotransformations of 1 M *n*-Hexanaloxime, *n*-Octanaloxime, and *n*-Decanaloxime Using OxdB as a Whole-Cell Catalyst on a 10 mL Scale



no.	substrate	reaction time (h)	conversion[a] (%)	isolated yield (%)
1	*n*-hexanaloxime	24	>99	92
2	*n*-octanaloxime	24	>99	89
3	*n*-decanaloxime	24	87	

[a]Conversion of aldoximes to the relating nitriles were determined by GC after extraction of the reaction mixture using ethyl acetate.

Within a reaction time of 24 h, a full conversion was reached for *n*-hexanaloxime as well as *n*-octanaloxime, whereas in case of *n*-decanaloxime a conversion of 87% was detected. In part, these biotransformations running on a 10 mL scale showed improved results in comparison to the 0.5 mL scale experiments, especially for the C_6-aldoxime. In the latter case, on a 0.5 mL scale less than 10% of was converted within 24 h at a substrate concentration of 1 M, whereas a full conversion was found for the same substrate concentration when carrying out the reaction on a 10 mL scale. In the case of C_8- and C_{10}-aldoxime also on a 0.5 mL scale ∼90% conversion was reached, which corresponds to the results obtained from the 10 mL scale biotransformations. The improved conversion of the aldoximes to the corresponding nitriles in the 10 mL scale experiments can be rationalized by a better mixing in the round-bottom flask in comparison to the microreaction tubes used in the case of the 0.5 mL scale experiments. Thus, further experiments were performed using the 10 mL scale reaction setup. On the basis of the results, which were obtained from the 1 M biotransformations, higher substrate concentrations were also tested. Due to the low solubility of the aldoxime and potential stirring and mixing problems, the substrates were added in portions (portions correspond to 143 g substrate per liter of aqueous reaction medium for C_8-aldoxime, 171 g/L for C_{10}-aldoxime, and 115 g/L for C_6-aldoxime).

Since we could not obtain full conversion of 1 M *n*-decanaloxime, as a next step, C_{10}-feeding experiments were conducted (Table 2). The first experiment was done at a 0.5 M substrate concentration, and after 1 and 9 h further substrate was added in portions, being equivalent to 0.5 M, to the reaction mixture (resulting in a 1.5 M overall concentration).

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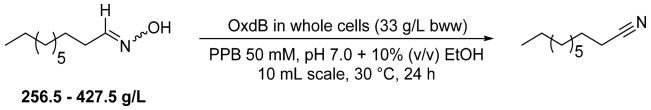
Table 2. Biotransformation of *n*-Decanaloxime with Whole Cells of OxdB to the Relating Nitrile Using a 0.5 M Feeding Approach on a 10 mL Scale



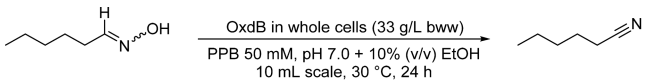
no.	final concn (g/L)	no. of portions[a]	conversion[b] (%)	isolated yield (%)
1	256.5	2	>99	85
2	342	3	>99	83.5
3	427.5	4	93	

[a]Added portions of *n*-decanaloxime during the reaction. Each portion corresponds to a substrate concentration of 0.5 M. [b]Conversion of aldoximes to the relating nitriles were determined by GC after extraction of the reaction mixture using ethyl acetate.

After 24 h reaction time, a conversion of >99% and an isolated yield of 85% of the desired product was obtained (Table 2, no. 1). In the next step, the substrate loading was further increased to 2 M, leading to a full conversion to the corresponding nitrile within 24 h as well (Table 2, no. 2). When the substrate concentration was further increased to 2.5 M of *n*-decanaldoxime, 93% conversion was obtained after 24 h reaction time at a catalyst loading of OxdB whole cells of 33 g/L bww. It is noteworthy that this concentration corresponds to an amount of approximately 430 g/L of *n*-decanaloxime.

Furthermore, an experiment with *n*-hexanaloxime running at a substrate concentration of 2.5 M was also conducted, and by means of a substrate feeding approach we were pleased to find that this process results in a full conversion and isolation of *n*-hexanenitrile in a high yield of 81% (Scheme 1).

Scheme 1. Synthesis of *n*-Hexanenitrile at a Substrate Loading of 287.5 g/L Using OxdB in Whole Cells on a 10 mL Scale Using a Substrate-Feeding Approach



Since *n*-octanaloxime also turned out to be a promising substrate for the enzyme OxdB in the 0.5 mL scale screening experiment and as the resulting product *n*-octanenitrile represents a chemical of commercial interest, a detailed investigation on the biocatalytic nitrile synthesis at high substrate loading was done using this substrate. Therefore, we performed 10 mL scale biotransformations of *n*-octanaloxime to the corresponding nitrile using a feeding approach, thus adding portions being equivalent to a substrate concentration of 1 M to the stirred reaction solution (Table 3).

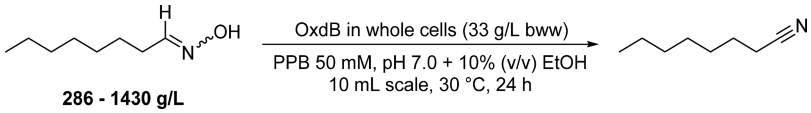
At first, we studied the biotransformation with an overall concentration of 2 M of the aldoxime, which resulted in full conversion and an isolated yield of 97% (Table 3, no. 1). In a next step, we raised the substrate concentration to 4.65 M. This concentration corresponds to a substrate loading of 665 g/L, and also in this case we could obtain a full conversion within 24 h as well as an isolated yield of 97.5% (Table 3, no. 2). Photos of the reaction mixture at various stages of the reaction are shown in Figure 2.

It is noteworthy that while a homogeneous emulsion is formed during the reaction through intensive stirring (thus enabling rapid mass transfer) after the reaction and without mixing then a clear phase separation is achieved, which simplifies product isolation tremendously as the organic phase exclusively consists of crude product without any organic solvent. To complete the product isolation, in the current, non-optimized process subsequently a further extraction step with ethyl acetate was done (which might be avoided in the future within the process optimization work).

However, it should be added that even this high substrate loading turned out not to be the maximal substrate concentration, which can be converted by means of a biomass of 33 g/L bww OxdB within a reaction time of 24 h. When we conducted a further 10 mL scale experiment utilizing an overall substrate loading of ca. 1.4 kg of *n*-octanaloxime per L of aqueous medium, which corresponds to a substrate concentration of 10 M, a conversion of 93% after 24 h was achieved (Table 3, no. 3). This experiment demonstrates that substrate loadings exceeding 1 kg/L can be converted by OxdB in the case of *n*-octanaloxime. To the best of our knowledge, this substrate loading belongs to the highest ever reported in biocatalysis in general and represents the highest substrate loading, which has been applied for the synthesis of a water-immiscible product in aqueous medium.[1,2,3] It is noteworthy that the biotransformation at such a high substrate loading was achieved by means of a metalloprotein bearing an iron-containing heme subunit in the active site.

In order to demonstrate the robustness of this process as well as the feasibility of this technology to be conducted on larger scale, an initial scale-up of this *n*-octanenitrile synthesis with an OxdB in whole cells as a biocatalyst was performed at an elevated lab scale utilizing a 250 mL aqueous reaction

Table 3. Biotransformation of *n*-Octanaloxime to the Relating Nitrile Using a 0.5 M Feeding Approach in the Presence of OxdB-Containing Whole Cells



no.	final concn (g/L)	no. of portions[a]	conversion[b] (%)	isolated yield (%)
1	286	2	>99	97
2	665	4[c]	>99	97.5
3	1430	9	93	

[a]Added portions of *n*-octanaloxime during the reaction. Each portion corresponds to a substrate concentration of 1 M. [b]Conversion of aldoximes to the corresponding nitriles were determined by GC after extraction of the reaction mixture using ethyl acetate. [c]Last added portion corresponds to a substrate concentration of 0.65 M *n*-octanaloxime (instead of 1 M).

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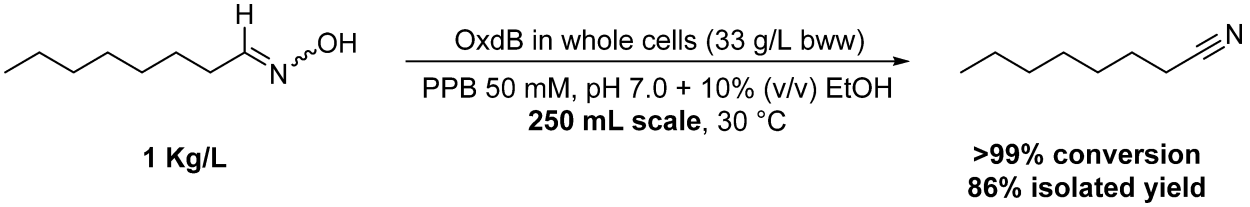
Figure 2. Photos of C8-aldoxime biotransformation using ∼665 g/L overall substrate loading (a) after 15 min reaction time, (b) after 24 h reaction time while stirring, and (c) after 24 h reaction time (without stirring for 5 min).

Scheme 2. Synthesis of *n*-Octanenitrile Using OxdB in Whole Cells on a 250 mL Scale Using a Substrate-Feeding Approach



solution consisting of buffer, 10% ethanol as cosolvent and whole cells (Scheme 2).

In total, ∼250 g *n*-octanaloxime was used as a substrate, dosed stepwise to the stirred reaction mixture (7 steps, ∼ 36 g per step, corresponding to addition of 1 M of substrate per step). Within this experiment we synthesized 188 g of *n*-octanenitrile using 8.25 g of wet cell mass (∼1.9 g bio dry weight) as a catalyst, which represents a product formation of 23 g of *n*-octanenitrile per g of bio wet mass. This corresponds to an excellent product formation of ∼113 g of *n*-octanenitrile using 1 g of dry whole cells as catalyst. Taking into account the low production costs of a biocatalyst when being manufactured by means of high-cell density fermentation starting from a recombinant *E. coli* strain, this excellent product to biomass ratio also illustrates the enormous potential of such a biotransformation for technical purposes and even for the field of specialty chemicals such as nitrile-based solvents.

■ CONCLUSION

In conclusion, we reported a biocatalytic synthetic approach toward linear aliphatic nitriles, which runs at high substrate loadings of up to 1.4 kg per liter of reaction medium as demonstrated for the synthesis of *n*-octanenitrile. In this process, recombinant whole cells containing an aldoxime dehydratase from *Bacillus* sp. (being a heme protein) were used as a biocatalyst, which catalyze the dehydration of aldoximes as readily available starting materials in aqueous medium. The biocatalyst productivity is excellent with a product formation exceeding 100 g per gram of dry whole cells as biocatalyst, and the simple work up by only separation of the organic product phase represents another advantage.

■ EXPERIMENTAL SECTION

Experimental Information. *n*-Hexanal, *n*-octanal, *n*-decanal, and *n*-dodecanal were purchased by Sigma-Aldrich and used without further purification. Chemicals for aldoxime and nitrile synthesis were purchased from VWR Chemicals, Carl Roth, and Fluka Chemicals, respectively, and used without further purification. *n*-Hexanenitrile, *n*-octanenitrile, *n*-decanenitrile, and *n*-dodecanenitrile as reference compounds for GC analytics were purchased from Sigma-Aldrich or TCI Chemicals. NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^{1}H) or 125 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$). Accurate mass nano-ESI measurements are performed using a Q-IMS-TOF mass spectrometer Synapt G2Si (Waters GmbH, Manchester, UK) in resolution mode, interfaced to a nano-ESI ion source. Nitrogen serves both as the nebulizer gas and the dry gas for nano-ESI. Nitrogen is generated by a nitrogen generator NGM 11. Helium 5.0 is used as buffer gas in the IMS entry cell, nitrogen 5.0 is used for IMS separations. 1,3-Dicyanobenzene is used as an electron-transfer reagent in ETD experiments. Samples were dissolved in acetonitrile and introduced by static nano-ESI using in-house pulled glass emitters. Conversion of the biotransformations was determined by GC measurements (Shimadzu GC-2010 Plus) in comparison to a calibration curve. Measurements were conducted on a chiral SGE Analytik B6B-174 column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) with nitrogen as carrier gas. An injector temperature of 220 °C in a split injection mode was used and a sample amount of 1 μL was injected in this method. The following temperature gradient was used for C_6-aldoxime (retention time (t_r) = 4.2 min) and -nitrile (t_r = 4.0 min): 60 °C starting temperature, in 20 °C/min to 150 °C (hold 0.5 min). The following temperature gradient was used for C_8- (t_r = 2.7 min) and C10-aldoximes (t_r = 3.8 min), and -nitriles (t_r (C_8) = 2.4 min; t_r (C_{10}) = 3.4 min): 140 °C starting temperature (hold 1 min), in 20 °C/min to 190 °C (hold 0.5 min) and in 50 °C/min to 200 °C. The following temperature gradient was used for C_{12}-aldoxime (t_r = 2.6 min) and -nitrile (t_r = 3.0 min): 190 °C start temperature (hold 1 min), in 10 °C/min to 200 °C (hold 0.5 min) and in 50 °C/min to 220 °C (hold 2 min). Accuracy of the GC-analytical method: the error of the determined conversions to the corresponding nitriles is in the range of up to 5% depending on the concentration of the aldoximes due to temperature-depending degradation of the aldoximes at the GC-column.

Aldoxime Synthesis from Aldehydes. Sodium carbonate (0.75 equiv) was dissolved in distilled (dist.) water including 5% (v/v) EtOH. Afterward, hydroxylamine hydrochloride (1.5 equiv) was added to the stirred solution. The aldehyde (1 equiv; *n*-hexanal: 1.62 mol; *n*-octanal: 3.10 mol; *n*-decanal: 1.05 mol; *n*-dodecanal: 0.08 mol) was added dropwise to the stirred aqueous solution. The reaction mixture was stirred at room temperature for 12 h, while the aldoximes

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precipitated as colorless solids from the reaction solution. The reaction progress was monitored by TLC. After completion of the reaction, the crude product was isolated as a colorless solid by filtration. The crude product was rinsed with dist. water (50 mL per mol of substrate) before drying in vacuo. Pure aldoximes were obtained after recrystallization from *n*-hexane.

n-Hexanaloxime. Yield: 96% (178.7 g; *E*/*Z* ratio: 1.00:0.22). ^{1}H NMR (500 MHz, $CDCl_3$): *Z*-**2**: δ 7.42 (t, J = 6.1 Hz, 1H), 2.18 (td, J = 7.5, 6.2 Hz, 2H), 1.49 (m, 2H), 1.31 (m, 4H), 0.91 (m, 3H, $H_3C(CH_2)_4CH_2-$), *E*-**2**: δ 6.72 (t, J = 5.5 Hz, 1H), 2.37 (td, J = 7.6, 5.5 Hz, 2H), 1.49 (m, 2H), 1.31 (m, 4H), 0.91 (m, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$): *Z*-**2**: δ 152.4, 31.4, 25.8, 31.6, 22.5, 14.1, *E*-**2**: δ 153.0, 31.7, 26.3, 25.1, 22.5, 14.1; IR (neat): 1660.2 cm^{-1}; HRMS (ESI, positive ions) m/z: $[M + H]^+$ calcd for $C_6H_{13}NOH$ 116.1070, found 116.1069.

n-Octanaloxime. Yield: 67% (307.1 g; *E*/*Z* ratio: 1.00:0.48). ^{1}H NMR (500 MHz, $CDCl_3$): *Z*-**5**: δ 7.41 (t, J = 6.1 Hz, 1H), 2.18 (td, J = 7.5, 6.2 Hz, 2H), 1.48 (m, 2H), 1.31 (m, 8H), 0.87 (m, 3H), *E*-**5**: δ 6.71 (t, J = 5.5 Hz, 1H), 2.37 (td, J = 7.6, 5.5 Hz, 2H), 1.48 (m, 2H), 1.31 (m, 8H), 0.87 (m, 3H). $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$): *Z*-**5**: δ 152.4, 31.4, 25.8, 29.6, 22.5, 14.1. *E*-**5**: δ 153.0, 31.7, 26.3, 25.1, 22.5, 14.0. IR (neat): 1669.7 cm^{-1}. HRMS (ESI, positive Ions) m/z: $[M + H]^+$ calcd for $C_8H_{17}NOH$ 144.1383, found 144.1383.

n-Decanaloxime. Yield: 63% (114.3 g; *E*/*Z* ratio: 1.00:0.43). ^{1}H NMR (500 MHz, $CDCl_3$): *Z*-**8**: δ 7.42 (t, J = 6.1 Hz, 1H), 2.19 (td, J = 7.5, 6.1 Hz, 2H), 1.48 (m, 2H), 1.29 (m, 12H), 0.88 (m, 3H). *E*-**8**: δ 6.72 (t, J = 5.5 Hz, 1H), 2.38 (td, J = 7.6, 5.5 Hz, 2H), 1.48 (m, 2H), 1.29 (m, 12H), 0.88 (m, 3H). $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$): *Z*-**8**: δ 152.4, 29.6–29.2, 26.2, 29.6, 22.8, 14.2, *E*-**8**: δ 153.1, 29.6–29.2, 26.7, 25.1, 22.8, 14.2. IR (neat): 1666.5 cm^{-1}. HRMS (ESI, positive Ions) m/z: $[M + H]^+$ calcd for $C_{10}H_{21}NOH$ 172.1696, found 172.1694.

n-Dodecanaloxime. Yield: 69% (10.8 g; *E*/*Z* ratio: 0.77:1.00). ^{1}H NMR (500 MHz, $CDCl_3$): *Z*-**11**: δ 7.42 (t, J = 6.1 Hz, 1H), 2.20 (td, J = 7.5, 6.1 Hz, 2H), 1.48 (m, 2H), 1.29 (m, 8H), 0.88 (m, 3H), *E*-**11**: δ 6.71 (t, J = 5.5 Hz, 1H), 2.38 (td, J = 7.6, 5.5 Hz, 2H), 1.48 (m, 2H), 1.29 (m, 8H), 0.88 (m, 3H). $^{13}C\{^1H\}$-NMR (125 MHz, $CDCl_3$): *Z*-**11**: δ 152.5, 29.7–29.2, 26.7, 29.5, 22.8, 14.2, *E*-**11**: δ 153.2, 29.7–29.2, 26.2, 25.1, 22.8, 14.2. IR (neat): 1663.3 cm^{-1}. HRMS (ESI, positive Ions) m/z: $[M + H]^+$ calcd for $C_{12}H_{25}NOH$ 200.2009, found 200.2005.

Biotransformations of Aldoximes to Nitriles. *Biotransformations on a 0.5 mL Scale*. Standard activity assays of Oxds in whole cells using fatty aldoximes were performed using whole cells (bww) (33 or 50 mg/mL) in a total volume of 0.5 mL in 1.5 mL microreaction tubes in triplicate. The reaction was conducted in 50 mM PPB (pH 7.0) containing 10% (v/v) ethanol as cosolvent. The activity assay was performed at 30 °C and 1400 rpm in an Eppendorf ThermoMixer. The reaction was stopped by addition of 500 μL of ethyl acetate (EtOAc) and extraction of substrates and products into the organic phase. The phase separation was simplified by centrifugation for 5 min at 14000*g* at room temperature. The organic phase was analyzed by GC (Supporting Information, Table S1).

Cosolvent Study. The cosolvent study was performed on a 0.5 mL scale using OxdB in whole cells (16.5 mg/mL bww) and an *n*-octanaloxime concentration of 5 mM. The whole cell catalyst was incubated at 30 °C for 15, 30, 60, 120, or 150 min in 10%, 20%, or 30% of each solvent in PPB (50 mM, pH 7.0). After the incubation, the substrate, dissolved in the related solvent (200 mM, 12.5 μL), was added. After 15 min at 30 °C, 500 μL ethyl acetate was added, and the phases were mixed and separated afterward. The ethyl acetate phase was analyzed by GC. The results of the cosolvent study are shown in Figure S2a–c (Supporting Information). The 100% reference value is referring to the activities of OxdB in whole cells without incubation in cosolvent.

Biotransformations on 10 mL Scale. In the case of the 10 mL biotransformations, the required starting amount of aldoxime was added to a 100 mL round-bottom flask. One milliliter of ethanol was added as well as 9 mL of 50 mM PPB (pH 7.0) to the aldoxime and heated to 30 °C with stirring (magnetic stirrer). One milliliter of a 333 mg/mL wet whole cell solution (OxdB overexpressing *E. coli* BL21(DE3)Codon+RIL cells) was then added to start the reaction. After different reaction times, in several experiments further portions of the substrate were added to the stirred reaction mixture until the desired overall substrate concentration was achieved. After 24 h, the reaction mixture was centrifuged at 4000*g* for 5 min, and the nitrile was collected by decanting. The aqueous phase was extracted with EtOAc (2 × 5 mL) and centrifuged again. The EtOAc phases and the nitrile were combined, the solvent was removed in vacuo, and the product was dried in high vacuum (not in case of *n*-hexanenitrile due to volatility reasons). The purity of the nitriles was determined by GC and ^{1}H NMR analysis in comparison to the one of the commercially available pure nitriles obtained by TCI. In case of >99% conversion a further purification of the nitriles was not necessary as also the purity then is >99% in these cases. In Tables S2–S5 (Supporting Information) are listed specific data for the 10 mL reactions.

Biotransformation on a 250 mL Scale. *n*-Octanaloxime (35.75 g, 0.25 mol) was weighed into a round-bottom flask, and ethanol (25 mL) was added under stirring with a magnetic stirrer at 30 °C. PPB (200 mL, pH 7.0) and 333 mg/mL whole cell catalyst suspension (25 mL; thus, leading to a 1:10 dilution when preparing the reaction mixture) were added. After several reaction times, further portions of *n*-octanaloxime (each dosage 35.75 g, 0.25 mol) were added to the stirred reaction mixture to reach an overall amount of 1 kg/L of reaction medium (250.25 g, 1.75 mol). After 48 h of reaction time, the reaction mixture was transferred into several 50 mL falcon tubes and centrifuged at 4000*g* for 15 min. The organic layer (product) was transferred to another round-bottom flask, and the aqueous phase was extracted with ethyl acetate (total 100 mL) using centrifugation at 4000*g* for 15 min for phase separation. The organic layers were combined, the solvent was removed in vacuo, and the product was analyzed by GC and ^{1}H NMR. *n*-Octanenitrile (182.11 g, 1.5 mol, 86%) was obtained as a yellowish oil. In case of >99% conversion a further purification of this nitrile was not necessary as also the purity then is >99% in these cases. Additional experimental information is listed in Table S6 (Supporting Information).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.9b00184.

Gene and protein sequence of the used enzyme, experimental descriptions of the preparation of the enzyme, as well as the biotransformations and NMR spectra of the aldoximes and nitriles (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Enzymatic Synthesis of Aliphatic Nitriles at a Substrate Loading of up to 1.4 Kg/L: A Biocatalytic Record Achieved with a Heme Protein

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1 Aldoxime Dehydratase (Oxd) Sequences, Plasmids and Expression

1.1 Oxd Sequences and Plasmids

1.1.1 Oxd from *Bacillus* sp. OxB-1 (OxdB)
(Accession number: GenBank: AP013294.1)

Base sequence (codon-optimized for *E. coli*):
ATGAAAAATATGCCGGAAAATCACAATCCACAAGCGAATGCCTGGACTGCCGAATTTCC
TCCTGAAATGAGCTATGTAGTATTTGCGCAGATTGGGATTCAAAGCAAGTCTTTGGATCA
CGCAGCGGAACATTTGGGAATGATGAAAAAGAGTTTCGATTTGCGGACAGGCCCCAAA
CATGTGGATCGAGCCTTGCATCAAGGAGCCGATGGATACCAAGATTCCATCTTTTTAGC
CTACTGGGATGAGCCTGAAACATTTAAATCATGGGTTGCGGATCCTGAAGTACAAAAGT
GGTGGTCGGGTAAAAAAATCGATGAAAATAGTCCAATCGGGTATTGGAGTGAGGTAACG
ACCATTCCGATTGATCACTTTGAGACTCTTCATTCCGGAGAAAATTACGATAATGGGGTT
TCACACTTTGTACCGATCAAGCATACAGAAGTCCATGAATATTGGGGAGCAATGCGCGA
CCGCATGCCGGTGTCTGCCAGTAGTGATTTGGAAAGCCCCCTTGGCCTTCAATTACCG
GAACCCATTGTCCGGGAGTCTTTCGGAAAACGGCTAAAAGTCACGGCGCCGGATAATAT
TTGCTTGATTCGAACCGCTCAAAATTGGTCTAAATGTGGTAGCGGGGAAAGGGAAACGT
ATATAGGACTAGTGGAACCGACCCTCATAAAAGCGAATACGTTTCTTCGTGAAAATGCTA
GTGAAACAGGCTGTATTAGTTCAAAATTAGTCTATGAACAGACCCATGACGGCGAAATA
GTAGATAAATCATGTGTCATCGGATATTATCTCTCCATGGGGCATCTTGAACGCTGGAC
GCATGATCATCCAACACATAAAGCGATCTACGGAACCTTTTATGAGATGTTGAAAAGGCA
TGATTTTAAGACCGAACTTGCTTTATGGCACGAGGTTTCGGTGCTTCAATCCAAAGATAT
CGAGCTTATCTATGTCAACTGCCATCCGAGTACTGGATTTCTTCCATTCTTTGAAGTGAC
AGAAATTCAAGAGCCTTTACTGAAAAGCCCTAGCGTCAGGATCCAGTGA

Amino acid sequence:
MKNMPENHNPQANAWTAEFPPEMSYVVFAQIGIQSKSLDHAAEHLGMMKKSFDLRTGPKH
VDRALHQGADGYQDSIFLAYWDEPETFKSWVADPEVQKWWSGKKIDENSPIGYWSEVTTI
PIDHFETLHSGENYDNGVSHFVPIKHTEVHEYWGAMRDRMPVSASSDLESPLGLQLPEPIV
RESFGKRLKVTAPDNICLIRTAQNWSKCGSGERETYIGLVEPTLIKANTFLRENASETGCISS
KLVYEQTHDGEIVDKSCVIGYYLSMGHLERWTHDHPTHKAIYGTFYEMLKRHDFKTELALW
HEVSVLQSKDIELIYVNCHPSTGFLPFFEVTEIQEPLLKSPSVRIQ

1.2 Oxd Expression in *E.coli* BL21-CodonPlus(DE3)-RIL

E.coli BL21-CodonPlus(DE3)-RIL cells harboring the plasmids with the OxdB-gene (Table S2) were stored as glycerol stocks at -80 °C.

A sample from the glycerol stocks was plated on LB-agar containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol and incubated for 12 to 18 h at 37 °C.

Pre-cultures were prepared in 5 mL LB-medium containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol using a single colony from the LB-agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm.

Main cultures for the expression of OxdB was performed using TB-autoinduction medium. Sterile 20 g/L Lactose solution in MilliQ water (160 mL) and sterile 50 g/L D-glucose solution in MilliQ water (16 mL) was added to 1424 mL sterile TB-medium (Carl Roth) in a 2 L Erlenmeyer flask. 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol were added to the medium. Main cultures were inoculated with 1% (16 mL) of the OxdB pre-culture and incubated for 1 h at 37 °C and 150 rpm. After 1 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 150 rpm.

Cell harvest was performed at 4,000 xg for 15 min and 4 °C. The supernatant was discarded and cells were washed three times with 50 mM potassium phosphate buffer (PPB, KP_i) at pH 7.0. The biomass was determined (bio wet weight (bww)) and cells were resuspended in 50 mM PPB (pH 7.0) to a final concentration of 333 mg/mL cells in buffer. Cell suspensions were stored at 4 °C or on ice before usage in biotransformations.

Table S2. Used vector constructs, origins of the Oxd-genes, provider of the vector constructs and marker-resistance of the constructs.

Entry	Origin of Oxd-gene	Vector construct	Oxd	Provider	Resistance
1	*Bacillus* sp. OxB-1	pUC18_OxdB	OxdB	Asano group	Carbenicillin

Overexpression of Oxds in *E. coli* Bl21-CodonPlus(DE3)-RIL was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after cell disruption and denaturation of the proteins in the crude extracts (Figure S1).

Crude extracts of 33% wet cell suspensions were obtained by sonication (5x 1 min, 10 – 15% Output, Bandelin Sonopuls®) and subsequent centrifugation at 21,500 xg for 45 min at 4 °C. The pellet including the cell debris was discarded. Protein concentrations in crude extracts were determined by Bradford assay using a bovine serum albumin (BSA)-standard curve (1.4 mg/mL, 0.7 mg/mL, 0.35 mg/mL, 0.175 mg/mL, 0.0875 mg/mL) as reference. Protein dilutions of 1 µg/µL whole cell protein concentration were obtained by dilution of the crude extracts in water and Laemmli-buffer. 10 µL of these samples were transferred to a 12% SDS-PAGE.

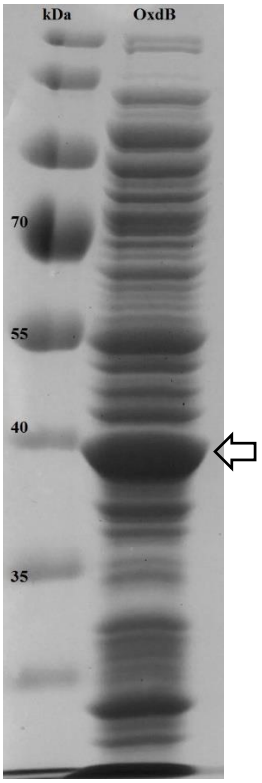


Figure S1. 12% SDS-PAGE of crude extracts of OxdB-overexpressing cells. The molecular weights of the overexpressed Oxd correlates with the molecular weights determined from the amino acid sequences.

2 Biotransformations of aldoximes to nitriles

Concentrations of aldoxime substrates are defined as follows:
A substrate concentration of “1 M” corresponds to 1 mol of substrate per liter of aqueous reaction medium (and does not refer to the total volume of the resulting mixture). This type of definition for the concentration was used for all experiments described in the article and in the Supporting Information.

2.1 Biotransformations on 0.5 mL-scale

Table S1. Conversions of C_6- **2**, C_8- **5**, C_{10}- **8** and C_{12}-aldoximes **11** to the relating nitriles determined by GC.

#	Substrate	Amount	Concentration	Conversion (GC)* /%
1	**2**	0.6 mg	10 mM	>99
2	**2**	2.9 mg	50 mM	>99
3	**2**	5.8 mg	100 mM	>99
4	**2**	11.5 mg	200 mM	>99
5	**2**	28.8 mg	500 mM	22
6	**2**	57.6 mg	1 M	4
7	**2**	115.2 mg	2 M	1
8	**5**	0.7 mg	10 mM	>99
9	**5**	3.6 mg	50 mM	>99
10	**5**	7.2 mg	100 mM	>99
11	**5**	14.4 mg	200 mM	>99
12	**5**	36 mg	500 mM	>99
13	**5**	72 mg	1 M	92
14	**5**	144 mg	2 M	35
15	**8**	0.9 mg	10 mM	98
16	**8**	4.5 mg	50 mM	99
17	**8**	9.0 mg	100 mM	>99
18	**8**	18.0 mg	200 mM	90
19	**8**	45.0 mg	500 mM	88
20	**8**	90.0 mg	1 M	93
21	**8**	180.0 mg	2 M	16
22	**11**	1.0 mg	10 mM	54
23	**11**	5.0 mg	50 mM	45
24	**11**	10.0 mg	100 mM	42

* Accuracy of the GC-analytical method: the error of the determined conversions to the corresponding nitriles is in the range of up to 5% depending on the concentration of the aldoximes due to temperature-depending degradation of the aldoximes at the GC-column.

2.2 Biotransformations on 10 mL-scale

Experimental data to Table 1.

Table S2. Conversions of C_6-, C_8- and C_{10}-aldoximes to the relating nitriles determined by GC and isolated yields.

#	Substrate	Starting concentration	Addition	Overall substrate concentration	Conversion* /%	Isolated yield /% (g)
1	**2**	1 M	--	1 M	>99 (24 h)	92 (0.89)
2	**5**	1 M	--	1 M	>99 (24 h)	89 (1.11)
3	**8**	1 M	--	1 M	87.5 (24 h)	--

* Conversions were determined by GC and validated by ^{1}H-NMR analytics.

Experimental data to Table 2.

Table S3. Conversions of C_{10}-aldoxime to the relating nitrile determined by GC and isolated yields.

#	Substrate	Starting concentration	Addition	Overall substrate concentration	Conversion* /%	Isolated yield /% (g)
1	**8**	0.5 M (85.5 g/L)	0.5 M (5 h) 0.5 M (6.5 h)	1.5 M (256.5 g/L)	>99 (24 h)	85 (1.30)
2	**8**	0.5 M (85.5 g/L)	0.5 M (5.5 h) 0.5 M (7 h) 0.5 M (8.5 h)	2 M (342 g/L)	>99 (24 h)	83.5 (1.28)
3	**8**	0.5 M (85.5 g/L)	0.5 M (2 h) 0.5 M (5 h) 0.5 M (7 h) 0.5 M (9 h)	2.5 M (427.5 g/L)	93 (24 h)	--

* Conversions were determined by GC and validated by ^{1}H-NMR analytics.

Experimental data to Scheme 1.

Table S4. Conversions of C_6-aldoxime to the relating nitrile determined by GC and isolated yields.

#	Substrate	Starting concentration	Addition	Overall substrate concentration	Conversion* /%	Isolated yield /% (g)
1	**2**	1 M (115 g/L)	1 M (0.5 h) 0.54 M (1 h)	2.54 M (287.5 g/L)	>99 (24 h)	81 (2.00)

* Conversions were determined by GC and validated by ^{1}H-NMR analytics.

Experimental data to Table 3.

Table S5. Conversions of C_8-aldoxime to the relating nitrile determined by GC and isolated yields.

#	Substrate	Starting concentration	Addition	Overall substrate concentration	Conversion* /%	Isolated yield /% (g)
1	**5**	1 M (143 g/L)	1 M (1 h) 1 M (3 h) 1 M (0.5 h)	2 M (280 g/L)	>99 (24)	97 (2.78)
2	**5**	1 M (143 g/L)	1 M (1 h) 1 M (1.5 h) 0.65 M (2 h)	4.65 M (665 g/L)	>99 (23 h)	97.5 (5.68)
3	**5**	1 M (143 g/L)	1 M (1.5 h) 1 M (2 h) 1 M (3 h) 1 M (5 h) 1 M (7 h) 1 M (8 h) 1 M (9.5 h) 1 M (11 h) 1 M (12 h)	10 M (1.4 Kg/L)	93 (24 h)	--

* Conversions were determined by GC and validated by ^{1}H-NMR analytics.

2.3 Biotransformation on 250 mL-scale

Experimental data to Scheme 2.

Table S6. Conversions of C_8-aldoxime to the relating nitrile in 250 mL-scale determined by GC and isolated yields.

#	Substrate	Starting concentration	Addition	Overall substrate concentration	Conversion* /%	Isolated yield /% (g)
1	**5**	1 M (143 g/L)	1 M (0.5 h) 1 M (1 h) 1 M (1.5 h) 1 M (2 h) 1 M (2.5 h) 1 M (4 h)	7 M (1 Kg/L)	>99 (24 h)	86 (188.44)

* Conversions were determined by GC and validated by ^{1}H-NMR analytics.

2.4 Cosolvent study

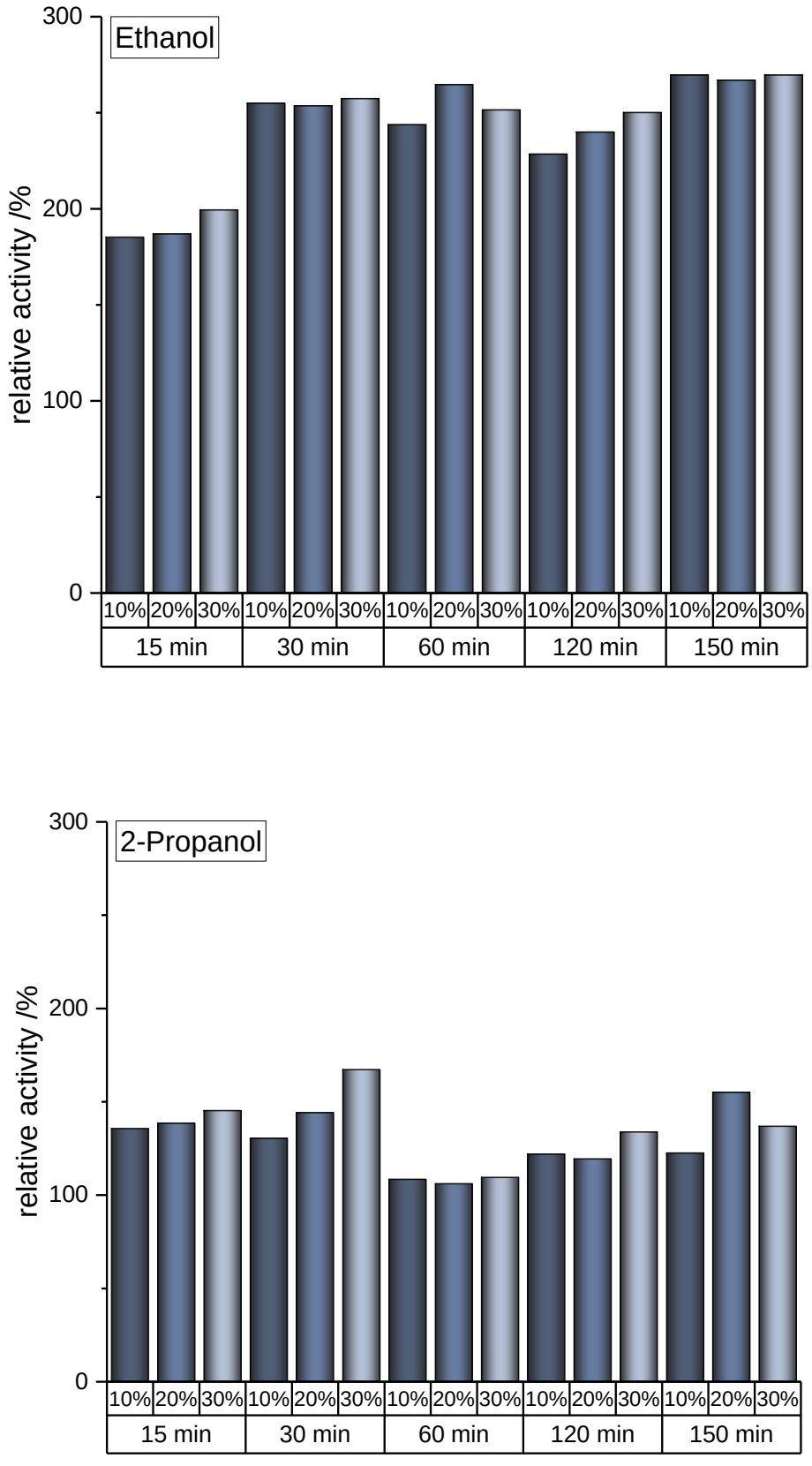


Figure S2a. Results of the cosolvent study of OxdB using 2-propanol or ethanol as solvent.

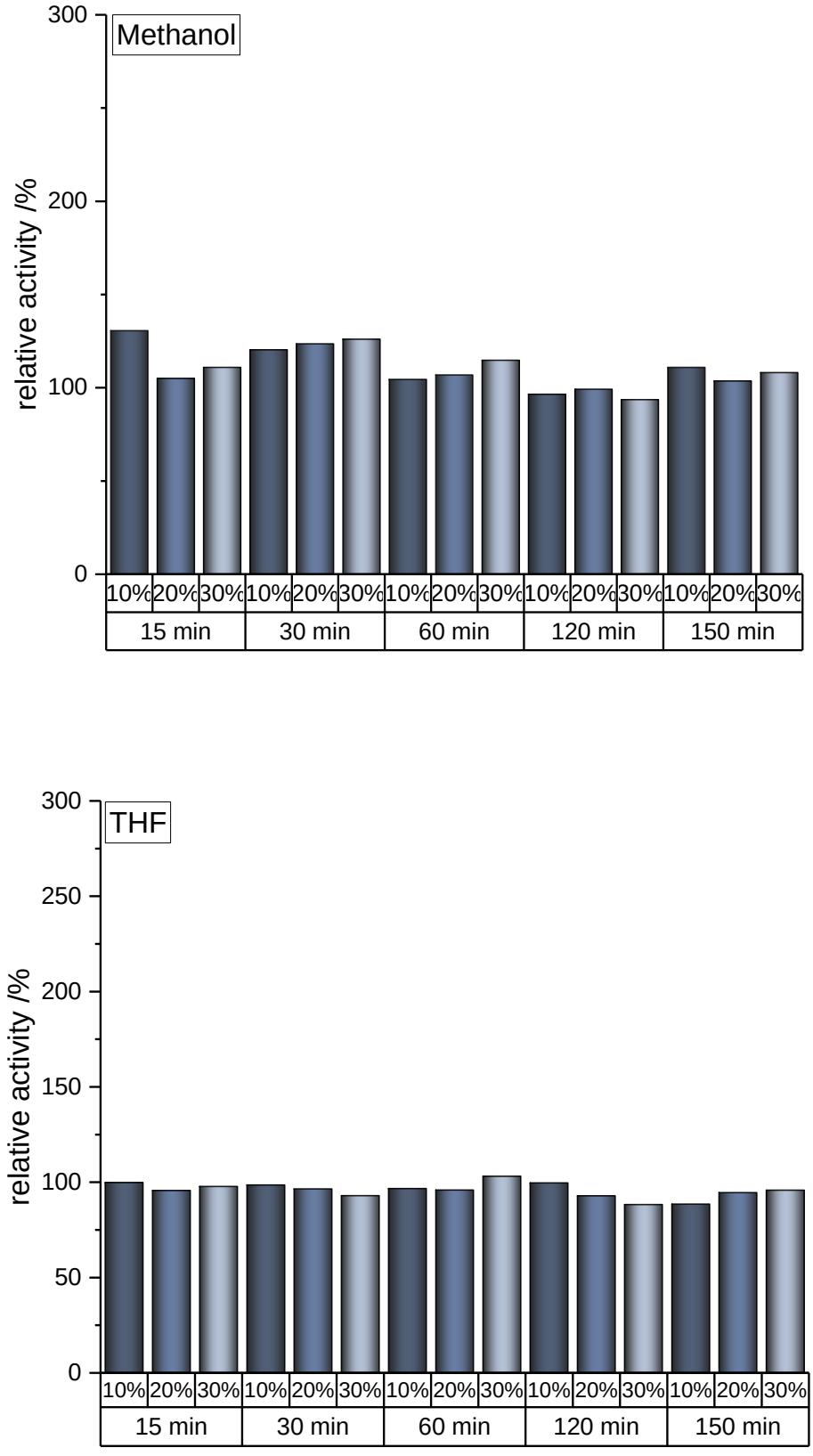


Figure S2b. Results of the cosolvent study of OxdB using methanol or tetrahydrofuran (THF) as solvent.

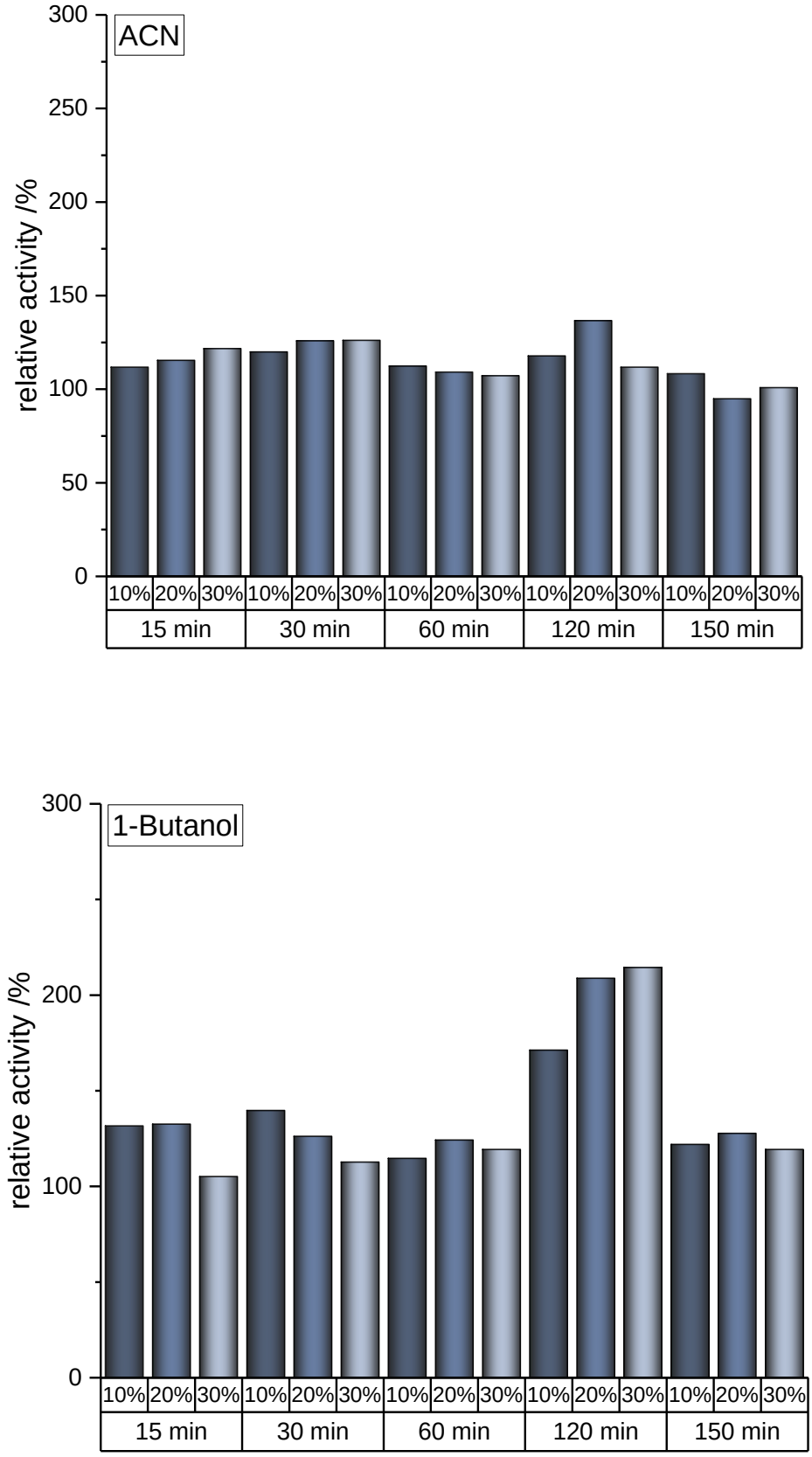
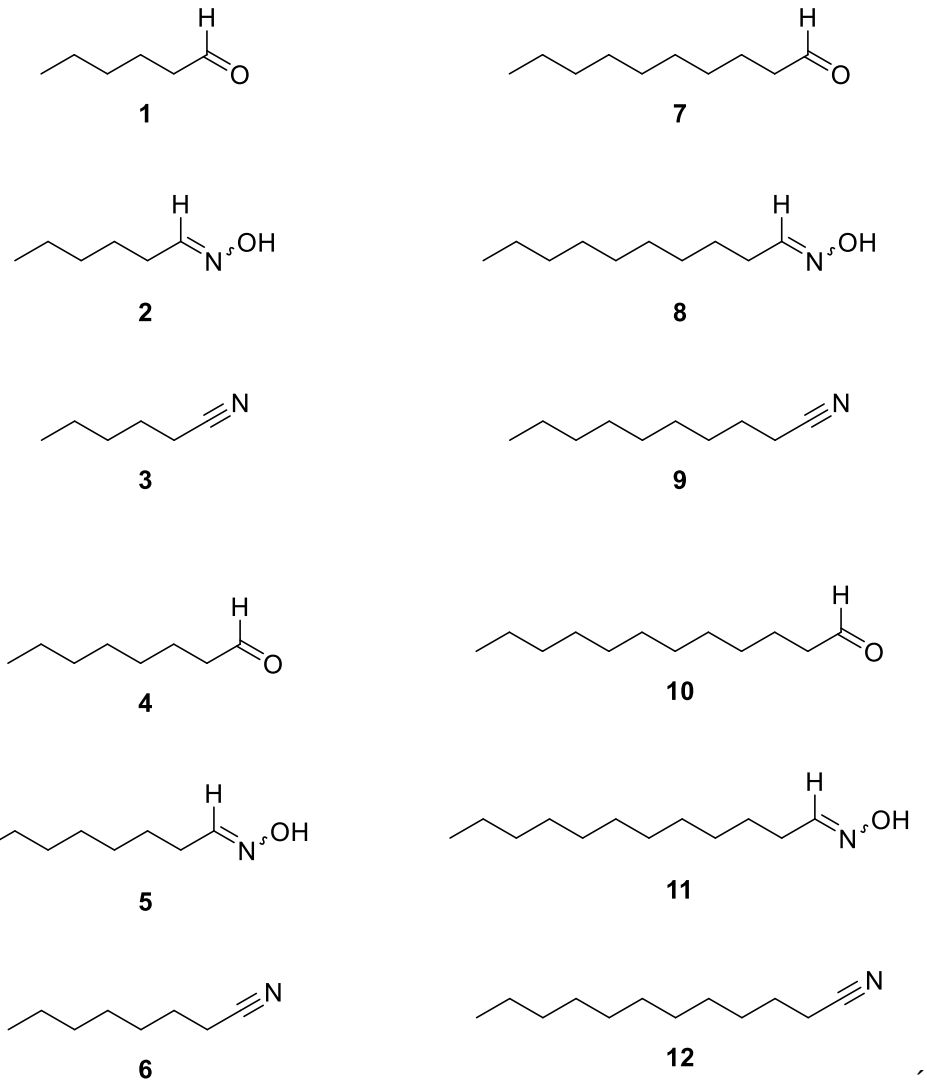


Figure S2c. Results of the cosolvent study of OxdB using acetonitrile (ACN) or 1-butanol as solvent.

3. Attachment

3.1 List of molecules

The structures of the aldehyde, aldoxime and nitrile molecules used in this study are shown in Scheme S1.



Scheme S1. List of molecules.

3.2 NMR-spectra

3.2.1 *n*-Hexanaloxime (**2**)

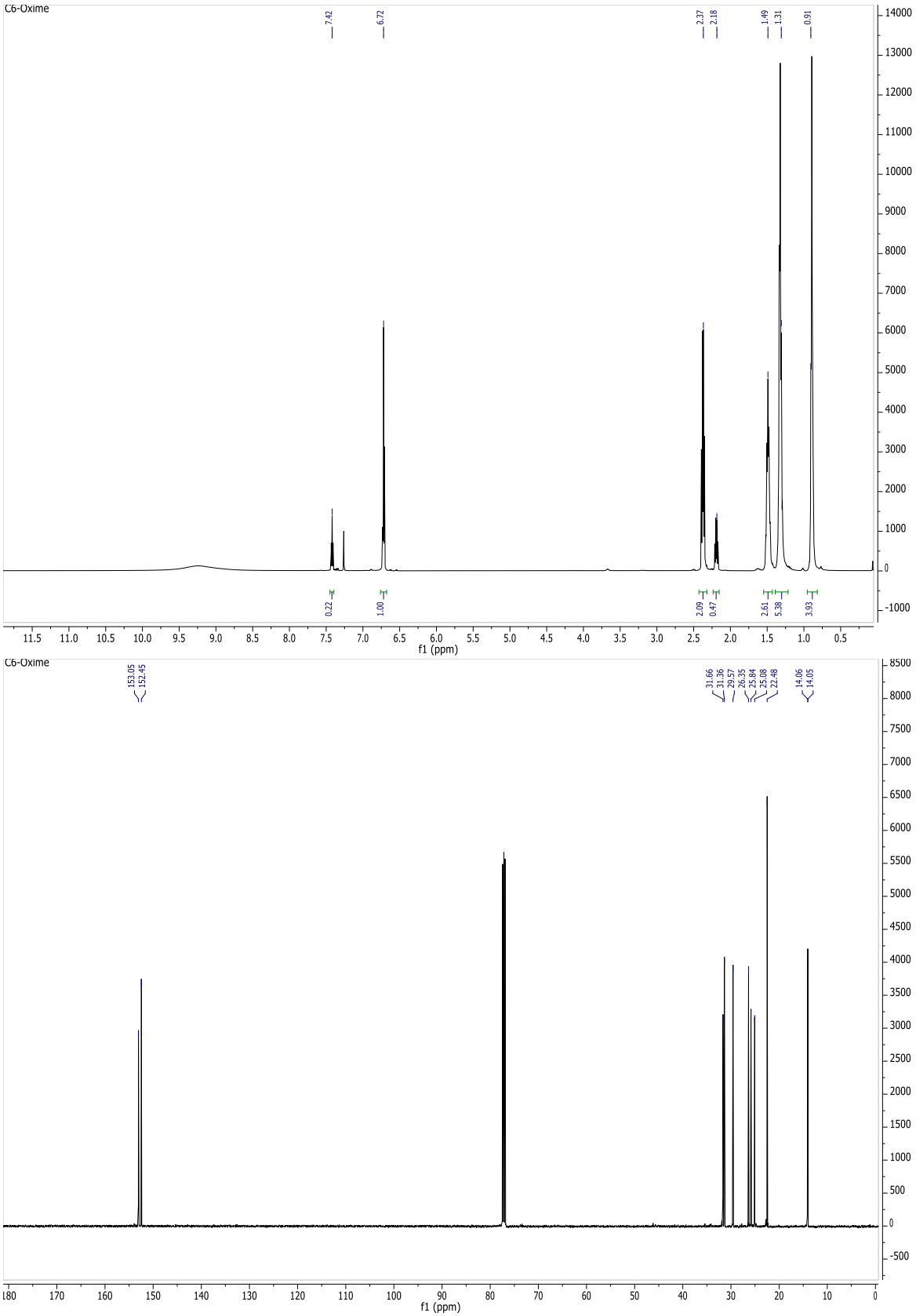


Figure S3. ^{1}H- and $^{13}C\{^{1}H\}$-NMR spectra of *n*-hexanaloxime (**2**) (C_6-Aldoxime).

3.2.2 *n*-Octanaloxime (**5**)

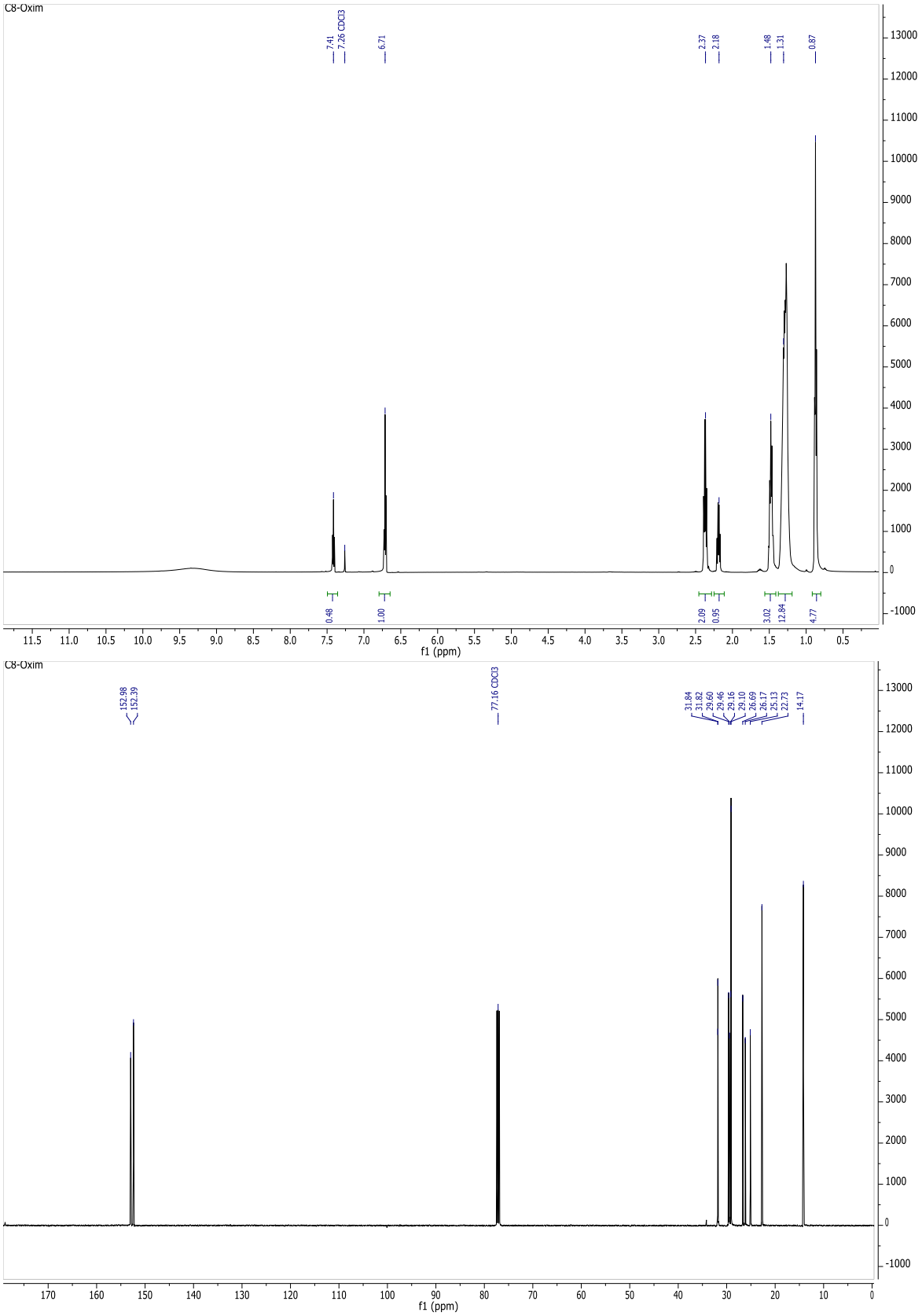


Figure S4. ^{1}H- and ^{13}C{^{1}H}-NMR spectra of *n*-octanaloxime (**5**) (C_8-Aldoxime).

3.2.3 *n*-Decanaloxime (**8**)

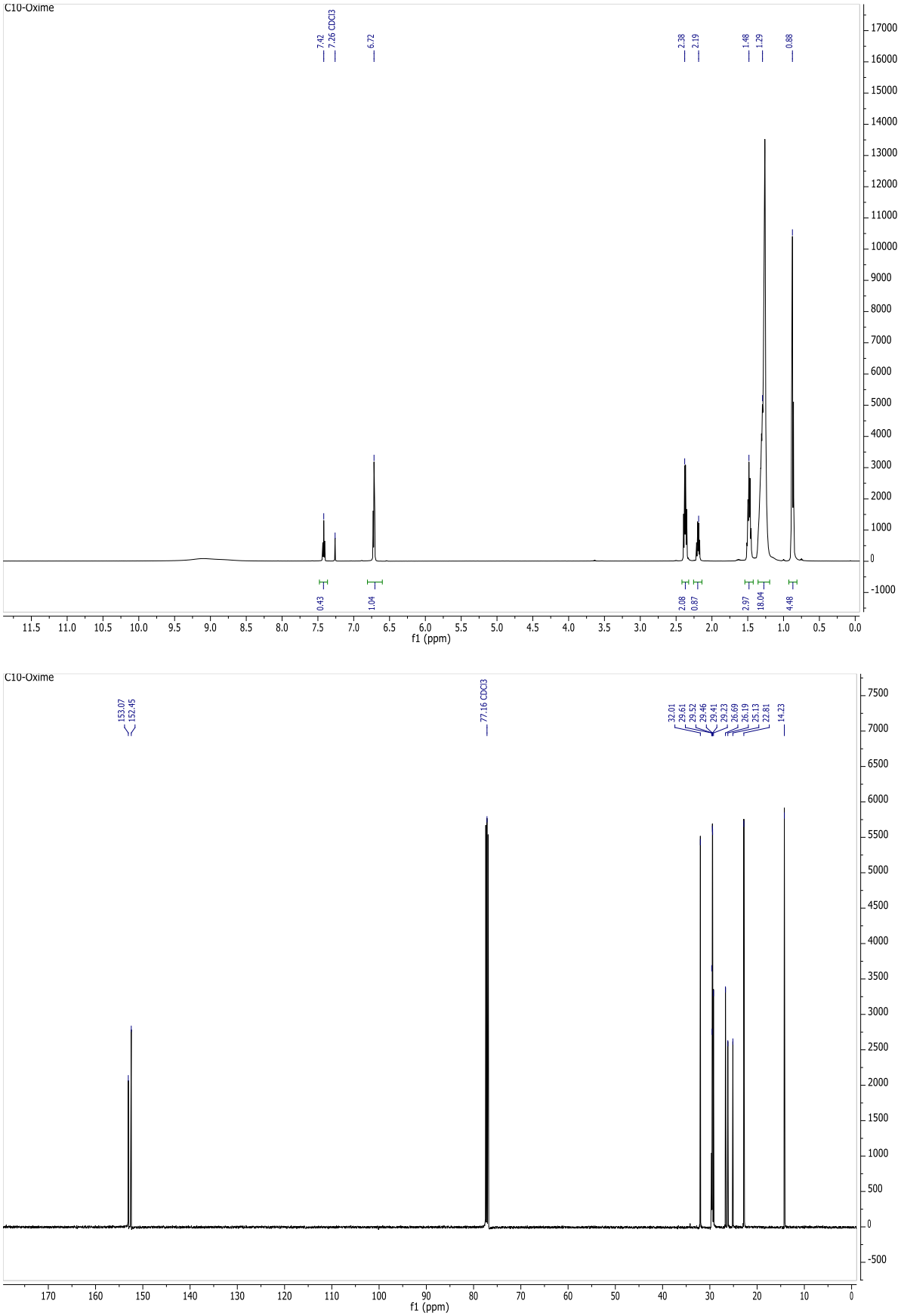


Figure S5. ^{1}H- and ^{13}C{^{1}H}-NMR spectra of *n*-decanaloxime (**8**) (C_{10}-Aldoxime).

3.2.4 *n*-Dodecanaloxime (**11**)

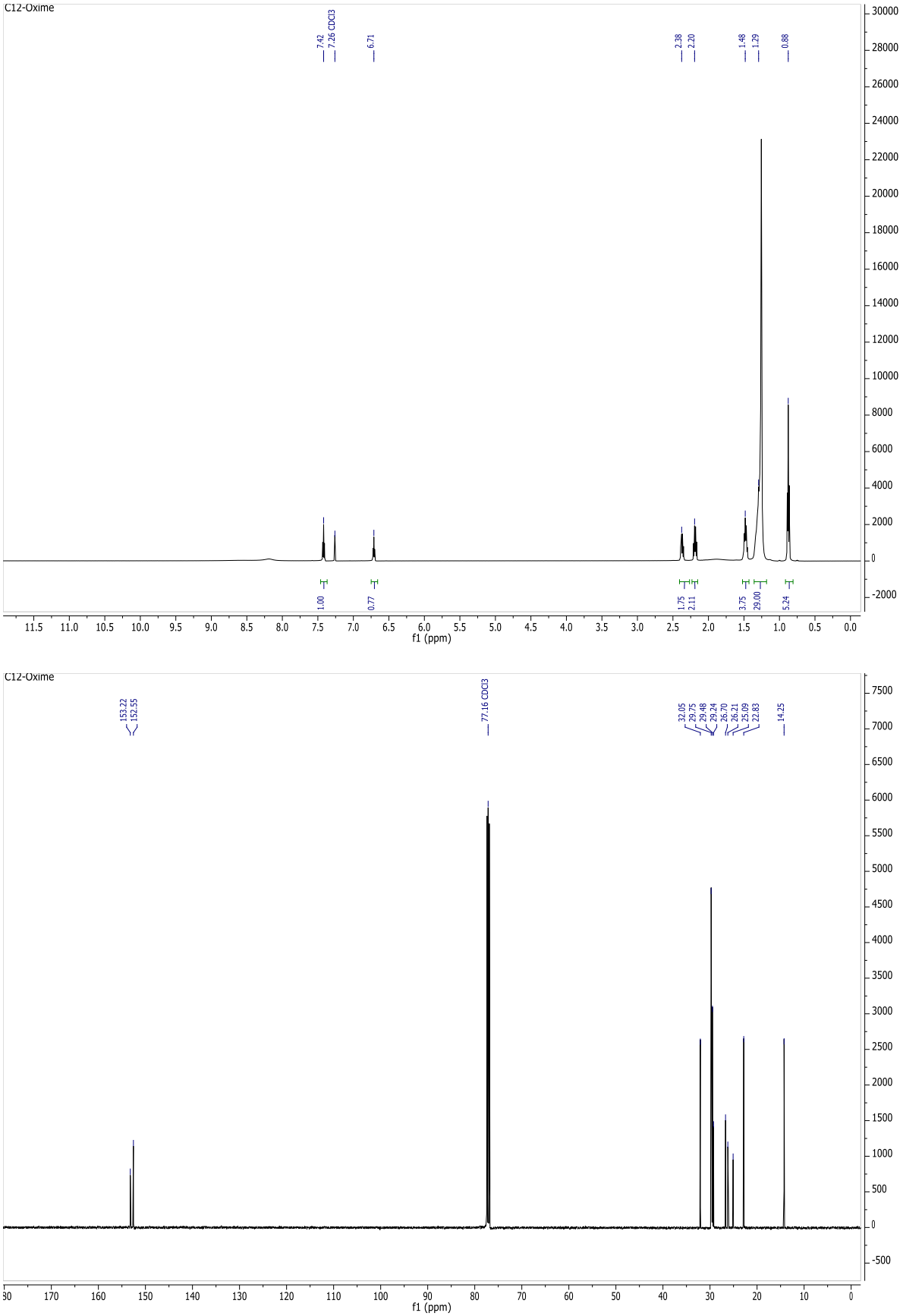


Figure S6. ^{1}H- and ^{13}C{^{1}H}-NMR spectra of *n*-dodecanaloxime (**11**) (C_{12}-Aldoxime).

3.2.5 *n*-Hexanenitrile (**3**)

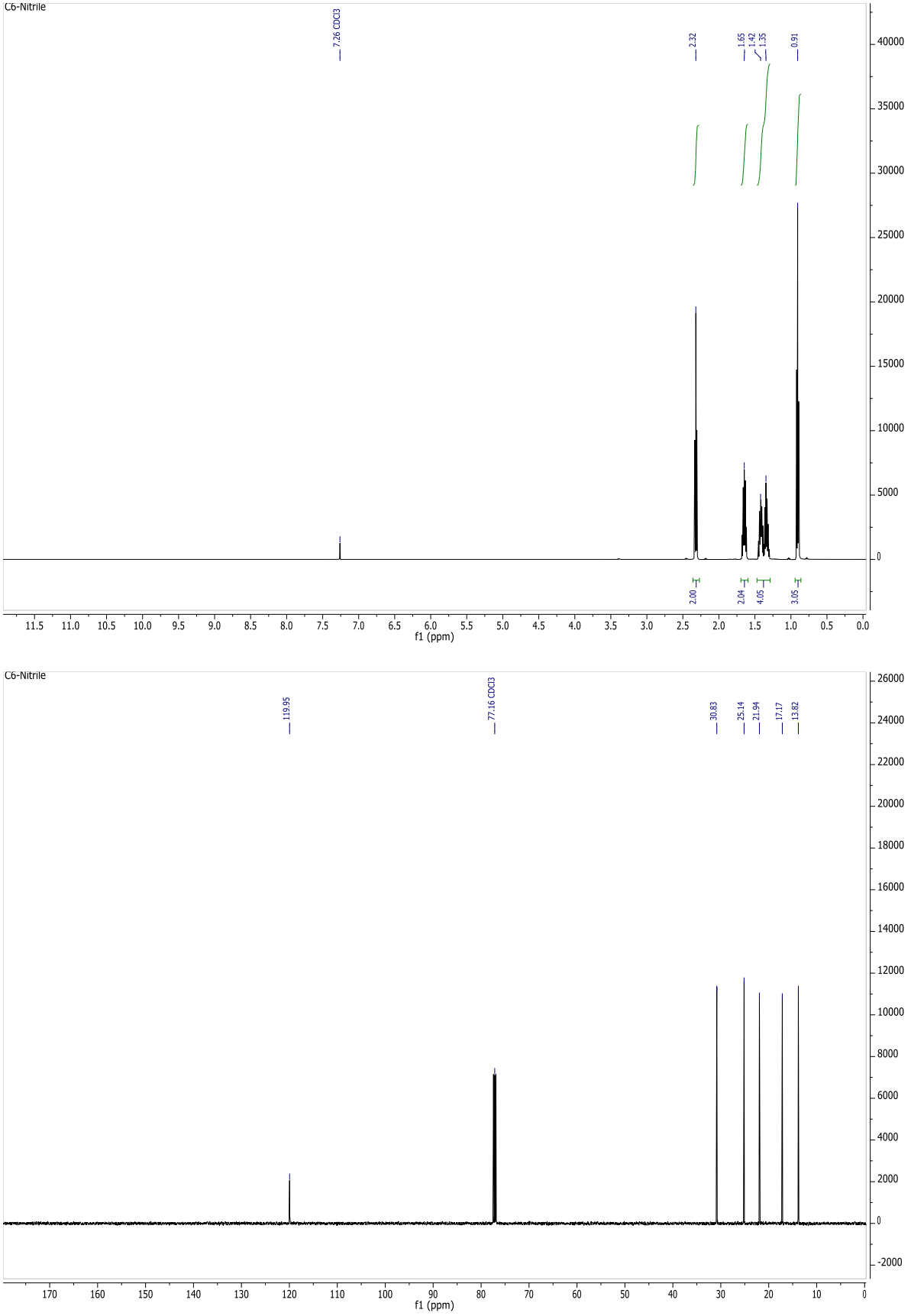


Figure S7. ^{1}H- and ^{13}C{^{1}H}-NMR spectra of *n*-hexanenitrile (**3**) (C_6-Nitrile). NMR spectra were recorded of the product of experiment described in Table S5#1.

3.2.6 *n*-Octanenitrile (**6**)

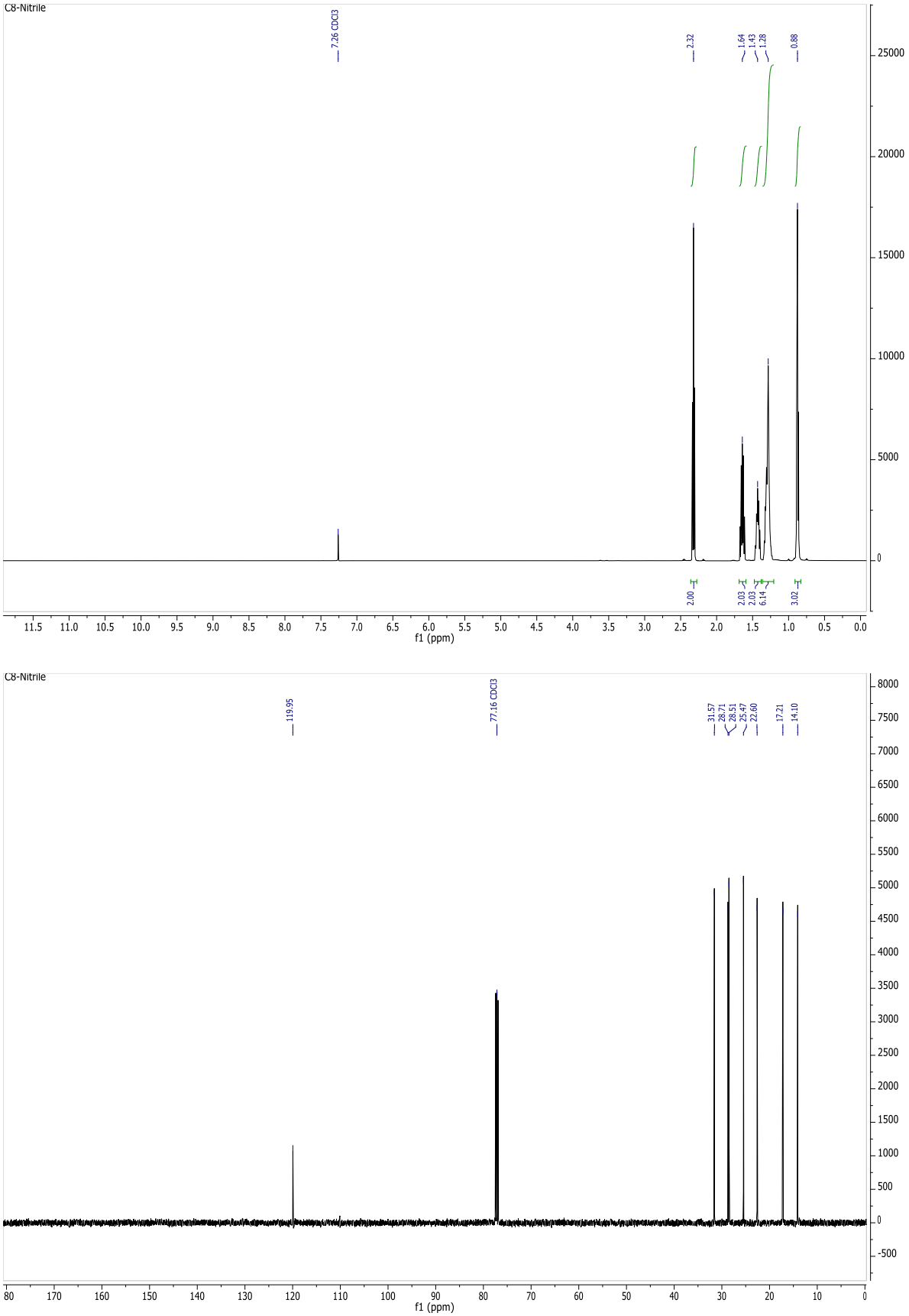


Figure S7. ^{1}H- and ^{13}C{^{1}H}-NMR spectra of *n*-octanenitrile (**6**) (C_8-Nitrile). NMR spectra were recorded of the product of experiment described in Table S6#2.

3.2.6 *n*-Decanenitrile (**9**)

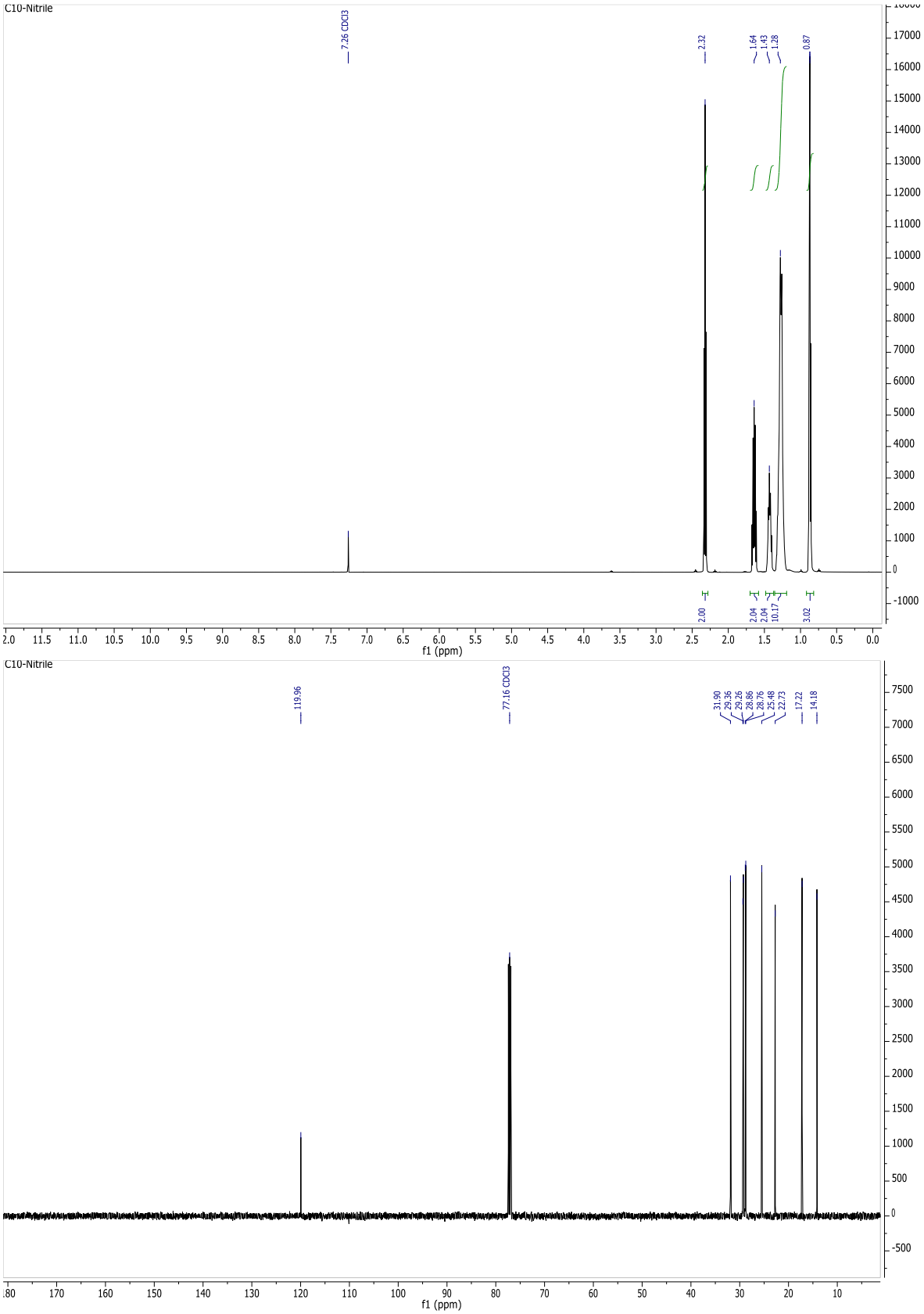


Figure S8. ^{1}H- and $^{13}C\{^{1}H\}$-NMR spectra of *n*-decanenitrile (**9**) (C_{10}-Nitrile). NMR spectra were recorded of the product of experiment described in Table S4#2.

Article 4 *Galley Proof*

Biocatalytic Synthesis of *n*-Octanenitrile Using an Aldoxime Dehydratase from *Bacillus* sp. OxB-1

A. Hinzmann, H. Gröger

Book Chapter in: *Applied Biocatalysis: The Chemist's Enzyme Toolkit*

Author contribution

AH wrote the manuscript. AH and HG read and edited the manuscript.

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9.4.4 Conclusion

The described procedure allows the separation of crystalline (*S*)-1,2-dodecanediol (*S*)-**1** of very high chemical and stereochemical purity in high yield from a commercial racemic mixture of modest chemical purity. Lipase-catalysed kinetic resolution is used, followed by chemo- and stereoselective crystallisation of the target compound from the crude product dissolved in a limited amount of chloroform. The procedure is easy to scale up due to the technical simplicity and high reliability of all steps and the fact that no chromatographic separation is needed.

In addition to 1,2-dodecanediol, the described method has been successfully used for semipreparative separation of 1,2-octanediol enantiomers [5] and for the separation of all three stereoisomers of 1,2,7,8-octanetetrol (unpublished results). In principle, the method is suitable for the separation of stereoisomers of terminal vicinal alkanediol and alkanetetrol homologues that are amenable to crystallisation but whose corresponding perbutyrates remain soluble in acetonitrile.

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9.5 Biocatalytic Synthesis of *n*-Octanenitrile Using an Aldoxime Dehydratase from *Bacillus* sp. OxB-1

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Nitriles represents a class of compounds in organic chemistry that have gained importance for a wide range of applications from different industrial segments such as pharmaceuticals and specialty and bulk chemicals [1]. For example, fatty acid-derived nitriles are used as intermediates in the production of fatty amines, which find use in many industrial applications, including as emulsifiers, corrosion inhibitors, dispersants and lubricating additives [1c, 2]. In general, a typical prerequisite for efficient and cost-attractive production on scale is running processes with high substrate loading and simple downstream processing, enabling high space–time yields.

In continuation of our ongoing studies [3] on the chemoenzymatic synthesis of nitriles, we recently reported an efficient synthesis of various *n*-alkylnitriles that proceeds at very high substrate loading of up to 1.4 $kg.L^{-1}$ of aqueous buffer as reaction medium [3e]. This process, which is based on an aldoxime dehydratase-catalysed dehydration reaction,

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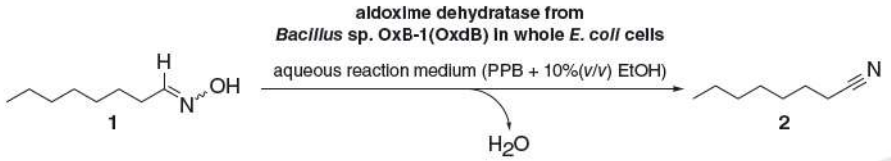

Scheme 9.6 *Conversion of* n-*octanaloxime* ***1*** *to* n-*octanenitrile* ***2*** *using the aldoxime dehydratase OxdB in whole cells as a biocatalyst.*

is exemplified for the synthesis of *n*-octanenitrile **2**, resulting in >99% conversion when operating at a substrate loading of 665 $g.L^{-1}$ (Scheme 9.6) [3e]. Even at a further increased substrate loading of 1.4 $kg.L^{-1}$ of aldoxime **1** with aqueous buffer, the reaction proceeds well, leading to 93% conversion.

9.5.1 Procedure 1: Recombinant Expression of Aldoxime Dehydratase from *Bacillus* sp. OxB-1 (OxdB) in *E. coli* BL21(DE3)CodonPlus-RIL

9.5.1.1 Materials and Equipment

- Lysogenic broth (LB) medium (sterile)
- terrific broth (TB) medium (sterile)
- D-Lactose (20 $g.L^{-1}$ solution in dH_2O, sterile)
- D-Glucose (50 $g.L^{-1}$ solution in dH_2O, sterile)
- K_2HPO_4/KH_2PO_4-buffer (PPB; 50 mM, pH 7.0)
- Distilled water (dH_2O)
- Carbenicillin (100 $mg.mL^{-1}$ in dH_2O, filter-sterilised)
- Chloramphenicol (25 $mg.mL^{-1}$ in ethanol, filter-sterilised)
- LB agar plate with colonies of *E. coli* BL21(DE3)CodonPlus-RIL harbouring the vector pUC18 bearing the gene encoding OxdB inserted [3e]
- Schott bottles with screw caps
- 100 mL and 2 L Erlenmeyer flasks with alumina caps
- Orbital shaker (InforsHT Multitron 2 Standard)
- Autoclave (Tuttnauer 5075 EL)
- Cooling centrifuge (min. 4500× g)

9.5.1.2 Procedure

1. Premixed LB medium (25 g) was dissolved in dH_2O (1 L). Premixed TB medium (80.8 g) and glycerol (5 g) were dissolved in dH_2O (1 L). D-Lactose (20 g) was dissolved in dH_2O (1 L). D-Glucose (50 g) was dissolved in dH_2O (1 L). Solutions were autoclaved at 121 °C for 20 min to give sterile media (components).
2. To prepare the pre-culture, sterile LB medium (20 mL) was placed into a sterile 100 mL Erlenmeyer flask. Stock solutions of carbenicillin (20 µL) and chloramphenicol (20 µL) were added to reach final concentrations of 100 and 25 $\mu g.mL^{-1}$, respectively. The solution was inoculated with a single colony of *E. coli* BL21(DE3)CodonPlus-RIL harbouring pUC18_OxdB and shaken at 180 rpm and 37 °C overnight.

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3. TB autoinduction medium (1.6 L total volume) was prepared from D-lactose solution (160 mL), D-glucose solution (16 mL) and TB medium (1.424 L) in a sterile 2 L Erlenmeyer flask. Stock solutions of carbenicillin (1.6 mL) and chloramphenicol (1.6 mL) were added prior to inoculation with the pre-culture (16 mL).
4. The main culture was shaken at 150 rpm and 37 °C for 2 hr. Afterwards, the temperature was decreased to 30 °C and the culture was shaken at 150 rpm for a further 72 hr (typical OD_{600} ~13).
5. The cells were harvested by centrifugation at 5000× g and 4 °C for 15 min. The supernatant was discarded and the cells were washed with PPB (2 × 15 mL). Finally, the cells (10 g) were resuspended in PPB (20 mL) and stored at 4 °C for later use. Cells produced this way can be used for approximately 6 weeks before a (significant) loss of activity is observed.

9.5.2 Procedure 2: Preparation of *n*-Octanaloxime 1

9.5.2.1 Materials and Equipment

- Hydroxylamine hydrochloride (334 g, 4.8 mol)
- Sodium carbonate (254 g, 2.4 mol)
- *n*-Octanal (500 mL, 3.2 mol)
- Ethanol (100 mL)
- dH_2O (1.9 L)
- *n*-Hexane (250 mL)
- 4 L round-bottom flask
- Magnetic stirrer (IKA RCT Classic)
- Magnetic stirring bar

9.5.2.2 Procedure

1. Hydroxylamine hydrochloride was dissolved in dH_2O (2 L) containing 5% v/v ethanol.
2. Sodium carbonate was added to the stirred aqueous solution.
3. *n*-Octanal was added dropwise whilst *n*-octanaloxime **1** started precipitating.
4. The reaction mixture was stirred for 12 hr at room temperature.
5. *n*-Octanaloxime **1** was filtered and washed with dH_2O (~160 mL).
6. *n*-Octanaloxime **1** (307 g, 2.1 mol, 67%) was obtained as colourless crystals after recrystallisation from *n*-hexane (250 mL).

9.5.3 Procedure 3: Biocatalytic Conversion of *n*-Octanaloxime 1 into *n*-Octanenitrile 2

9.5.3.1 Materials and Equipment

- *n*-Octanaloxime **1** from Procedure 2 (250.25 g, 1.75 mol)
- PPB, 50 mM, pH 7.0 (200 mL)
- Ethyl acetate (EtOAc; 100 mL)
- Resting cells from Procedure 1 (25 mL)
- 50 mL Falcon tubes
- 4 L round-bottom flask

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- Magnetic stirrer (IKA RCT Classic)
- Magnetic stirring bar
- 2× 50 mL syringe
- 2× sterile filter (Filtropur S 0.2, Sarstedt AG & Co. KG)
- Gas chromatography (GC) system with flame ionisation detector (FID; Shimadzu GC-2010 Plus)
- Chiral GC column (SGE Analytik B6B-174: 30 m × 0.25 mm ID, 0.25 µm film)
- Cooling centrifuge (min. 5000× g)
- Rotary evaporator (Büchi)

9.5.3.2 Procedure

1. *n*-Octanaloxime **1** (35.75 g, 0.25 mol) was placed into a 1 L round-bottom flask.
2. Ethanol (25 mL) was added and *n*-octanaloxime **1** was dissolved partly by stirring using a magnetic stirring bar.
3. PPB (200 mL) was added whilst stirring at 30 °C.
4. Resting cell suspension (25 mL of 333 $g.L^{-1}$ stock solution (Procedure 1), 33 $g.L^{-1}$ final wet whole-cell concentration) was added.
5. After reaction times of 0.5, 1, 1.5, 2, 2.5 and 4 hr, additional *n*-octanaloxime **1** portions (each portion 35.75 g, 0.25 mol; total amount after all additions 250.25 g, 1.75 mol) were added.
6. The reaction mixture was stirred at 30 °C for 24 hr.
7. The magnetic stirrer was stopped and the phases (aqueous PPB phase and organic *n*-octanenitrile **2** phase) were separated.
8. *n*-Octanenitrile **2** was filled into a 50 mL syringe and filter-sterilised through a 0.2 µm filter (Filtropur S 0.2, Sarstedt AG & Co KG).
9. The aqueous phase was extracted using EtOAc (2 × 50 mL). The EtOAc phases were combined and dried over $MgSO_4$ and the solvent was removed *in vacuo*, resulting in *n*-octanenitrile **2**, which was filled into a 50 mL syringe, filter-sterilised through a 0.2 µm filter (Filtropur S 0.2, Sarstedt AG & Co KG) and combined with the *n*-octanenitrile **2** from Step 8.
10. *n*-Octanenitrile **2** (188 g, 86%) was isolated as a yellowish oil.

9.5.4 Analytical Method

The method and results of GC analysis are given in Tables 9.6 and 9.7, respectively.

Table 9.6 *GC method.*

Temperature program (r = °C/min)	Duration
140 °C 1 min – 20 r → 190 °C 0.5 min – 50 r → 200 °C	4.2 min

Table 9.7 *Retention times for GC analysis.*

Substance	Retention (min)
n-Octanaloxime **1**	2.4
n-Octanenitrile **2**	2.7

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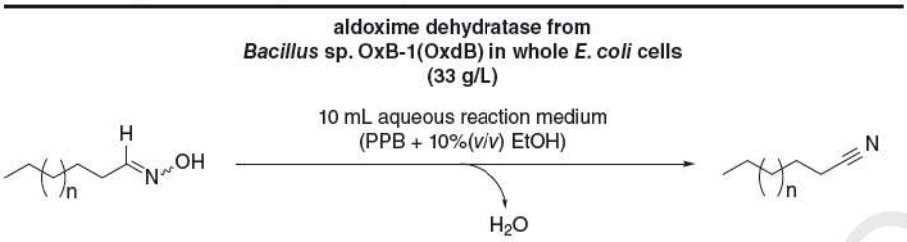
Table 9.8 *Aliphatic nitrile synthesis using OxdB in 10 mL scale for different substrates.*



Entry	n	Final conc ($g.L^{-1}$)	Conversion /%	Isolated yield /%
1	1	288	>99	81
2	3	665	>99	98
3	3	1430	93	
4	5	342	>99	83.5
5	5	428	93	

9.5.5 Conclusion

The described procedure enabled a practical and highly productive synthesis of *n*-octanenitrile **2** using aldoxime dehydratases as biocatalyst. In an aqueous reaction medium, *n*-octanaloxime **1** as a water-insoluble substrate was converted into the corresponding desired nitrile product **2** via enzymatic dehydration [3e]. Furthermore, this method enabled a simple product isolation by phase separation of the aqueous reaction medium, consisting of buffer and whole-cell catalyst and the organic nitrile phase, followed by extraction of the aqueous phase with an organic solvent. A successful extension of the substrate scope of this method towards related substrates with different alkyl chain lengths such as *n*-hexanaloxime or *n*-decanaloxime has been also demonstrated, albeit at somewhat lower substrate loadings to date [3e].

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6.2 Article 5 *submitted*

Immobilization of Aldoxime Dehydratase for the use in Aqueous Reaction Media

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Catalysts **2020**, submitted manuscript

Manuscript-ID: catalysts-900547

Author contribution

AH and MS initiated the project. Experiments were designed by AH and MS and performed by AH. AH wrote the manuscript with the help of MS. AH, MS and HG read and edited the manuscript.

Immobilization of Aldoxime Deydratases and their Use as Biocatalysts in Aqueous Reaction Media

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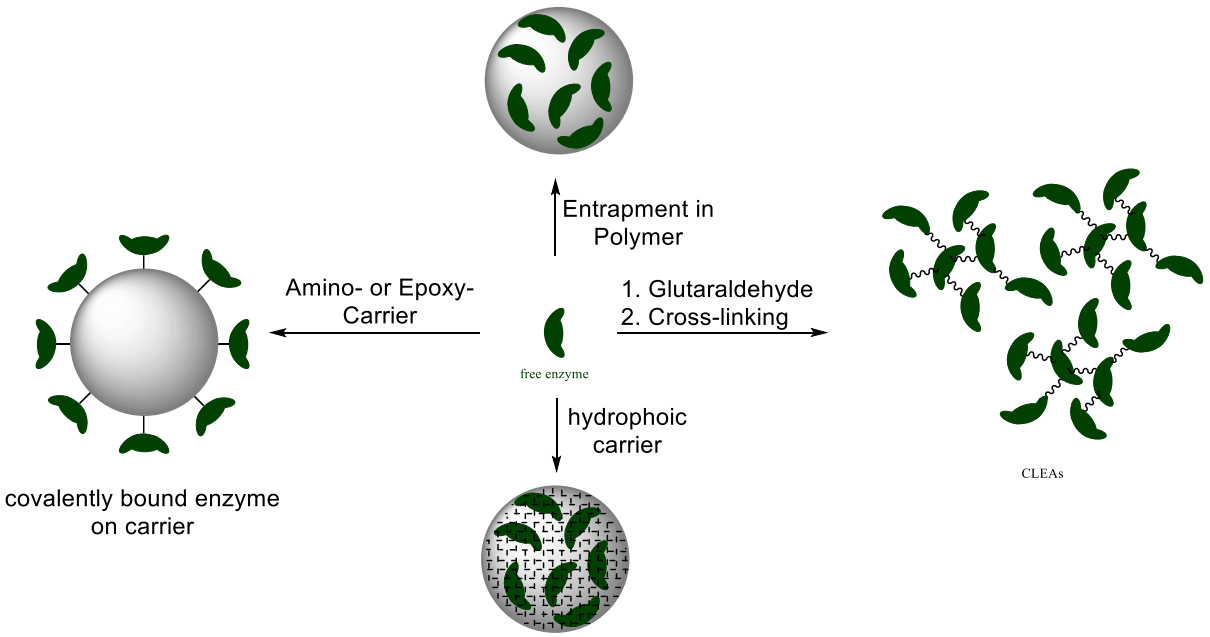
Abstract

Immobilization of biocatalysts is a current topic in research enabling the easy recovery of catalysts from the reaction medium after the reaction and is often accompanied by a stabilization of the catalysts, which enables a recycling. Within our ongoing research on the utilization of aldoxime dehydratases in the cyanide-free synthesis of nitriles through dehydration of readily available aldoximes, a screening of different immobilization methods for free enzymes was performed. The applied immobilization methods are based on covalent binding and hydrophobic interactions of the enzyme with the carrier material and whole cell immobilization in calcium alginate beads with and without subsequent coating. Within our study we found that the immobilization of purified free aldoxime dehydratases, even at high immobilization efficiencies, leads to a strong loss of activity with a residual activity of <20%, regardless of the carrier material used. However, we could show that calcium alginate beads are a suitable immobilization method for the heterogenization of aldoxime dehydrate whole cell catalysts with residual activities of up to ~75% and higher stability compared to free whole cells.

Introduction

Since biocatalysts have emerged as a valuable alternative to metal- and organo-catalysts,[1–6] it is of major interest to develop highly efficient and stable biocatalysts, which in the best case can be reused. This can for example be realized by immobilization of the biocatalyst on a carrier or by entrapment in polymers or hydrogels. Such a heterogenized catalyst can be separated from the reaction medium more easily, usually by filtration. In comparison, whole cell catalysts are often separated by centrifugation[7–9] or dissolved enzymes in an aqueous reaction medium which usually cannot be isolated. The free enzymes often lead to problems during the isolation of the product, especially if extraction is used. Extraction often lead to precipitation of the enzymes due to the denaturating effect of organic solvents and the precipitate forms an interphase between organic and aqueous phase.[1–6,10,11][12] This often makes downstream-processing tedious, thus causing lower isolated yields. In addition, in many cases the biocatalyst cannot be reused after such an extractive work-up. Thus, for an improved process, a suitable immobilization method for the use of most biocatalysts is desirable. There are many different immobilization methods known and different biocatalyst formylations can be used. On the one hand, free enzymes (used as crude extract or in purified form) can be used while on the other hand also whole cell catalysts can be immobilized by various techniques. The application of whole cell catalysts often has the positive effect that the cell membrane shields the enzymes from disturbing influences such as pH or solvents in the

reaction medium. Immobilization of free enzymes can be performed using different strategies. An overview of different established immobilization-techniques for enzymes is shown in Scheme 1.



Scheme 1. Immobilization strategies of enzymes.

The first strategy is a heterogenization by crosslinking of the enzymes. The most prominent example are CLEAs (cross-linked enzyme aggregates),[13] which are formed by a preactivation of primary amino functionalities on the enzyme surface, such as the side-chain of lysine, by glutaraldehyde and subsequent crosslinking of free primary amino groups with glutaraldehyde-bound amino groups to form heterogenized enzyme clusters. A second strategy of enzyme-immobilization is a covalent binding of enzymes to carriers.[14] This can be performed for example by either usage of epoxy-carrier material which reacts with side chains of amino acids of the enzyme surface or by usage of amino carrier which are preactivated by glutaraldehyde and reacts with amino side-chains similar to the formation of CLEAs. A third technique is a strategy in which enzymes are bound non-covalently for example by hydrophobic interactions to carrier material with hydrophobic residues[14] or His-tagged enzymes on specific carriers[15], which often has the benefit that this immobilization method has not such an strong impact than covalent binding. Besides free enzyme immobilization also whole cells catalysts can be immobilized. Many methods are known, for example immobilization of wholes cells by entrapment in polymers, hydrogels, or other materials. To give an example for the entrapment of biocatalysts in polymers, von Langermann *et al.* developed a technique of whole-cell immobilization in polyurethane being suitable heterogenized biocatalysts with a higher stability compared to free whole cells.[16–18] Whole cell catalyst (or free enzymes[19,20]) in an aqueous medium can also be immobilized by entrapment in superabsorber, yielding in a solid aqueous phase which can be used in organic solvent as reaction medium and easily separated by filtration.[21] Although this technique allows biocatalytic reactions in organic solvents, this technique has the disadvantage that this immobilized biocatalyst cannot be used in aqueous medium because an aqueous medium would lead to a leaching of the absorbed biocatalyst out of the superabsorber. Furthermore, the organic solvent has usually a deactivating effect on the enzymes, wherefore the heterogenized catalyst cannot be reused.[22] Enzyme immobilization can also be performed by entrapment in calcium alginate beads. This immobilization technique was recently applied by the Patel group to combine a pig liver

esterase with a Grubbs catalyst, which both need different reaction medium.[23–26] In this study presented, we focused on the immobilization of aldoxime dehydratase (Oxd), which only have been immobilized in superabsorber so far. This limits the use of Oxd-immobilizates to pure organic mediums. Our purpose in this project was to broaden the usability of immobilized Oxds also to aqueous reaction systems by usage of other immobilization techniques.

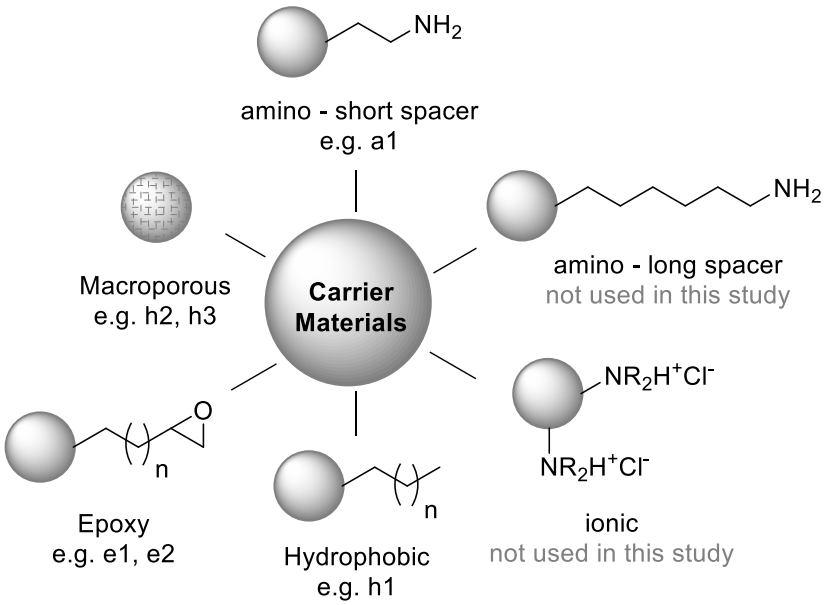
Results

The investigation of suitable immobilization strategies for Oxds was performed with two different enyzmes, namely OxdB from *Bacillus* sp. OxB-1 and OxdRE from *Rhodococcus erythropolis*, which already emerged as efficient catalysts for aldoxime dehydration, especially for aliphatic substrates.[21,27,28] We focused on the dehydration of *n*-octanaloxime to *n*-octanenitrile for our standard activity assays, since this substrates is accepted by both enzymes. In the first step, we investigated the immobilization of isolated enzymes (purified by Ni-NTA affinity chromatography) on different carriers, listed in Table 1.

Table 1. Immobilization carrier used for the immobilization of OxdB or OxdRE purified enzymes.

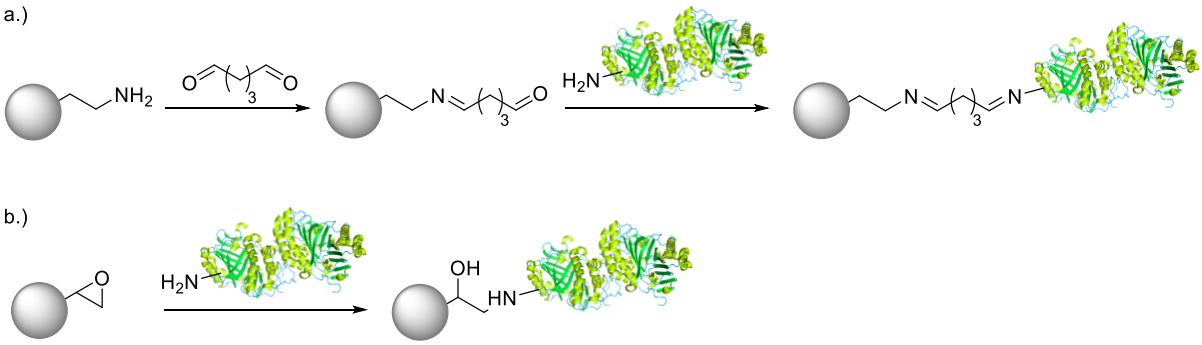
#	Carrier material	Abbreviation	Pore size /Å	Purolite no.
1	Octadecyl methacrylate	h1	500-700	Lifetech ECR8806
2	Macroporous styrene	h2	900-1100	Lifetech ECR1090
3	Divinylbenzene/Methyacrylate	h3	200-300	Lifetech ECR1030M$^{\neq}$
4	Amino C2 methacrylate	a1	600-1200	Lifetech ECR8309F
5	Epoxy methacrylate	e1	300-600	Lifetech ECR8204
6	Epoxy/butyl methacrylate	e2	400-600	Lifetech ECR8285

Depending on the carrier material, the immobilization is based on different interactions between the carrier and the enzyme. A schematic presentation of the interactions between immobilization carriers and enzymes is summarized in Scheme 2.



Scheme 2. Schematic overview of carrier materials used in this study.

Carriers h1 – h3 are hydrophobic resins which non-covalently immobilize proteins via physical adsorption. We chose h1, h2 and h3 as hydrophobic carriers because they differ in surface area (h1: >80 m^2/g, h2: >750 m^2/g and h3: >90 m^2/g) and the pore sizes of the resins (see Table 1). Besides non-covalent immobilization we also tested carrier, which covalently bind proteins. We chose one amino carrier (a1), which has a short ethylene spacer between resin and amino functionality. The amino group is preactivated by glutaraldehyde, which afterwards can covalently react with (for example) amino functions of the protein (Scheme 3, (a)). Lastly, two epoxy carrier were tested. Here we chose epoxy methacrylate (e1) and epoxy/butyl methacrylate (e2), which differ in the spacer length between resin and epoxy functionality and pore size. Epoxy resins react under formation of a covalent bond with (for example) amino functionalities of the protein (Scheme 3, (b)).



Scheme 3. (a) Protein immobilization on amino carrier after preactivation of the resin with glutar aldehyde and (b) protein immobilization on epoxy carriers. X-ray structure shown is the crystal structure of OxdRE.[29]

After immobilization of purified OxdB and OxdRE on the different carrier, the immobilization efficiency and residual activity of the protein in comparison to the purified Oxds were determined. Immobilization efficiencies were measured by determination of protein concentration of the purified enzyme solution in PPB used for the immobilization and of the supernatant after immobilization (Figure 1).

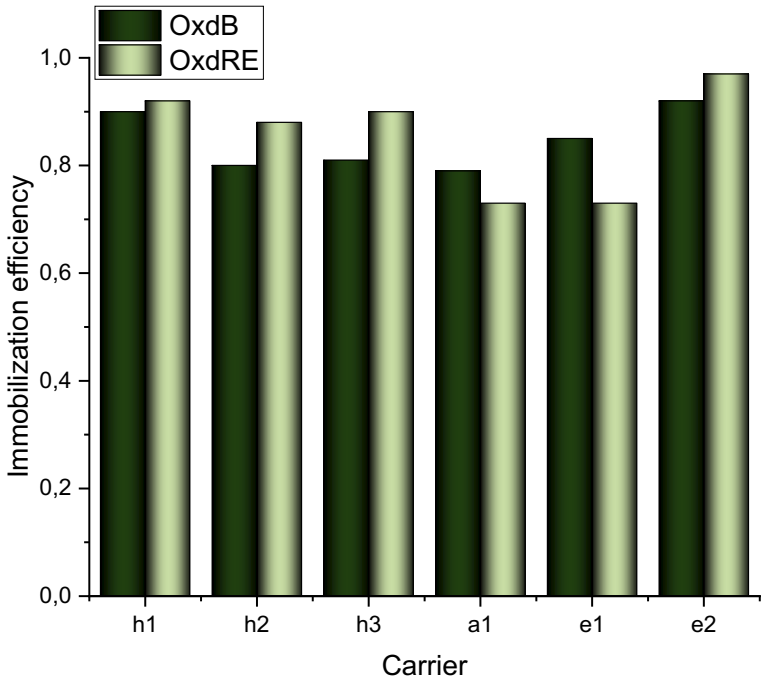


Figure 1. Immobilzation efficiency of purified OxdB and OxdRE on different carrier.

Immobilization efficiencies were found to be very high in all cases and for both proteins. These immobilization efficiency values between ~80 – 90% indicate that nearly all the protein in solution was immobilized on the carriers. *n*-Octanaloxime was used as substrate to determine the activity of the immobilized enzymes in comparison to purified OxdB and OxdRE (Figure 2).

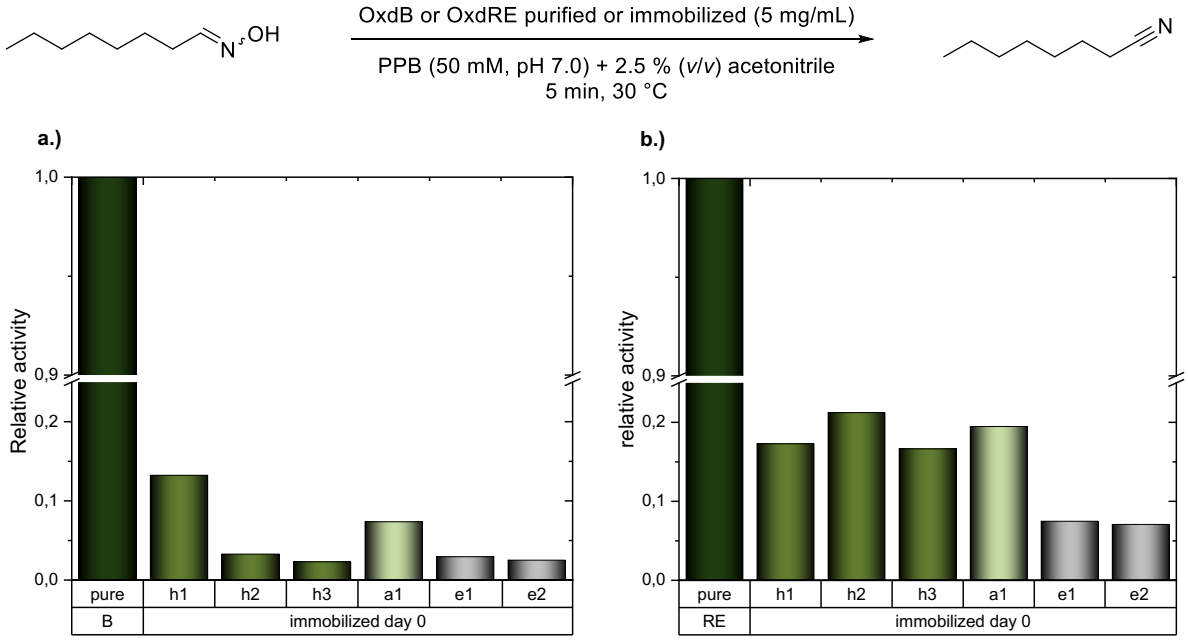


Figure 2. Residual activity of (a) OxdB and (b) OxdRE immobilizates in comparison to purified OxdB or OxdRE.

Residual activities after immobilization on different carrier materials were found to be in ranges of ~5 – 10 % of the activity of purified OxdB or OxdRE. Especially in case of OxdB the carrier material shows a strong impact on the residual activity. In case of h1 and a1 the residual activity was still approximately 10 % of the activity of purified enzyme, whereas the epoxy carrier e1 and e2 as well as h2 and h3 showed lower residual activities of approximately 5 %. In case of OxdRE the impact of the carrier material was found to be less strong compared to OxdB. Only the epoxy resins e1 and e2 showed lower residual activities of ~7 %. The amino carrier a1 and the hydrophobic and macroporous carriers h1 – h3 showed residual activities of ~15 – 20 % compared to the purified enzyme. Since h1 and a1 carriers showed acceptable results for OxdB and OxdRE these two carriers were chosen for further experiments.

A storage stability assay was performed using OxdB and OxdRE purified enzyme and h1- and a1-immobilizates of both. Purified enzyme and immobilizates were stored at 4 °C for 6 and 32 days and the activity was examined (Figure 3).

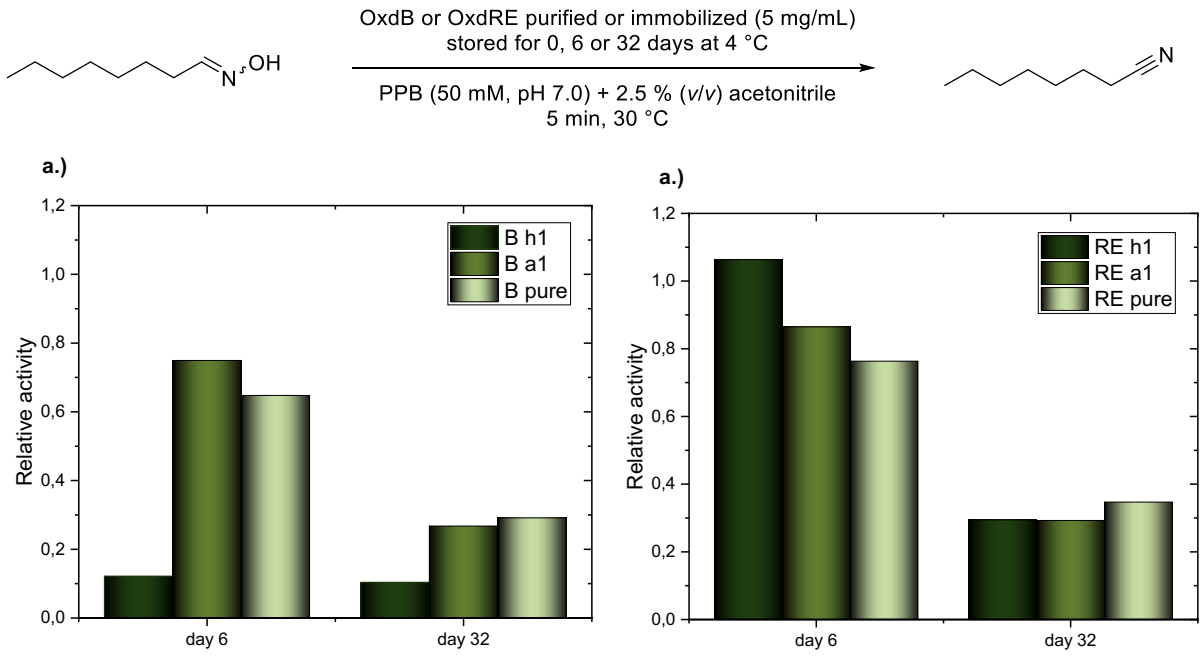


Figure 3. Storage stability assay of (a) OxdB and (b) OxdRE purified enzymes and h1- and a1-immobilizates. The relative activity data are referring to the activity measured at the first day (day 0).

The storage stability assay was performed in triplicates and the results show that the residual activity after different storage times at 4 °C for 6 days is often higher for the immobilized enzyme compared to the one for the purified enzyme. In detail, the a1-immobilizates of OxdB are more stable, while for OxdRE h1-immobilization seem to be more beneficial. After 32 days storage time, however, the activity dramatically decreases in all cases to ~20% or lower.

As next, a cosolvent study was performed using OxdB and OxdRE purified enzymes and h1- and a1-immobilizates (Figure 4). The effects of immobilization on the stability of OxdRE and OxdB against organic solvents should be tested. Therefore, the purified enzymes or immobilizates were incubated for different times in different cosolvents (20 % (*v*/*v*)) or PPB, afterwards the activity for the conversion of *n*-octanaloxime to *n*-octanenitrile was determined.

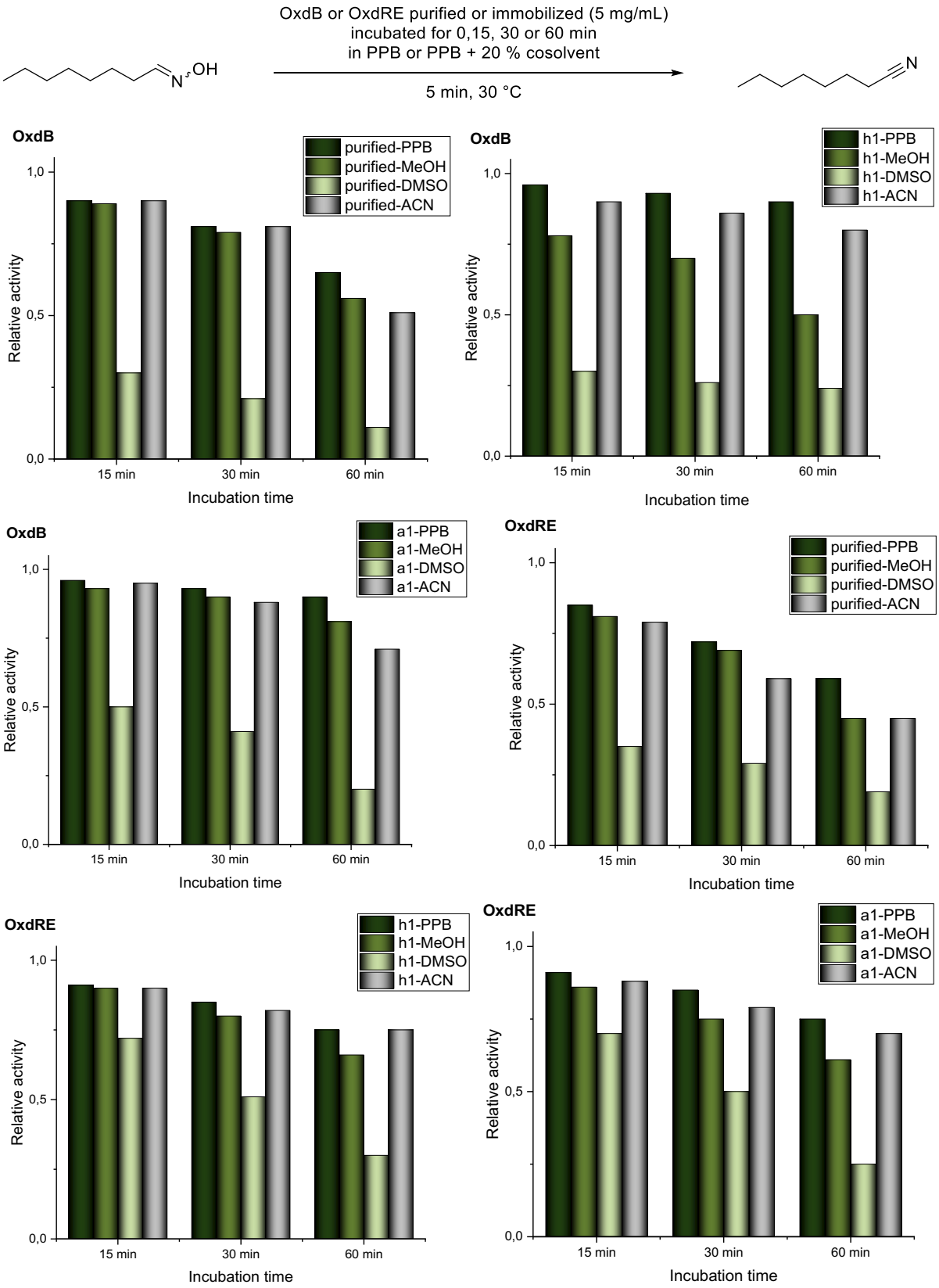


Figure 4. Cosolvent study of immobilized OxdB (top) and OxdRE (bottom).

In this cosolvent study, the activity of beads or purified enzyme without incubation was normalized to a relative activity of 1 and all measured activities after incubation were compared to this value. We found, that OxdB and OxdRE immobilized enzymes were not much more

stable after incubation in different cosolvents than purified enzymes. DMSO seems to be the most unsuitable solvent for both enzymes and acetonitrile (ACN) the most favorable, while methanol deactivates the enzymes more. Unfortunately, even after a short incubation time of 1 h a decrease of activity is found in all cases. Even without any co-solvent a strong decrease in activity, with ~65% residual activity for OxdB and ~50% for OxdRE, of the purified enzymes was observed already after 1 h. The immobilizates show a stabilizing effect for all solvents. However, the deactivation of DMSO is strong even when using immobilizates. Although the immobilization seems to have a stabilizing effect on the enzymes the activity of the immobilizates is very low in comparison to the free enzymes (~20% residual activity). These low activities are even decreased by the usage of organic solvents, which makes the use of purified enzyme or the tested immobilizates not suitable for application in organic synthesis.

Therefore, we further investigated immobilization of OxdB and OxdRE whole cell catalysts. Our group already found that Oxd whole cells can successfully be immobilized in superabsorber and used in organic media very efficiently.[22] A disadvantage of this system is that superabsorber-immobilized cells cannot be used in aqueous reaction medium due to leaching of the cells out of the superabsorber. Another immobilization-technique which is also suitable for the immobilization of whole cells is based on sodium alginate, which can be premixed in an aqueous solution with the cells of interest and dropped into $CaCl_2$-solution which hardens alginate. Applying this immobilization-technique to OxdB and OxdRE-whole cells, first, we needed to perform a buffer screening because Oxds are normally used in PPB as buffer which destabilize alginate beads. After we switched the standard buffer PPB to HEPES-buffer, which showed similar activity to PPB, we immobilized OxdB and OxdRE whole cells in alginate beads. They were prepared by dropping sodium alginate solution premixed with the whole cell catalysts and buffer (HEPES, 50 mM, pH 7) into $CaCl_2$-solution to harden the beads. The beads were filtered, washed with HEPES-buffer and used afterwards for biotransformation of *n*-octanaloxime to *n*-octanenitrile (Figure 5).

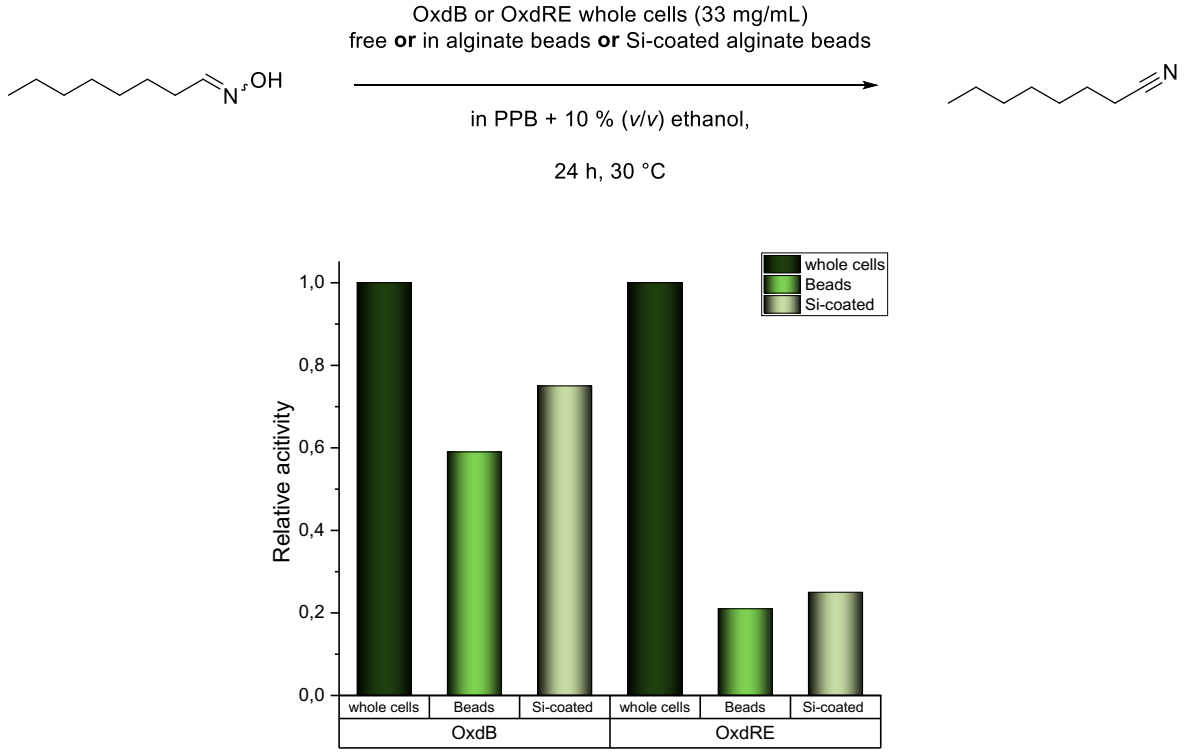
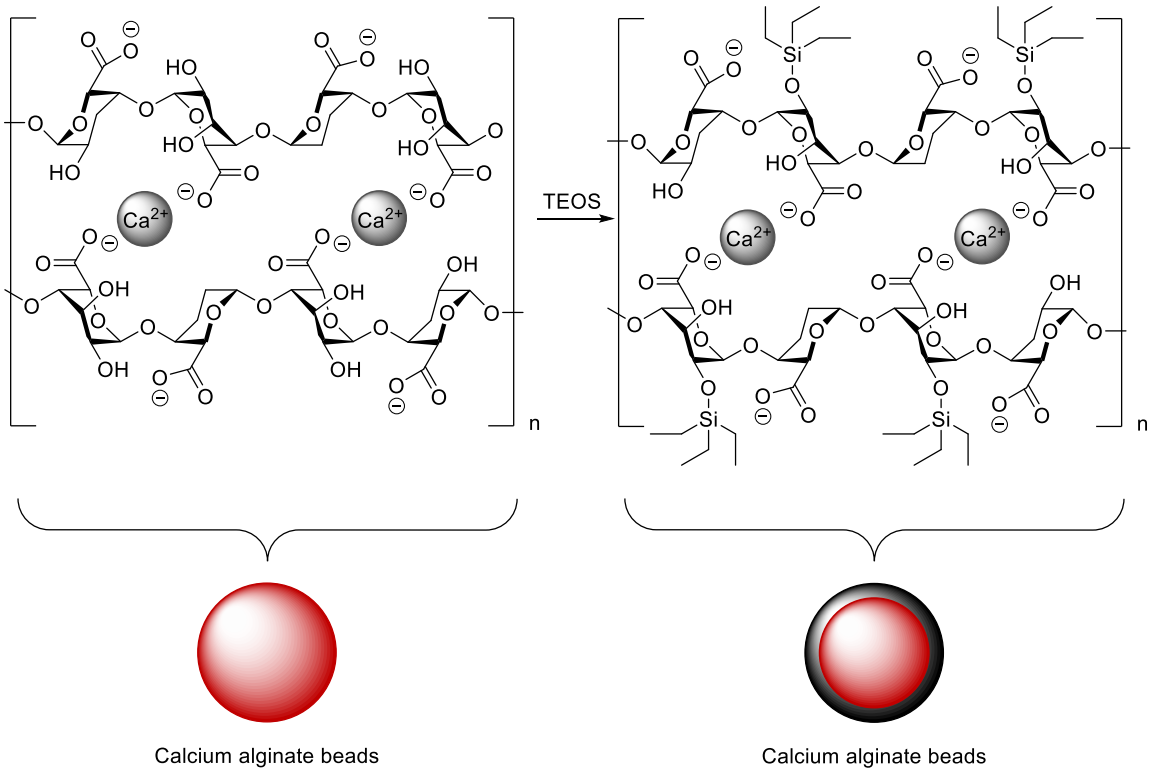


Figure 5. Comparison of OxdB and OxdRE whole cells and OxdB and OxdRE whole cells immobilized in alginate beads and immobilized in alginate beads coated with TEOS (Si-coated). Activities of OxdB and OxdRE free whole cell catalyst were set as 100%.

The activity of OxdB whole cells and immobilized OxdB whole cells were determined. As shown in Figure 5, both whole cell catalysts are still active after immobilization in alginate beads, however, the activity drops to approx. 60% in case of OxdB and approx. 20% in case of OxdRE in comparison to the free whole cell catalysts.

Since the surface of calcium alginate is very hydrophilic and might negatively influence the diffusion of *n*-octanaloixime into the beads, we furthermore investigated a coating of the beads with tetraethylorthosilicate (TEOS) (Scheme 4).



Scheme 4. Silica-coating of calcium alginate beads using TEOS.

This coating is performed after immobilization of OxdB or OxdRE in alginate beads as described before by stirring the beads overnight in a solution of TEOS in *n*-hexane. After this "silica-coating", the surface of the beads is more hydrophobic and the substrate might easier diffuse into the beads. The silica-coated (Si-coated) beads were also tested for the conversion of *n*-octanaloxime and compared to the results of free whole cells and uncoated alginate-immobilized whole cells (Figure 5).

Indeed, the silica-coating of the beads seems to have an impact on the activity of the immobilized whole cells. For both enzymes, the residual activity is higher after silica-coating compared to the uncoated beads, however, the residual activity of OxdRE is still much lower compared to OxdB (~20% residual activity for OxdRE and ~75% residual activity for OxdB). This finding is very surprising because normally Oxds (also in whole cells) were found to be unstable in organic media, while in this case after stirring of the beads overnight in pure *n*-hexane with TEOS leads to still active immobilized cells. Thus, this result induced us to test the activity of the beads also in pure organic solvent (Figure 6).

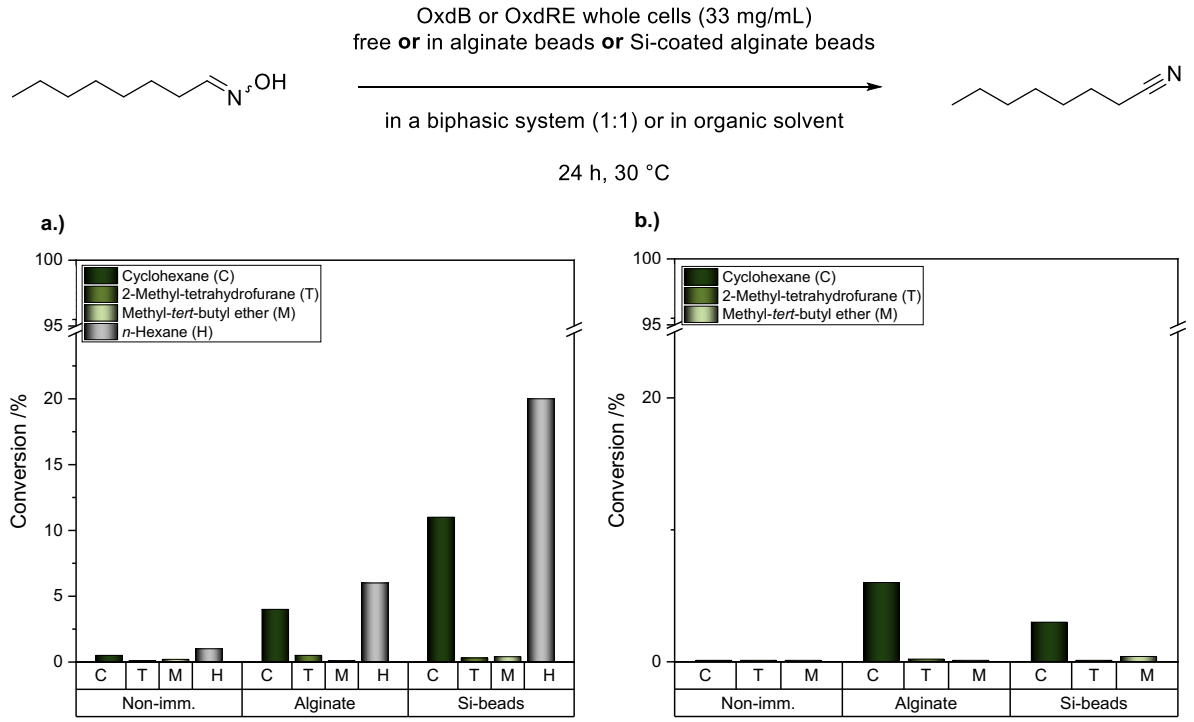


Figure 6. Dehydration of n-octanaloxime using immobilized whole cells in calcium alginate beads (Alginate) or Si-coated calcium alginate beads (Si-beads) in pure organic medium compared to free whole cells in a biphasic reaction medium of OxdB (a.) and OxdRE (b.). In the y-axis an interruption from 25 to 95% is inserted.

It is already known, that free Oxd whole cells lose their activity by usage in pure organic solvent or in a biphasic reaction medium.[22] For comparison reasons, we applied also free Oxd whole cells in a biphasic reaction medium compared to immobilized cells in alginate beads and Si-coated beads. As expected, we could not obtain any conversion in the biphasic approach in all tested solvents for free Oxd whole cells, namely cyclohexane (C), 2-methyl-tetrahydrofurane (T), methyl-*tert*-butyl ether (M) and *n*-hexane (H). In case of the OxdRE-immobilizates, we found conversion in cyclohexane as solvent, however, the conversions were very low and no difference between the coated and the uncoated beads were observed. In case of OxdB-immobilizates we could reach ~10% conversion by usage of cyclohexane as solvent for Si-coated beads. Even with the uncoated beads a conversion of ~5% was obsvered. Since we used *n*-hexane as solvent for the TEOS-coating, we further investigated *n*-hexane as solvent and found a conversion of 20% by using Si-coated OxdB immobilizate. This result shows in principle that the beads are more suitable in organic solvents than free whole cells, especially in very unipolar solvents like cyclohexane or *n*-hexane. This might be caused by a shielding effect of the alginate beads. Generally, we were interested in utilizing the whole cell immobilisates in an aqueous reaction medium, since we already established a method for the usage of Oxd whole cells in organic medium with superabsorber. Therefore, we investigated the stability of the beads (calcium alginate beads and Si-coated beads) in buffer containing ethanol as cosolvent. Normally, aldoximes are not very good soluble in an aqueous reaction medium, wherefore a cosolvent is needed. In previous studies it was found, that ethanol is a very suitable solvent for the use in combination with Oxd whole cell catalysts.[21] The activity of free Oxd whole cells and immobilized with alginate and Si-coated were tested in HEPES-buffer with 10% or 50% ethanol as solvent. The free whole cells or the beads were incubated for 24 h in this mixture and then biotransformations with *n*-octanaloxime were performed with these pretreated catalysts (Figure 7). We normalized the results on the activity of the free whole cells or immobilized whole cells without incubation.

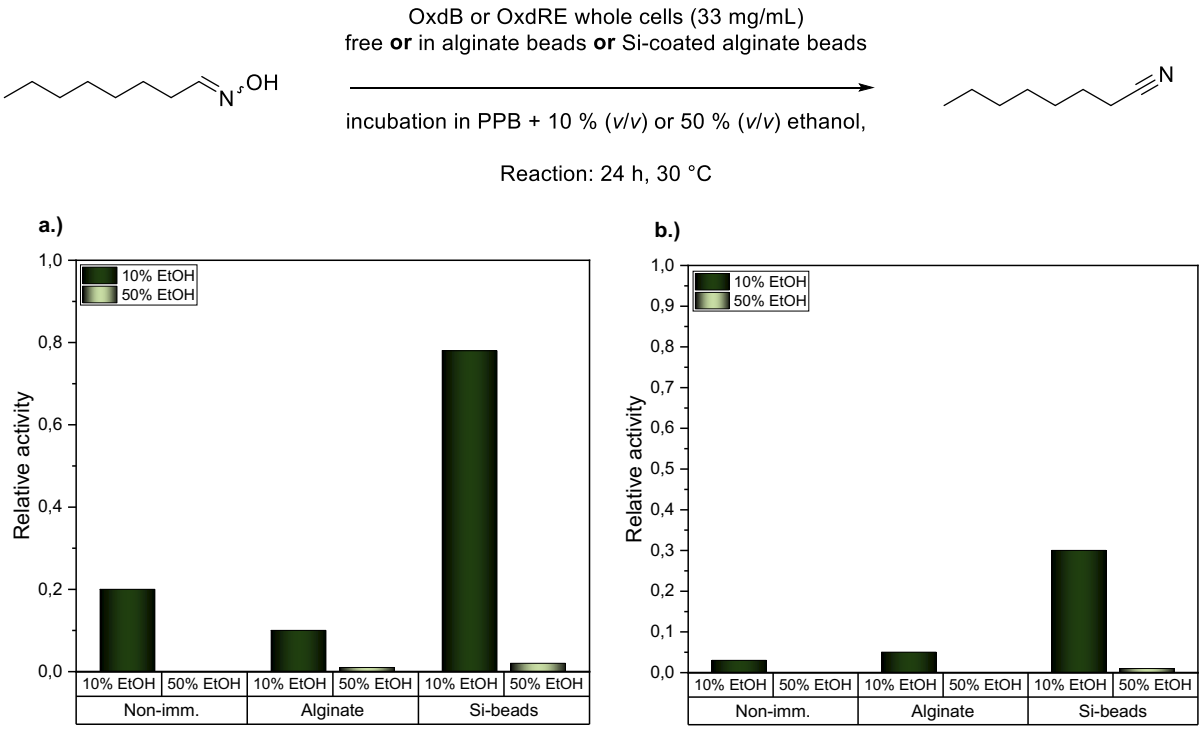


Figure 7. Incubation of OxdB and OxdRE whole cells (free (non-imm.), immobilized in calcium alginate (Alginate) or in Si-coated calcium alginate (Si-beads)) in 10% or 50% ethanol in HEPES. Shown is the relative activity in comparison to standard activity of the dehydration.

We found a very interesting stabilizing effect of the Si-beads in comparison to the free whole cells. While for the whole cells only 20% (OxdB) or ~5% (OxdRE) residual activity was observed after 24 h incubation time in buffer with 10% ethanol, ~80% (OxdB) or ~30% (OxdRE) residual activity was found for the Si-beads. These experiments show that a stabilizing effect of the beads, especially of the coated beads is obtained. It is very surprising that after the TEOS-coating overnight in *n*-hexane residual activity and an enhanced stability for both of the catalysts (OxdB and OxdRE) is observed, whereas usually only a few minutes incubation of the whole cells in organic solvents leads to a dramatical loss of activity.[22]

Based on these findings, we then performed a recycling study using OxdB whole cells immobilized in Si-coated alginate beads. We did not perform a recycling study for the OxdRE-immobilizates because the previous experiments demonstrated the higher stability and residual activity of OxdB after immobilization. In our recycling experiment, the conversion of *n*-octanaloxime to *n*-octanenenitrile in the first round (88%) was normalized to 1 and all experiments with the recycled catalyst were compared to this value (Figure 8).

As shown in Figure 8 a loss of activity during the recycling-study is observed, however, within the first three recycling steps more than 85% residual activity is obtained. After five rounds of recycling only ~20% residual activity of the catalyst is left.

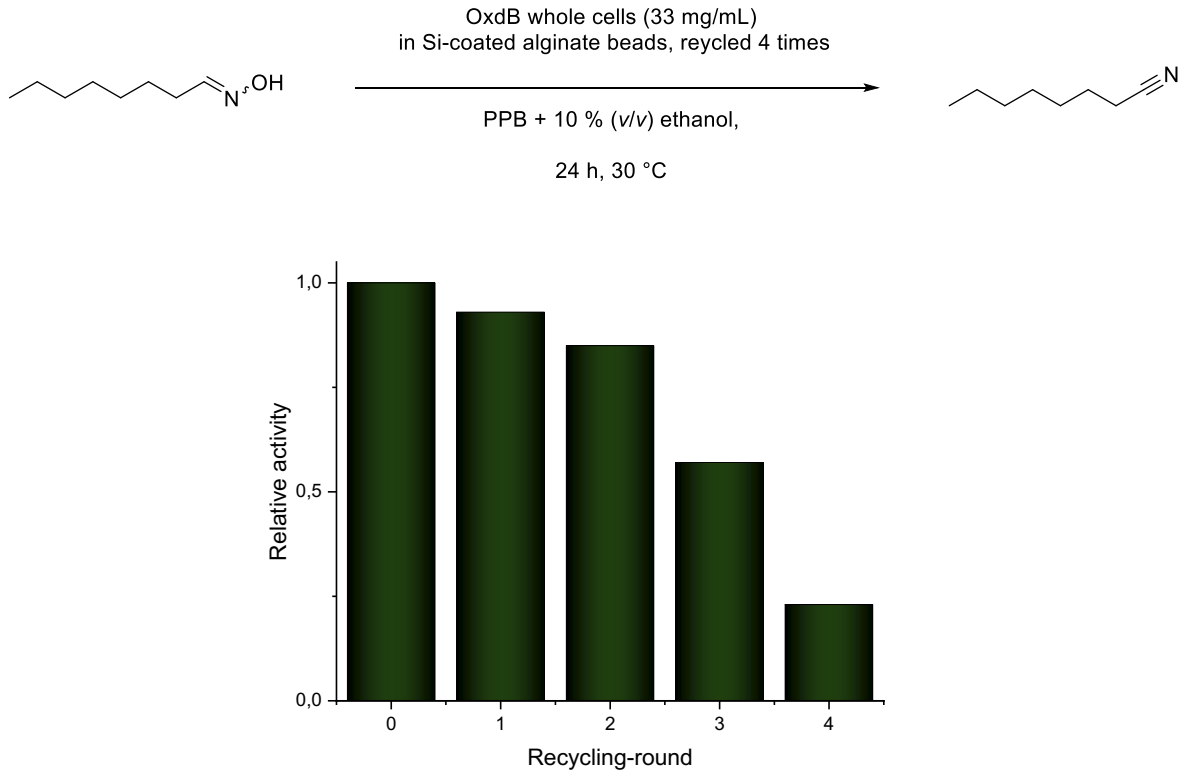


Figure 8. Recycling-study of OxdB immobilized in calcium alginate beads, coated with TEOS (Si-beads).

Discussion

Different immobilization techniques of Oxds were tested using purified Oxds and Oxds in whole cells. We focused on two enzymes, namely OxdB from *Bacillus* sp. OxB-1 and OxdRE from *Rhodococcus erythropolis*. Both are active for the dehydration of *n*-octanaloxime to *n*-octanenitrile, which was used as a standard substrate in every activity assay. First, we investigated different immobilization carriers, which immobilize free enzymes, by hydrophobic interactions or covalently. We chose three different hydrophobic carriers (h1, h2 and h3), one aminocarrier (a1) which is preactivated by glutaric aldehyde and two epoxy carrier (e1 and e2). It was found that the residual activity after immobilization with high immobilization efficiencies (~80-90%) were <20% in all cases. The strong loss of activity for all carriers is probably due to the instability of the purified enzyme at elevated temperatures. We could show that even an incubation of the purified enzymes for 1 h at 30°C in buffer leads to a loss of activity if 50%. During immobilization, the enzymes are shaken for 24 h hours at temperatures above rt (see supporting information), which partly explains the severe loss of activity. Although, the remaining activities after immobilization of the free enzymes were very low, an additional cosolvent screening was performed to determine the stability of the immobilizates in comparison to free purified enzyme. The immobilization of the enzymes seems to have a small stabilizing effect with and without co-solvent. These results led us to the conclusion that purified Oxds are not very stable and immobilization of the enzymes has a positive effect on the stability, while the activity is drastically decreased. However, the strong decrease of activity during immobilization makes it difficult to use in organic chemistry and we decided to switch our investigations to immobilization of whole cells, since it was already found that Oxds are more stable in the whole cells compared to the purified enzyme.[30] Using an immobilization technique with calcium alginate beads compared to calcium alginate beads coated with TEOS, we successfully immobilized OxdB and OxdRE in whole cells with residual activities of up to

~70%. Compared to the immobilization of purified enzyme, this residual activity is remarkable. For both enzymes we could observe higher activities for the Si-coated beads in comparison to the uncoated beads, which is probably due to a better diffusion of the substrate in the beads. The alginate beads have a very hydrophilic surface, which makes a diffusion of the hydrophobic substrate *n*-octanaloxime difficult. The Si-coating seems to mask the hydrophilic surface and make them more hydrophobic, which makes a diffusion easier. Since TEOS-coating to gain Si-coated alginate beads is performed in *n*-hexane as solvent, we performed reactions in pure organic solvents using immobilized cells compared to free cells. Only in case of OxdB in *n*-hexane or cyclohexane as solvent conversion was found, leading us to the conclusion that the alginate beads are not suitable for the use in organic solvent. Superabsorber-immobilization of Oxd whole cells were already successfully established for the use in organic solvent, wherefore we did not further investigate the use of alginate beads in organic media.[22] It was found that the alginate beads, especially the Si-coated beads, are significantly more stable in ethanol-containing buffer with ~80% residual activity after 24 h incubation time in 10% ethanol of OxdB in Si-coated beads compared to ~20% for the free cells. Recycling of the beads led to decreased residual activity, however, the immobilizates can be used three times before a decrease of the activity to <85% is observed.

Conclusion

Within this study, we tested several immobilization techniques for enzymes such as epoxy- and amino-substituted resins and hydrophobic carriers, however, the residual activity for both tested enzymes OxdB and OxdRE were found to be very low. Therefore, we switched to the immobilization of OxdB and OxdRE in whole cells by entrapment in calcium alginate beads. After this immobilization and coating with TEOS high residual activities with up to ~75% were obtained. Using these immobilizates a stability study in ethanol-containing buffer was performed and a higher stability to free whole cells were found. In a recycling study of OxdB whole cell immobilized in calcium alginate beads covered with TEOS over three cycles the activity remains at >80% compared to the starting activity. Thus, in conclusion, we found an applicable immobilization technique after which the immobilizates are usable in aqueous reaction medium, without dramatical loss of activity and higher stability compared to free whole cells, making it possible to reuse the biocatalyst and enable a simple work-up.

Material and Methods

See Supporting Information

Acknowledgement

We gratefully acknowledge generous support from the Fachagentur Nachwachsende Rohstoffe (FNR) and the German Federal Ministry of Food and Agriculture (BMEL), respectively, within the funding program on the utilization of biorenewables (Grant No. 22001716). We also thank Hannah Bork and Jendrik Wagner for technical assistance.

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Supporting Information

Immobilization of Aldoxime Deydratases and their Use as Biocatalysts in Aqueous Reaction Media

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General experimental information

Chemicals used

Material for cell cultivation and aldoxime dehydratase (Oxd) expression was purchased by Carl Roth (Antibiotics, LB- and TB-premixed medium, D-glucose, D-lactose). Buffer salts (KH_2PO_4 and K_2HPO_4) were obtained from VWR chemicals. Immobilization carrier were purchased by Purolite®, Sodium alginate and TEOS were purchased by Sigma Aldrich. Solvents were purchased by VWR Chemicals in HPCL-grade and used without further purification.

Analytical methods

Conversions were determined by GC measurements (Shimadzu GC-2010 Plus) in comparison to a calibration curve. Measurements were conducted on a chiral SGE Analytik B6B-174 column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with nitrogen as carrier gas. An injector temperature of 220 °C in a split injection mode was used and a sample amount of 1 µL was injected in this method. The following temperature gradient was used:): 140 °C starting temperature (hold 1 min), in 20 °C/min to 190 °C (hold 0.5 min) and in 50 °C/min to 200 °C. Retention times of the substances of interest are: t_R (octanaloxime) = 2.7 min; t_R (octanenitrile) = 2.4 min. Accuracy of the GC-analytical methods: the error of the determined conversions to the corresponding nitriles is in the range of up to 5% depending on the concentration of the aldoximes due to temperature depending degradation of the aldoximes on the GC-column.

Oxd sequences

Aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) (Accession number: GenBank: AP013294.1)

His-tagged variant in pET22a

ACAGGGAACGGGGGCGGAAATTCCCTCTAGAATAATTTTGGTTTAACTTTAAGGAAGGA
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GAGCACCACCACCACCACCACTGAGATCCGGCTGCTAACAAAGCCCGAAAGAAGTTTTT
T

Without His-tag in pUC18

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CGAGCTTATCTATGTCAACTGCCATCCGAGTACTGGATTTCTTCCATTCTTTGAAGTGAC
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Aldoxime dehydratase from *Rhodococcus erythropolis* N-771 (OxdRE) (Accession number: GenBank: AB094201.1)

His-tagged variant in pET28a

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGC
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AA

Oxd expression, purification and immobilization

Oxd expression and purification

E.coli BL21-CodonPlus(DE3)-RIL (aldoxime dehydratase from *Rhodococcus erythropolis* (OxdRE)) or BL21(DE3) (aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB)) cells harboring the plasmids with the Oxd-genes (for sequences see Supporting Information) were stored as glycerol stocks at -80 °C. A sample from the glycerol stocks for each Oxd was plated on LB-agar containing 50 µg/mL Kanamycin and 34 µg/mL Chloramphenicol (OxdRE) or 100 µg/mL Carbenicillin (OxdB) and incubated for 12 to 18 h at 37 °C. Pre-cultures were prepared in 5 mL LB-medium containing 50 µg/mL Kanamycin and 34 µg/mL Chloramphenicol (OxdRE) or 100 µg/mL Carbenicillin (OxdB) using a single colony from the LB-agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm. Main cultures for Oxd expression were performed using TB-autoinduction medium (50 mL 20 g/L lactose solution, 5 mL 50 g/L D-glucose solution and 445 mL TB-medium (purchased from Carl Roth) in a 500 mL Erlenmeyer flask). 50 µg/mL Kanamycin and 34 µg/mL Chloramphenicol (OxdRE) or 100 µg/mL Carbenicillin (OxdB) were added to the medium. Main cultures were inoculated with 1 % (5 mL) of the relating pre-cultures

and incubated for 1 h at 37 °C and 120 rpm. After 1 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 120 rpm and OxdRE-cultures were cultivated at 15 °C for 72 h and 120 rpm. Cell harvest was performed at 4,000 xg for 15 min at 4 °C. Cells were washed three times with lysis buffer (20 mM Tris-HCl, 300 mM NaCl, 10 mM imidazole) and afterwards resuspended in 2x mass of the bio wet weight (bww) of the cells in lysis buffer. Cells were disrupted by sonication (5x 1 min, 15 – 20 % output*, Sonoplus Ultraschall-Homogenisator HD 2070* (Bandelin electronic GmbH & Co. KG)) and crude extracts were obtained by centrifugation for 45 min at 25,000 xg and 4 °C. Oxd-purification was performed using Ni-NTA affinity chromatography. Oxds were eluted using elution buffer (20 mM Tris-HCl, 300 mM NaCl, 150 mM imidazole) and rebuffered to 50 mM potassium phosphate buffer (PPB) (pH 7.0). Protein concentration of the purified enzyme and crude extracts were determined by Bradford-assay in comparison to a bovine serum albumin (BSA) standard curve. Expression and purification success was analyzed by SDS-PAGE using a 12 % separation gel in comparison to a protein marker (*PageRuler Prestained* (Thermo Fisher)).

(a) (b)

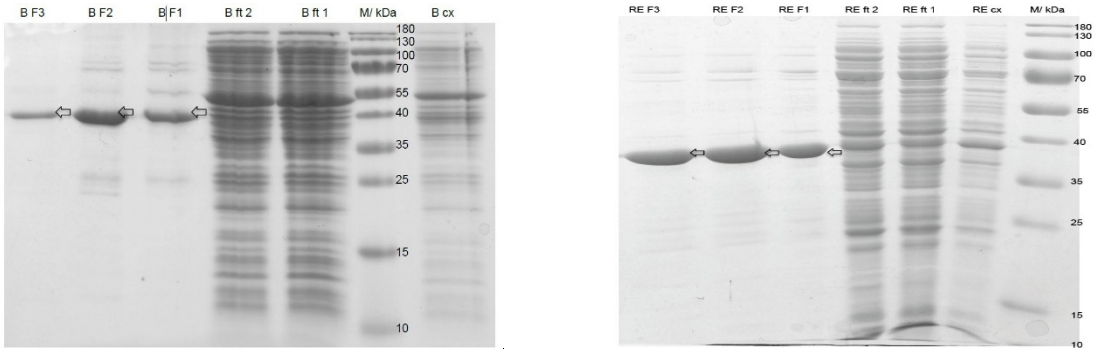

Figure 1. SDS-PAGEs of OxdB (a)- and OxdRE (b)-crude extracts (cx) and fractions of protein purification (flowthrough (ft), fractions of elution (F))**.**

Pure fractions of protein purification were combined and rebuffered to PPB (50 mM, pH 7.0) before usage for immobilization studies.

Oxd immobilization, determination of immobilization efficiency and standard activity assay

Immobilization

Different carriers were chosen for the immobilization study of OxdB and OxdRE (Table 1).

Table 1. Immobilization carrier used for the immobilization of OxdB or OxdRE purified enzymes.

#	Carrier material	Abbreviation	Pore size /Å	Purolite no.[1]
1	Octadecyl methacrylate	h1	500-700	Lifetech ECR8806
2	Macroporous styrene	h2	900-1100	Lifetech ECR1090
3	Divinylbenzene/Methyacrylate	h3	200-300	Lifetech ECR1030M$^{\neq}$
4	Amino C2 methacrylate	a1	600-1200	Lifetech ECR8309F
5	Epoxy methacrylate	e1	300-600	Lifetech ECR8204

6	Epoxy/butyl methacrylate	e2	400-600	Lifetech ECR8285

Except for minor deviations in the incubation time, the immobilization according to the instructions of Purolite.[1] For immobilization, the respective resin (46 mg) was placed in an Eppendorf tube and washed with PPB (46 µL, 50 mM, pH 7.0). Then in the case of the hydrophobic resins and the epoxyresins, the purified enzyme solution (184 µL) was added. The aminoresin, however, had to be activated before the enzyme solution was added. For this purpose, glutaraldehyde solution (184 µL of a 25% aqueous solution) was added to the washed aminoresin (46 mg), 2% in 50 mM PPB pH 7.0) and incubated at 600 rpm and 20 °C for 1 h. The activated aminoresin was then diluted with PPB (pH 7.0) and the enzyme solution (184 µL) was added. The enzyme solution was previously immersed in PPB (pH 7.0) is re-buffered and concentrated to such an extent that 2.76 mg enzyme is incorporated into the 184 µL of the enzyme solution were contained and thus the protein load was 6 %. The preparations were then incubated at approx. 300 rpm and 20 °C. After the immobilization, the supernatant was removed and the immobilizates washed with PPB (pH 7.0) and NaCl-solution. For each carrier material a different concentration of PPB for washing the carrier, for re-buffering the enzyme solution and for washing of the immobilisate was used. The used buffers and the different incubation times are shown in Table 2.

Table 2. Used buffer for washing oft he carrier material, the enzyme solution and washing after immobilization.

Carrier material	PPB for washing of the carrier	PPB of the enzyme solution	PPB for washing the immobilizates	Incubation time /h
Hydrophobic	50 mM	50 mM	50 mM	21-23
Amino	50 mM	50 mM	10 mM, afterwards 0.5 mM NaCl in 50 mM PPB	18
Epoxy	0.5 mM	0.5 mM	3x 10 mM, afterwards 1x 0.5 mM in 50 mM PPB	20, then further 25

Determination of immobilization efficiencies

Immobilization efficiencies were determined by comparison of the protein concentration, determined by Bradford assay, of the purified enzyme solution used for the immobilization and the supernatant after the immobilization.

Determination of water amount in immobilizates

To determine the solvent content, approx. 5 mg of each immobilizate of OxdB and OxdRE are balanced and lyophilized after freezing for approx. 24 h. The immobilizates were then balanced again. The determination was performed in triplicates and results shown in Table 3.

Table 3. Mass of immobilizates before and after drying.

Enzyme	Immobilizate	Mass before drying /mg	Mass after drying /mg
OxdB	h1	5.11	2.96
	h2	5.33	1.97
	h3	6.37	3.26
	a	4.32	1.80

	e1	4.59	2.14
	e2	5.31	1.87
OxdRE	h1	4.20	3.15
	h2	5.24	2.74
	h3	5.62	3.33
	a	4.24	2.46
	e1	5.59	3.33
	e2	5.68	2.14

Activity assay for purified or immobilized enzymes

Octanaloxime was prepared as described in previous publication.[2] To the enzyme (purified enzyme or immobilizates, 50 µL, 5 mg/mL final concentration) PPB (437.5 µL, 50 mM, pH=7.0) was added. After an incubation time of 5 min at 30 °C and 1400 rpm octanaloxime (12.5 µL, final concentration 5 mM) in acetonitrile (final concentration 2.5% (*v*/*v*)), was added. The conversion to octanenitrile was determined after 5 min at 30 °C and 1400 rpm by extraction with ethyl acetate (500 µL) and analyzation of the organic phase by gas chromatography. The data were compared to standard curves of octanaloxime and octanenitrile, givening conversions from which activities in $U/mg_{protein}$ were determined.

Table 4. Activities of OxdB and OxdRE purified enzymes ans immobilizates.

Enyzme	Formulation	Activity /$U \cdot mg^{-1}$
OxdB	purified	1.51
	h1	0.19
	h2	0.04
	h3	0.02
	a	0.11
	e1	0.04
	e2	0.03
OxdRE	purified	0.65
	h1	0.11
	h2	0.14
	h3	0.11
	a	0.13
	e1	0.03
	e2	0.03

Storage stability of enzymes and immobilizates were performed by comparison of the activity for octanaloxime after storage of the enzymes or immobilizates for 6 or 32 days at 4 °C.

Cosolvent study

To the enzyme solution (50 µL, 5 mg/mL final concentration) or the equivalent amount of immobilized enzyme PPB (350 µL, 50 mM, pH=7.0) and cosolvent (87.6 µL and 98 µL) were added. After different incubation times of 0 min, 15 min, 30 min and 1 h at 30 °C and 1400 rpm, octanaloxime (12.5 µL, final concentration of 5 mM) in the respective cosolvent (2.5% (*v*/*v*) final concentration) was added. After 5 min reaction time ethyl acetate (500 µL) was added, vortex and the organic phase analyzed by GC. As cosolvent, methanol, dimethyl sulphoxide and acetonitrile were used

Whole cell immobilization in calcium alginate beads, coating with TEOS and activity assay

Buffer screening

The measurements of the activities of the selected buffers were recorded by GC analytic and performed with OxdB in whole cells . For the buffer activity, 50 µl of the cell-suspension (333 mg/ml in PPB) were placed into Eppendorf tubes. The suspension was centrifuged (1 min, 17000 g), the PPB removed and resuspend in the selected buffer to a final concentration of 333 mg/mL. The selected buffers were HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid), TRIS-HCl (2-Amino-2-(hydroxymethyl)propane-1,3-diol), MOPS (3-Morpholinopropane-1-sulfonic acid) and ammonium acetate all in a concentration of 50 mM and pH of 7.0. 400 µL of the selected buffer was placed into an Eppendorf tube and 50 µL of the cell suspension in the resepective buffer was added (final concentration of cells 33 mg/mL). After incubation (30 °C, 1000 rpm, 5 min), 50 µL of an octanaloxime solution in ethanol solution (1 M, 100 mM final concentartion) was added. The samples were incubate for 15 min at 1000 rpm and 30 °C. Afterwards ethyl acetate (500 µl) was added, the mixture was vortexed and centrifuged. The organic phase was analyzed by GC. The conversions are shown in Table 5.

Table 5. Conversion of octanaloxime in different buffers using OxdB in whole cells.

Buffer	Conversion /%
PPB	80
HEPES	90
MOPS	91
TRIS-HCl	91
Ammium acetate	89

Immobilization in calcium alginate beads

The cell suspension of OxdB and OxdRE (333 g/L, 9 ml) in HEPES buffer (50 mM, pH 7.0) were mixed with sodium alginate solution (9 ml; 4% (w/w) in HEPES). The solution was dropped into a calcium chloride solution (200mM) in HEPES buffer using a flow rate of 2 mL/min. After this, the beads were stirred for further 30 min before they were washed with HEPES to remove residual $CaCl_2$. The sodium alginate beads were stored in HEPES buffer at 4 °C.

Coating of calcium alignate beads with TEOS

The calcium alginate beads were produced as described before. Accordingly, the beads were covered with *n*-hexan before tetraethyl orthosilicate (TEOS) was added (7.5 ml). The suspension was stirred for 22 h at room temperature. The beads were filtered and washed with n-hexan and HEPES to remove residual TEOS.

Activity assay of free whole cells and immobilized whole cells

Octanaloxime was prepared as described in previous publication.[2] The activity assay of whole cells is performed in 10 ml scale in round-bottom flasks. HEPES buffer (9 ml; 50mM; pH = 7) and and octanaloxime in ethanol solution (1 M; 1 ml, 100 mM final concnetration) was added. While stirring, the biocatalyst OxdB (free whole cells 16.5 mg/mL final concentration; immobilized 33 mg/mL final concentration) or OxdRE (free and immobilized whole cells

33 mg/mL final concentration) were added. The reaction was performed at 30 °C for 24 h. After the reaction whole cell biocatalysts were removed by centrifugation (5000 g, 2 min) and aqueous phase and cells were extracted with ethyl acetate (3x 15 mL). The combined organic phases were analyzed by GC. Immobilized whole cells were filtered and washed with HEPES buffer and ethanol. The filtrate was extracted with ethyl acetate (3x 15 mL) and the organic phase was analyzed by GC.

In case of the recycling study, the immobilized cells were isolated as described above and directly used for another biotransformation using the same conditions.

Biotransformation in pure organic medium

Free whole cells were used in a biphasic reaction medium consisting of HEPES buffer (7.5 mL) and organic solvent (7.5 mL). A final concentration of the free whole cells of 33 mg/mL was used. In case of the immobilizates (alginate beads or Si-coated beads) they were directly applied to organic solvent (15 mL) with a concentration of whole cells of 33 mg/mL. The reactions were performed for 24 h at 30 °C with an octanaloxime concentration of 100 mM. before the organic phases were analyzed by GC.

Stability assay of free whole cells and immobilized whole cells in the presence of ethanol as cosolvent in HEPES buffer

Free whole cells (33 mg/mL final concentration) or immobilizates (33 mg/mL final concentration) were incubated in HEPES buffer (50 mM, pH 7.0) including 10% or 50% (*v*/*v*) ethanol (total volume 15 mL) for 24 h at 30 °C before addition of octanaloxime (final concentration of 100 mM). The reaction was performed for 24 h and 30 °C, before the same work-up was performed as described above. The organic phases after extraction were analyzed by GC.

References

[1] Purolite, *User Man. Immobil. Carr.* **2015**.

[2] A. Hinzmann, S. Glinski, M. Worm, H. Gröger, *J. Org. Chem.* **2019**, *84*, 4867–4872.

6.3 Article 6

Biotransformations in Pure Organic Medium: Organic Solvent-Labile Enzymes in the Batch and Flow Synthesis of Nitriles

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Author contribution

AH initiated the project. Experiments were planned and performed by AH, except for the flow experiment, which was performed by NA. Initial experiments (not shown in the manuscript) on aldoxime dehydratases in biphasic reaction medium were performed by TB and ML (bachelor student, supervised by TB). AH wrote the manuscript with the help of NA (flow experiment). AH, NA and HG read and edited the manuscript.

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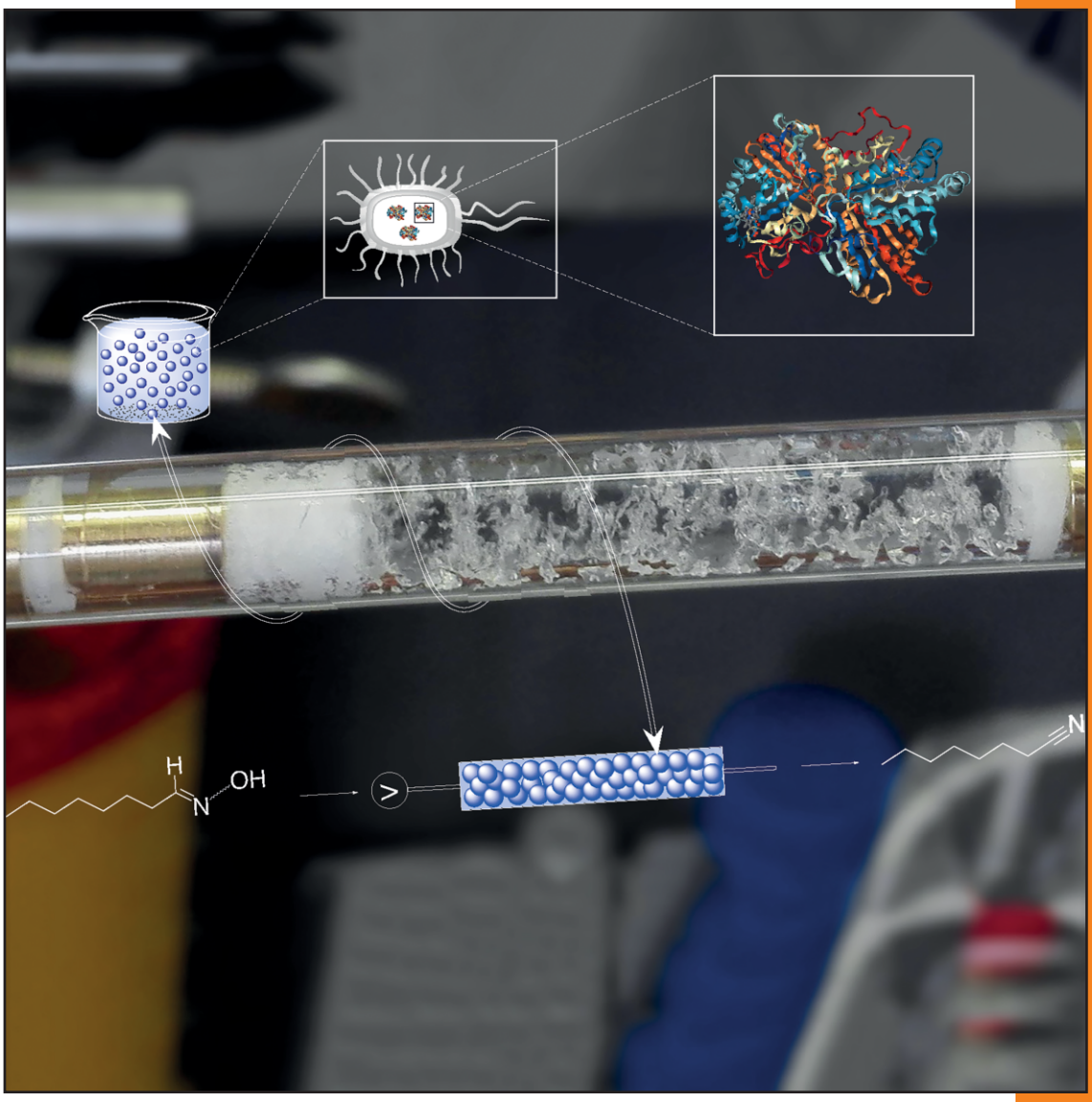

Front Cover

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Communication

Enzyme Catalysis

Biotransformations in Pure Organic Medium: Organic Solvent-Labile Enzymes in the Batch and Flow Synthesis of Nitriles

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Abstract: In recent years, there has been an increasing tendency to use biocatalysts in industrial chemistry, especially in the pharma and fine chemical sector. Preferably, enzymes or whole cells, applied as catalysts for a specific biotransformation, are utilized in aqueous reaction media since water is the natural medium for enzymes. In numerous examples of biocatalytic systems, however, a major problem is the insolubility of hydrophobic substrates in such aqueous reaction media. Apart from lipases, many enzymes are highly sensitive to organic solvents and are inactivated by an organic medium. Therefore, a change of solvent for biotransformations from water to organic solvents is usually challenging. In this study, we investigated the synthesis of nitriles by an organic solvent-labile aldoxime dehydratase in pure organic solvents, exemplified for the dehydration of *n*-octanaloxime to *n*-octanenitrile. We present a method for applications in batch as well as flow mode based on an "immobilized aqueous phase" bearing the whole cells in a superabsorber as solid phase, thus enabling the use of a purely organic solvent as "mobile phase" and reaction medium.

Introduction

In recent decades biotransformations have emerged towards a valuable tool in organic synthesis on both lab and industrial scale.[1–6] In spite of tremendous success illustrated, e.g., by numerous applications in industry, however, a general challenge related to the reaction medium remained: Typically, enzyme catalysis is conducted in aqueous medium whereas in contrast substrates and products are mostly hydrophobic.[1–8] Thus, the option to run biotransformations in purely organic medium would offer significant advantages. Besides the benefits from solubilizing hydrophobic substrates in a pure organic medium, emulsions as a result of two-phase aqueous/organic solvent systems could be avoided. Such emulsions often lead to a tedious work-up due to, e.g., difficulties in phase separation. In contrast, when using pure organic medium[9–11] the work-up could consist of a simple filtration or decantation of the organic solvent from a heterogenized biocatalyst. Such a biocatalytic process would be particularly interesting for producing bulk chemicals due to simplification of downstream-processing and minimization of the unit operation steps therein.[12] A major task for obtaining high conversions and productivities with hardly water-miscible substrates in enzyme catalysis is to overcome limitations in terms of enzyme stability when being in contact with hydrophobic reaction media. Whereas for lipases their use in solely organic media is widely known (due to their high affinity "by definition" to organic solvents), for most other enzymes general methodologies for their utilization in pure organic medium rarely exist.[3,4,9,10,13] Enzymes are often unstable in organic solvents, biphasic reaction systems consisting of water and an organic solvent and in many cases even in aqueous reaction media when containing a large portion of a water-soluble organic co-solvent.[1,5,14] Furthermore, enzymes in crude extracts and in particular in purified form might be even more unstable compared to whole-cell catalysts, which benefit from the cellular matrix protecting the enzymes. But also for whole-cell catalysts, examples are rare in which they are used in pure organic medium or biphasic systems.[15] At the same time various strategies to overcome these stability problems of biocatalysts in contact with organic solvents have been studied. One opportunity is compartmentalization of the reaction medium. This concept enables the combination of non-compatible reactions comprising biocatalytic steps and, e.g., polydimethylsiloxane (PDMS) membranes were successfully used for this purpose.[16–18] Another solution to overcome the instability problems of biocatalysts in organic media is immobilization. Besides immobilization of enzymes in crude extracts or purified enzymes themselves,[19–22] techniques of whole cell immobilization are known as well. Usually, immobilization of "free" enzymes without protection of the cells might not be sufficient to make these biocatalysts usable in pure organic solvent. In case of whole cell immobilization[15,23,24,25] some methods showed stabilizing effects and biocatalysts were found to be more stable in presence of organic solvents.[15,23] Encapsulation of whole cells in polyurethanes, as shown by von Langermann *et al.*, turned out as a highly suitable technique to prevent contact of the biocatalyst with the organic medium, thus resulting in a significant higher stability of the catalyst in such me-

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dia.[23,26] In continuation of our ongoing study on the use of enzymes being immobilized in superabsorber materials as a technique for immobilization of "free" enzymes,[22,27,28] and our research work on aldoxime dehydratase and their application for the synthesis of nitrile bulk chemicals in the presence of aldoxime dehydratases,[29–31] we became interested if such superabsorber-immobilized whole cell catalysts can be successfully used in pure organic medium. In the following we report the development of such a solution for utilizing whole-cell catalysts being immobilized in superabsorbers in pure organic media, which also provides the perspective for a broad general applicability in biocatalysis.

Results and Discussion

We chose an aliphatic aldoxime, namely *n*-octanaloxime (**1**), as substrate because firstly, substrate **1** and product **2** are insoluble in water and secondly, based on a previous study of Asano *et al.*[32–35] recently we developed a process for transforming aliphatic aldoximes to the corresponding nitriles at high substrate loading in the presence of an aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) as a catalyst.[30,36] Our first approach towards conducting aldoxime dehydratase-catalyzed reactions in pure organic medium consisted of using wet *E. coli* cells with overexpressed OxdB as biocatalyst (Scheme 1) since previously[30] we utilized such whole cells in aqueous reaction media.

In our experiments, we utilized *n*-octanaloxime (**1**) at a concentration of 0.5 M in combination with various types of organic solvents (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene and dichloromethane (DCM)) as reaction medium and 33 mg of wet cell mass (wcm) of *E. coli* cells including OxdB as biocatalyst per mL of reaction medium. A control reaction was performed using potassium phosphate buffer (PPB) as reaction medium. The reactions were performed at 30 °C reaction temperature for 24 h in round-bottomed flasks and 20 mL scale.

During the chosen reaction time, the whole cell catalyst behaved very different in each solvent, as visualized in the photos shown in Figure 1. In case of cyclohexane and toluene, the cells were suspended in the solvent, but in case of DCM and especially MTBE, the cells precipitated and were not suspended in the organic medium (Figure 1). After a reaction time of 24 h, the conversion of *n*-octanaloxime (**1**) to the corresponding nitrile **2** was analyzed by GC (Table 1). In case of the control reaction in phosphate buffer, the reaction was extracted with ethyl acetate (EtOAc) beforehand.

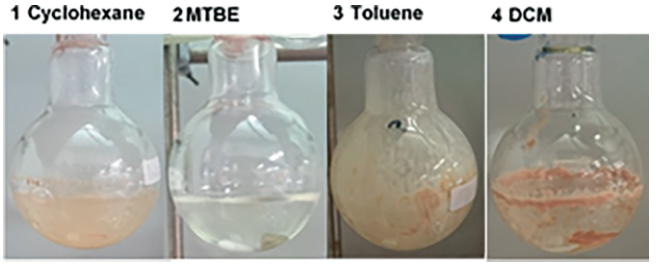


Figure 1. Pictures of reaction mixtures with OxdB in wet whole cells in different organic solvents.

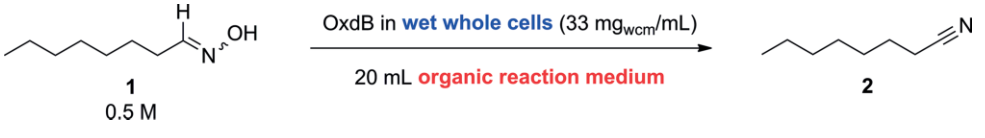
Table 1. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in pure organic medium.

#	Reaction medium/solvent	Conversion to **2** /%[a]
1	Cyclohexane	2
2	Methyl-*tert*-butyl ether (MTBE)	1
3	Toluene	2
4	Dichloromethane (DCM)	<1
5	Potassium phosphate buffer (PPB)	61[b]

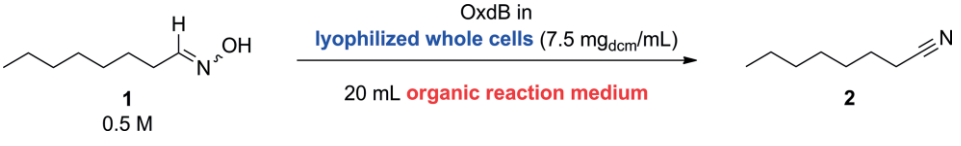
[a] 33 mg_{wcm}/mL OxdB in whole cells were suspended in 20 mL organic solvent or PPB including 500 mM *n*-octanaloxime (**1**). The reactions were performed at 30 °C for 24 h and conversions to *n*-octanenitrile (**2**) were determined by GC analysis. [b] Conversion was determined after extraction of the reaction mixture with ethyl acetate (EtOAc). The EtOAc phase was analyzed by GC for determination of conversion.

The use of any type of organic solvent as reaction medium directly led to a strong drop of activity, indicating the high degree of deactivation of the enzyme in these cases. Thus, we found very low conversions in the range of <1 % to 2 % of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) when using OxdB in pure organic medium (Table 1, entries 1–4). In contrast, in the control reaction using the aqueous buffer as reaction medium a conversion of 61 % was observed (entry 4). These experiments revealed that wet cells are not applicable in pure organic solvent. Thus, as a next step we studied the use of lyophilized cells containing OxdB as a catalyst in pure organic medium (Scheme 2).

Again, we used *n*-octanaloxime (**1**) in a concentration of 0.5 M in different solvents (cyclohexane, MTBE, toluene and DCM) and at 30 °C reaction temperature for 24 h. In this case,



Scheme 1. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in pure organic medium.



Scheme 2. OxdB in lyophilized cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in pure organic medium.

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7.5 mg dry cell mass (dcm) per mL of reaction medium was used, corresponding to 33 mg bio wet mass. We again performed a control reaction using resuspended dry cells in aqueous buffer. As shown in Figure 2, in all solvents the lyophilized whole cells were homogeneously suspended. After 24 h reaction time, the conversion of *n*-octanaloxime (**1**) to the corresponding nitrile was analyzed by GC (Table 2). In case of the control reaction in aqueous buffer the reaction was extracted with EtOAc beforehand.

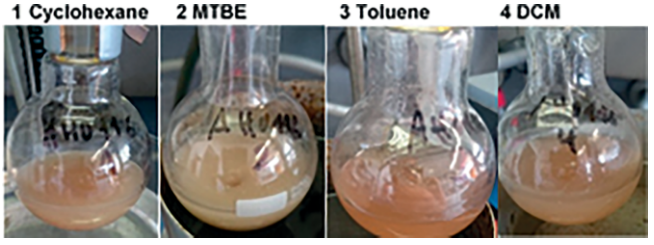


Figure 2. Pictures of reaction mixtures with OxdB in lyophilized cells in different organic solvents.

Table 2. OxdB in dry whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in pure organic medium.

#	Reaction medium/solvent	Conversion to **2** /%[a]
1	Cyclohexane	<1
2	Methyl-*tert*-butyl ether (MTBE)	<1
3	Toluene	<1
4	Dichloromethane (DCM)	<1
5	Potassium phosphate buffer (PPB)	23[b]

[a] 7.5 mg_{dcm}/mL OxdB in whole cells were suspended in 20 mL organic solvent or PPB including 500 mM *n*-octanaloxime (**1**). The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis. [b] Conversion was determined after extraction of the reaction with ethyl acetate (EtOAc). The EtOAc phase was analyzed by GC for determination of conversion.

However, in spite of a homogeneous biocatalyst suspension also in these cases a strong deactivation of the Oxd-enzyme was observed as soon as the reaction is conducted in an organic solvent, thus underlining that these enzymes are labile towards the presence of an organic medium (Table 2, entries 1–4). In detail, we found very poor conversions below 1 % of *n*-octanaloxime (**1**) to the corresponding nitrile **2** independent of the type of organic solvent as reaction medium. In contrast, the control reaction gave 23 % conversion showing that the enzyme in the lyophilized cells is still active after lyophilization.

Since we found that the use of any type of whole cells in a pure organic solvent does not lead to significant conversions, as a next step we studied this type of biotransformation running in a biphasic aqueous-organic media as this media has been utilized often in enzyme catalysis.[4,5] In these experiments, we used wet whole cells containing OxdB as a biocatalyst at a concentration of 33 mg_{wcm}/mL of total reaction medium (1:1 (v/v) aqueous/organic, total 20 mL). As aqueous phase, a phosphate buffer containing the whole cells was used. The organic phase consisted of the organic solvents cyclohexane, MTBE, toluene or DCM, containing 1 M of the substrate *n*-octanaloxime (**1**) (thus, leading to an overall concentration of 500 mM of **1**). The reactions were performed in a batch mode utilizing a round-bottomed flask with magnetic stirrer at 30 °C for 24 h (Scheme 3).

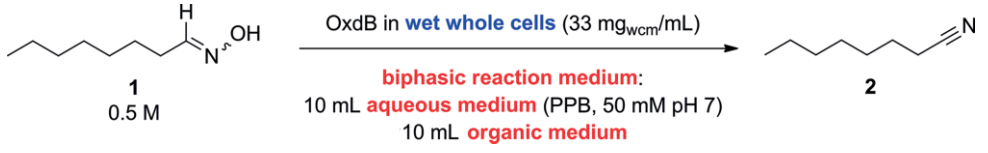
After a reaction time of 24 h, the organic phases were analyzed by GC for the determination of the conversion of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) (Table 3). While MTBE, toluene and DCM again gave very low conversions not exceeding 4 % (Table 3, entries 2–4), the use of cyclohexane led to 9 % conversion of *n*-octanaloxime (**1**) to the corresponding nitrile **2** (entry 1), which at that time was the best result for this biotransformation in the presence of an organic solvent. Furthermore, this experiment illustrates that an aqueous phase is necessary to keep OxdB active in the cells (see comparison of experiments shown in entry 1 of the Table 1, Table 2, and Table 3). However, still such a biphasic system has a negative impact on the activity and/or stability of the enzyme.

Table 3. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in biphasic systems.

#	Organic phase	Conversion to **2** /%[a]
1	Cyclohexane	9
2	Methyl-*tert*-butyl ether (MTBE)	4
3	Toluene	3
4	Dichloromethane (DCM)	<1

[a] 33 mg_{wcm}/mL OxdB in whole cells were suspended in 10 mL PPB and an organic solvent including 500 mM *n*-octanaloxime (**1**) was added. The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis.

As a concept to overcome these limitations, we envisioned that encapsulating the whole cells in a solid and immobilized aqueous phase could make such biotransformations possible, although being conducted in a pure organic solvent as mobile phase. As such an "immobilized aqueous phase" we used superabsorber, which is basically polyacrylic acid having a very high affinity to bind water. It was already shown, that this technique is useful for the immobilization of alcohol dehydrogenases as a crude extract for ketone reduction.[22,28,37] Although such enzymes are known to be tolerant to organic solvents, we became interested to evaluate this methodology also for the entrapment of whole cells bearing enzymes which are labile against organic solvents such as the Oxd-enzyme used in this study. By means of such an immobilized aqueous phase we expected that the contact between the organic solvent and the Oxd-containing whole cells is minimized, thus leading to a stabilizing



Scheme 3. OxdB in whole cells catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) in a biphasic system.

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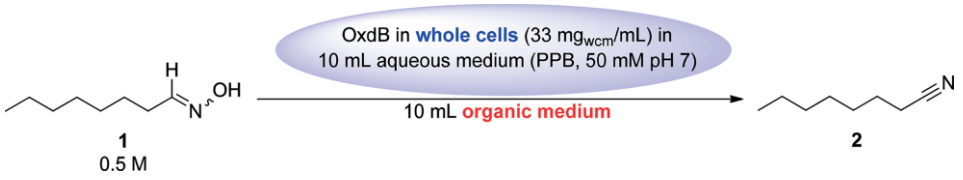

Scheme 4. OxdB in whole cells immobilized in superabsorber catalyzed dehydration of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) using a pure organic solvent as reaction medium.

effect on the whole cells and enzymes therein, and to the opportunity to conduct biotransformations with organic solvent-labile enzymes in a pure organic reaction medium as liquid phase. Furthermore, separation of the reaction mixture from the biocatalyst would be simplified. The concept of this approach is visualized in Scheme 4.

In the resulting experiment, 33 mg$_{wcm}$ whole cells containing OxdB per mL of total reaction medium (1:1(*v*/*v*) aqueous/organic, total 20 mL) were dissolved in 10 mL of aqueous buffer, and the resulting aqueous phase was immobilized by addition of the superabsorber whilst stirring in a round-bottomed flask. Then, 10 mL organic phase containing 1 M *n*-octanaloxime (**1**) (0.5 M overall concentration) was added, and the reaction was performed at 30 °C for 24 h. We were pleased to find that after 24 h reaction time with cyclohexane as organic solvent an excellent conversion of >99 % of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) was achieved (Table 4, entry 1). When utilizing toluene, a conversion of 11 % was detected and in case of MTBE (5 %) and DCM (<1 %) lower conversions were obtained (entries 2–4), thus indicating a significant impact on the type of organic solvent and the high suitability of cyclohexane as organic reaction medium.

Table 4. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) in the "superabsorber system".

#	Organic phase	Conversion to **2** (isolated yield) /%[a]
1	Cyclohexane	>99 (82)
2	Methyl-*tert*-butyl ether (MTBE)	5
3	Toluene	11
4	Dichloromethane (DCM)	<1

[a] 33 mg$_{wcm}$/mL OxdB in whole cells were suspended in 10 mL PPB and immobilized in superabsorber. An organic solvent including 500 mM *n*-octanaloxime (**1**) was added. The reactions were performed at 30 °C for 24 and conversions to *n*-octanenitrile (**2**) were determined by GC analysis.

These results showed that this "superabsorber approach" in combination with the right choice of organic solvent component is useful for the biocatalytic synthesis of nitriles in organic medium although using an enzyme being labile against organic solvents. We found cyclohexane to be the most suitable solvent for our purpose. In comparison to the simple use of whole cells in pure organic solvent, immobilization in superabsorber gave a much more stabilized catalyst, thus leading to an excellent conversion. This also indicates that the superabsorber stabilizes the whole cells by protecting them from direct contact with the solvent. The positive impact of cyclohexane can be rationalized by its high log P value, thus ensuring a low concentration in the aqueous super-absorber phase as well as a negligible "dry out"-effect of removing water from the superabsorber to the organic solvent. As we were interested to explore the suitability of this method to operate at higher substrate loading as a desired criterion for a technical process, we investigated substrate concentrations of 500 mM, which corresponds to a substrate loading of approximately 75 g/L reaction medium. We were pleased to find that the biocatalyst remains stable in the superabsorber system over several hours and that quantitative conversions were obtained in the biotransformation. The time-course of this reaction with the superabsorber-immobilized whole cells using cyclohexane as solvent as well as a photo of this reaction mixture is shown in Figure 3.

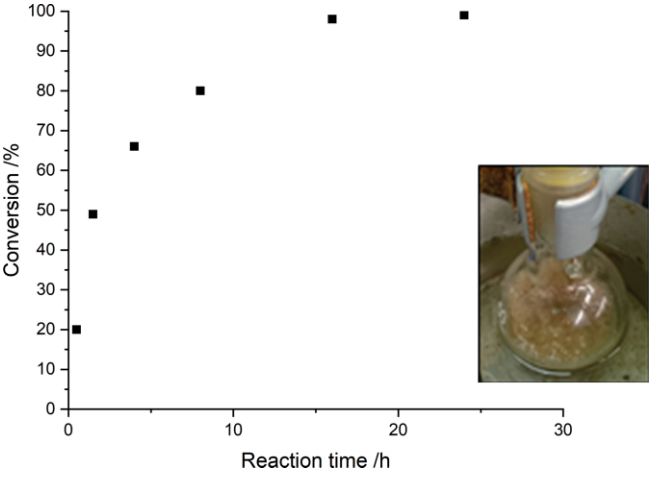

Figure 3. Picture and time-course measurement of the "superabsorber-system" with cyclohexane as organic phase.

The time-course measurement indicates that the reaction further proceeds even after 8 h reaction time. This result shows that the enzymes within the whole cell-catalyst maintain their activity (at least to a large extent) in the superabsorber when using cyclohexane as organic solvent. In order to get further insight into the stability of the OxdB biocatalyst under the chosen reaction conditions, we performed a stability assay of OxdB whole cells in superabsorber and in biphasic reaction medium using cyclohexane as organic solvent. After different incubation times of the OxdB(-immobilisate) in an organic solvent or biphasic system, *n*-octanaloxime (**1**) was added and the conversion to *n*-octanenitrile (**2**) was determined after 15 min. The results are shown in Figure 4.

As shown in Figure 4, the activity of OxdB-containing whole cells immobilized in superabsorber does not decrease when being incubated for one hour. After 5 hours of incubation in cyclohexane, still approximately 80 % activity was left. In the case of the biphasic reaction medium, OxdB showed already lower activity without incubation beforehand. The activity is approximately 60 % of the activity of superabsorber-immobilized cells

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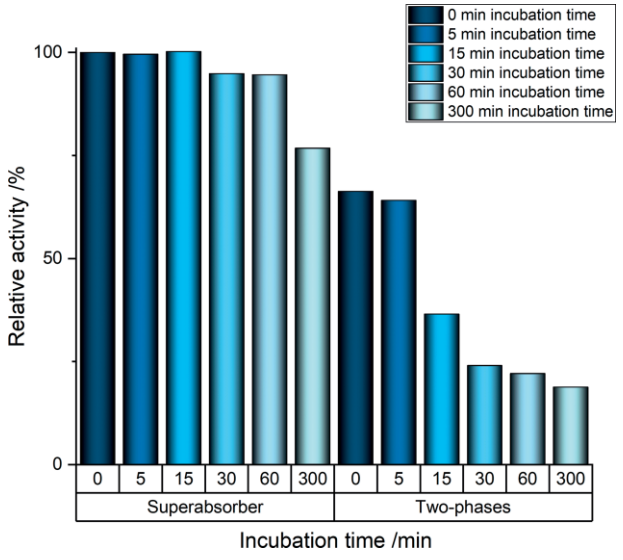

Figure 4. Stability assay of OxdB whole cells immobilized in superabsorber in cyclohexane and OxdB whole cells in a biphasic system consisting of buffer and cyclohexane. The superabsorber-approach without previous incubation in cyclohexane was set as 100 %.

without incubation. The activity drops very fast in case of the biphasic approach, indicating again that the superabsorber-immobilization protects the biocatalyst against the organic solvent.

Having such an efficient biotransformation in batch mode now in hand and stimulated by the impressive recent achievements and increasing importance of flow processes,[38–45] our next goal was to develop a flow process for this superabsorber-based synthesis of *n*-octanenitrile (**2**). Recently, biocatalysis in continuous processes getting more and more popular due to high reproducibility and productivity of these processes.[43] Thus, we were interested in the behavior and performance of our Oxd-superabsorber immobilizates in flow reactions. We chose a packed bed reactor bearing the superabsorber-immobilized whole cells, thus enabling an easy separation and re-use of the catalyst. Toward this end, the reactor was loaded first with a small cotton layer (5 mm) and then with the superabsorber granulate, followed by addition of 1 mL suspension of 66 mg_{wcm}/mL of enzyme. After all liquid was absorbed by the superabsorber, a second cotton layer (5 mm) was added and the reactor was sealed. In Figure 5 the set-up of this reactor is shown.

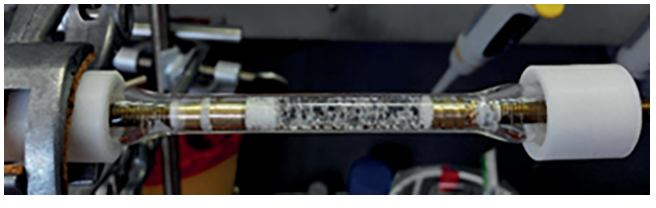

Figure 5. Picture of the packed bed reactor charged with superabsorber gel.

The flow process then was conducted by means of a syringe pump, and a solution of 100 mM of *n*-octanaloxime (**1**) in cyclohexane was pumped through the packed bed reactor. Due to the limited solubility of **1**, the concentration was reduced to 100 mM in comparison to 500 mM in batch experiments in order to ensure a completely dissolved substrate which is needed for running the reaction in such a flow-reactor without blocking effects. The reaction was performed at room temperature with a residence time of 30 min. After an equilibration time of two residence times, a high conversion of >95 % for the formation of *n*-octanenitrile (**2**) was obtained for at least 3 h reactor run time for this flow system (Figure 6). Although within the chosen reaction time some loss in activity was observed, thus underlining at least to a low extent a deactivation effect of the organic solvent on the enzyme, this superabsorber-based whole cells immobilization method in combination with a pure organic solvent also turned out to be robust and suitable to be operated in a flow mode.

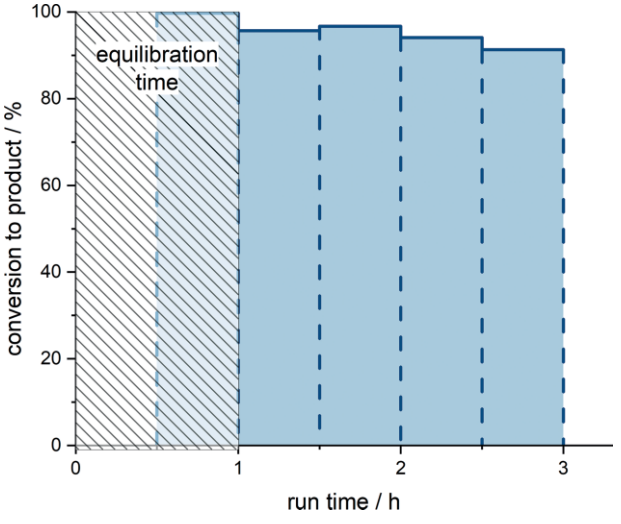

Figure 6. OxdB in wet whole cells catalyzed dehydration of *n*-octanaloxime (**1**) using a superabsorber packed bed approach.

Conclusion

In conclusion, a biocatalytic process running in pure organic medium has been reported in which an enzyme being labile towards this organic solvent is used. The process concept is based on the encapsulation of the enzyme as a whole-cell catalyst in a superabsorber as a "solid aqueous phase" in combination with an organic solvent with a high log P value as "mobile phase". This approach has been exemplified for the aldoxime dehydratase-catalyzed dehydration of *n*-octanaloxime (**1**) with formation of *n*-octannitrile (**2**), leading to excellent conversion of >99 %. In contrast, very low or no conversion was observed when using other solvents or other methods such as the direct use of whole-cell catalysts in pure organic medium or biphasic solvent mixtures. Furthermore, an efficient flow-setup with a packed-bed reactor containing superabsorber-immobilized whole cells was developed, which turned out to be also very suitable for the biotransformation leading to high conversion and low catalyst deactivation.

Acknowledgments

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Keywords: Aldoximes · Enzyme catalysis · Flow chemistry · Immobilization · Nitriles

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Supporting Information

Biotransformations in Pure Organic Medium: Organic- Solvent-Labile Enzymes in the Batch and Flow Synthesis of Nitriles

Alessa Hinzmann, Niklas Adebar, Tobias Betke, Monja Leppin, and Harald Gröger*

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1 Experimental information

n-Octanal was purchased by Sigma Aldrich and used without further purification. Chemicals for aldoxime synthesis were purchased by VWR Chemicals, Carl Roth and Fluka Chemicals and used without further purification, respectively.

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^{1}H). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$).

Conversion of the biotransformations was determined by GC measurements (Shimadzu GC 2010) in comparison to a calibration curve with the following settings. Column: chiral BGB-174 (0.25 mm ID, 0.25 µm film, 30 m length) by BGB Analytik AG. Column oven temperature program: 140 °C for 1 min, 140 °C to 190 °C with 20 °C min^{-1}, 190 °C for 1 min, 190 °C to 200 ◦C with 50 °C min−1. Settings: SPL1: 250 °C, pressure: 175.2 kPa, total flow: 24.8 mL min^{-1}, column flow: 1.98 mL min^{-1}, linear velocity: 46.9 cm s^{-1}, purge flow: 3.0 mL min^{-1}, split ratio: 10.0, FID: 250 °C. Retention times: n-Octanaloxime 2.41 min, octanenitrile 2.72 min. Accuracy of the GC-analytical method: the error of the determined conversions to the corresponding nitriles is in the range of up to <1% depending on the concentration of the aldoximes due to temperature-depending degradation of the aldoximes at the GC-column

2 Aldoxime dehydratase (Oxd) sequences, plasmids and expression

2.1 Oxd sequences and plasmids

The gene for the aldoxime dehydratase from *Bacillus* sp. OxB-1[1] was cloned into a pUC18 vector using HindIII and PstI restriction sites.

Oxd from *Bacillus* sp. OxB-1 (OxdB)
(Accession number: GenBank: AP013294.1)

Base sequence (codon-optimized for *E. coli*):
ATGAAAAATATGCCGGAAAATCACAATCCACAAGCGAATGCCTGGACTGCCGAA
TTTCCTCCTGAAATGAGCTATGTAGTATTTGCGCAGATTGGGATTCAAAGCAAGT
CTTTGGATCACGCAGCGGAACATTTGGGAATGATGAAAAAGAGTTTCGATTTGC
GGACAGGCCCCAAACATGTGGATCGAGCCTTGCATCAAGGAGCCGATGGATAC
CAAGATTCCATCTTTTTAGCCTACTGGGATGAGCCTGAAACATTTAAATCATGGG
TTGCGGATCCTGAAGTACAAAAGTGGTGGTCGGGTAAAAAAATCGATGAAAATA
GTCCAATCGGGTATTGGAGTGAGGTAACGACCATTCCGATTGATCACTTTGAGA
CTCTTCATTCCGGAGAAAATTACGATAATGGGGTTTCACACTTTGTACCGATCAA
GCATACAGAAGTCCATGAATATTGGGGAGCAATGCGCGACCGCATGCCGGTGT
CTGCCAGTAGTGATTTGGAAAGCCCCCTTGGCCTTCAATTACCGGAACCCATTG
TCCGGGAGTCTTTCGGAAAACGGCTAAAAGTCACGGCGCCGGATAATATTTGCT
TGATTCGAACCGCTCAAAATTGGTCTAAATGTGGTAGCGGGGAAAGGGAAACGT
ATATAGGACTAGTGGAACCGACCCTCATAAAAGCGAATACGTTTCTTCGTGAAAA
TGCTAGTGAAACAGGCTGTATTAGTTCAAAATTAGTCTATGAACAGACCCATGAC
GGCGAAATAGTAGATAAATCATGTGTCATCGGATATTATCTCTCCATGGGGCATC
TTGAACGCTGGACGCATGATCATCCAACACATAAAGCGATCTACGGAACCTTTT
ATGAGATGTTGAAAAGGCATGATTTTAAGACCGAACTTGCTTTATGGCACGAGG
TTTCGGTGCTTCAATCCAAAGATATCGAGCTTATCTATGTCAACTGCCATCCGAG
TACTGGATTTCTTCCATTCTTTGAAGTGACAGAAATTCAAGAGCCTTTACTGAAA
AGCCCTAGCGTCAGGATCCAGTGA

Amino acid sequence:
MKNMPENHNPQANAWTAEFPPEMSYVVFAQIGIQSKSLDHAAEHLGMMKKSFDLR
TGPKHVDRALHQGADGYQDSIFLAYWDEPETFKSWVADPEVQKWWSGKKIDENS
PIGYWSEVTTIPIDHFETLHSGENYDNGVSHFVPIKHTEVHEYWGAMRDRMPVSAS
SDLESPLGLQLPEPIVRESFGKRLKVTAPDNICLIRTAQNWSKCGSGERETYIGLVEP
TLIKANTFLRENASETGCISSKLVYEQTHDGEIVDKSCVIGYYLSMGHLERWTHDHP
THKAIYGTFYEMLKRHDFKTELALWHEVSVLQSKDIELIYVNCHPSTGFLPFFEVTEI
QEPLLKSPSVRIQ

2.2 Oxd expression in *E.coli* BL21-CodonPlus(DE3)-RIL

E.coli BL21-CodonPlus(DE3)-RIL cells harboring the plasmids with the Oxd-genes were stored as glycerol stocks at -80 °C.

A sample from the glycerol stocks for each Oxd was plated on LB-agar containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol and incubated for 12 to 18 h at 37 °C.

Pre-cultures were prepared in 5 mL LB-medium containing 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol (OxdB in pUC18) using a single colony from the LB-agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm.

Main cultures for Oxd expression were performed using TB-autoinduction medium. Sterile 20 g/L Lactose solution in MilliQ water (40 mL) and sterile 50 g/L D-glucose solution in MilliQ water (4 mL) was added to 356 mL sterile TB-medium (Carl Roth) in a 500 mL Erlenmeyer flask. 100 µg/mL Carbenicillin and 34 µg/mL Chloramphenicol were added to the medium. Main cultures were inoculated with 1% (5 mL) of the OxdB pre-culture and incubated for 1 h at 37 °C and 120 rpm. After 1 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 120 rpm.

Cell harvest was performed at 4,000 xg for 15 min and 4 °C. The supernatant was discarded and cells were washed three times with 50 mM potassium phosphate buffer (PPB, KP_i) at pH 7.0. The biomass was determined (bio wet weight (bww)) and cells were resuspended in 50 mM PPB (pH 7.0) to a final concentration of 333 mg/mL cells in buffer. Cell suspensions were stored at 4 °C or on ice before usage in biotransformations.

Table S1. Used vector constructs, origins of the Oxd-genes, provider of the vector constructs and marker-resistance of the constructs.

Entry	Origin of Oxd-gene	Vector construct	Oxd	Provider	Resistance
1	*Bacillus* sp. OxB-1	pUC18_OxdB	OxdB	Asano group	Carbenicillin

Overexpression of Oxds in *E. coli* Bl21-CodonPlus(DE3)-RIL was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after cell disruption and denaturation of the proteins in the crude extracts.

Crude extracts of 33% wet cell suspensions were obtained by sonication (5x 1 min, 10 – 15% Output, Bandelin Sonopuls®) and subsequent centrifugation at 21,500 xg for 45 min at 4 °C. The pellet including the cell debris was discarded. Protein concentrations in crude extracts were determined by Bradford assay using a bovine serum albumin (BSA)-standard curve (1.4 mg/mL, 0.7 mg/mL, 0.35 mg/mL, 0.175 mg/mL, 0.0875 mg/mL) as reference. Protein dilutions of 1 µg/µL whole cell protein concentration were obtained by dilution of the crude extracts in water and Laemmli-buffer. 10 µL of these samples were transferred to a 12% SDS-PAGE.

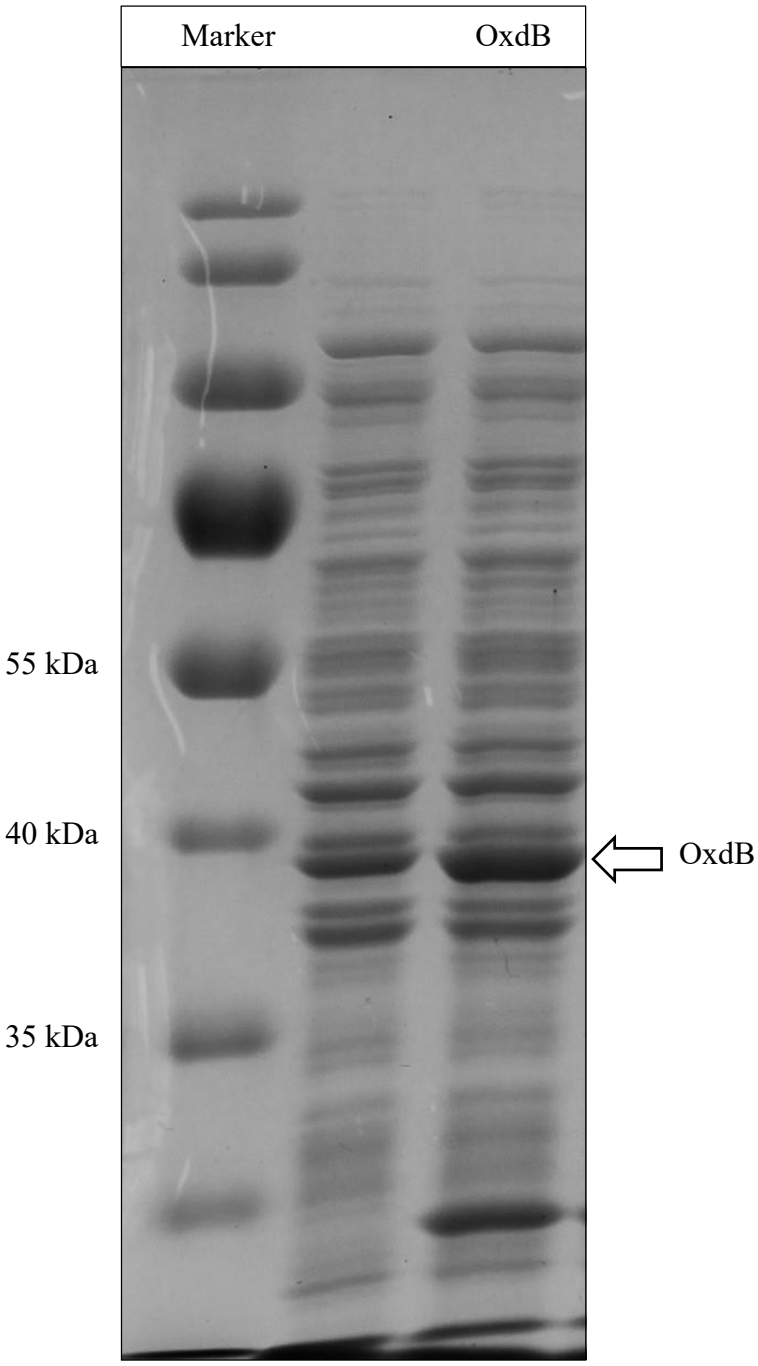

Figure 1. 12% SDS-PAGE of crude extracts of OxdA-, OxdB-, OxdFG-, OxdRE- and OxdRG-overexpressing cells. The molecular weights of the overexpressed Oxds correlates with the molecular weights determined from the amino acid sequences.

3 Biocatalytic conversion of *n*-octanaloxime to *n*-octanenitrile

3.1 Usage of OxdB in wet whole cells in pure organic medium

666 mg *E. coli* cells including overexpressed OxdB were transferred to a 50 mL round bottom flask. 20 mL organic solvent (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene or dichloromethane (DCM)) containing 500 mM *n*-octanal aldoxime (1.43 g, 10 mmol) were added. A control reaction with 20 mL potassium phosphate buffer (PPB) (50 mM, pH 7.0) was conducted using the same amount of substrate. The reaction mixtures were stirred at 30 °C for 24 h. The conversion of *n*-octanaloxime to the corresponding nitrile in the organic solvents was analyzed by GC. In case of the aqueous control reaction, the reaction mixture was extracted with ethyl acetate (EtOAc) (1x 20 mL) and the EtOAc-phase was afterwards analyzed by GC.

Table S2. Results of the biotransfomation of *n*-octanaloxime to the corresponding nitrile using wet whole cells in pure organic medium.

#	Reaction medium/solvent	Conversion after 3 h	Conversion after 24 h
1	Cyclohexane	<1%	2%
2	MTBE	<1%	<1%
3	Toluene	<1%	3%
4	DCM	<1%	<1%
5	PPB	51%	61%

3.2 Usage of OxdB in lyophilized whole cells in pure organic medium

666 mg *E. coli* cells including overexpressed OxdB were transferred to a 50 mL round bottom flask. The cells were lyophilized overnight until the cells were completely dry. 20 mL organic solvent (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene or dichloromethane (DCM)) containing 500 mM *n*-octanaloxime (1.43 g, 10 mmol) were added. A control reaction with 20 mL potassium phosphate buffer (PPB) (50 mM, pH 7.0) was conducted using the same amount of substrate. The reaction mixtures were stirred at 30 °C for 24 h. The conversion of *n*-octanaloxime to the corresponding nitrile in the organic solvents was analyzed by GC. In case of the aqueous control reaction, the reaction mixture was extracted with ethyl acetate (EtOAc) (1x 20 mL) and the EtOAc-phase was analyzed by GC.

Table S3. Results of the biotransfomation of *n*-octanaloxime to the relating corresponding using lyophilized whole cells in pure organic medium.

#	Reaction medium/solvent	Conversion after 3 h	Conversion after 24 h
1	Cyclohexane	<1%	<1%
2	MTBE	<1%	<1%
3	Toluene	<1%	<1%
4	DCM	<1%	<1%
5	PPB	19%	23%

3.3 Usage of OxdB in wet whole cells in biphasic systems

666 mg wet *E. coli* cells including overexpressed OxdB were transferred to a 50 mL round bottom flask. The cells were suspended in 10 mL PPB (50 mM, pH 7.0). 10 mL organic solvent (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene or

dichloromethane (DCM)) containing 1 M *n*-octanaloxime (1.43 g, 10 mmol) were added. The reaction mixtures were stirred at 30 °C for 24 h. The conversion of *n*-octanaloxime to the corresponding nitrile in the organic solvents was analyzed by GC.

Table S4. Results of the biotransfomation of *n*-octanaloxime to the corresponding nitrile using wet whole cells in biphasic systems.

#	Organic phase	Conversion after 16 h	Conversion after 24 h
1	Cyclohexane	6%	9%
2	MTBE	4%	4%
3	Toluene	3%	3%
4	DCM	<1%	<1%

3.4 Usage of OxdB in wet whole cells immobilized in superabsorber

666 mg wet *E. coli* cells including overexpressed OxdB were transferred to a 50 mL round bottom flask. The cells were suspended in 10 mL PPB (50 mM, pH 7.0) and immobilized in superabsorber Favor SXM 9155 (Evonik) at room temperature for 15 min. 10 mL organic solvent (cyclohexane, methyl-*tert*-butyl ether (MTBE), toluene or dichloromethane (DCM)) containing 1 M *n*-octanaloxime (1.43 g, 10 mmol) were added. The reaction mixtures were stirred mechanically at 30 °C for 24 h. The conversion of *n*-octanoxime to the corresponding nitrile in the organic solvents was analyzed by GC.Time-course measurements were performed by taking samples of the reaction medium at different reaction times and analysis of the conversion by GC.

Table S5. Results of the biotransfomation of *n*-octanaloxime to the corresponding nitrile using wet whole cells in superabsorber systems.

#	Organic phase	Conversion after 4 h	Conversion after 16 h	Conversion after 24 h
1	Cyclohexane	66%	98%	99%
2	MTBE	2%	4%	5%
3	Toluene	2%	6%	11%
4	DCM	<1%	<1%	<1%

3.5 Usage of OxdB in wet whole cells immobilized in superabsorber in a packed bed reactor (PBR)

A glass reactor was filled with cotton (5 mm) and superabsorber (40 mg). A suspension of *E. coli* cells including overexpressed OxdB (100 µL, 333 mg/mL) was diluted with PPB (900 µL, 50 mM, pH 7.0) and filled in the reactor to be immobilized at room temperature for 20 min. The reactor was filled with an additional cotton layer (5 mm) and sealed. A solution of *n*-octanaloxime (71.6 mg, 0.5 mmol) in MTBE (5mL) was transferred into a syringe (5 mL, S.G.E., gas tight), which was attached to a syringe pump. The pump was connected to the reactor and set to a flow rate of 0.23 mL/h corresponding to a residence time of 30 min. Fractions were collected and analyzed via GC.

Table S6. Results of the biotransfomation of *n*-octanaloxime to the corresponding nitrile using wet whole cells in superabsorber systems in a packed bed reactor.

#	Reactor run time / h	Conversion after 24 h
1	0.5 - 1	>99%
2	1 – 1.5	96%
3	1.5 - 2	97%
4	2 – 2.5	94%
5	2.5 - 3	91%

4 Synthesis of *n*-octanaloxime from *n*-octanal

Condensation of *n*-octanal with hydroxylamine hydrochloride was performed analogue to the protocol described by our group, previously.[2] Sodium carbonate (0.75 eq.) was dissolved in distilled (dist.) water including 5% (v/v) EtOH. Afterwards, hydroxylamine hydrochloride (1.5 equiv) was added to the stirred solution. *n*-Octanal (1 eq., 3.2 mol) was added dropwise to the stirred aqueous solution. The reaction mixture was stirred at room temperature for 12 h, while the aldoxime precipitated as colorless solids from the reaction solution. The reaction progress was monitored by TLC. After completion of the reaction, the crude product was isolated as a colorless solid by filtration. The crude product was rinsed with dist. water (50 mL per mol of substrate) before drying *in vacuo*. Pure octanaloxime (307.1 g, 2.15 mol, 67%) was obtained by recrystallization from *n*-hexane.

^{1}H-NMR (500 MHz, $CDCl_3$): *Z*-octanaloxime: δ 7.41 (t, J = 6.1 Hz, 1H), 2.18 (td, J = 7.5 Hz, 6.2 Hz, 2H), 1.48 (m, 2H), 1.31 (m, 8H), 0.87 (m, 3H); *E*-octanaloxime: δ 6.71 (t, J = 5.5 Hz, 1H), 2.37 (td, J = 7.6 Hz, 5.5 Hz, 2H), 1.48 (m, 2H), 1.31 (m, 8H), 0.87 (m, 3H).

5 Stability assay of OxdB whole cells in a biphasic reaction medium or immobilized in superabsorber in pure organic medium

OxdB whole cells (33 mg/mL(bww), 250 µL) in PPB (50 mM, pH 7) were immobilized in superabsorber Favor SXM 9155 (Evonik) (5 mg) or directly treated with cyclohexane (250 µL). The mixtures were incubated at 30 °C and 1000 rpm for 0, 5, 15, 30, 60 or 300 mM before addition of 1 M *n*-octanaloxime solution in ethanol (50 µL, final concentration 90 mM). After 15 min reaction time 10 µL of the organic phase were dissolved in 990 µL ethyl acetate and analyzed by GC. Stability assay experiments were performed in triplicates. Conversion of *n*-octanaloxime to the corresponding nitrile after all different incubation times are listed in Table S7.

Table S7. Results of the stability assay of OxdB whole cells in a biphasic reaction medium or immobilized in superabsorber in pure cyclohexane.

Reaction system	Incubation time /min	Conversion	Relative activity
Superabsorber	0	42	100
	5	42	100
	15	42	100
	30	40	95
	60	40	95
	300	32	77
Biphasic system	0	28	66
	5	27	64
	15	15	36
	30	10	24
	60	9	22
	300	8	19

6 References

[1] Y. Kato, K. Nakamura, H. Sakiyama, S. G. Mayhew, Y. Asano, *Biochemistry* **2000**, *39*, 800–809.
[2] A. Hinzmann, S. Glinski, M. Worm, H. Gröger, *J. Org. Chem.* **2019**, *84*, 4867–4872.

6.4 Article 7 *submitted*

Improving Activity and Stability of Aldoxime Dehydratase OxdRE from *Rhodococcus erythropolis* by Directed Evolution

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Author contribution

AH initiated the project, designed and performed the experiments. HY performed calculation studies using MOE. AH wrote the manuscript and HY provided the text of the calculation study. AH, HY, HG and YA read and edited the manuscript.

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Full Paper

Improving Activity and Stability of Aldoxime Dehydratase OxdRE from *Rhodococcus erythropolis* by Directed Evolution

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Abstract

In the past 20 years, aldoxime dehydratases were found to be interesting enzymes in organic synthesis of nitriles without need of cyanide salts. This enzyme class dehydrates aldoximes to the corresponding nitriles in aqueous media under ambient reaction conditions. In the present work, a color assay for the determination of conversion of an aliphatic aldoxime to the nitrile using the cofactor-independent aldoxime dehydratases was investigated and developed. This color assay was used as screening method for a directed evolution study of OxdRE, an aldoxime dehydratase from *Rhodococcus erythropolis*. By means of an error-prone PCR approach improved OxdRE-mutants were identified, which were found to be more stable in the presence of acetonitrile as cosolvent and at higher reaction temperatures. In addition, the impact of the mutations has been rationalized by means of molecular modelling with MOE.

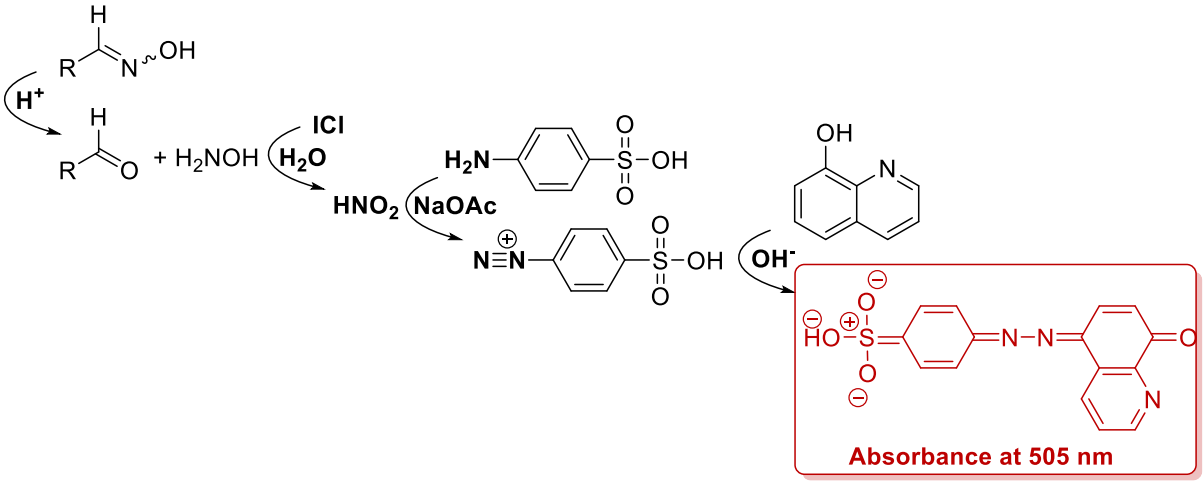
Introduction

Biocatalysis has emerged to a valuable tool in organic synthesis for different products. For example, enzymes are nowadays often used for the synthesis of chiral alcohols or amines due to their high selectivity.[1–3] Also in other areas of organic chemistry, the importance of biocatalysts is more and more increasing. Recently aldoxime dehydratases (Oxds) were found to be highly suitable catalysts for, e.g., aliphatic nitrile synthesis showing a high productivity and stability in presence of high amounts of aldoximes and nitriles.[4–7] For a stable process using biocatalyst, however, also the stability of the catalyst besides the activity and productivity. Especially in the bulk chemical sector, the catalyst costs have to be very low, thus making a recyclability of the catalyst desirable.[8,9] Improved stability of enzymes can be evolved by many methods.[10–12] A very effective method is directed evolution of enzymes as underlined by the

Nobel Prize in Chemistry in 2018 for Arnold.[13,14] Directed evolution can be performed by many methods.[10] One example is error-prone PCR, which is based on the error-rate of polymerases to include random mutations into a gene.[14–16] The error-rate of polymerases are usually increased in this method by addition of manganese to the PCR, which replace the magnesium in the polymerase active center.[16] When using this method, randomly mutations are included to a gene. As this might also occur at amino acid positions, which are necessary for the enzyme activity, a high throughput assay (HTA) is useful to minimize screening expenditure for finding improved variants. Many enzymes can be screened by well-known HTAs.[17–19] For enzymes, which are dependent on a cofactor, such as NAD(P)H, UV/Vis-spectroscopy can be used to determine activity of enzymes and randomly obtained mutants.[19] However, for enzymes such as aldoxime dehydratases,[20–26] which are not cofactor-dependent, this method cannot be used. For these enzymes, alternatives need to be found to develop a simple screening method for directed evolution. In the present work, we are presenting a color assay for aldoxime dehydratases and directed evolution of one enzyme by error-prone PCR as well as the rationalization of the impact of the identified beneficial mutations by means of molecular modelling with the software tool MOE.

Results and Discussion

Development of a color assay for aldoxime conversion to nitriles. To minimize the screening effort by means of a directed evolution approach to optimize a biocatalyst, a high-throughput assay (HTA) or at least an easily detectable assay to determine conversions is necessary. In the present work we focusing on an aldoxime dehydratase from *Rhodococcus erythropolis* (OxdRE), which catalyzes the dehydration reaction from aldoximes to nitriles.[26,27] We decided to use *n*-octanaloxime (**1**) as a model substrate as this substrate turned out to be easily converted by Oxds. [5,7] However, Oxds are neither cofactor-dependent (which would allow an indirect UV-detection of substrate consumption) nor their substrate exhibit a change of absorbance by transformation to the nitriles. Especially in case of aliphatic nitriles, such as the model substrate *n*-octanaloxime (**1**), a direct detection of conversion by UV is not possible. Therefore, the first task of this work was to develop a suitable assay, which allows an easy detection of the conversion of aldoximes into nitriles. Typically, the conversion of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) is determined by GC-analysis[5] but this method is time-intensive and therefore not suitable as HTA for this purpose. Thus, we developed a color assay, which is based on a spot-assay by Verma reported in 1979.[28]



Scheme 1. Color assay for the detection of aldoximes by UV-spectroscopy.

In the first step of this cascade, the reaction mixture is mixed with HCl after removal of the whole cell-catalyst. In this step, the aldoxime is cleaved into hydroxylamine and the corresponding aldehyde. When subsequently adding an iodine chloride solution, hydroxylamine is oxidized to nitrous acid, which then reacts with sodium acetate and 4-aminobenzenesulfonic acid to 4-sulfobenzenediazonium. In the final step, this azonium ion is treated with quinolin-8-ol to form an azo compound, which absorbs at 505 nm and produces a reddish color. By means of this color assay, a standard curve for different concentrations of *n*-octanaloxime (**1**) was recorded (Figure 1).

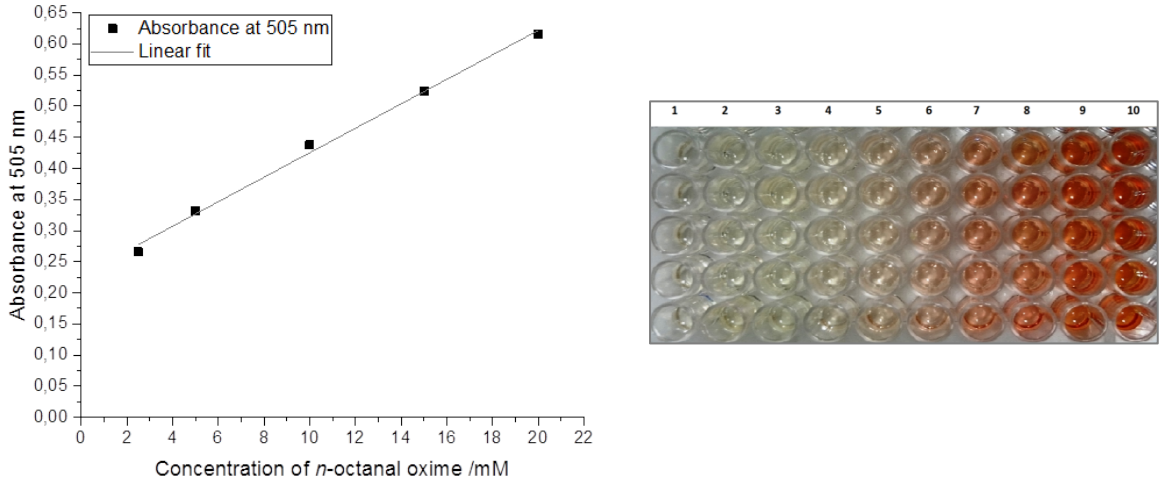
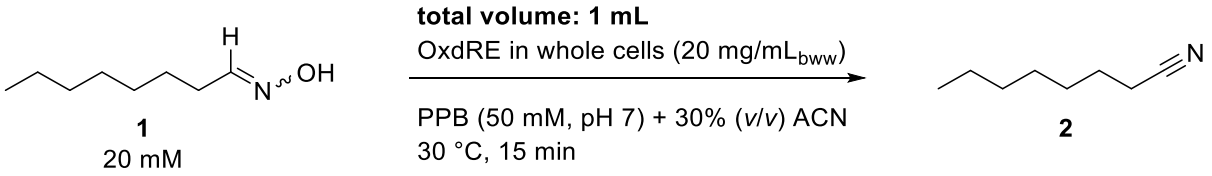


Figure 1. Left: Standard curve of different *n*-octanaloxime (**1**) concentrations using the color assay. Right: Picture of the 96-well plate in which the standard curve was recorded.

As shown in Figure 1, the absorbance is linear with the concentration of aldoxime **1**. In order to check if the nitrile **2** has an impact on the assay result, we used 5 mM *n*-octanenitrile (**2**) in

the solution and applied the same assay. In comparison to a blank experiment (PPB without aldoxime **1** or nitrile **2**), no difference in the absorbance was detected (Figure 1, right; entry 1: 5 mM *n*-octanenitrile (**2**); entry 2: blank), thus indicating that the color assay is suitable for the purpose of determining different aldoxime **1** concentrations in biotransformations. Accordingly, by comparing the concentrations of aldoxime **1** determined by the color assay, the conversion of aldoxime **1** to nitrile **2** in biotransformations can be calculated. As a next step, such a standard activity screening method of OxdRE(-mutants) in whole cells was then tested using the OxdRE wild-type enzyme. In Scheme 2, the conditions for the activity screening are shown.



Scheme 2. Standard-activity assay for OxdRE using *n*-octanaloxime (**1**) as substrate.

In detail, the reaction mixture was shaken at 30 ˚C and after different reaction times, the cells were removed by centrifugation. The supernatant was used for determining the conversion via this color assay after extraction with ethyl acetate (EtOAc) by GC-analysis. In Figure 2 the results of the activity screening using OxdRE wild-type enzyme in whole cells are shown.

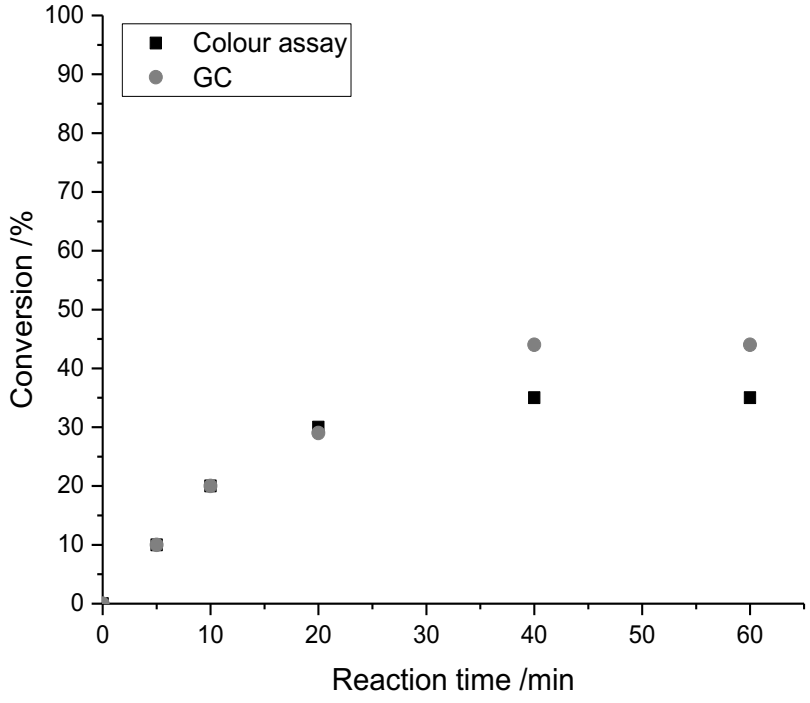


Figure 2: Conversion of *n*-octanaloxime (**1**) to *n*-octanenitrile (**2**) using OxdRE-wild type in whole cells. The conversion was determined by GC (grey) and color assay (black).

As shown in Figure 2, the conversion determined by GC and by color assay does not differ more than 5% from each other. Thus, the development of a color assay was reached, and in the next step, an error-prone PCR of the aldoxime OxdRE for directed evolution was performed.

Directed evolution of OxdRE using error prone PCR. Error-prone PCR of N-terminal His_6-tagged OxdRE in pET28(a)+ (for the sequence, see Supporting Information) was used to improve OxdRE activity and stability via directed evolution. A taq-polymerase was used for amplification of wild type-OxdRE DNA in the PCR reaction samples. Different concentrations of $MnCl_2$ were used to increase the error-rate of the taq-polymerase.

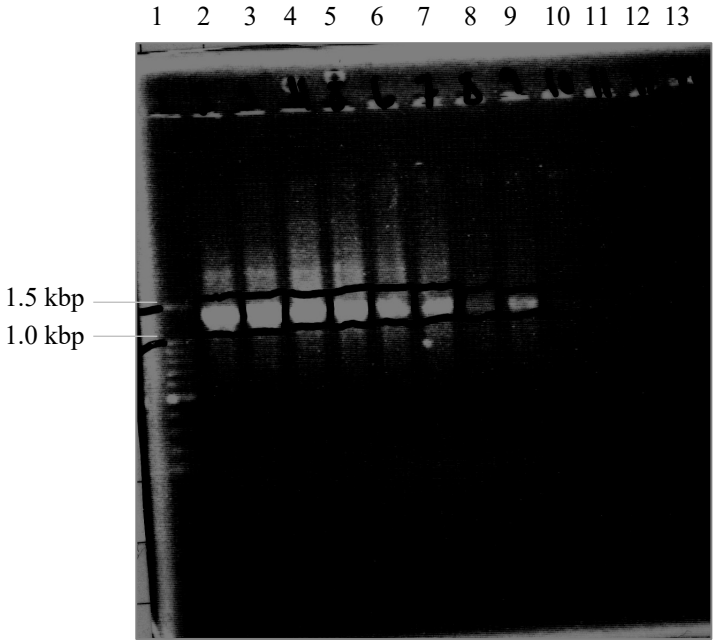


Figure 3: Picture 1.5% agarose gel after ethidium bromide coloring of PCR-products of N-His-OxdRE amplification using T7 primer and taq-polymerase in buffer including different Mn^{2+} concentrations.

Key	**1**	DNA ladder	**2,3**	0.2 mM Mn^{2+}	**4,5**	0.4 mM Mn^{2+}
	6,7	0.6 mM Mn^{2+}	**8,9**	0.8 mM Mn^{2+}	**10,11**	1.0 mM Mn^{2+}

In Figure 3 it is shown, that different concentrations of Mn^{2+} in the PCR reaction solutions leads to different amounts of amplification product. For further PCR-experiments 0.2 mM to 0.8 mM Mn^{2+} were used because this experiment showed, that higher concentrations lead to insufficient amplification of the template-DNA. The OxdRE-amplification products from error-prone PCR were cloned into pET28(a)+ vector using NdeI and HindIII restriction enzymes. After ligation the vector including the insert (OxdRE-gene) were transformed into *E. coli* BL21(DE3) to create recombinant *E. coli* cells harboring a plasmid including OxdRE(-variant). Besides the “classical” cloning technique, also the In-Fusion® technique from Takara company

was used (see Supporting Information).[29] The vector pET28a(+) was amplified using KOD-Plus-polymerase and In-Fusion® primers, without use of manganese in the PCR. The OxdRE-gene was amplified by error-prone PCR using taq-polymerase in different Mn^{2+}-concentrations and In-Fusion® primer. In Figure 4 the agarose gel after amplification is shown.

1.) **2.)**

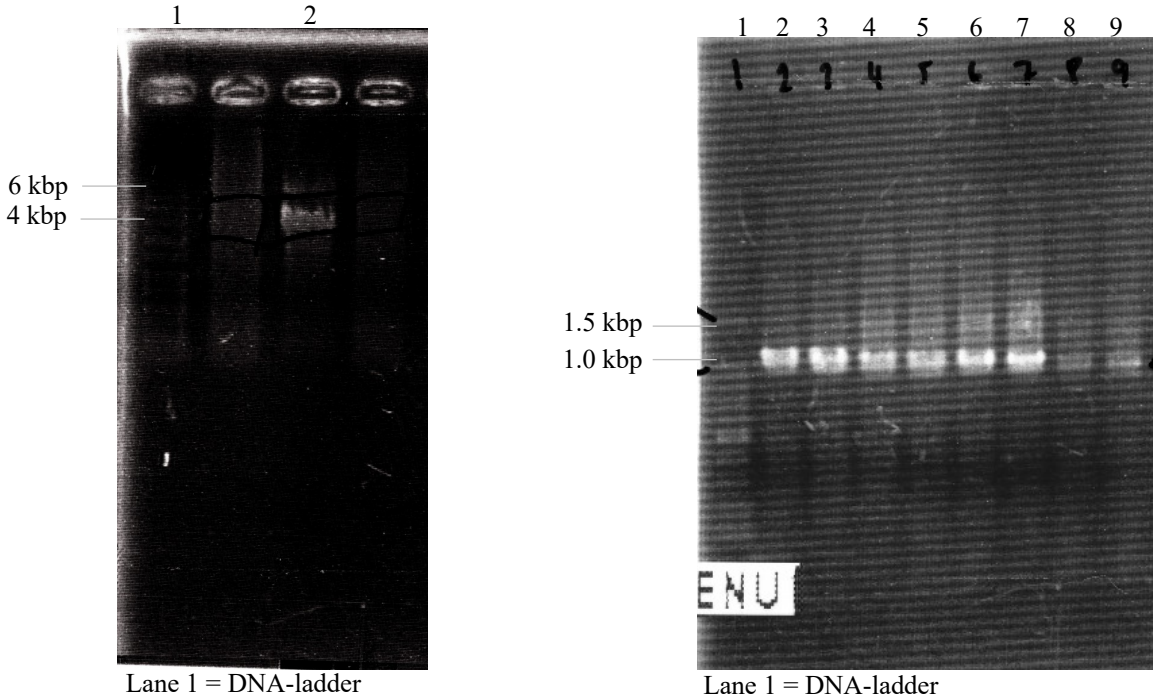
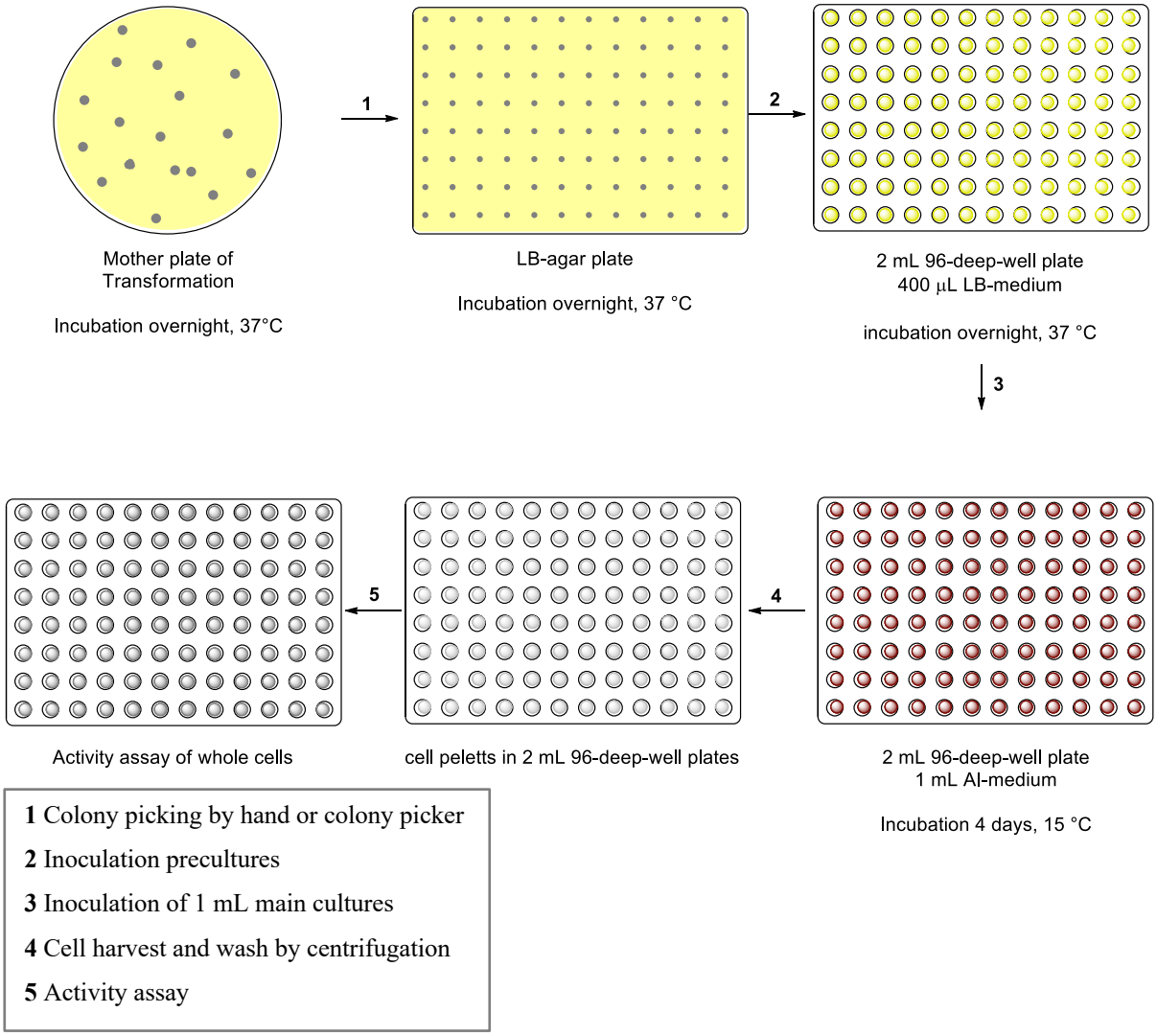


Figure 4: 1.5% agarose gel of 1.) pET28(a)+ amplification and 2.) of OxdRE error-prone PCR using different Mn^{2+}-concentrations.

Key 2.) **2,3:** 0.2 mM Mn^{2+} **4,5:** 0.4 mM Mn^{2+} **6,7:** 0.6 mM Mn^{2+} **8,9:** 0.8 mM Mn^{2+}

The amplification of pET28(a)+-DNA as well as the error-prone PCR of OxdRE-gene was successful, and the OxdRE-amplification products were cloned into pET28(a)+ and transformed into *E. coli* BL21(DE3). OxdRE wild type and varaints were first tested by color assay in whole cells. Afterwards selected enzymes were purified and studied in more detail.

Activity Screening of OxdRE(-variants). After transformation of the error-prone PCR products in pET28(a)+ vectors into *E. coli* BL21(DE3) and incubation over night at 37 ˚C on LB-agar containing kanamycin, the single colonies from the transformation mother plate were transferred to a new LB-agar plate including kanamycin. The transfer was performed using a colony picker or by hand. After incubation of the agar plates, precultures in LB-medium containing kanamycin were inoculated in 96-deepwell plates. 1 mL autoinduction (AI) medium in deepwell plates were inoculated with precultures of OxdRE-mutants and incubated at 15 ˚C for 3-4 days. Cell harvest and washing was performed in deepwell plates as well. In Scheme 5 a summarizing figure for this procedure is shown.



Scheme 3. From transformation to activity screening of OxdRE-mutants.

The activity screening was performed as used before in a total volume of 1 mL (Scheme 2). The screening plate was first analyzed by color assay and selected examples were analyzed by GC afterwards. In Figure 5 the screening-results (GC-analysis) of the four best and four worst mutants in comparison to the wild type are shown.

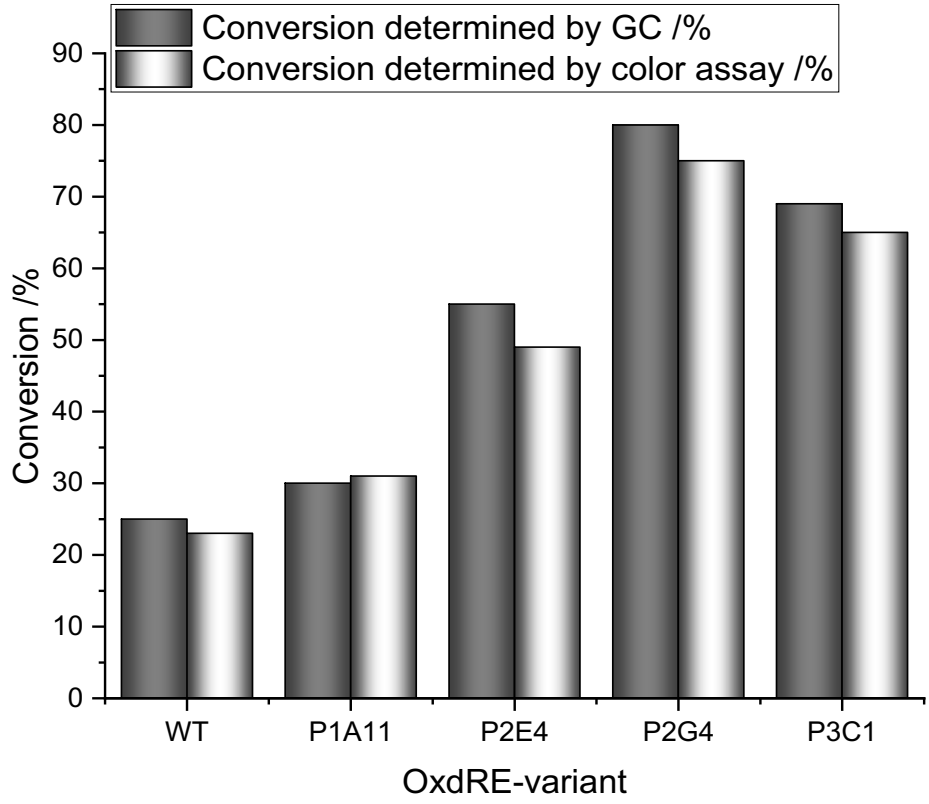
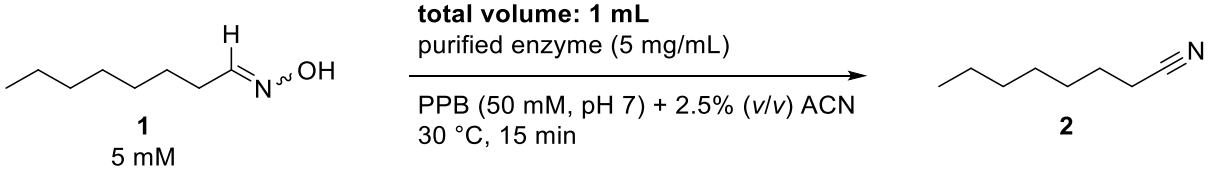


Figure 5: Screening results of the 4 best OxdRE-mutants compared to OxdRE-wild type. Conversions were determined, first by color assay (screening) (bright grey) and validated by GC (dark grey).

Since the exact cell mass and the amount of active protein in the cells are not fixed in this screening assay, the results only give a qualitative statement. As shown in Figure 5 the results of the best mutants of OxdRE differ very much from those of OxdRE-wild type, for what reason these mutants were characterized more detailed.

OxdRE-wild type and the mutants carry a N-terminal His_6-tag, which was used to purify OxdRE wild type and the 4 variants, shown in Figure 5. After purification the activity of the enzymes for *n*-octanaloxime (**1**) were measured. In Scheme 4 the reaction conditions for the determination of specific activity is shown.



Scheme 4. Conditions of activity assay using purified OxdRE(-mutants).

Using this activity assay, the specific activities of OxdRE(-variants) for the conversion of *n*-octanaloxime (**1**) were determined (Figure 7).

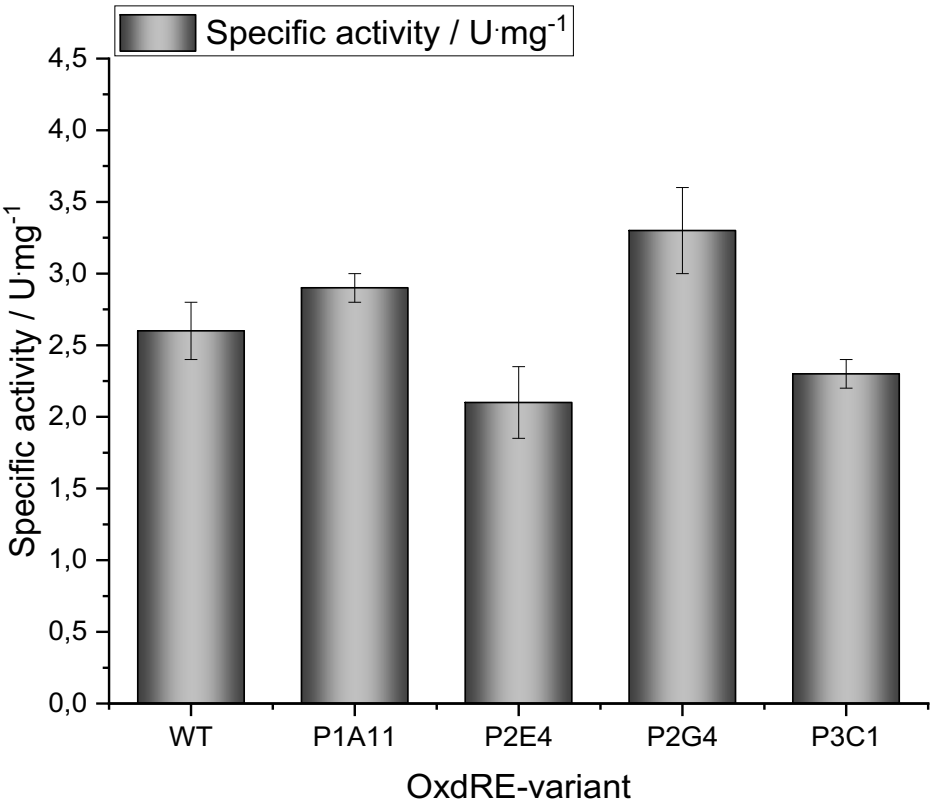


Figure 6. Specific activities in U/mg of OxdRE-wild type and -mutants for *n*-octanaloxime (1).

As shown in Figure 6 the specific activities of OxdRE-wild type and OxdRE-mutants do not differ in a high manner. P1A11- and P2G4- show slightly higher activities and P2E4- and P3C1-variants slightly lower specific activities, however, the variation seems to be in range of the error of the triplicates. Besides the activity also the stability of OxdRE-wild type and -mutants in acetonitrile (ACN) and at 50 °C were determined. For this reason, purified enzymes were incubated for 30 min at 50 °C of in 30% (*v*/*v*) ACN. After incubation, the same activity assay was performed as used before for the determination of specific activities. In Figure 11 the results of the stability determination are shown.

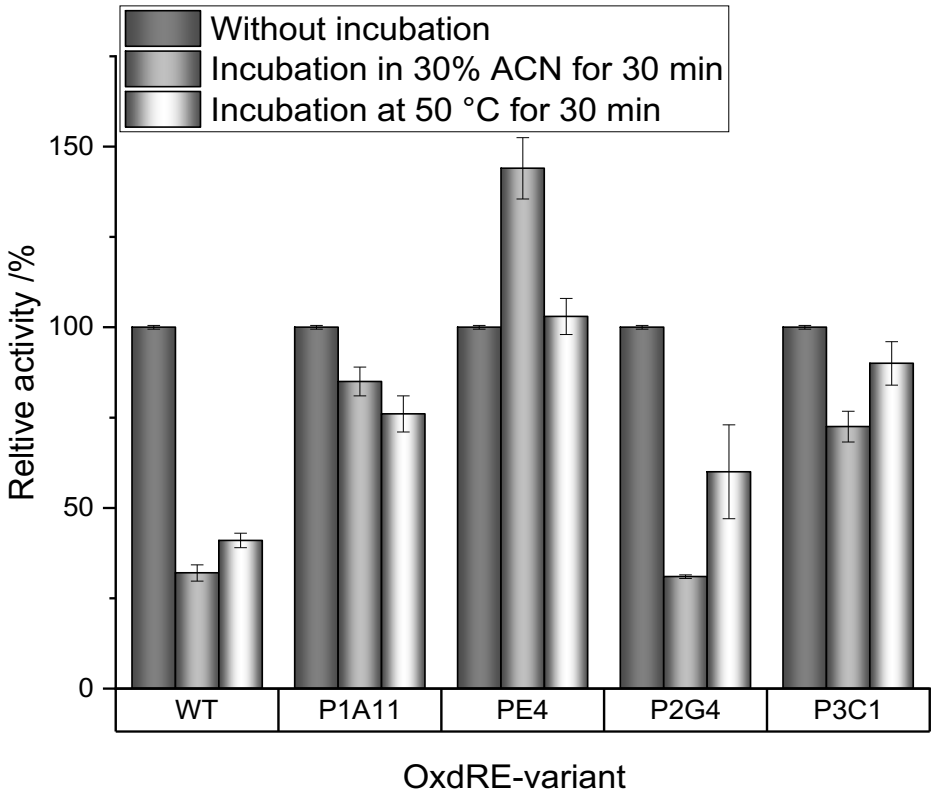


Figure 7. Relative activities of OxdRE-wild type and -mutans after incubation in 30% (v/v) ACN or at 50 °C for 30 min. 100% marks are the specific activities without incubation in ACN or at 50 °C.

A strong enhancement of the thermo- and solvent-stability of the OxdRE-mutants was detected. In particular, the P2E4-, P1A11- and P3C1- mutants showed the highest residual activity after incubation in 30% (*v*/*v*) ACN or at 50 °C. In case of P2G4, only the thermostability was improved, the solvent-stability was detected to be in the same range as for the wild type. P2E4 seems to be a special case because in this case a higher relative activity after incubation in 30% ACN was detected compared to the measurement without incubation. After sequencing these four variants, it was found that P1A11 is an E150D-mutant of OxdRE wild type, P2E4 a variant with ten different mutations (L60P, D65G, I85V, L145P, I214V,D229N, R231C, D247H, N272S, H296L), P2G4 a double mutant (D36A, R308G) and P3C1 a W117T-mutant. Mutant P1A11 was obtained using 0.2 mM Mn^{2+} in error-prone PCR reaction and P2E4, P2G4 and P3C1 were obtained using 0.4 mM Mn^{2+} in the error-prone PCR. It is noteworthy, that we were able with only one round of error-prone PCR to obtain variants, which show improved stability in comparison to the wild-type. It is even more surprising, that we found a variant with 10 mutations, which shows specific activities in the range of the wild-type enzyme and improved solvent- and thermostability. Since the X-ray structure of OxdRE is known, we searched for the positions of each mutation in the structure (Figure 8).

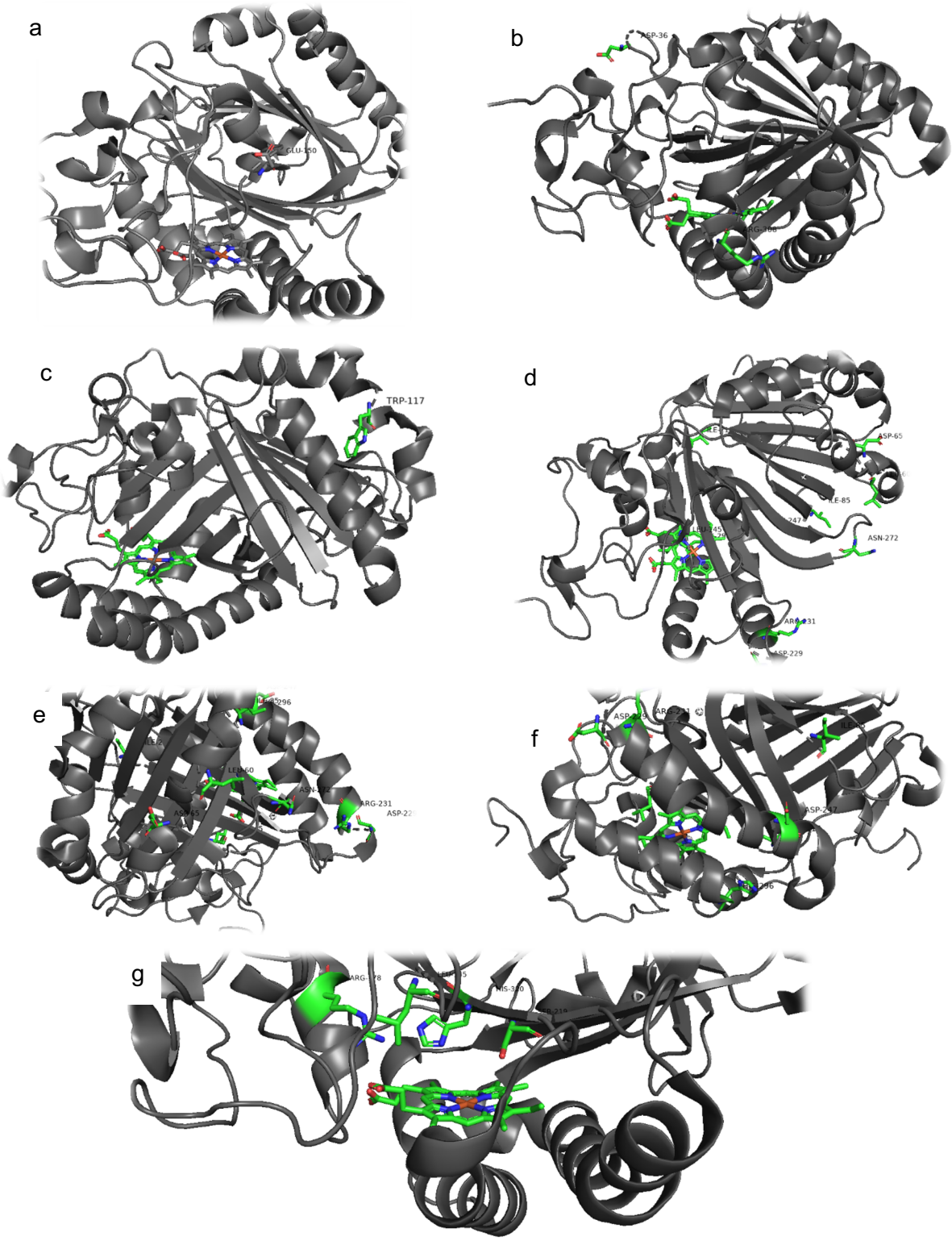


Figure 8. Pictures of OxdRE structure in cartoon model. The figures always show the heme centre in sticks.

a.E150 marked (exchanged in P1A11 variant to E150D); b. D36 and R308 marked (exchanged in P2G4-variant to D36A and R308G); c. W117 marked (exchanged in P3C1 variant to Q117T); d,e and f. L60, D65, I85, L145, I214, D229, R231, D247, N272 and H296 marked (exchanged in P2E4 variant to L60P, D65G, I85V, L145P, I214V, D229N, R231C, D247H, N272S, H296L); g. Zoom into the active site of OxdRE with marked catalytic triade (H320, S219 and R178) and L145 (exchanged in variant P2E4 to L145P).

As can be seen from these figures of the structure of the OxdRE mutants, in most cases of the mutated amino acids the position is at the outside of the enzyme and far away from the active site. It is surprising that many mutations are found in secondary structures like α-helices, e.g. in case of the P1A11 variant (Figure 8, a), R308G mutation in P2G4 (Figure 8, b) or in the P3C1 variant. Usually, exchanges of amino acids in the secondary structures lead to destabilization of the secondary structure element, depending on the replaced amino acid position and functionality. Only in case of the P2E4-variant, a replacement of an amino acid in the active site was detected, namely the L145P mutation. As visible in Figure 8 part g, this leucine is in the center of the active site and is involved in the positioning of the aldoxime substrate, which must bind to the active site to be converted to the nitrile.

Especiallly in case of the P2E4-variant we were interested in finding an explanation why this mutant is more stable compared to the wild-type. Therefore, the mutants were analyzed by the modeling software MOE.[30] The *in silico* analysis was performed to identify the stabilitzing mutation points and to rationalize the effects. For the study the crystal structure of OxdRE (3a17)[26] was used. The structure of the mutant P2E4 was generated using MOE. Both structures, were prepared and optimized using a standard protocol. With the use of the protein design function the single point mutations could be generated and tested for stability. This application calculates stability with the assumption that the stability is only dependent on the residual change using the scoring function from *Guerois et al*.[31] The energy and protein property changes can be determined for the defined mutations as single point or as combinational mutations. With this protocol we identified the mutation points, which did lead to a stabilization of the protein, but also mutations that had no or a negative effect on the stability of the protein. The mutations I85V and I214V showed no influence on protein stability because valine is comparable to isoleucine in terms of structure and physicochemical properties. The mutations L60P, L145P, R231C and H296L showed a deterioration of the protein stability. While R231C and H296L increase the hydrophobicity on the surface of the protein, the proline mutations lead to a structural change which in turn leads to lower hydrogen bonding. The mutations D65G, D229N, D247H and N272S increase the stability and these 4 positions are the reason for the increased stability of the mutant. All four mutants increase the energy difference between the folded and unfolded state. Besides that, all four mutations are on the surface and increase the hydrophobic moment and reduce the dipole moment (D247H as exception), which has the effect that the water accessible hydrophilic area is increased and the water accessible hydrophobic area decrease. To further increase the stability of the P2E4 it might be an option to change the four negative mutations back to the wild type or to better variants.

In Figure 9 the positive mutations are shown. It is noteworthy that all these mutations have one feature in common, namely that the number of hydrogen bonds within the protein is reduced

and thus free functional groups can interact with water molecules. This increases the surface area for hydrophilic interaction with water and the flexibility of the residue, which is also stabilizing feature since it increases the entropy of the protein folded form.[32] The hydrophobic moment is a value, which can also be considered for the stability of the protein in this matter.[33] For example, the D65G mutation leads to G61 having a free amino group to coordinate water (Figure 7, a). Although the loss of a hydrogen bond contributes to destabilization, this effect is negligible if it increases hydrophobicity to higher amount within the protein, which is crucial for the stability of the protein and the driving force for the folding.[34,35] This is not directly evident from the protein structures shown in Figure 7, but the values in Table 1 show this effect.

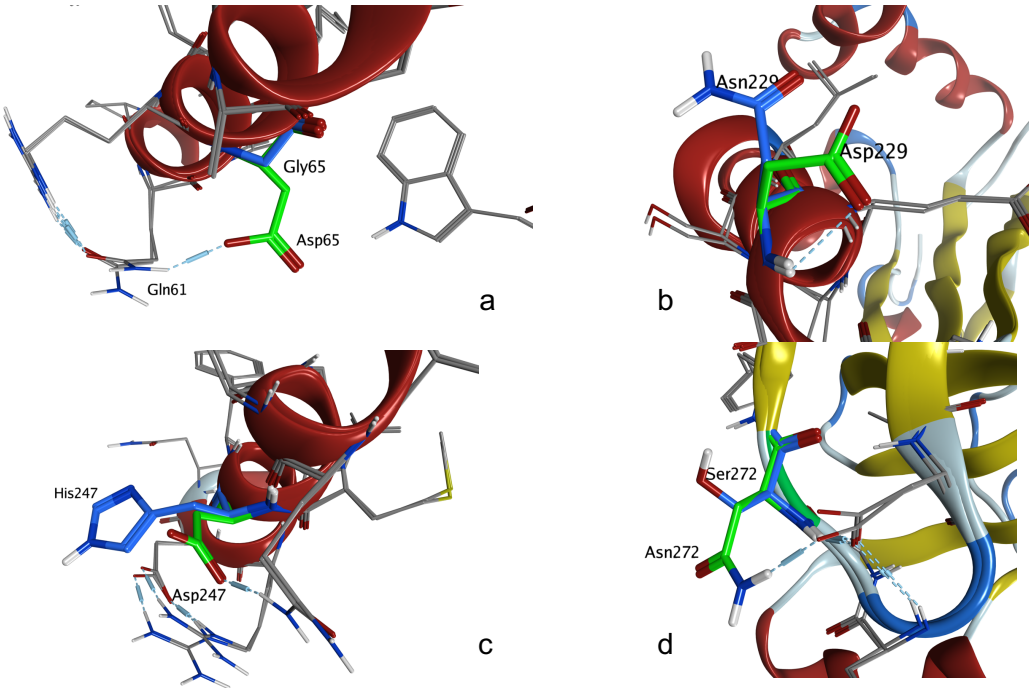


Figure 9. Comparison of positive mutation sites in an Alignment of P2E4 and OxdRE-WT. In each frame the wild type residue is shown in green, while the mutant is shown in blue. D65G mutant (frame a), D229N mutant (frame b), D247H mutant (frame c) and N272S mutant (frame d).

Considering the protein design application, the positive mutation (Table 1, Entry 1) increase the protein stability by -1.8 kcal/mol and as described before the hydrophobic moment is increased, but the dipole moment as well. The increase in the dipole moment is only caused by the mutation D247H. However, the histidine shields the hydrophobic area behind it, thereby reducing the water accessible hydrophobic area. In total the positive mutations increase the water accessible hydrophilic surface area by 28.8 $Å^2$ and decrease the water accessible hydrophobic area by 66.8 $Å^2$. While the neutral mutations (Table 1, Entry 2) I85V and I214V do not have a significant influence on the stability, the negative mutations (Table 1, Entry 3)

have a very strong negative influence on stability, as the data shows. The stability is decrease by 2 kcal/mol and the hydrophobic moment is strongly decreased by 159 D.

Table ?. Results of the protein design application. Showing stability change (ΔS in kcal·mol-1), change in water accessible hydrophilic surface area (Δhyd) change in water accessible hydrophobic surface area (Δhyp), change in dipole moment (Δdipol) and change in hydrophobic moment (Δhyp) for positive, neutral and negative mutation in P2E4 with P2E4 as comparison.

Entry	Protein	ΔS /kcal·mol^{-1}	Δhyd /Å^2	Δhyp /Å^2	Δdipol. /D	Δhyp. /D
1	D65G-D229N-D247H-N272S	-1.8	28.8	-66.8	76.2	48.1
2	I85V-I214V	0.1	-16.0	12.0	2.9	2.5
3	L60P-L145P-R231C-H296L	2.0	-13.2	-27.3	-5.8	-159.0
4	AHI(L60P-D65G-L145P-I85V-I214V-D229N-R231C-D247H-N272S-H296L)	-2.3	60.3	-87.5	17.5	-65.6

The complete mutant P2E4 is with 2.3 kcal/mol more stable than the WT, it is noteworthy that this value is highly dependent on the conformation and with 10 mutations quite inaccurate, but since the data are consistent with the experimental findings, we can assume the presence of a good and robust model. With that we conclude the improved thermostability due to the improved properties in terms of water accessible hydrophobic/hydrophilic area caused by the mutations in D65G, D229N, D247H and N272S.

Conclusion

We successfully developed a colorassay, which can be used for the determination of conversion of *n*-octanaloxime (**1**) to the corresponding nitrile **2** by simple UV-measurement. To the best of our knowledge this is the first color assay, which was developed for aldoxime dehydratases, converting aliphatic aldoximes to nitriles. We further performed directed evolution of OxdRE, an aldoxime dehydratase from *Rhodococcus erythropolis*, using error-prone PCR and obtained mutants, which were found to be more stable in presence of cosolvent and at higher temperatures.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Improving Activity and Stability of Aldoxime Dehydratase OxdRE from *Rhodococcus erythropolis* by Directed Evolution

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1 Materials

Chemicals including *n*-octanal, hydroxylamine hydrochloride, sodium carbonate and chemicals for the color assay were purchased from commercial ressources (Japan).

2 Alignment tool for comparison of OxdRE-genes and amino acids sequences

Constraint-based Multiple Alignment Tool from BLAST was used for alignment and comparison of identities of OxdRE-variants.

3 Analytical Methods

3.1 Absorbance measurement (color assay)

The color assay was performed using the following pipette sequence.

Table 1: Pipette sequence of the color assay for activity screening of OxdRE-mutants. The reaction samples were centrifuged beforehand to remove the cell mass.

Step	Solution	Concentration	Volume
1	*n*-Octanaloxime in reaction sample (supernatant)	0 - 20 mM	60 µL
2	HCl in water	500 mM	5 µL
3	Sodium actetate in dis. water	100 g/L	5µL
4	Sulphanilic acid in 0.1 M HCl	1%	5µL
5	ICl in 0.1 M HCl	100 mM	5µL
6	8-Hydroxy-quinoline in 0.1 M HCl	1%	5µL
7	KOH in dis. water	50%	20µL

The absorbance was measured at 505 nm using a Tecan Microplate reader (Infinite M200Pro).

3.2 GC-analytics

For conversion determination using GC the reaction samples were extracted with EtOAc (0.5x volume of sample volume) by vortexing and subsequent centrifugation. The organic phase was used for the GC analytics. In following table the temperature gradient used for this GC method is shown. A GC-2014 instrument from the company Shimadzu and a DB-WAX column from Agilent Technologies was used with a flame detector. A sample volume of 8 µL was used.

Table 2: Temperature gradient of the GC-method.

Start /°C	End /°C	Hold /min
--	110	0
110	125	0.5
125	140	0.5
140	180	0.5
180	240	4

Retention times *n*-octanenitrile (**2**) t_r = 3.8 min

n-octanaloxime (**1**) t_r = 5.2 min

Table 3: GC-settings for the separation of *n*-octanaloxime (**2**) and *n*-octane nitrile (**3**).

GC-settings	
Column	DB-WAX Agilent Technologies (ID: 0.250 mm, film thickness): 0.5 µm, length: 30 m)
Injection volume	8 µL
SPL1	300 °C
Pressure	102.5 kPa
Total flow	52.8 mL/min
Column flow	0.98 mL/min
Linear velocity	27.5 cm/s
Purge flow	3.0 mL/min
Split ratio	50

4 Error-prone PCR of OxdRE

4.1 Error-prone PCR with subsequent standard cloning

Error-prone PCR were performed using a total reaction volume of 50 µL. The ingredients and concentrations are shown in following table.

Table 4: Concentrations of ingredients in error-prone PCR of N-His-OxdRE.

Ingredient	Concentration/Amount
LA-taq buffer	1x
dNTPs	0.2 mM
$MgSO_4$	1 mM
FW Primer (T7)	1 pM
RV Primer (T7)	1 pM
DNA-Template	2 ng/µL
Taq-polymerase	5 U
$MnCl_2$	0.2 – 1.2 mM

The temperature profile of the PCR is shown in following table. PCRs were performed in a thermos cycler (C1000 Touch™ Thermal Cycler, BioRad).

Table 5: Temperature profile of error-prone PCR using T7-primer.

Temperature	Hold time
95 °C	2 min
95 °C	30 s
45 °C	30 s
72 °C	1 min
72 °C	10 min

After error-prone PCR the PCR-products as well as the vector pET28(a)+ were cut by the restriction enzymes NdeI and HindIII. Therefore 10 µg PCR product or 1 µg pET28(a)+ vector DNA were mixed with 1 µL NdeI-solution (Takara, 10 U/mL*) and 1 µL HinIII-solution (Takara, 15 U/mL*) in a total volume of 10 µL. The restriction enzyme digestion was performed

for 1 h at 37 °C. 1 µL sample buffer for agarose gel was added. Total amounts of the vector and insert restriction enzyme reaction sample were transferred to a 1.5% agarose gel. The gel was ran for 15 minutes before staining in ethydiumbromide solution for 10 min. The gel was analyzed using UV-light and the products (vector-DNA and OxdRE-gene amplification product, cut by restriction enzymes) were cut from the gel. The DNA was purified from the gel using Promega® gel extraction kit.

Ligation of pET28(a)+ vector DNA and error-prone PCR products were performed using the "Protocol-at-a-Glance: DNA Ligation Kit, Mighty Mix" kit from Takara. Therefore, ~100 ng insert and ~85 ng vector DNA in a volume of 5 µL were mixed with 5 µL premixed ligation solution. The ligation samples were incubated for 30 min at 16 °C.

5 µLligation reaction were used for transformation into *E. coli* BL21(DE3). Commercially available chemical competent cells (One Shot™ BL21(DE3) Chemically Competent *E. coli*, Thermo Fisher Scientific) were mixed with the ligation solution and incubated on ice for 30 min. After heat-shock for 30 s at 42 °C 500 µL SOC-medium was added and the cells incubated at 37 °C for 1 h. The cell suspension was transferred to LB-agar plates containing 50 µg/mL kanamycin and incubated over night at 37 °C.

* 1 U = enzyme amount which converts 1 µg template DNA in 1 h.

4.2 Error-prone PCR with In-Fusion® primers

Error-prone PCR of OxdRE was performed using the In-Fusion® cloning kit from Takara as a second method. In **Figure 12** the method is described.

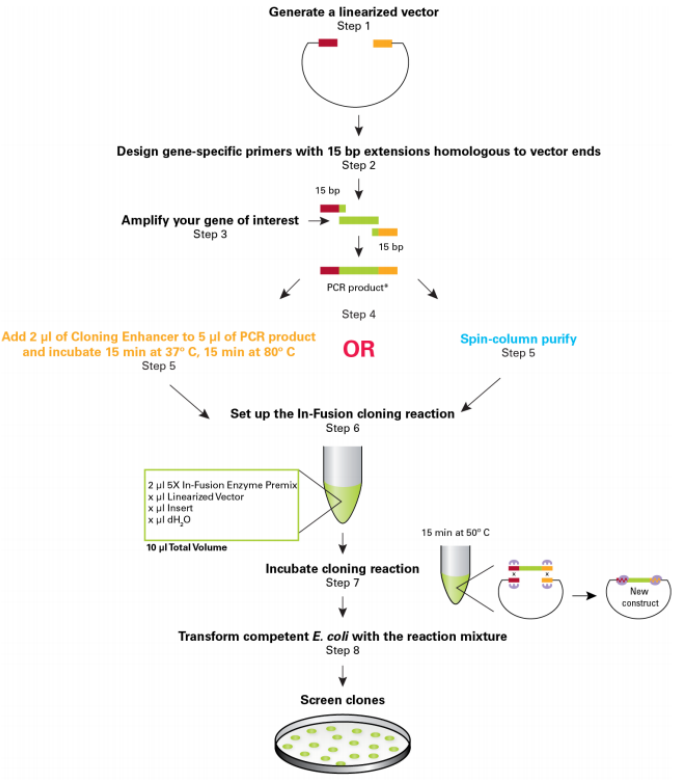

Figure 1: Cloning strategy using the In-Fusion® strategy from Takara.

Picture taken from: Takara Bio USA, Inc., *In-Fusion® HD Cloning Kit User Manual – Clontech.* [1]

First, the linearized vector was produced by PCR. Therefore pET28(a)+ primer were used with an overhang complementary to OxdRE (**Table 9**).

Table 6: Concentrations of ingredients in PCR of pET28(a)+ vector-DNA.

Ingredient	Concentration
iPCR buffer	1x
dNTPs	0.2 mM
$MgSO_4$	1 mM
FW Primer	3 pM
RV Primer	3 pM
DNA-Template	50 ng/µL
KOD-Plus-polymerase	5 U
Total volume 50 µL	

The temperature profile of the PCR is shown in following table. PCRs were performed in a thermos cycler (C1000 Touch™ Thermal Cycler, BioRad).

Table 7: Temperature profile of the PCR of pET28(a)+.

Temperature	Hold time
94 °C	2 min
98 °C	10 s
58 °C	15 s
68 °C	6 min
4 °C	∞

Secondly, error-prone PCR of OxdRE-wild type was performed using primers with an overhang complementary to pET28(a)+-sequence (see **Table 15**). The ingredients and concentrations are shown in following table.

Table 8: Concentrations of ingredients in error-prone PCR of OxdRE using In-Fusion® primer.

Ingredient	Concentration
LA-taq buffer	1x
dNTPs	0.2 mM
$MgSO_4$	1 mM
FW Primer	1 pM
RV Primer	1 pM
DNA-Template	2 ng/µL
Taq-polymerase	5 U
$MnCl_2$	0.2 – 0.8 mM
Total volume 50 µL	

The temperature profile of the PCR is shown in following table.

Table 9: Temperature profile of error-prone PCR using In-Fusion®-primer.

Temperature	Hold
95 °C	2 min
95 °C	30 s

58 °C	30 s
72 °C	1 min
72 °C	10 min

5 Cell cultivation, protein expression and purification via Ni-NTA affinity chromatography

Clones from LB agar plate were inoculated into 5 mL or 400 µL (deep well-plates (2 mL max. volume)) LB medium containing 50 µg/mL kanamycin and shaken at 250 rpm at 37 °C for ~15 h. The 5 mL or 100 µL of the preculture was inoculated into 500 mL (500 mL Erlenmeyer flask) or 1 mL (deep well-plates (2 mL max. volume)) autoinduction medium containing kanamycin (50 µg/mL). The main culture was shaken at 15 °C at 200 rpm for ~72 h.
Cell harvest was performed by centrifugation at 4,000 xg for 10 min. Cells were washed with potassium phosphate buffer (50 mM, pH 7.0) three times (each ~3x mass of cell pelett).
For protein purification by Ni-NTA affinity chromatographie the cells were suspended in lysis buffer (20 mM Tris-HCl, 10 mM Imidazole, 300 mM NaCL, pH 8.0) and disrupted at 0 °C by sonication (30 min, 15 – 20% output, 50% duty cycle). Cell debris were removed by centrifugation for 10 min at 8,000x g and 4 °C. Crude extracts were filtered through a 0.45 µm pore filter before loading to a lysis buffer washed Ni-NTA sepharose column. Bound OxdRE(-variants) on the column were washed with lysis buffer (5 column volumes) and eluted with elution buffer (20 mM Tris-HCl, 150 mM Imidazole, 300 mM NaCl, pH 8.0). The activity of freshly purified Oxds were measured as described below.

6 Determination of protein concentration via Bradford-assay and analysis of overexpression and purity of purified enzymes by SDS-PAGE

Protein concentrations in crude extracts or purified enzyme solutions were determined by Bradford assay using a bovine serum albumin (BSA)-standard curve (1.4 mg/mL, 0.7 mg/mL, 0.35 mg/mL, 0.175 mg/mL, 0.0875 mg/mL) as reference. Protein dilutions of 1 µg/µL whole cell protein concentration were obtained by dilution of the crude extracts in water and Laemmli-buffer. 10 µL of these samples were transferred to a 12% SDS-PAGE. Overexpression and

purity was analyzed after staining the SDS-PAGE in Coomassie Blue R250 stain solution (0.1% Coomassie in 10% acetic acid, 50% methanol and 40% H_2O) for one hour in comparison to a protein marker. The SDS-page was destained in 10% acetic acid, 50% methanol and 40% H_2O-solution.

7 Activity assay of OxdRE-wild type and OxdRE-mutants

Screening of OxdRE-wild type and –mutant activity for the conversion of *n*-octanaloxime (**2**) to *n*-octane nitrile (**3**) was performed using a whole cell assay in an aqueous reaction system including 30% (*v*/*v*) ACN. The reaction conditions are shown in following table.

Table 10: Whole-cell screening assay.

Ingredient	Concentration
Wet cell mass	20 mg/mL
n-Octanaloxime	20 mM
Cosolvent (ACN)	30% (*v*/*v*)
KP_i	50 mM, pH 7
Total volume 1 mL	

After different reaction times, samples were taken and extracted with EtOAc for GC-analytics or used for the color assay without extraction. Cells were removed by centrifugation (4,000 xg, 10 min) beforehand.

Stability and specific activities were calculated using purified enzyme. Stability of OxdRE-wild type and –mutants were determined by incubation at 50 °C or in 30% (*v*/*v*) ACN. Therefore, the reaction mixture as described in Table 11 was used without the substrate solution. After 30 min incubation time the substrate solution was added and the activity measurement was performed. The activity assay itself is performed at 30 °C at a total volume of 1 mL. After 15 min reaction time 500 µL EtOAc was added and the reaction mixture was vortexed 5 min. After centrifugation (15,000 xg, 2 min) the organic phase was measured by GC.

Table 11: Activity and stability assay for purified OxdRE-wild type and –mutants.

Ingredient	Concentration
Purified Enzyme	5 mg/mL
n-Octanaloxime (**2**) (200 mM in ACN)	5 mM
KP_i	50 mM, pH 7
Cosolvent ACN	2.5% (*v*/*v*)
Total volume 1 mL	

8 *In silico* Study

8.1 Experimental

The crystal structure of OxdRE-WT (3a17)[2] was loaded from PDB (protein data bank) into the modelling software MOE (molecular operating environment)[3]. The wild type was then mutated to have the 10 point mutation P2E4. Both Wild-Type and mutant were used for the protein design application.

8.2 General procedure protein design application (GPP)

The screening was performed using AMBER10:EHT force field using Born-solvation model with dielectric constant of 1 and exterior constant of 80. The cut-off for non-bonded interaction were set to 8-10 Å. The mutation sites were picked and only the target mutation was chosen. The mutants were refined with a RMSD-gradient of 0.5 and always with an environmental repack with a 4.5 Å cut off. The mutation site was varied. If not mentioned all experiments were done with this set up.

8.3 Protein design OxdRE-WT towards P2E4 single point mutation

The single point mutations of OxdRE-WT towards P2E4 were done according to GPP and shown in Table 12.

Table 12. Results of single point mutation of OxdRE-WT towards P2E4 using GPP.

Mutation	Stability	ΔS /kcal·mol^{-1}	Δhyd /Å^2	Δhyp /Å^2	Δdipol. /D	Δhyp. /D
L60L	-1354.5	0.0	0.0	0.0	0.0	0.0
L60P	-1351.7	2.8	15.9	-2.9	10.5	-87.7
D65D	-1349.7	0.0	0.0	0.0	0.0	0.0
I85I	-1354.9	0.0	0.0	0.0	0.0	0.0
I85V	-1354.0	0.9	-16.9	10.5	-0.7	-2.2
L145L	-1353.4	0.0	0.0	0.0	0.0	0.0
L145P	-1351.0	2.4	15.0	2.6	-0.3	56.5
I214I	-1357.6	0.0	0.0	0.0	0.0	0.0
I214V	-1356.3	1.3	-0.9	0.0	-0.7	1.2
D229D	-1348.8	0.0	0.0	0.0	0.0	0.0
D229N	-1348.5	0.4	-2.9	-7.6	26.7	-0.2
R231R	-1343.2	0.0	0.0	0.0	0.0	0.0
R231C	-1341.2	2.0	-4.0	9.4	35.6	-23.6
D247D	-1349.0	0.0	0.0	0.0	0.0	0.0
D247H	-1348.0	1.0	27.6	-28.5	77.2	-1.7
N272N	-1348.7	0.0	0.0	0.0	0.0	0.0
N272S	-1348.1	0.6	21.5	-40.0	4.3	30.2
H296H	-1353.1	0.0	0.0	0.0	0.0	0.0
H296L	-1352.5	0.6	30.2	-16.5	-8.0	-99.2

8.4 Protein design P2E4 towards OxdRE-WT single point mutation

The single point mutations of P2E4 towards OxdRE-WT were done according to GPP and shown in Table 13.

Table 13. Results of single point mutation of OxdRE-WT towards P2E4 using GPP.

Mutation	Stability	ΔS /kcal·mol^{-1}	Δhyd /Å^2	Δhyp /Å^2	Δdipol. /D	Δhyp. /D
P60P	-1324.5	0.0	0.0	0.0	0.0	0.0
P60L	-1325.1	-0.6	-0.2	-10.0	-6.5	117.4
G65G	-1323.5	0.0	0.0	0.0	0.0	0.0
G65D	-1323.2	0.3	-33.8	21.0	94.5	-54.4
V85V	-1322.5	0.0	0.0	0.0	0.0	0.0
V85I	-1322.0	0.5	16.1	-9.2	2.9	5.1
P145P	-1325.0	0.0	0.0	0.0	0.0	0.0
P145L	-1325.3	-0.4	-21.9	-6.5	-0.7	-46.2
V214V	-1324.9	0.0	0.0	0.0	0.0	0.0
V214I	-1324.8	0.1	0.3	-1.5	0.5	-2.5
N229N	-1317.1	0.0	0.0	0.0	0.0	0.0
N229D	-1316.2	0.9	7.1	-11.9	-1.7	0.2
C231C	-1325.0	0.0	0.0	0.0	0.0	0.0
C231R	-1324.9	0.0	20.8	5.6	1.0	14.5
H247H	-1321.3	0.0	0.0	0.0	0.0	0.0
H247D	-1320.8	0.5	-5.3	20.6	-78.1	-1.4
S272S	-1323.5	0.0	0.0	0.0	0.0	0.0
S272N	-1322.3	1.2	-29.5	53.7	-3.9	-47.0
L296L	-1323.3	0.0	0.0	0.0	0.0	0.0
L296H	-1322.6	0.7	29.0	3.7	11.8	78.5

8.5 Protein design application of P2E4 towards OxdRE-WT of triple point mutation with potentially positive effect

The triple point mutations of P2E4 towards OxdRE-WT were done according to GPP and shown in Table 14. L60, L145 and I214 were not included since they were already clarified at this point to either have negative impact or no impact at all.

Table 14. Results of triple point mutation with potentially positive effect of P2E4 towards OxdRE-WT using GPP.

Mutation	Stability	ΔS /kcal·mol^{-1}	Δhyd /Å^2	Δhyp /Å^2	Δdipol. /D	Δhyp. /D
G65G -V85V -N229N	-1317.8	0.0	0.0	0.0	0.0	0.0
G65D-V85I-N229D	-1317.5	0.3	-17.8	0.5	85.0	-48.6
G65G-V85V-H247H	-1322.5	0.0	0.0	0.0	0.0	0.0
G65D-V85I-H247D	-1322.5	0.0	-29.7	28.0	30.1	-51.2
G65G-N229N-H247H	-1316.7	0.0	0.0	0.0	0.0	0.0
G65D-N229D-H247D	-1316.1	0.6	-40.1	35.1	28.1	-56.7
V85V-N229N-H247H	-1316.1	0.0	0.0	0.0	0.0	0.0
V85I-N229D-H247D	-1315.5	0.5	8.8	11.6	-64.3	3.6
G65G-V85V-S272S	-1324.2	0.0	0.0	0.0	0.0	0.0
G65D-V85I-S272N	-1323.5	0.6	-51.1	72.2	94.4	-95.6
G65G-N229N-S272S	-1318.8	0.0	0.0	0.0	0.0	0.0
G65D-N229D-S272N	-1317.8	1.0	-57.2	61.8	79.4	-100.8
V85V-N229N-S272S	-1317.8	0.0	0.0	0.0	0.0	0.0
V85I-N229D-S272N	-1316.6	1.2	-7.9	26.1	-4.2	-41.8
G65G-H247H-S272S	-1323.0	0.0	0.0	0.0	0.0	0.0
G65D-H247D-S272N	-1322.3	0.7	-77.3	100.4	17.7	-103.8
V85V-H247H-S272S	-1322.4	0.0	0.0	0.0	0.0	0.0
V85I-H247D-S272N	-1321.8	0.6	-30.5	62.4	-80.6	-43.5
N229N-H247H-S272S	-1316.6	0.0	0.0	0.0	0.0	0.0
N229D-H247D-S272N	-1315.4	1.2	-36.6	65.4	-65.5	-48.2
G65G-V85V-L296L	-1324.1	0.0	0.0	0.0	0.0	0.0
G65D-V85I-L296H	-1323.9	0.2	2.3	11.4	110.6	18.8
G65G-N229N-L296L	-1318.7	0.0	0.0	0.0	0.0	0.0
G65D-N229D-L296H	-1318.2	0.5	-3.3	9.4	94.5	15.1
V85V-N229N-L296L	-1317.6	0.0	0.0	0.0	0.0	0.0
V85I-N229D-L296H	-1316.9	0.7	34.8	-17.1	8.2	82.3
G65G-H247H-L296L	-1322.4	0.0	0.0	0.0	0.0	0.0
G65D-H247D-L296H	-1322.7	-0.3	-29.8	47.0	35.3	15.8
V85V-H247H-L296L	-1321.9	0.0	0.0	0.0	0.0	0.0

V85I-H247D-L296H	-1322.0	-0.2	35.4	17.9	-63.9	82.3
N229N-H247H-L296L	-1315.9	0.0	0.0	0.0	0.0	0.0
N229D-H247D-L296H	-1315.7	0.2	27.3	8.7	-51.0	79.5
G65G-S272S-L296L	-1325.3	0.0	0.0	0.0	0.0	0.0
G65D-S272N-L296H	-1324.2	1.1	-35.9	62.0	117.2	-26.7
V85V-S272S-L296L	-1323.7	0.0	0.0	0.0	0.0	0.0
V85I-S272N-L296H	-1323.1	0.6	33.0	6.7	13.3	40.4
N229N-S272S-L296L	-1318.2	0.0	0.0	0.0	0.0	0.0
N229D-S272N-L296H	-1317.4	0.8	20.7	12.0	5.0	37.1
H247H-S272S-L296L	-1322.2	0.0	0.0	0.0	0.0	0.0
H247D-S272N-L296H	-1322.0	0.1	-6.7	39.6	-72.5	38.8

8.6 Protein design combinational mutations in comparison

Chosen combinational mutations of OxdRE designed according to GPP and shown in Table 15. They were sorted in three categories: positive mutation (Entry 1), neutral mutations (Entry 2) and negative mutations (Entry 3). Also, the 10-point mutation P2E4 is shown (Entry 4). For the 10-point mutation the refinement was turned off, because the conformational change was too great.

Table 15: Results of the protein design application. Showing stability change (ΔS in kcal·mol^{-1}), change in water accessible hydrophilic surface area (Δhyd) change in water accessible hydrophobic surface area (Δhyp), change in dipole moment (Δdipol) and change in hydrophobic moment (Δhyp) for positive, neutral and negative mutation in P2E4 with P2E4 as comparison.

Entry	Protein	ΔS /kcal·mol^{-1}	Δhyd /Å^2	Δhyp /Å^2	Δdipol. /D	Δhyp. /D
1	D65G-D229N-D247H-N272S	-1.8	28.8	-66.8	76.2	48.1
2	I85V-I214V	0.1	-16.0	12.0	2.9	2.5
3	L60P-L145P-R231C-H296L	2.0	-13.2	-27.3	5.8	-159.0
4	AHI(L60P-D65G-L145P-I85V-I214V-D229N-R231C-D247H-N272S-H296L)	-2.3	60.3	-87.5	17.5	-65.6

References

[1] "Takara - In-Fusion Coloning Technique," can be found under https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-faqs, **2019**.

[2] H. Sawai, H. Sugimoto, Y. Kato, Y. Asano, Y. Shiro, S. Aono, *J. Biol. Chem.* **2009**, *284*, 32089–32096.

[3] *Molecular Operating Enviroment (MOE)*, Chemical Computing Group ULC, Montreal, QC, Canada, **2019**.

Appendix

Primer Sequences

Table 16: Sequences of primer.

Primer	Sequence (5'-3')
T7 Forward	TAATACGACTCACTATAGGG
T7 Reverse	GCTAGTTATTGCTCAGCGG
pET28(a)+ In-Fusion® Forward	TGGCTGCCGCGCGGCACCAGGCC
pET28(a)+ In-Fusion® Reverse	AGCTTGCGGCCGCACTCGAGCACC
OxdRE In-Fusion® Forward	GCCGCGCGGCAGCCATATGGAATCTGCAAT
OxdRE In-Fusion® Reverse	GTGCGGCCGCAAGCTTTCAGTGCTCGGCGA

Sequences of OxdRE-wild type and OxdRE-variants

Sequences of aldoximdehydratase N-His_6-OxdRE from *Rhodococcus erythropolis* and N-His_6-OxdRE-mutants

OxdRE accession-no.: BAD17969.1

OxdRE-wild type

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADD
ALQQVVMGYLGVQFRDEDQRPAALQAMRDIVAGFDLPDGPAHHDLTHHIDNQGYE
NLIVVGYWKDVSSQHRWSTSTPIASWWESEDRLSDGLGFFREIVAPRAEQFETLYAF
QEDLPGVGAVMDGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGG
RVVVRGHDNIALIRSGQDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSN
RFVRNIDIDGNFLDLSYNIGHWASLDQLERWSESHPTHLRIFTTFFRVAAGLSKLRLY
HEVSVFDAADQLYEYINCHPGTGMLRDAVTIAEH

OxdRE-variant P1A11 (E150D)

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADD
ALQQVVMGYLGVQFRDEDQRPAALQAMRDIVAGFDLPDGPAHHDLTHHIDNQGYE
NLIVVGYWKDVSSQHRWSTSTPIASWWESEDRLSDGLGFFREIVAPRAEQFETLYAF

QDDLPGVGAVMDGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGG
RVVVRGHDNIALIRSGQDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSN
RFVRNIDIDGNFLDLSYNIGHWASLDQLERWSESHPTHLRIFTTFFRVAAGLSKLRLY
HEVSVFDAADQLYEYINCHPGTGMLRDAVTIAEH

OdRE-variant P2E4 (L60P, D65G, I85V, L145P, I214V, D229N, R231C, D247H, N272S, H296L)

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADD
ALQQVVMGYLGVQFRDEDQRPAAPQAMRGIVAGFDLPDGPAHHDLTHHVDNQGYE
NLIVVGYWKDVSSQHRWSTSTPIASWWESEDRLSDGLGFFREIVAPRAEQFETPYAF
QEDLPGVGAVMDGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGG
RVVVRGHDNVALIRSGQDWADAEANECSLYLDEILPTLQSGMHFLRDNGPAVGCYS
NRFVRNIDIDGSFLDLSYNIGHWASLDQLERWSESLPTHLRIFTTFFRVAAGLSKLRLY
HEVSVFDAADQLYEYINCHPGTGMLRDAVTIAEH

OxdRE-variant P2G4 (D36A, R308G)

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADA
ALQQVVMGYLGVQFRDEDQRPAALQAMRDIVAGFDLPDGPAHHDLTHHIDNQGYE
NLIVVGYWKDVSSQHRWSTSTPIASWWESEDRLSDGLGFFREIVAPRAEQFETLYAF
QEDLPGVGAVMDGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGG
RVVVRGHDNIALIRSGQDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSN
RFVRNIDIDGNFLDLSYNIGHWASLDQLERWSESHPTHLRIFTTFFGVAAGLSKLRLY
HEVSVFDAADQLYEYINCHPGTGMLRDAVTIAEH

OxdRE-variant P3C1 (W117T)

Amino acid sequence:

MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADD
ALQQVVMGYLGVQFRDEDQRPAALQAMRDIVAGFDLPDGPAHHDLTHHIDNQGYE
NLIVVGYWKDVSSQHRWSTSTPIASTWESEDRLSDGLGFFREIVAPRAEQFETLYAFQ
EDLPGVGAVMDGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGGR
VVVRGHDNIALIRSGQDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSNR

FVRNIDIDGNFLDLSYNIGHWASLDQLERWSESHPTHLRIFTTFFRVAAGLSKLRLYH
EVSVFDAADQLYEYINCHPGTGMLRDAVTIAEH

6.5 Article 8 *submitted*

Chemoenzymatic cascades towards aliphatic nitriles starting from biorenewable feedstocks

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ACS Sustainable Chemistry & Engineering **2020**, submitted manuscript

Manuscript-ID: sc-2020-04981j

Author contributions

HG initiated the project. AH designed and performed all experiments for mononitrile synthesis, MS for dinitrile synthesis. AH wrote the manuscript with the help of MS. AH, MS and HG read and edited the manuscript.

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Chemoenzymatic cascades towards aliphatic nitriles starting from biorenewable feedstocks

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Aldoxime dehydratase, Nitriles, Cascade reactions, TEMPO-oxidation, Biorenewables

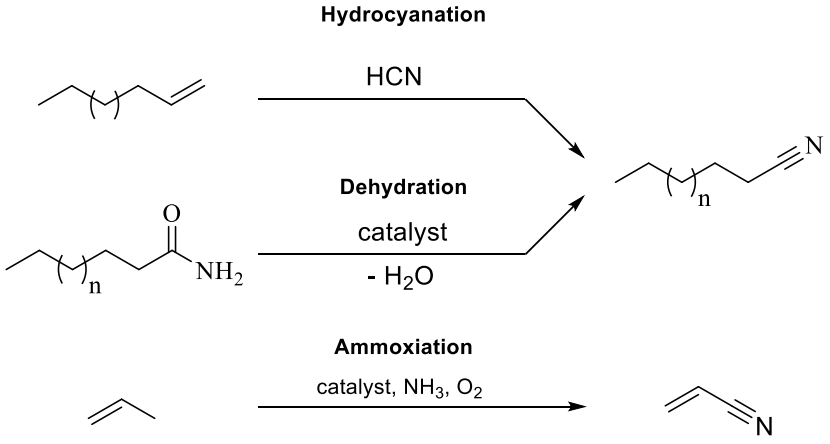
Abstract

A modern concept of producing nitriles involves aldoxime dehydratases, which are capable of dehydrating aldoximes to the corresponding nitriles without need of a cofactor or toxic cyanide salts. Aldoximes as starting material for nitrile synthesis are easily prepared by condensation of hydroxylamine with the corresponding aldehydes. In this contribution we present chemoenzymatic cascade reactions towards nitriles consisting of an initial nitroxyl-radical catalyzed oxidation of aliphatic alcohols to aldehydes using sodium hypochlorite as oxidation agent, subsequent condensation of the aldehyde with hydroxylamine and a biocatalytic dehydration using aldoxime dehydratases to the corresponding nitriles without isolation of intermediates and using the product nitrile as a solvent. These formal "solvent-free" cascades open up the possibility to use

biorenewable sources, namely fatty acids, as starting material for a chemoenzymatic nitrile synthesis. We were also able to apply this cascade concept for the synthesis of aliphatic dinitriles, which are used as, e.g., precursor for polymer building blocks. Overall yields without isolation of intermediates of up to 70% with very simple product isolation were achieved.

Introduction

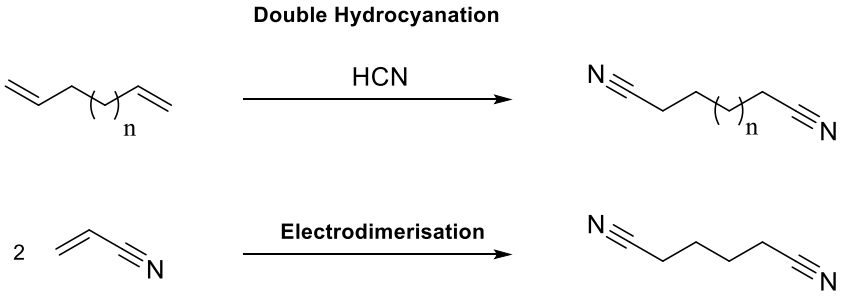
Aliphatic nitriles are valuable products of the chemical industry and are broadly used, for example, as solvents (e.g. acetonitrile) or as precursors for other chemical products such as amines.[1–4] These nitriles are high-volume-low-price products and currently synthesized in particular by means of hydrocyanation, ammoxidation or dehydration of amides (Scheme 1).[1,3]



Scheme 1. Examples for chemical methods to synthesize nitriles.

However, not only aliphatic nitriles with one terminal nitrile functionality are valuable products, but also the linear dinitriles. Especially adiponitrile is of interest due to its use as precursor for 1,6-

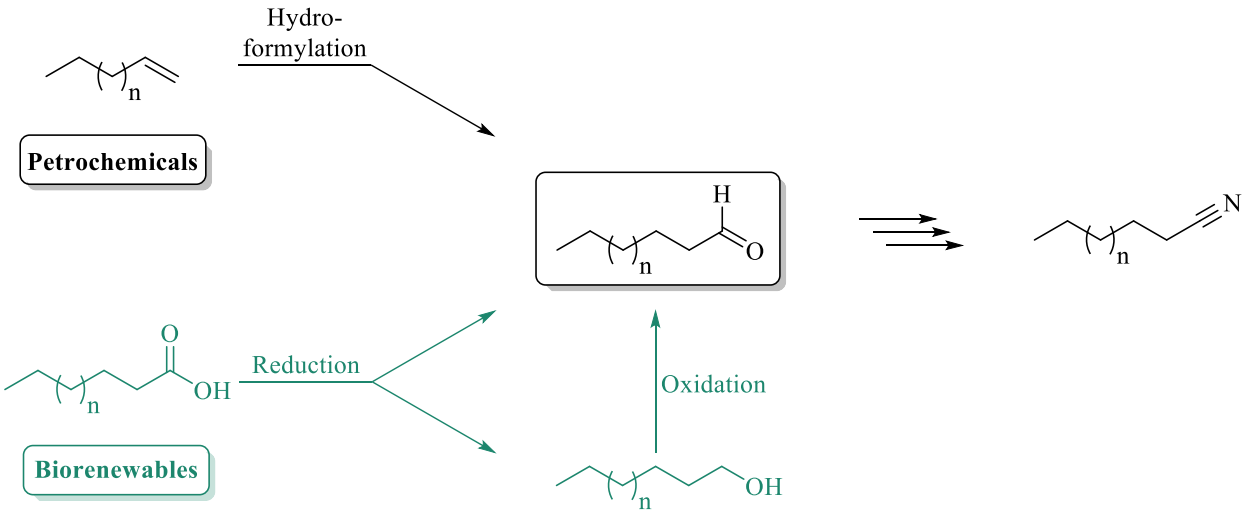
hexanediamine,[1,5,6] which is needed for the manufacture of polyamides like nylon-6,6.[7,8] Also in this case the dinitrile is a high-volume-low-price product and needs to be efficiently produced based on a cost-effective methodology. Besides adiponitrile also other linear, aliphatic dinitriles are interesting compounds, such as octanedinitrile, which can be used as non-classical solvent or as precursor for the synthesis of 1,8-octanediamine or other compounds.[9–11] Dinitriles are currently preferably synthesized by means of a double hydrocyanation of dienes or electrodimerization of acrylonitrile, which is applied for the production of adiponitrile (Scheme 2).[12–14]



Scheme 2. Examples for chemical methods to synthesize aliphatic dinitriles.

These methods to synthesize mono- and dinitriles chemically as shown in Scheme 1 and Scheme 2 have several drawbacks. Not only highly toxic cyanide salts have to be used in some of these methods, but also very high reaction temperatures are often needed.[3] Amides as precursor for nitriles require tedious synthesis of the amides and ammoxidation reactions are performed in gas phase at very high temperatures and might lead to selectivity concerns. As an alternative to these chemical methods, aldoxime dehydratases (Oxds) as biocatalyst can be used for the synthesis of nitriles.[15–36] These enzymes catalyze the dehydration of aldoximes to nitriles without need of a cofactor and at moderate reaction temperatures in water. Oxds have recently be found to be efficient catalysts for the synthesis of aliphatic mono- and dinitriles.[24,25] Especially OxdB, an Oxd from *Bacillus* sp. OxB-1, turned out to be a highly suitable catalyst for preparing these nitrile

products.[25] Aldoximes as precursors in such nitrile syntheses are easily accessible by condensation of aldehydes with hydroxylamine. This reaction can be performed at room temperature and proceeds spontaneously without the need of a catalyst. Since aldoximes can be obtained from aldehydes easily, a very important issue of producing nitriles using Oxds is the access to the linear aldehydes. Here, different methods to produce aldehydes are conceivable (Scheme 3).



Scheme 3. Possible chemical routes towards aliphatic aldehydes.

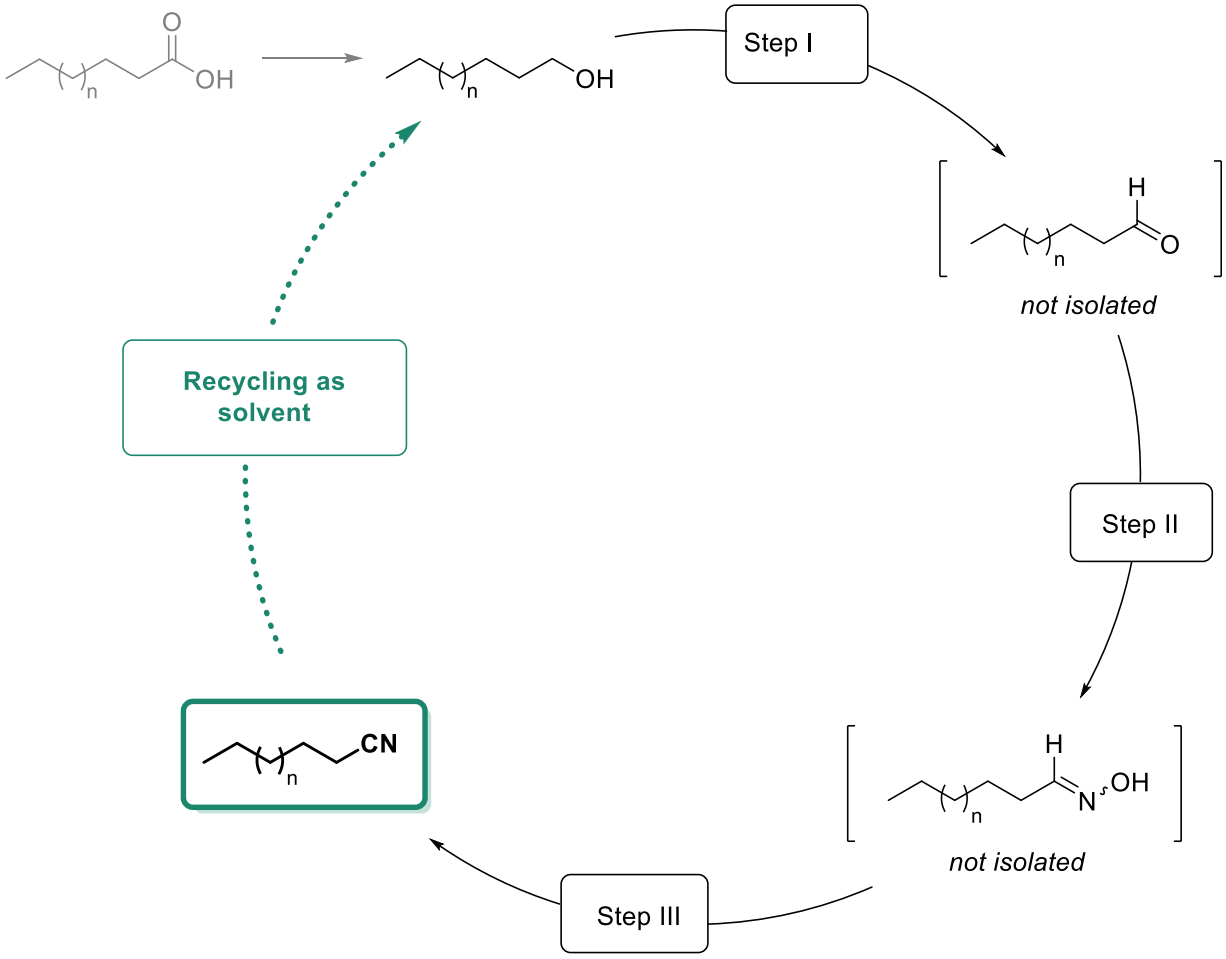
Starting from petrochemical resources, aldehydes are easily accessible by hydroformylation of refinery-based alkenes.[26,37] Our group recently developed a chemoenzymatic cascade towards aliphatic nitriles starting from 1-octene.[26] In this cascade 1-octene is hydroformylated in a biphasic reaction medium to a mixture of *n*- and *iso*-aldehydes. Both aldehydes were then condensed with hydroxylamine hydrochloride after a phase separation. Residual hydroxylamine hydrochloride was decomposed by heating overnight, since hydroxylamine is very toxic for Oxds. In a last step the aldoxime is biocatalytically dehydrated to the corresponding nitrile by Oxds. Another, more sustainable source for the production of such aldehydes are biorenewable raw materials such as

fatty acids. Acids themselves cannot be easily reduced to aldehydes directly. Therefore, a more favored way to get aldehydes from fatty acids is a two-step process consisting of an initial reduction of the acids to alcohols, followed by their selective oxidation to the aldehyde. The oxidation of mono- and dialcohols in aliphatic nitriles as a solvent to (di)aldehydes was recently published by our group based on a nitroxyl radical-catalyzed oxidation methodology.[38] In this method sodium hypochlorite is used as oxidation agent and moderate reaction temperatures at 0 °C were applied. It was shown that nitriles can be used as solvents instead of dichloromethane, which makes this method even more attractive as the same nitrile being the desired product can also be applied as a solvent. Within this project our goal was to perform chemoenzymatic cascade reactions towards nitriles starting from biorenewable raw materials. Since it was already shown that the oxidation of alcohols to aldehydes can be performed selectively in aliphatic nitriles as solvent, we investigated the possibility to perform a chemoenzymatic cascade in the nitrile product as solvent for each step.

Results and Discussion

In the present work, an alternative access towards aliphatic nitriles through chemoenzymatic cascade reactions starting from biorenewable resources was investigated (Scheme 4). We decided to start our chemoenzymatic cascade reactions with the oxidation of primary aliphatic alcohols to the corresponding aldehydes, since such alcohols are, at least in part, accessible from biorenewable fatty acids as shown in many examples.[3,39,40] After this selective alcohol oxidation to aldehydes, the products were converted with hydroxylamine hydrochloride to the corresponding aldoximes. In the last step of the cascade, the aldoximes are dehydrated to the nitriles using a biocatalyst,

namely an aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB). Our goal was to develop a cascade without isolation of the aldehydes and aldoximes formed as intermediates. One major challenge for this cascade was the replacement of dichloromethane as unfavorable organic solvent used in a biphasic mixture with water for the TEMPO-catalyzed oxidation of alcohols to aldehydes in the presence of hypochlorite as oxidation agent. Besides its detrimental effects on the environment and its toxicity, dichloromethane typically cannot be used as solvent for biocatalysis due to inactivation effects on enzymes.[41] Another hurdle for the combination of all three steps is the toxicity of hydroxylamine for both, the TEMPO-oxidation (results not shown) and the aldoxime dehydratase-catalyzed dehydration reaction.[26] Since our group recently found that the TEMPO-derivative catalyzed oxidation of alcohols to aldehydes can selectively be performed in aliphatic nitriles[38] and aldoxime synthesis can be performed in biphasic systems with nearly every organic solvent together with water, we first focused on the development of a two-step one-pot transformation of alcohols into aldoximes and a subsequent separate step towards the nitriles as final step of this cascade, which would allow the removal of the residual amounts of hydroxylamine hydrochloride from the aldoximes. An ideal cascade for the synthesis of nitriles using the three steps oxidation, condensation and dehydration would be a one-pot reaction in which all reactions can be carried out in the same solvent. Since the TEMPO-oxidation can be performed in aliphatic nitriles as solvent and the condensation can be performed in biphasic reaction media, the solvent of choice for such an ideal cascade would be the product itself. The development of such a cascade was the major goal of the present work.

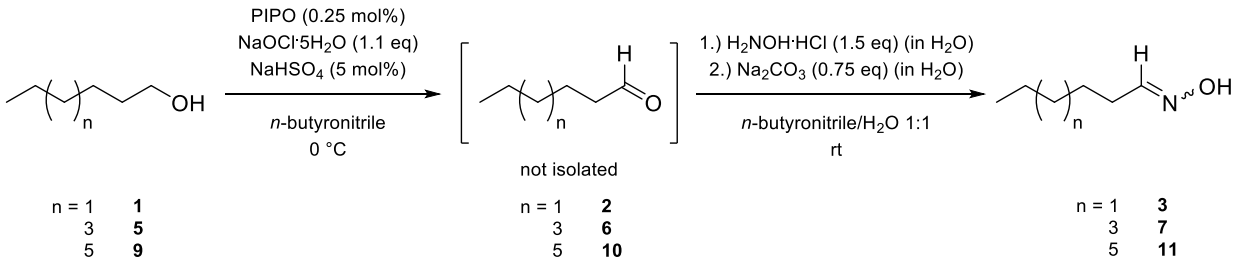


Scheme 4. Chemoenzymatic cascade towards aliphatic (di)nitriles starting from fatty acids as biorenewable source.

Two-step one-pot cascade of alcohols to aldoximes and subsequent biotransformation to nitriles

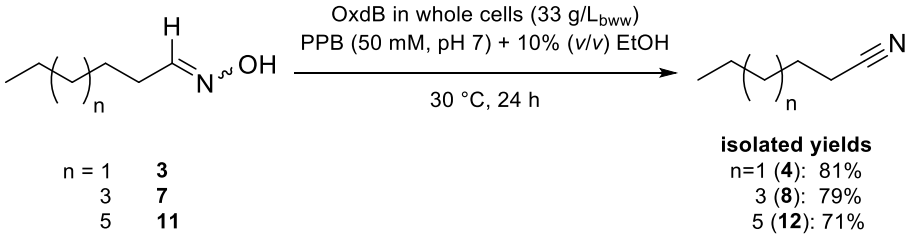
The two-step one-pot process towards the needed aldoximes is performed by addition of an aqueous hydroxylamine hydrochloride solution to the TEMPO-oxidation mixture after its completion. After phase separation and removal of the solvent from the aldoximes, they are converted to the nitriles using OxdB in whole cells as a biocatalyst (Table 1).

Table 1. Synthesis of *n*-hexanaloxime (**3**), *n*-octanaloxime (**7**) and *n*-decanaloxime (**11**) in a two-step one-pot cascade reaction.



n =	**Conversion alcohol**	**Conversion aldehyde to aldoxime**	**Isolated yield (2 steps)**
1	97% aldehyde (3% acid)	>99%	64%
3	99% aldehyde (1% acid)	>99%	81%
5	99% aldehyde (1% acid)	>99%	70%

For the first step of the cascade we could reach full conversion with only small amounts of by-products for all alcohols tested. In a second step all aldehydes were quantitatively converted to the corresponding aldoximes with isolated yields of 64-81%. After the aldoxime synthesis, the biotransformation was performed as reported previously by us using the enzyme OxdB in *E. coli* BL21-CodonPlus(DE3)-RIL+pUC18-OxdB whole cells as catalyst in an aqueous reaction medium consisting of potassium phosphate buffer (PPB) (pH 7) and 10% (*v*/*v*) of ethanol as a co-solvent (Scheme 5).[25]



Scheme 5. Biotransformation of aldoximes to nitriles using OxdB in whole cells in aqueous reaction medium.

These biotransformations of *n*-hexanaloxime (**3**), *n*-octanaloxime (**7**) and *n*-decanaloxime (**11**) as substrates led to full conversions and 71-81% isolated yields of the desired nitrile products.

T able 2. Isolated yields over all three steps consisting of a two-step one-pot synthesis of aldoximes from alcohols and subsequent biocatalytic dehydration in aqueous reaction media.

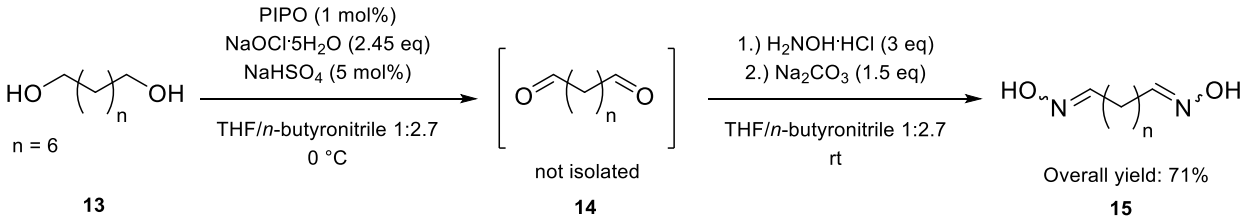
n =	**Isolated yield of aldoxime (2 steps)**	**Isolated yield of nitrile (3. step)**	**Isolated yield of nitrile (Overall yield)**
1	64%	81%	52%
3	81%	79%	64%
5	70%	71%	50%

Thus, for the overall process consisting of the initial two-step one-pot sequence from alcohols to aldoximes and subsequent enzymatic nitrile formation, overall yields of 52% for C_6, 64% for C_8 and 50% for C_{10} were reached (Table 2).

Synthesis of aliphatic dinitriles using the two-step one-pot cascade followed by a biotransformation

Besides the synthesis of monofunctionalized aliphatic nitriles, we also were interested in synthesizing aliphatic dinitriles, which serve as precursors for polymers. As a first step in such a two-step one-pot cascade, a TEMPO-catalyzed oxidation of *n*-octandiol (**13**) to *n*-octandial (**14**)

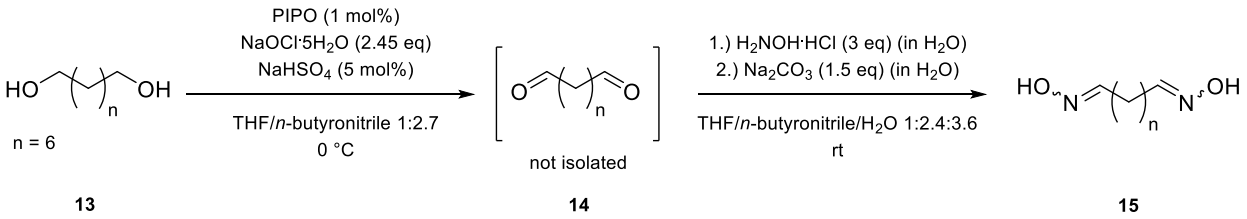
was performed, and after completion of the reaction hydroxylamine hydrochloride and sodium carbonate solution was added (Scheme 6).



Scheme 6. Reaction scheme of the initial two-step-one-pot *n*-octanedialdoxime (**15**) synthesis.

Shortly after addition of the hydroxylamine salt, we observed already precipitation of a colorless solid. The reaction was then further stirred until complete consumption was confirmed and the colorless solid was filtered and washed with water and ethanol to remove residual hydroxylamine and PIPO. An analysis via GC and NMR proved that the colorless solid was the product *n*-octanedialdoxime (**15**), which could be isolated in 58% yield. Although full conversion was achieved in both steps, only a moderate yield was obtained. To increase the yield, a study on the optimization of the work-up was conducted, in which the amount of water was varied and different work-up methods were tested. Since we could not change the reaction conditions of the TEMPO-oxidation without a decrease in selectivity, the only option for optimization of the solvent system was the amount of water in the reaction. The amount of water was subsequently decreased until no water was added after the TEMPO-oxidation. We were pleased to find that even without the addition of water the condensation with hydroxylamine hydrochloride can be performed with full conversion at a prolonged reaction time of 24 h (see Supplementary information). After the water content was optimized, an analysis of the product loss during work-up was carried out. It was found that the major loss of product is due to the presence of *n*-butyronitrile and the washing step

with ethanol. Therefore, we decided to remove the solvent after the reaction and crush the residual solid with a mortar. The solid was then washed with water and a small amount of ethanol. The washing step with ethanol cannot be avoided since the PIPO has to be removed after the reaction.

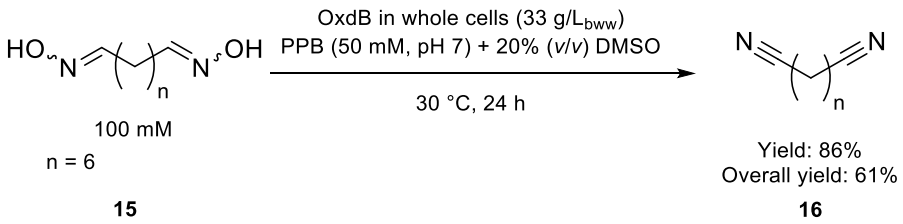


Scheme 7. Reaction scheme of the two-step-one-pot *n*-octanedialdoxime (**15**) synthesis with optimized conditions.

With this work-up method in hand, we then could convert *n*-octanediol (**13**) to *n*-octanedialdoxime (**15**) with full conversion and a selectivity of 83% for the TEMPO-oxidation and full conversion for the condensation with hydroxylamine hydrochloride leading to the desired aldoxime in 71% a yield.

The next step towards the desired dinitriles was the biocatalytic conversion of *n*-octanedialdoxime (**15**) to *n*-octanedinitrile (**16**) with OxdB as a whole cell catalyst. Since we expected a lower activity of OxdB towards *n*-octanedialdoxime (**15**) due to the very low solubility of substrate **15**, the substrate loading was reduced to 100 mM and DMSO (20%) was added as co-solvent. We could isolate 86% of the dinitrile after 24 h reaction time with a purity of 98% (Scheme 8). Although only small amounts of the non-converted dialdoxime were observed, a conversion could not be determined due to the very low solubility of the substrate in nearly all organic solvents used for extraction. Thus, even at low substrate concentration, full extraction of the substrate was not achieved. In a next step the substrate loading was increased to 200 mM. However, already at

a substrate loading of 200 mM and 48h reaction time solid substrate was still present in the reaction medium. Thus, additional OxdB whole cell catalyst was added and the reaction was extended to 72 h. After work-up, the dinitrile **16** was obtained in 68% yield at a purity of 86%. The major impurity was residual *n*-octanedialdoxime (**15**), which underlines the challenge to increase substrate loading even at higher catalyst loadings.



Scheme 8. Biotransformation of *n*-octanedialdoxime (**15**) using OxdB in whole cells in aqueous reaction medium (overall yield from diol **13**).

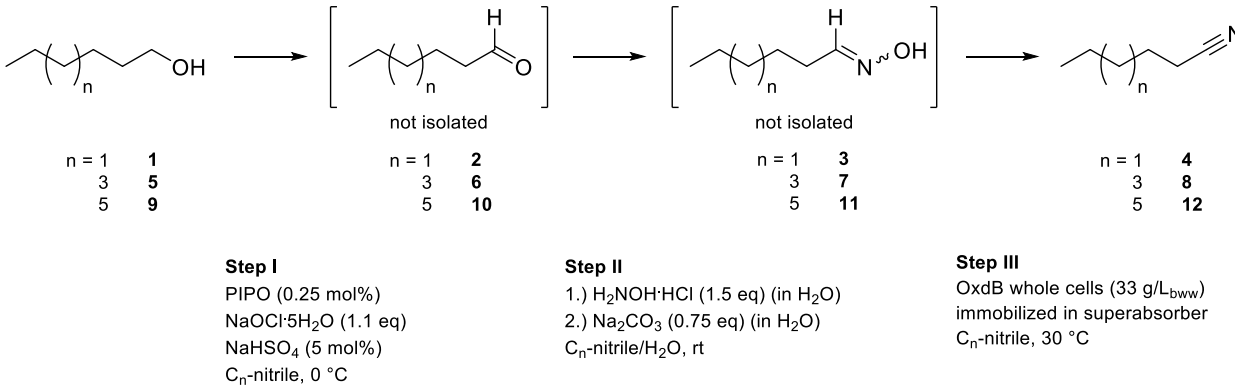
Taking the high yield of 86% achieved in the experiment at 100 mM substrate concentration into account, over all three steps *n*-octanediol (**13**) was converted to *n*-octanedinitrile (**16**) with an overall yield of 61% (Scheme 8).

Three-step chemoenzymatic cascade towards aliphatic nitriles without isolation of intermediates

Since our goal was the development of a chemoenzymatic cascade from alcohols to nitriles without isolation of the intermediates aldehyde and aldoximes, we further improved our cascade by skipping the isolation step of the aldoxime. Taking into account that the PIPO-catalyzed oxidation can also be performed in longer chain aliphatic nitriles as solvents, e.g., in *n*-hexanenitrile (**4**),

n-octanenitrile (**8**) or *n*-decanenitrile (**12**), which are the final products of our cascades and as aldoxime dehydratases turned out as suitable catalysts in organic media when immobilizing them as whole cells in a superabsorber,[41] we investigated a cascade in the corresponding nitrile products as solvent (Table 3). Accordingly, the PIPO-catalyzed oxidation has been performed in the related nitrile product as a solvent, followed by addition of a hydroxylamine hydrochloride solution in water after completion of the oxidation reaction (GC-control) to the reaction medium and stirring at room temperature until complete conversion of the aldehyde to the aldoxime. Subsequently, the phases were separated and the organic phase containing the aldoxime was then directly used for the biotransformation. Toward this end, OxdB whole cells in PPB were immobilized in the superabsorber and then treated with the aldoxime dissolved in the corresponding nitrile solvent. The reaction was monitored by GC and conversion was determined by following the decrease of the aldoxime peak and comparison to standard curves.

Table 3. Chemoenzymatic cascade reaction towards aliphatic nitriles without isolation of aldehyde and aldoxime intermediates using immobilized OxdB whole cells.

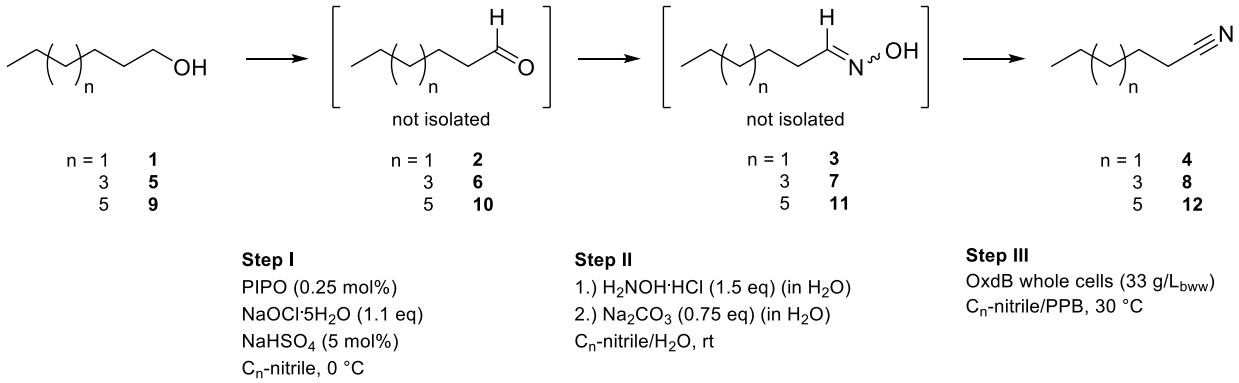


n =	**Conversion of alcohol**	**Conversion aldehyde to aldoxime**	**Conversion aldoxime to nitrile**	**Isolated yield (3 steps)**
1	99% aldehyde (1% acid)	>99%	>99%	63%
3	99% aldehyde (1% acid)	>99%	>99%	70%
5	99% aldehyde (1% acid)	>99%	>99%	67%

As shown in Table 3, very high conversions for every step of the cascade and high isolated yields of up to 70% over three steps were obtained. Another advantage of this cascade is the simple isolation of the product. The cascade is performed in the nitrile product as organic solvent, thus a

removal of solvent is not required at the end of the process and only a simple filtration of the superabsorber bearing the catalyst is needed to isolate the product. Due to the strategy to use the nitrile product at the same time as a solvent, the resulting nitrile product solution already represents the pure product. Since we were operating under "solvent-free" conditions, washing of the superabsorber and all other materials was not possible. Thus, washing of the superabsorber-immobilized catalyst and the used glass material could further increase the isolated yields. The immobilization of the Oxd cells in a superabsorber matrix was chosen as catalyst formulation since Oxd-whole cell catalysts were found earlier to be unstable in organic solvents or aqueous-organic biphasic reaction systems.[41] However, our group recently found that in the absence of organic solvents aliphatic nitrile synthesis in aqueous reaction medium of chain length between C_6 and C_{10} can be performed with substrate loadings of up to 1.4 kg/L, resulting in a biphasic reaction medium of buffer and the product nitrile as organic phase. Since the biocatalyst seem to accept the product nitriles as second phase in these reactions, next we performed our cascade reaction using non-immobilized cells to validate the hypothesis that such a process can be also conducted without the need to immobilize the biocatalyst (Table 4).

Table 4. Chemoenzymatic cascade reaction towards aliphatic nitriles without isolation of aldehyde and aldoxime intermediates using OxdB free whole cells.



n =	**Conversion of alcohol**	**Conversion aldehyde to aldoxime**	**Conversion aldoxime to nitrile**	**Isolated yield (2 steps)**
1	99% aldehyde (1% acid)	>99%	92%	60%
3	99% aldehyde (1% acid)	>99%	95%	71%
5	99% aldehyde (1% acid)	>99%	93%	63%

We were pleased to find that nearly quantitative conversions could be reached. The resulting organic phases were afterwards purified via automated column chromatography to remove the residual aldoximes yielding in the pure products. With this approach we could achieve nearly the same yields in comparison to using immobilized whole cells. Besides the high efficiency of the

developed cascade, this study also reveals that nitriles are unusual and at the same time promising solvents not only for biocatalysis, but also for chemocatalytic and "classic" non-catalyzed organic reactions. Since we found that aliphatic nitriles have only a minor deactivating effect on aldoxime dehydratase whole cell catalysts, such a beneficial effect might be also found for other whole cell catalysts, which cannot be used in biphasic reaction systems with other organic solvents.

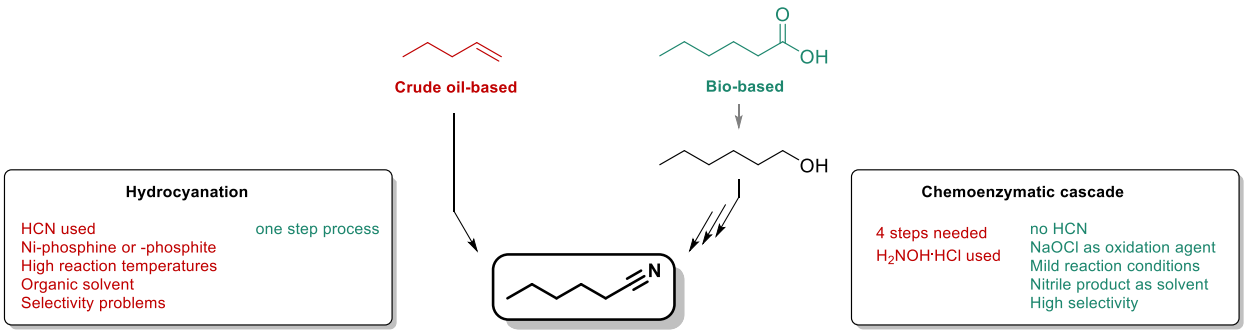
In order to transfer the conditions of this cascade to the synthesis of analogous dialdoximes, these substrates would have to be dissolved in the corresponding dinitrile after the TEMPO oxidation. However, this has turned out to be difficult for *n*-octanedialdoxime (**15**). The replacement of *n*-butyronitrile with *n*-octanedinitrile (**16**) leads to a drastic reduction of the selectivity and furthermore the product *n*-octandialdoxime (**15**) precipitates during the condensation, which makes a filtration step necessary (results not shown).

Apart from this exception, both cascades based on the use of immobilized and non-immobilized biocatalysts, are suitable for the chemoenzymatic three-step-two-pot syntheses of aliphatic nitriles in the product nitriles as solvent, thus leading to high overall yields of up to 70% demonstrated for three different synthetic examples.

Evaluation of different nitrile synthesis concepts

Our developed three-step two-pot cascade without isolation of the intermediates by performing all steps in the nitrile product as a solvent appears to be (at least on longer term) a sustainable alternative for the synthesis of nitriles in comparison to today's industrial scale reactions like hydrocyanation. This outlook should be underlined by an evaluation of this cascade in terms of applicability and sustainability comprising a comparison with existing routes (Scheme 9). One advantage of this cascade is that alcohols being easily accessible from biorenewable resources

serve as starting materials. In contrast, the alkenes for hydrocyanation and other methods are typically based on petrochemical feedstocks. However, it also has to be stated that the hydrocyanation leads directly to nitriles from alkenes, while our cascade needs four steps. On the other hand, three of these four steps can be performed in a three-step two-pot reaction under mild reaction conditions without isolation of the intermediates and the nitrile product as a solvent. Furthermore, the isolation of the product is performed by a simple filtration and no further purification is needed due to the very high selectivity for all steps. This is usually not the case for other nitrile syntheses, which often require some kind of purification to eliminate by-products.[42] Since the intermediates are not isolated and the whole cascade is performed in only one solvent except for an addition of water at the aldoxime stage, only low amounts of waste are produced. This waste is the aqueous phase, which is discarded after the aldoxime formation and could possibly be recycled. Besides this liquid waste, also the solid biocatalyst in case of the cascade using immobilized OxdB cannot be recycled as well as the free OxdB whole cells. The polymer-based TEMPO-derivative PIPO, which we are using as catalyst for the oxidation step, can potentially be recycled after filtration of the heterogeneous catalyst.



Scheme 9. Comparison of nitrile syntheses via a chemoenzymatic cascade reaction starting from biorenewables with the hydrocyanation approach based on petrochemical feedstocks.

Another disadvantage of hydrocyanation is the use of toxic cyanide and nickel-phosphine or nickel-phosphite catalysts. Within our cascade reaction we do not need to use cyanide or metal-catalysts, but it has to be mentioned that other harmful ingredients are used. The first step, namely the oxidation of alcohols, requires sodium hypochlorite as oxidation agent which is known as green alternative for many other oxidation agents.[43] As solvent for the TEMPO-oxidation in the first cascade with isolation of the aldoxime butyronitrile was used as solvent. This solvent is toxic by exposure and flammable.[44] However, at least for the mono-nitriles this solvent could be replaced by the product nitrile as solvent for all steps. In the second step of the cascades, the aldehydes are condensed with hydroxylamine hydrochloride, which is explosive and harmful to humans and the environment.[45] Furthermore, hydroxylamine is produced by reducing nitric oxides with hydrogen,[46] but there is potentially an alternative for the production of hydroxylamine with microorganisms since in some nitrogen-fixating organisms hydroxylamine is formed as a metabolite.[24,47,48] It should be added that today hydroxylamine is utilized on large scale for the manufacture of ε-caprolactam being produced on a multi-million tons scale annually, thus serving as a readily available bulk chemical.

According to our opinion our chemoenzymatic cascade represents a promising sustainable concept towards nitriles, thus serving as a long-term alternative to the "classic" nitrile synthetic approaches. Advantageous are the use of a bio-based starting material and "by definition" the avoidance of toxic cyanide salts and hydrogen cyanide, respectively. Other advantages of the developed chemoenzymatic method are mild reaction conditions, high selectivity, no usage of organic solvents (except the product nitrile) and low amounts of waste due to only one needed isolation step as well as no purification of the product (since the process is carried out in the product nitrile serving as a solvent). However, there are also some challenges ahead which need to be addressed

in the future. In particular, a recycling of the immobilized biocatalyst after the last step of the reaction would decrease the amount of waste significantly and toward this end a sufficient stability of the biocatalyst for re-use still has to be demonstrated. Furthermore, the use of hydroxylamine hydrochloride is problematic. On a long term, this issue could be circumvented by implementing a tailor-made microorganism, which is capable of producing hydroxylamine *in situ* starting from molecular nitrogen being present in air. Such a microorganism could potentially then be used in combination with our Oxd-whole cell catalyst to condense aldehydes with hydroxylamine *in situ* followed by a direct dehydration to the nitriles catalyzed by Oxds.

Conclusion

In conclusion, we investigated different variations of chemoenzymatic cascades starting from alcohols being readily accessible from biorenewable fatty acids. These cascades start with a nitroxyl radical-catalyzed oxidation using hypochlorite as oxidation agent. In a subsequent reaction, without isolation and in a one-pot fashion the aldehyde undergoes a condensation with hydroxylamine, thus forming the corresponding aldoxime. The final step consists of an enzymatic dehydration by means of an aldoxime dehydratase, leading to the desired nitriles. This cascade was implemented for aliphatic nitriles with a chain length of C_6, C_8 and C_{10}. Overall yields of up to 70% could be obtained without isolation of the aldehyde and aldoxime intermediates and with just one phase separation step at the aldoxime stage. Utilizing the product at the same time as solvent, no product purification is needed at the final stage of the cascade. Thus, this cascade concept provides a new and alternative access to aliphatic nitriles starting from fatty acids as bio-based material, which gives these desired products with high conversion, selectivity and yield.

ASSOCIATED CONTENT

Supporting Information. Experimental description and data (.pdf)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. ‡

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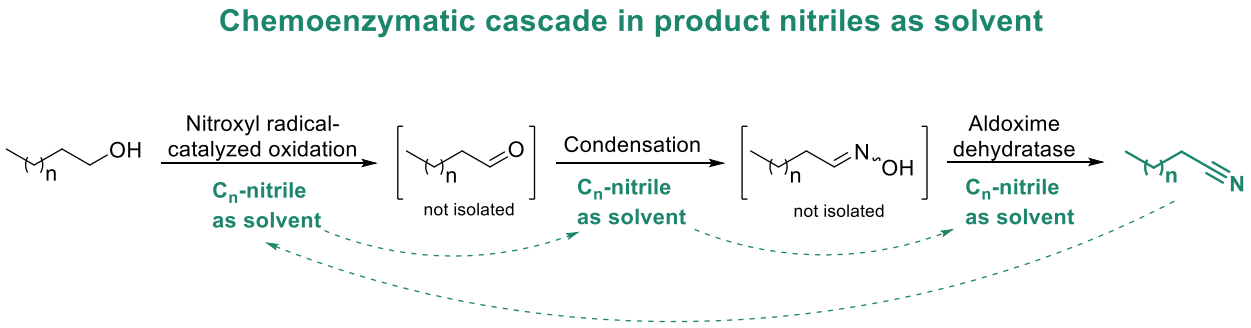
SYNOPSIS

Chemoenzymatic cascades towards nitriles were developed using fatty acids as biorenewable resources instead of petrochemicals. All steps are carried out under mild reaction conditions and in the product as solvent, producing low amounts of waste.

TOC



Supplementary Information

Chemoenzymatic cascade towards aliphatic mono- and dinitriles starting from biorenewable feedstocks

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Biocatalytic dehydration of *n*-octanedialdoxime (15) using *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cell catalyst 200 mM substrate loading 13

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1. Experimental information

1.1 Chemicals used

Material for cell cultivation and aldoxime dehydratase (Oxd) expression was purchased by Carl Roth (Antibiotics, LB- and TB-premixed medium, D-glucose, D-lactose). Buffer salts (KH_2PO_4 and K_2HPO_4) were obtained from VWR chemicals.

n-Hexan-1-ol, *n*-ctanan-1-ol and *n*-decan-1-ol were purchased by Sigma Aldrich and the oxidant $NaOCl \cdot 5H_2O$ was purchased by TCI Chemicals (Germany) and the chemicals were used without further purification. *n*-Hexanal, *n*-octanal, *n*-decanal, and *n*-dodecanal as analytical standard were purchased by Sigma-Aldrich and used without further purification. Chemicals for aldoxime and nitrile synthesis were purchased by VWR Chemicals, Carl Roth, and Fluka Chemicals, respectively, and used without further purification. *n*-hexanenitrile, *n*-octanenitrile, *n*-decanenitrile, and *n*-dodecanenitrile as reference compounds for GC analytics were purchased by Sigma-Aldrich or TCI Chemicals.

1.2 Analytical methods

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^1H) or 125 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$ or DMSO-d6).

Conversions were determined by GC measurements (Shimadzu GC-2010 Plus) in comparison to a calibration curve. Measurements were conducted on a chiral SGE Analytic B6B-174 column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with nitrogen as carrier gas. An injector temperature of 220 °C in a split injection mode was used and a sample amount of 1 µL was injected in this method. The following temperature gradient was used for C_6-cascade (retention time alcohol t_r = 2.0 min, aldehyde t_r = 1.8 min, aldoxime t_r = 4.2 min and nitrile t_r = 4.0 min): 60 °C starting temperature, in 20 °C/min to 150 °C (hold 0.5 min). The following temperature gradient was used for C_8- (alcohol t_r = 2.0 min, aldehyde t_r = 1.8 min. Aldoxime t_r = 2.7 min and nitrile t_r = 2.4 min) and C_{10}-cascades (alcohol t_r = 3.0 min, aldehyde t_r = 2.5 min, aldoxime t_r = 3.8 min and nitrile t_r = 3.4 min): 140 °C starting temperature (hold 1 min), in 20 °C/min to 190 °C (hold 0.5 min) and in 50 °C/min to 200 °C. Accuracy of the GC-analytical methods: the error of the determined conversions to the corresponding nitriles is

in the range of up to 5% depending on the concentration of the aldoximes due to temperature depending degradation of the aldoximes on the GC-column.

Conversion and selectivity for the PIPO catalyzed oxidation of octanediol **13** and the synthesis of octanedialdoxime **15** were determined by GC measurements (Shimadzu GC-2010 Plus). Measurements were conducted on an achiral Zebron Phenomenex ZB-5MSi column (30 m length, 0.25 mm inner diameter, 0.25 µm film thickness) with nitrogen as carrier gas. Selectivities of the oxidation reactions are defined as: $amount_{aldehyde}/(amount_{aldehyde}+amount_{by\text{-}products})$. An injector temperature of 250 °C in a split injection mode was used and a sample amount of 1 µL was injected in this method. The following temperature gradient was used (retention time octanediol **13** t_r = 2.8 min, octanedial **14** t_r = 2.1 min): 130 °C starting temperature for 2 min, in 30 °C/min to 230 °C (hold 0.5 min).

The purity of octanedinitrile **16** was determined with the same column and the following method. An injector temperature of 250 °C in a split injection mode was used and a sample amount of 5 µL was injected in this method. The following temperature gradient was used (retention time octanedialdoxime **15** t_r = 5.1 min, octanedinitrile **16** t_r = 4.2 min): 110 °C starting temperature for 2 min, in 30 °C/min to 260 °C (hold 2.5 min).

2. Aldoxime Dehydratase (Oxd) Sequences, Plasmids and Expression

2.1 Oxd sequence and plasmid

2.1.1 Oxd from *Bacillus* sp. OxB-1 (OxdB) (Accession number: GenBank: AP013294.1)

2.1.1.1 Base sequence (codon-optimized for *E. coli*)

ATGAAAAATATGCCGGAAAATCACAATCCACAAGCGAATGCCTGGACTGCCGAA
TTTCCTCCTGAAATGAGCTATGTAGTATTTGCGCAGATTGGGATTCAAAGCAAGT
CTTTGGATCACGCAGCGGAACATTTGGGAATGATGAAAAAGAGTTTCGATTTGCG
GACAGGCCCCAAACATGTGGATCGAGCCTTGCATCAAGGAGCCGATGGATACCA
AGATTCCATCTTTTTAGCCTACTGGGATGAGCCTGAAACATTTAAATCATGGGTTG
CGGATCCTGAAGTACAAAAGTGGTGGTCGGGTAAAAAAATCGATGAAAATAGTC
CAATCGGGTATTGGAGTGAGGTAACGACCATTCCGATTGATCACTTTGAGACTCT

TCATTCCGGAGAAAATTACGATAATGGGGTTTCACACTTTGTACCGATCAAGCAT
ACAGAAGTCCATGAATATTGGGGAGCAATGCGCGACCGCATGCCGGTGTCTGCC
AGTAGTGATTTGGAAAGCCCCCTTGGCCTTCAATTACCGGAACCCATTGTCCGGG
AGTCTTTCGGAAAACGGCTAAAAGTCACGGCGCCGGATAATATTTGCTTGATTCG
AACCGCTCAAAATTGGTCTAAATGTGGTAGCGGGGAAAGGGAAACGTATATAGG
ACTAGTGGAACCGACCCTCATAAAAGCGAATACGTTTCTTCGTGAAAATGCTAGT
GAAACAGGCTGTATTAGTTCAAAATTAGTCTATGAACAGACCCATGACGGCGAA
ATAGTAGATAAATCATGTGTCATCGGATATTATCTCTCCATGGGGCATCTTGAAC
GCTGGACGCATGATCATCCAACACATAAAGCGATCTACGGAACCTTTTATGAGAT
GTTGAAAAGGCATGATTTTAAGACCGAACTTGCTTTATGGCACGAGGTTTCGGTG
CTTCAATCCAAAGATATCGAGCTTATCTATGTCAACTGCCATCCGAGTACTGGATT
TCTTCCATTCTTTGAAGTGACAGAAATTCAAGAGCCTTTACTGAAAAGCCCTAGC
GTCAGGATCCAGTGA

2.1.1.2 Amino acid sequence

MKNMPENHNPQANAWTAEFPPEMSYVVFAQIGIQSKSLDHAAEHLGMMKKSFDLR
TGPKHVDRALHQGADGYQDSIFLAYWDEPETFKSWVADPEVQKWWSGKKIDENSPI
GYWSEVTTIPIDHFETLHSGENYDNGVSHFVPIKHTEVHEYWGAMRDRMPVSASSDL
ESPLGLQLPEPIVRESFGKRLKVTAPDNICLIRTAQNWSKCGSGERETYIGLVEPTLIKA
NTFLRENASETGCISSKLVYEQTHDGEIVDKSCVIGYYLSMGHLERWTHDHPTHKAIY
GTFYEMLKRHDFKTELALWHEVSVLQSKDIELIYVNCHPSTGFLPFFEVTEIQEPLLKS
PSVRIQ

2.1.1.3 Construct

Table 1. Used vector constructs, origins of the Oxd-genes, provider of the vector constructs and marker-resistance of the constructs.

Origin of Oxd-gene	Vector	Oxd	Provider	Resistance
Bacillus sp. OxB-1	pUC18	OxdB	Asano group	Carbenicillin

2.2 Oxd expression in *E. coli* BL21-CodonPlus(DE3)-RIL

Chemical competent *E- coli* BL21-CodonPlus(DE3)-RIL cells (100 µL) were transformed with pUC18-plasmid DNA containing the gene encoding OxdB analogue to standard protocols.[?]

Transformed *E. coli* cells were plated on LB-agar containing Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) and incubated overnight at 37 °C.

Pre-cultures were prepared in LB-medium (20 mL) in 100 mL Erlenmeyer flasks containing Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) using a single colony from the LB-agar plate. The cultures were incubated overnight at 37 °C and 180 rpm.

Main cultures for the expression of OxdB was performed using TB-autoinduction medium. Sterile 20 g/L D-Lactose solution in MilliQ water (160 mL) and sterile 50 g/L D-glucose solution in MilliQ water (16 mL) was added to 1424 mL sterile TB-medium (Carl Roth) in a 2 L Erlenmeyer flask. Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) were added to the medium. Main cultures were inoculated with 1% (16 mL) of the OxdB pre-culture and incubated for 2 h at 37 °C and 150 rpm. After 2 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 150 rpm.

Cell harvest was performed at 5,000 g for 15 min and 4 °C. The supernatant was discarded and cells were washed three times with 50 mM potassium phosphate buffer (PPB, KPi) at pH 7.0. The biomass was determined (bio wet weight (bww)) and cells were resuspended in 50 mM PPB (pH 7.0) to a final concentration of 333 mg/mL cells in buffer. Cell suspensions were stored at 4 °C or on ice before usage.

Overexpression of Oxds in E. coli Bl21-CodonPlus(DE3)-RIL was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after cell disruption and denaturation of the proteins in the crude extracts.

Crude extracts of 33% wet cell suspensions were obtained by sonication (5x 1 min, 10 – 15% Output, Bandelin Sonopuls®) and subsequent centrifugation at 21,500 xg for 45 min at 4 °C. The pellet including the cell debris was discarded. Protein concentrations in crude extracts were determined by Bradford assay using a bovine serum albumin (BSA)-standard curve (1.4 mg/mL, 0.7 mg/mL, 0.35 mg/mL, 0.175 mg/mL, 0.0875 mg/mL) as reference. Protein dilutions of 1 µg/µL whole cell protein concentration were obtained by dilution of the crude extracts in water and Laemmli-buffer. 10 µL of these samples were transferred to a 12% SDS-PAGE.

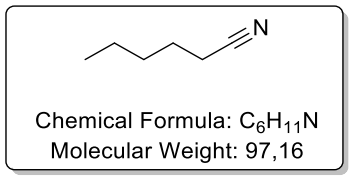
3.Chemoenzymatic cascade reactions

3.1 Mononitriles

3.1.1 Two-step-one-pot cascade of alcohols to aldoximes and subsequent biotransformation to nitriles

Butyronitrile (15 mL) was submitted to a round bottom flask. $NaHSO_4 \cdot H_2O$ (5 mol%, 0.75 mmol, 103 mg), PIPO (0.25 mol%, 0.04 mmol, 112 mg) and $NaOCl \cdot 5H_2O$ (1.1 eq, 30 mmol, 2.7 g) were added and the reaction mixture cooled to 0 °C while stirring. The alcohol (15 mmol, 1 M) was added and the reaction mixture was stirred at 0 °C until a change of the color from yellow to colorless was obtained and complete oxidation was detected by GC-analysis. The reaction was quenched by addition of hydroxylamine hydrochloride (1.5 eq, 22.5 mmol, 1.56 g) and sodium carbonate (0.75 eq, 11.25 mmol, 1.19 g) solution dest. water (15 mL). The biphasic system was stirred at room temperature until the aldehyde was converted completely to the aldoxime (GC-analysis of the organic phase). The phases were separated, the organic phase was washed with brine (1x 15 mL) and dried over $MgSO_4$ before the solvent was removed *in vacuo*. The aldoximes were used for the last step of the cascade without further purification. OxdB whole cells (33 mg/mL) in PPB (total 13.5 mL) were submitted to a round bottom flask and stirred at 30 °C. Ethanol (1.5 mL, 10% (*v*/*v*)) and the aldoxime isolated after step 2 of the cascade were added. The reaction mixture was stirred for 24 h at 30 °C. The nitrile products were isolated by centrifugation (15 min, 4,000 xg) of the reaction mixture and phase separation. After removal of residual ethanol in vacuo, the purity of the nitriles was determined by GC and ^{1}H-NMR analysis in comparison to the one of the commercially available pure nitriles obtained by TCI.

n-Hexanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime). **Yield:** 64%.
Conversion step 3: >99% (>99% nitrile **4**). **Yield:** 81%.
Overall isolated yield: 52%.

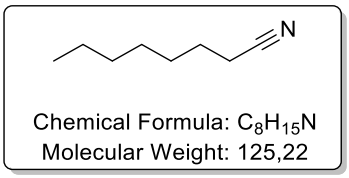
Purity (^{1}H-NMR): >99%.

^{1}H-NMR (*n*-hexanaloxime (*E*/*Z* ratio: 1.00:0.22), 500 MHz, $CDCl_3$) *Z*-aldoxime: δ/ppm = 7.42 (1H, t, C**H**-NOH), 2.18 (2H, td, -C**H$_2$**-CHNOH), 1.49 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.31 (4H, m, H_3C-(C**H$_2$**)$_2$-CH_2-CH_2-CHNOH), 0.91 (3H, m, C**H$_3$**-), *E*-aldoxime: δ/ppm = 6.72 (1H, t, C**H**-NOH), 2.37 (2H, td, -C**H$_2$**-CHNOH), 1.49 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.31 (4H, m, H_3C-(C**H$_2$**)$_2$-CH_2-CH_2-CHNOH), 0.91 (3H, m, C**H$_3$**-).

^{1}H-NMR spectra were found to be in accordance to the literature.[1]

^{1}H-NMR (*n*-hexanenitrile, 500 MHz, $CDCl_3$) δ/ppm = 2.32 (2H, t, -C**H$_2$**-CN), 1.65 (2H, p, -C**H$_2$**-CH_2-CN), 1.42 (2H, m, -C**H$_2$**-CH_2- CH_2-CN) 1.35 (4H, m, H_3C-(C**H$_2$**)$_3$-CH_2- CH_2-CN), 0.91 (3H, m, **H$_3$**C-).

n-Octanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime). **Yield:** 81%.
Conversion step 3: >99% (>99% nitrile). **Yield:** 79%.

Overall isolated yield: 64%.

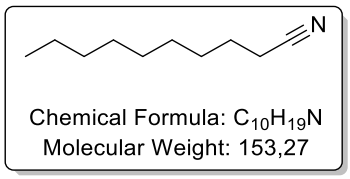
Purity (^{1}H-NMR): >99%.

^{1}H-NMR (*n*-octanaloxime (*E*/*Z* ratio: 1.00:0.48), 500 MHz, $CDCl_3$) *Z*-aldoxime: δ/ppm = 7.41 (1H, t, C**H**-NOH), 2.18 (2H, td, -C**H$_2$**-CHNOH), 1.48 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.31 (8H, m, H_3C-(C**H$_2$**)$_4$-CH_2-CH_2-CHNOH), 0.87 (3H, m, **H$_3$**C-); *E*-aldoxime: δ/ppm = 6.71 (1H, t, -C**H**-NOH), 2.37 (2H, td, -C**H$_2$**-CHNOH), 1.48 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.31 (8H, m, H_3C-(C**H$_2$**)$_4$-CH_2-CH_2-CHNOH), 0.87 (3H, m, **H$_3$**C-).

^{1}H-NMR spectra were found to be in accordance to the literature.[1]

^{1}H-NMR (*n*-octanenitrile, 500 MHz, $CDCl_3$) δ/ppm = 2.32 (2H, t, -C**H$_2$**-CN), 1.64 (2H, p, -C**H$_2$**-CH_2-CN), 1.42 (2H, m, -C**H$_2$**-CH_2- CH_2-CN) 1.28 (8H, m, H_3C-(C**H$_2$**)$_3$-CH_2- CH_2-CN), 0.87 (3H, m, **H$_3$**C-).

n-Decanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime). **Yield:** 70%.
Conversion step 3: >99% (>99% nitrile). **Yield:** 71%.

Overall isolated yield: 50%.

Purity (^{1}H-NMR): >99%.

^{1}H -NMR (*n*-decanaloxime (*E*/*Z* ratio: 1.00:0.43), 500 MHz, $CDCl_3$) *Z*-aldoxime: δ/ppm = 7.42 (1H, t, -C**H**-NOH), 2.19 (2H, td, -C**H$_2$**-CHNOH), 1.48 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.29 (12H, m, H_3C-(C**H$_2$**)$_4$-CH_2-CH_2-CHNOH), 0.88 (3H, m, C**H$_3$**-); *E*-aldoxime: δ/ppm = 6.72 (1H, t, -C**H**-NOH), 2.38 (2H, td, C**H$_2$**-CHNOH), 1.48 (2H, m, -C**H$_2$**-CH_2-CHNOH), 1.29 (12H, m, H_3C-(C**H$_2$**)$_4$-CH_2-CH_2-CHNOH), 0.88 (3H, m, C**H$_3$**-).
^{1}H-NMR spectrum was found to be in accordance to the literature.[1]

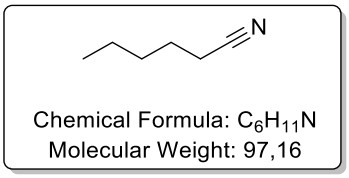
^{1}H-NMR (*n*-decanenitrile, 500 MHz, $CDCl_3$) δ/ppm = 2.33 (2H, t, -C**H$_2$**-CN), 1.65 (2H, p, -C**H$_2$**-CH_2-CN), 1.44 (2H, m, -C**H$_2$**-CH_2- CH_2-CN) 1.27 (12H, m, H_3C-(C**H$_2$**)$_5$-CH_2- CH_2-CN), 0.88 (3H, m, **H$_3$**C-$(CH_2)_4$-CH_2- CH_2-CN).

3.1.2 Three-step chemoenzymatic cascade towards aliphatic nitriles without isolation of intermediates using immobilized OxdB whole cells

The product nitrile (15 mL) was submitted to a round bottom flask. $NaHSO_4 \cdot H_2O$ (5 mol%, 0.75 mmol, 103 mg), PIPO (0.25 mol%, 0.04 mmol, 112 mg) and $NaOCl \cdot 5H_2O$ (1.1 eq, 30 mmol, 2.7 g) were added and the reaction mixture cooled to 0 °C while stirring. The alcohol (15 mmol, 1 M) was added and the reaction mixture was stirred at 0 °C until a change of the color from yellow to colorless was obtained. The reaction was quenched by addition of hydroxylamine hydrochloride (22.5 mmol, 1.56 g) and sodium carbonate (11.25 mmol, 1.19 g) solution dest. water (15 mL). The biphasic system was stirred at room temperature until the aldehyde was converted completely to the aldoxime (GC-analysis of the organic phase). The phases were separated and the organic phase was used for the next step. OxdB whole cells (33 mg/mL total concentration) and potassium phosphate buffer (50 mM, pH 7.0) in (total 15 mL) were attached to a round bottom flask and immobilized in superabsorber (FAVOR® Evonik) (~50 mg). The organic phase was added and the reaction mixture stirred at 30 °C for 24 h.

The nitrile products were isolated by filtration. The purity of the nitriles was determined by GC and ^{1}H-NMR analysis in comparison to the one of the commercially available pure nitriles obtained by TCI.

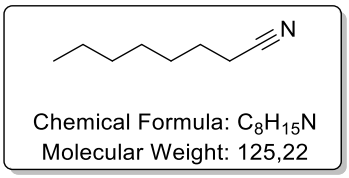
n-Hexanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime).
Conversion step 3: >99% (>99% nitrile).
Overall isolated yield: 63%.

Purity (^{1}H-NMR): >99%.

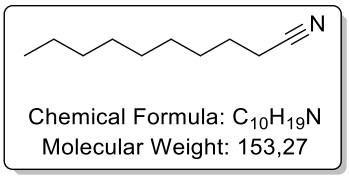
n-Octanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime).
Conversion step 3: >99% (>99% nitrile).
Overall isolated yield: 70%.

Purity (^{1}H-NMR): >99%.

n-Decanenitrile:



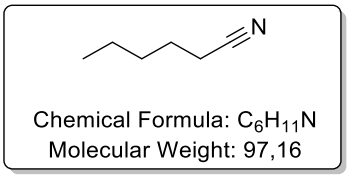
Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime).
Conversion step 3: >99% (>99% nitrile).
Overall isolated yield: 67%.

Purity (^{1}H-NMR): >99%.

3.1.2 Three-step chemoenzymatic cascade towards aliphatic nitriles without isolation of intermediates using free OxdB whole cells

The product nitrile (15 mL) was submitted to a round bottom flask. $NaHSO_4 \cdot H_2O$ (5 mol%, 0.75 mmol, 103 mg), PIPO (0.25 mol%, 0.04 mmol, 112 mg) and $NaOCl \cdot 5H_2O$ (1.1 eq, 30 mmol, 2.7 g) were added and the reaction mixture cooled to 0 °C while stirring. The alcohol (15 mmol, 1 M) was added and the reaction mixture was stirred at 0 °C until a change of the color from yellow to colorless was obtained. The reaction was quenched by addition of hydroxylamine hydrochloride (22.5 mmol, 1.56 g) and sodium carbonate (11.25 mmol, 1.19 g) solution dest. water (15 mL). The biphasic system was stirred at room temperature until the aldehyde was converted completely to the aldoxime (GC-analysis of the organic phase). The phases were separated and the organic phase was used for the next step. OxdB whole cells (33 mg/mL total concentration) and potassium phosphate buffer (50 mM, pH 7.0) in (total 15 mL) were attached to a round bottom flask. The organic phase was added and the reaction mixture stirred at 30 °C for 24 h. The nitrile products were isolated by centrifugation (15 min, 4,000 g) of the reaction mixture and phase separation. The purity of the nitriles was determined by GC and ^{1}H-NMR analysis in comparison to the one of the commercially available pure nitriles obtained by TCI. Nitriles were purified by automated column chromatography (Biotage Isolera One ®) using ethyl acetate and cyclohexane (gradient 5%-50% ethyl acetate) as solvent, SnapUltra cartridge (50 g) and a flow of 50 mL/min.

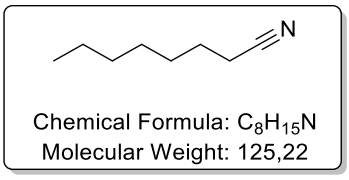
n-Hexanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime).
Conversion step 3: 92% (>99% nitrile).
Overall isolated yield: 60%.

^{1}H-NMR was found to be in accordance to the literature.[2]
Purity after column chromatography(^{1}H-NMR): >99%.

n-Octanenitrile:

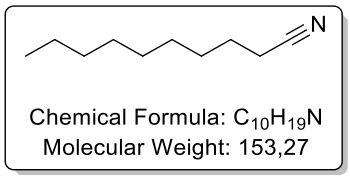


Conversion step 1: >99% (99% aldehyde, 1% acid).
Conversion step 2: >99% (>99% aldoxime).
Conversion step 3: 95% (>99% nitrile).
Overall isolated yield: 71%.

^{1}H-NMR was found to be in accordance to the literature. [2]

Purity after column chromatography (^{1}H-NMR): >99%.

n-Decanenitrile:



Conversion step 1: >99% (99% aldehyde, 1% acid).

Conversion step 2: >99% (>99% aldoxime).

Conversion step 3: 93% (>99% nitrile).

Overall isolated yield: 63%.

^{1}H-NMR was found to be in accordance to the literature. [2]

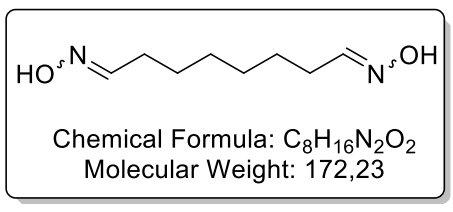
Purity after column chromatography (^{1}H-NMR): >99%.

3.2 Dinitriles

3.2.1 Two-step-one-pot *n*-octanedialdoxime (15) synthesis with addition of water (unoptimized)

$NaHSO_4 \cdot H_2O$ (0.375 mmol, 51.8 mg, 5 mol%) and sodium hypochlorite pentahydrate (18.4 mmol, 3.0 g, 2.45 eq) were suspended in *n*-butyronitrile (11 mL) and cooled to 0 °C. PIPO (0.075 mmol, 225 mg, 1 mol%) was added. The primary *n*-octanediol (7.5 mmol, 1.1 g, 0.5 M) was dissolved in THF (4 mL, 27% *v*/*v*) by gently warming. After cooling to rt the solution was added to the reaction mixture. The reaction mixture was stirred at 0 °C until a color change from red to colorless occurred. $H_2NOH \cdot HCl$ (22.5 mmol, 1.6 g, 3 eq) was dissolved in water (5 mL) and added to the reaction mixture and Na_2CO_3 (11.3 mmol, 1.2 g, 1.5 eq) was dissolved in water (5 mL) and added to the reaction mixture. The reaction was slowly warmed to rt under stirring. After 6 h full conversion was obtained (GC analysis of the organic phase). The suspension was then filtered and washed with water and ethanol. The product was dried *in vacuo* and analyzed using GC and ^{1}H-NMR spectroscopy in DMSO-d_6.

n-Octanedialdoxime:



Conversion step 1: >99%

Conversion step 3: >99%

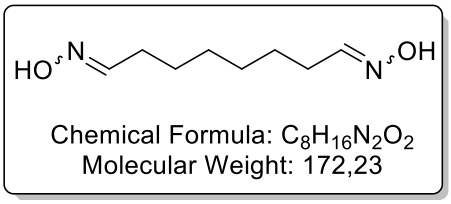
Overall yield: 58%

^{1}H-NMR was found to be in accordance to the literature. [2]

3.2.2 Two-step-one-pot synthesis of *n*-octanedialdoxime (15) without the addition of water and optimized work-up

$NaHSO_4 \cdot H_2O$ (0.375 mmol, 51.8 mg, 5 mol%) and sodium hypochlorite pentahydrate (18.4 mmol, 3.0 g, 2.45 eq) were suspended in *n*-butyronitrile (11 mL) and cooled to 0 °C. PIPO (0.075 mmol, 225 mg, 1 mol%) was added. The primary *n*-octanediol (7.5 mmol, 1.1 g, 0.5 M) was dissolved in THF (4 mL, 27% *v*/*v*) by gently warming. After cooling to rt the solution was added to the reaction mixture. The reaction mixture was stirred at 0 °C until a color change from red to colorless occurred. Solid $H_2NOH \cdot HCl$ (22.5 mmol, 1.6 g, 3 eq) and solid Na_2CO_3 (11.3 mmol, 1.2 g, 1.5 eq) was added to the reaction mixture. The reaction was slowly warmed to rt under stirring. After 24 h full conversion was obtained (GC analysis of the organic phase). The solvent was removed *in vacuo* and the red colored residue was crushed with a mortar to a fine powder. The residual solid was then washed with water (100 mL) and ethanol (50 mL). The product was dried *in vacuo* and analyzed using GC and ^{1}H-NMR spectroscopy in DMSO-d_6.

n-Octanedialdoxime:



Conversion step 1: >99% (84% selectivity)

Conversion step 3: >99%

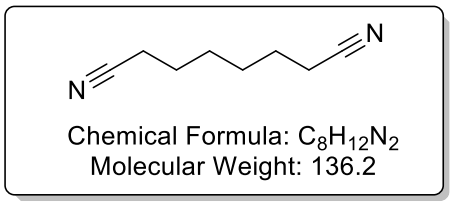
Overall yield: 71%

^{1}H-NMR was found to be in accordance to the literature. [2]

Purity (^{1}H-NMR): >99%.

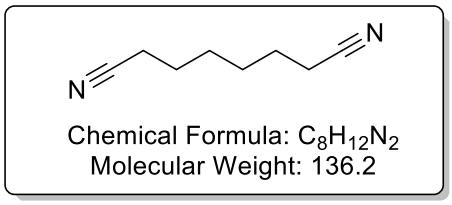
^{1}H NMR (*n*-octanedialdoxime (*E*/*Z* ratio: 1.0:6.7), 500 MHz, DMSO-d_6) *Z*-aldoxime: δ/ppm = 10.70 (2H, s, -CH-NO**H**), , 6.62 (2H, t, *J* = 5.4 Hz, -C**H**-NOH), 2.21 (4H, m, -C**H$_2$**-CH-NOH), 1.40 (4H, m, -C**H$_2$**-CH_2-CH-NOH), 1.28 (4H, m, -C**H$_2$**-CH_2-CH_2-CH-NOH); *E*-aldoxime: δ/ppm = 10.34 (2H, s, -CH-NO**H**), 7.28 (2H, t, *J* = 5.9 Hz, -C**H**-NOH), 2.09 (4H, m, -C**H$_2$**-CH-NOH), 1.40 (4H, m, -C**H$_2$**-CH_2-CH-NOH), 1.28 (4H, m, -C**H$_2$**-CH_2-CH_2-CH-NOH).

3.2.3 Biocatalytic dehydration of *n*-octanedialdoxime (15) using *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cell catalyst <u>100 mM</u> substrate loading



n-Octanedialdoxime (5 mmol, 861.2 mg, 100 mM) was suspended in DMSO (10 mL, 20% *v*/*v*). Potassium phosphate buffer 50 mM pH 7 (35 mL) and was added and the reaction mixture was warmed to 30 °C under stirring with a KPG stirrer. The reaction was started through addition of *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cells (5 mL, 333 mg/mL suspension). The reaction was stirred for 24 h at 30 °C with a KPG stirrer. After 24 h the cells were separated from the reaction mixture by centrifugation (10 min, 10000 xg). The aqueous phase was extracted with ethyl acetate (3x50 mL) and the merged organic layers were washed with saturated sodium chloride solution (1x 50 mL). The organic phase was dried with $MgSO_4$ and the solvent was removed *in vacuo* and the product analyzed using GC and ^{1}H-NMR spectroscopy in DMSO-d_6. The product *n*-octanedinitrile could be isolated as a liquid with 86% yield and a purity of 98% (GC analysis). **^{1}H-NMR** was found to be in accordance to the literature. [2]

Biocatalytic dehydration of *n*-octanedialdoxime (15) using *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cell catalyst <u>200 mM</u> substrate loading



n-Octanedialdoxime (2.5 mmol, 861.2 mg, 200 mM) was suspended in DMSO (5 mL, 20% *v*/*v*). Potassium phosphate buffer 50 mM pH 7 (17.5 mL) and was added and the reaction mixture was warmed to 30 °C under stirring with a KPG stirrer. The reaction was started through addition of *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cells (2.5 mL, 333 mg/mL suspension). The reaction was stirred for 48 h at 30 °C with a KPG stirrer. After 48 h additional *E. coli* BL21(DE3)-RIL+pUC18-OxdB whole cells (2.5 mL, 333 mg/mL suspension) were added and the suspension stirred for another 24 h at 30 °C (72 h overall reaction time). The work-up was according to 3.2.3. The product was analyzed using GC and ^{1}H-NMR spectroscopy in DMSO-d_6. *n*-Octanedinitrile could be isolated as a with 68% yield and a purity of 86% (GC analysis).

References

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2 T. Betke, M. Maier, H. Gruber-Wölfler and H. Gröger, *Nat. Commun.*, 2018, **9**, 1–9.

Article 9

Approaching Bulk Chemical Nitriles from Alkenes: A Hydrogen Cyanide-Free Approach through a Combination of Hydroformylation and Biocatalysis

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doi 10.1021/acscatal.8b05062

Author contribution

AV, TB and HG initiated the project. Experiments were planned by CP, AH, MT, AV and HG and performed by CP, AH and MT. Hydroformylation experiments were performed by MT. AH performed activity studies of aldoxime dehydratases for the hydroformylation products and cascade reactions together with CP. TB and WB (bachelor student, supervised by TB) performed initial experiments (not mentioned in the article) on the inhibition of aldoxime dehydratases with hydroxylamine hydrochloride. KO performed initial experiments (not mentioned in the article) on aliphatic aldoxime dehydration using aldoxime dehydratases. HG wrote the manuscript with the help of TB, CP, AH, MT and AV. CP, AH, MT, HY, AV and HG read and edited the manuscript.

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ACS Catalysis

Research Article

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Approaching Bulk Chemical Nitriles from Alkenes: A Hydrogen Cyanide-Free Approach through a Combination of Hydroformylation and Biocatalysis

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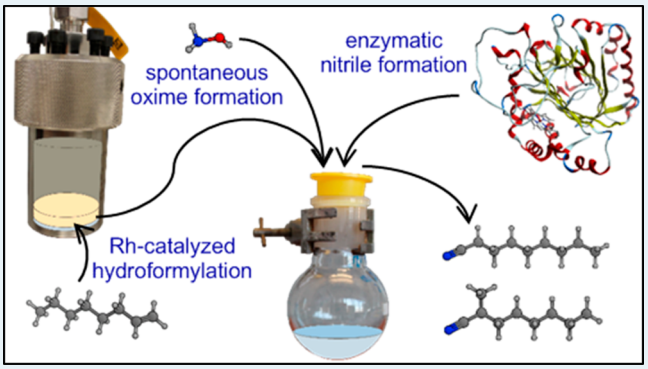
Supporting Information

ABSTRACT: A current challenge in catalysis is the development of methodologies for the production of bulk chemicals needed at levels of tens and hundreds of thousands of tons per year with the requirement to be produced at very low costs often being in the single-digit US dollar range. At the same time, such methodologies should address challenges raised by current manufacturing processes. Within this research area, a cyanide-free approach toward aliphatic nitriles used as industrial chemicals was developed starting from readily accessible *n*-alkenes as starting materials available in bulk quantities. This chemoenzymatic process concept is exemplified for the synthesis of nonanenitrile (as an *n*-/*iso*-mixture) and runs in water at low to moderate temperatures without the need for any types of cyanide sources. The process is based on a combination of a metal-catalyzed hydroformylation as the world-leading production technology for alkyl aldehydes with an emerging enzyme technology, namely, the recently developed transformation of aldoximes into nitriles through dehydration by means of aldoxime dehydratases. As a missing link, an efficient aldoxime formation with subsequent removal of remaining traces of hydroxylamine as an enzyme-deactivating component was found, which enabled the merging of these three steps, hydroformylation, aldoxime formation, and enzymatic dehydration, toward a nitrile synthesis without the need for purification of intermediates.



KEYWORDS: *aldoxime dehydratases, aldehydes, biocatalysis, hydroformylation, nitriles*

INTRODUCTION

Nitriles belong to the most important product classes within the product tree of industrial chemicals.[1] In addition to structurally complex stereoisomers of nitriles in the field of pharmaceuticals,[2] linear or branched alkyl nitriles play an important role in the field of specialty and bulk chemicals, which are needed at a level of tens and hundreds of thousands of tons per year with the requirement to be produced at very low costs often being in the single-digit US dollar range.[1,3] Prominent examples of bulk nitrile chemicals include *n*-hexanenitrile, *n*-nonanenitrile, or higher homologues thereof. Such long-chain alkyl nitriles find, e.g., utilizations as solvents and serve as intermediates for amines in the surfactant area.[4] More recently, the application as a jet fuel was reported as a further future option for utilization of representatives of this product class.[5] Today, three major synthetic approaches are known which, however, all raise challenges for improvement.[1−6] As for an organic chemist, introducing a nitrile moiety into an organic framework is often carried out through substitution or addition reactions with hydrogen cyanide or cyanides; this popular method has been widely used also for alkyl nitrile synthesis, in particular on the lab scale.[5] A severe drawback, however, is the need for stoichiometric amounts of highly toxic cyanide although established processes on a large scale exist. Further matured technologies on the industrial scale are ammoxidation,[6] as well as amide dehydration.[4] Although

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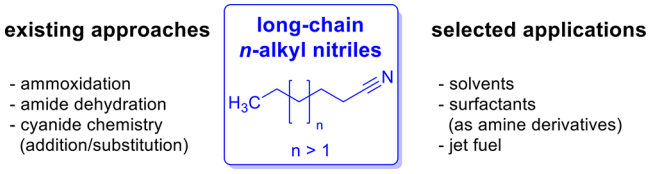
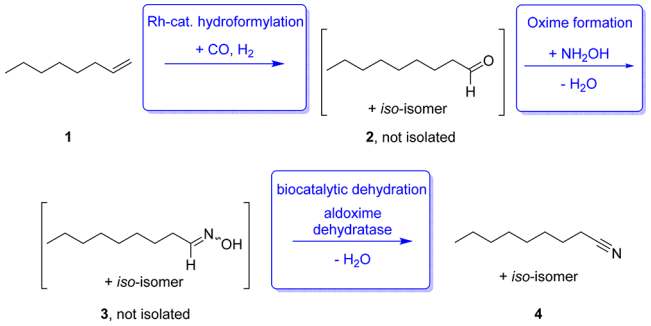
these have been applied successfully for decades, limitations are the high required temperatures, which cause a high energy demand and raise selectivity concerns (Scheme 1).

Scheme 1. Production Method for Long-Chain *n*-Alkyl Nitriles and Applications Thereof



In the following, we report an alternative, chemoenzymatic approach toward such long-chain alkyl nitriles, which starts from simple and readily available alkenes and proceeds in water at low to moderate temperatures and without the need for any types of cyanide sources (Scheme 2). In detail, this approach

Scheme 2. Reaction Sequence for the Synthesis of Long-Chain Alkyl Nitriles Starting from *n*-Alkenes Exemplified for Nonanenitrile (as an *n*-/*iso*-Mixture)



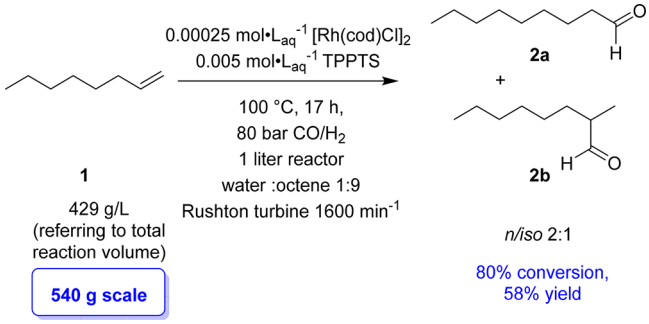
combines a world-leading production technology for aldehydes, namely, homogeneous metal-catalyzed hydroformylation[7] (being applied at a >12 million ton scale), with an emerging enzyme technology, namely, the recently developed transformation of aldoximes into nitriles through dehydration catalyzed by aldoxime dehydratases.[8,9] The link between these two reactions is the formation of the aldoximes from the aldehydes. This step is a simple and spontaneous condensation of the aldehyde with hydroxylamine as a further bulk chemical applied in ε-caprolactam production. Such a chemoenzymatic process concept is shown in Scheme 2, exemplified for nonanenitrile (as an *n*-/*iso*-mixture).

RESULTS AND DISCUSSION

A particular challenge was to identify suitable biocatalysts for this specific nitrile target product synthesis as well as to identify process conditions (*process windows*) under which these three steps can be combined without a need for intermediate isolation. Such a combination would give a perspective toward a three-step, one-pot-like process mode.

First, we focused on a hydroformylation process for 1-octene (**1**) as a model substrate, which is scalable and allows a simple separation of the linear/branched nonanal products from the reaction mixture. A previously developed protocol for the hydroformylation used a setup, which appeared to be very suitable for the envisaged combination with a subsequent biocatalytic step.[10] An aqueous-/organic-phase system with only substrate/product as the organic phase was applied to enable an intrinsic separation of catalyst and products after the reaction, thus securing a very low contamination of the aldehydes with the precious metal. As a catalyst system, a rhodium salt and TPPTS (tris(3-sulfophenyl)phosphine trisodium salt) as a cheap ligand, which is also used in the Ruhrchemie/Rhône Poulenc process, were used. The activity of the two-phase system containing the catalyst is highly dependent on the interfacial area being created between the water and the octene phase, which was shown by a kinetic term that implements the interfacial area.[10] This specific system was scaled up into a liter reactor. The reactivity is sensitive to the water to octene ratio (ϕ) and the stirrer type.[10] With a 10% water and 90% octene phase, a Rushton turbine as a stirrer, and pressure of 80 bar, an octene conversion of 80% with an overall yield of the two nonanal isomers of 58% was achieved (Scheme 3). The catalyst concentration was extremely low

Scheme 3. Hydroformylation of *n*-Octene to *n*-/*iso*-Nonanal



with 0.000 25 mol L^{-1}, and a high substrate loading of 429 g L^{-1} at a 540 g scale was used in our experiment. The resulting *n*/*iso*-ratio of the regioisomers *n*-nonanal (**2a**) and 2-methyloctanal (**2b**) was 2:1 over the whole reaction time. This value is characteristic for this catalyst system in the hydroformylation of long-chain alkenes.[10] The main side reaction was the formation of octene isomers, which lead to lower selectivities during the reaction process due to the accumulation of internal double-bonds. As the Rh-TPPTS catalyst gave a mixture of isomers with an *n*/*iso*-ratio of 2:1 (**2a**:**2b**), in our subsequent biocatalytic studies, we then investigated if the enzymes are capable of transforming both types of regioisomers. By means of the utilization of only a water/octene two-phase mixture, and because of the high difference in density and polarity, the leaching of the catalyst was below 1 ppm.

The subsequent step consists of the condensation of the aldehyde with hydroxylamine under formation of the aldoxime. This reaction was again carried out in water. Our specific interest focused on the optimization of this reaction by minimizing the excess of hydroxylamine, since we expected an enzyme deactivation by hydroxylamine, which has been reported by Oinuma et al.[11] We found in initial experiments with *E*/*Z*-phenylacetaldehyde oxime (which serves as the standard substrate for this enzyme class) that spontaneous formation of the aldoxime through condensation of the aldehyde with hydroxylamine also occurs with up to >99% conversion in an aqueous buffer solution at room temperature (see the Supporting Information). The same reaction type was found to proceed well when using the substrate nonanal (as the product being formed in the hydroformylation step). When

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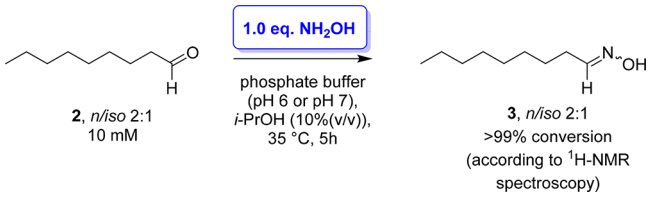
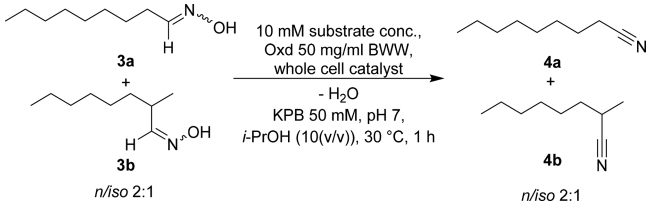
starting from a sample of nonanal isomers **2a** and **2b** (with an *n*/*iso*-ratio of 2:1 and at a substrate concentration of 10 mM), full conversion was achieved even without an excess of hydroxylamine. In detail, an equimolar amount of hydroxylamine led to a quantitative formation of the corresponding aldoximes within a reaction time of 5 h at 35 °C (according to ^{1}H NMR spectroscopy; Scheme 4). The increase in temperature was needed to achieve full conversion since the *iso*-isomer is converted more slowly than *n*-nonanal. Such an opportunity to form the aldoximes in aqueous media with excellent conversion now also enabled a perspective for combining this aldoxime formation and the enzymatic dehydration step within a one-pot process without the need to isolate and purify the aldoxime as an intermediate prior to a subsequent biotransformation.

Scheme 4. Optimized Spontaneous Aldoxime Formation Requiring Only an Equimolar Amount of Hydroxylamine



With this efficient method for an aldoxime preparation in an aqueous medium in hand, we next focused on the identification of a suitable aldoxime dehydratase for the desired dehydration step. Toward this end we studied five aldoxime dehydratases (Oxd; the origin of enzymes is described in Table S4), which are available in recombinant form and can be produced efficiently (Table 1). It is noteworthy that all five aldoxime dehydratases turned out to accept isolated *n*-nonanaldoxime, **3a**, as a substrate, which was utilized at a substrate concentration of 10 mM in this initial study in combination with 10%(v/v) of isopropanol (*i*-PrOH) for improving the substrate solubility (Table 1, entries 1−6). In addition, with exception of OxdFG, all Oxd enzymes led to the formation of the desired *n*-nonanenitrile (**4a**) with excellent conversion of >99%. However, utilizing an *n*/*iso*-nonanaldoxime mixture (with an *n*/*iso*-ratio of 2:1 for **3a** and **3b**) revealed that the *iso*-regioisomer **3b** is accepted to a less extent (entries 7−12). Nevertheless, also for this less reactive substrate **3b**, even a short reaction time of 60 min led to full conversion when using the enzymes OxdA and OxdRE (entries 7 and 11).

Table 1. Investigation of Aldoxime Dehydratases (Oxd) as Biocatalysts for the Synthesis of Nonanenitrile (4) as an *n*-/*iso*-Mixture



entry[a]	substrate	Oxd	conversion [%]
1	*n*-3	A	>99
2	*n*-3	B	>99
3	*n*-3	B[b]	>99
4	*n*-3	FG	42
5	*n*-3	RE	>99
6	*n*-3	RG	>99
7	*n*-/*iso*-3	A	>99 (*n*), >99 (*iso*)
8	*n*-/*iso*-3	B	96 (*n*), 32 (*iso*)
9	*n*-/*iso*-3	B[b]	>99 (*n*), 43 (*iso*)
10	*n*-/*iso*-3	FG	57 (*n*), 15 (*iso*)
11	*n*-/*iso*-3	RE	>99 (*n*), >99 (*iso*)
12	*n*-/*iso*-3	RG	99 (*n*), 88 (*iso*)

[a]Conditions: 50 mg mL^{-1} whole cells (which corresponds to 0.12−1.10 U mg^{-1} BWW, see Table S4), 10 mM **3**, 30 °C, pH = 7.0, 60 min. BWW = bio wet weight. Conversion determined by GC-analytics. [b]OxdB with C-terminal His-tag.

With the efficient three individual steps hydroformylation, spontaneous aldoxime formation, and enzymatic dehydration in hand, we focused on the combination of these reaction steps (as shown in Scheme 2) to avoid intermediate isolations. Accordingly, the compatibility of the enzyme with the components from the aldoxime formation step was studied as both (aldehyde and hydroxylamine) could be present in the reaction mixture at least in low amounts (if not fully consumed in the aldoxime forming step). Upon investigation of the effect of aldehyde **2** and hydroxylamine on the biocatalyst, *n*/*iso*-nonanal showed almost no negative effect on the enzyme in concentrations of up to 1 mM (see Supporting Information). In contrast, the activity of the tested enzymes was strongly decreased even in the presence of very low concentrations of hydroxylamine, which turned out to severely harm the Oxd enzymes (Figure 1). In detail, a nearly dramatic loss of activity was observed already at hydroxylamine concentrations of 0.5 and 1 mM, and even at 0.05 mM in part, a significant drop in activity was observed.

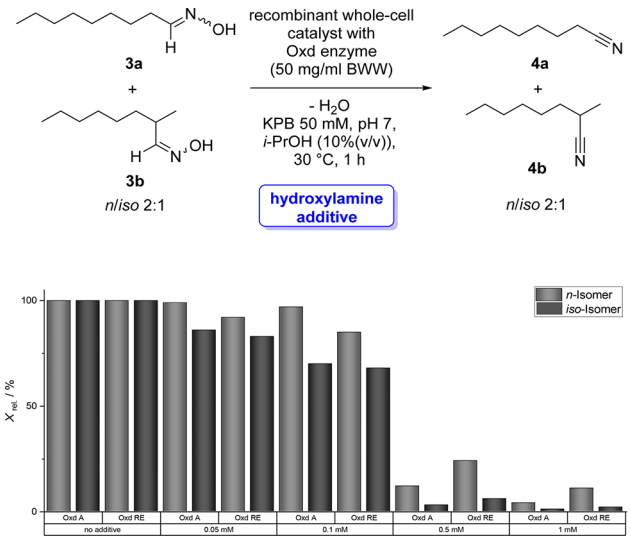


Figure 1. Inhibition of aldoxime dehydratases by hydroxylamine. BWW: bio wet weight; 50 mg mL^{-1} BWW corresponds to 0.12−1.10 U mg^{-1} BWW, see Table S4. OxdA: 10 mM substrate concentration. OxdRE: 20 mM substrate concentration. X_{rel}.: relative conversion referring to additive-free biotransformations.

These results are consistent with literature data reporting only a 30% activity for OxdA at a 0.01 mM hydroxylamine concentration.[11] Upon comparison of the two prioritized enzymes OxdA and OxdRE with each other, OxdRE showed a

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somewhat higher stability at elevated hydroxylamine concentrations of 0.5 and 1 mM, albeit these concentrations are still in a very low range.

As the stability of the Oxd enzymes is dramatically affected by very low amounts of hydroxylamine, even the encouraging finding (described above) that only an equimolar amount of hydroxylamine is needed for aldoxime formation with excellent conversion of >99% might not be sufficient to avoid an at least slight surplus of hydroxylamine in practical use (due to, e.g., weighing differences leading to a slight excess of hydroxylamine being sufficient to hamper enzyme stability). Thus, we were searching for a method which enables us to directly use the aldoxime prepared *in situ* but at the same time to ensure that no hydroxylamine remains when adding the enzyme.

We were pleased to find an elegant and easy-to-carry-out solution for this task which consists of heating the reaction mixture for 16 h up to 100 °C, thus leading to a decomposition of hydroxylamine while leaving the formed aldoxime product untouched. The decomposition of hydroxylamine in aqueous solution at an elevated temperature is described in the literature to proceed under formation of ammonia, nitrites, and nitrates.[12] However, it should be added that such a procedure has to be taken with high caution as in general thermal decomposition of hydroxylamine raises safety concerns since hydroxylamine has turned out to be a chemically instable compound, which also has led to tragic incidents in industry.[13] Thus, we only utilized this thermal decomposition method to remove trace amounts of hydroxylamine present in highly diluted concentration in an aqueous medium. The suitability of this methodology to remove a very low amount of hydroxylamine at low concentration is illustrated in Figure 2, which

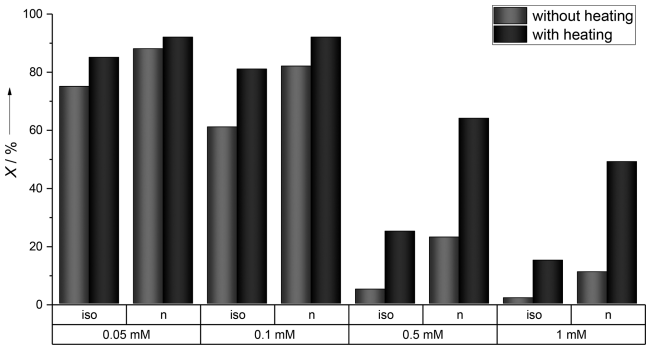
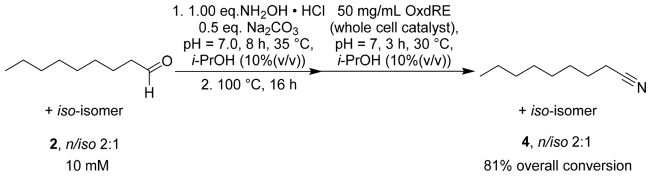


Figure 2. Effect of heating the hydroxylamine solution on the inhibition of the biotransformation. *X* = conversion. Conditions: 20 mM **3**, 50 mg mL^{-1} BWW corresponds to 0.80 U mg^{-1} BWW (see Table S4), OxdRE, potassium phosphate buffer (KPB, 50 mM, pH = 7), *i*-PrOH (10%(v/v)), 500 μL total volume, 30 °C, 1 h, 0.05−1 mM $H_2NOH{\cdot}HCl$ by addition of 1.2−25 μL of a 20 mM solution, either used directly or heated for 16 h to reflux.

shows a comparison of biotransformations starting from various aldoxime/hydroxylamine solutions, in which the hydroxylamine solution was used without and with heat-treatment according to the method described above. As can be seen from Figure 2, biotransformations utilizing the heat-treated hydroxylamine solutions as an additive gave significantly higher conversions. These results underline the positive impact of this method for removal of a trace amount of hydroxylamine present in an aqueous medium at a very low concentration through thermal *in situ*-decomposition.

Thus, this protocol also enabled a perspective toward a coupling of the aldoxime formation step with the biotransformation as a sequential one-pot process (Scheme 5).

Scheme 5. One-Pot Synthesis of *n*/*iso*-Nonanenitrile (4) Starting from a Mixture of *n*/*iso*-Nonanal (2; *n*-/*iso*-Ratio, 2:1)[a]



[a]50 mg mL^{-1} BWW corresponds to 0.80 U mg^{-1} BWW.

Accordingly, when starting from the aldehyde isomers as substrate (10 mM) and when carrying out the biotransformation directly with the reaction mixture from the spontaneous aldoxime formation after heat-treatment for removal of hydroxylamine, we were pleased to find that this one-pot process proceeds very efficiently furnishing the desired *n*/*iso*-nonanenitrile **4a**/**4b** with a high overall conversion of 81% over two synthetic steps. In contrast, a comparison experiment without heat-treatment of the reaction mixture resulting from aldoxime formation via condensation of *n*/*iso*-nonanal **2a**/**2b** and hydroxylamine gave a strongly decreased conversion of only 6% (see Supporting Information).

Taking into account that the aldehyde formed in the hydroformylation step does not require a purification but can be directly used after simple phase separation via extraction and solvent evaporation, this results in a straightforward three-step transformation of *n*-octene into nonanenitrile with an overall 65% conversion (as conceptually shown in Scheme 6). This overall conversion refers to all three steps starting from 1-octene and has been calculated from the conversion for the first step (80%, see Scheme 3) and the conversion of the second and third step being done in one pot (81%, see Scheme 5). It should be added that in principle also a process running without the phase-separation step would be conceivable. However, as the metal catalyst for the hydroformylation is the most expensive component in the overall process, the phase separation appeared to us as an elegant approach to achieve a simple catalyst separation from the aldehyde intermediates which then makes a reuse of the aqueous phase bearing this metal component possible.

In addition, we carried out such a three-step one-pot-related experiment with a subsequent product isolation (Scheme 6). Toward this end, we conducted our hydroformylation experiment at a reduced lab scale using 6 mL of 1-octene and 4 mL of aqueous phase, which contains a rhodium salt and TPPTS ligand with some slight changes in the setup compared to the experiment at the elevated lab scale. For this first step (hydroformylation), we obtained 74% conversion to the *n*/*iso*-aldehyde mixture in a ratio of ∼2.2:1 (*n*:*iso*; Scheme 6). The second step (condensation with hydroxylamine) was conducted using the organic phase from the first step after phase separation without purification in an amount which contains 1 mmol of aldehyde. After treatment with 1 equiv of hydroxylamine and heating the reaction mixture for 16 h to 100 °C, OxdRE whole cell catalyst was added (after cooling down) in an amount corresponding to a final concentration of 50 mg mL^{-1}, which is related to a standard activity of the catalyst of

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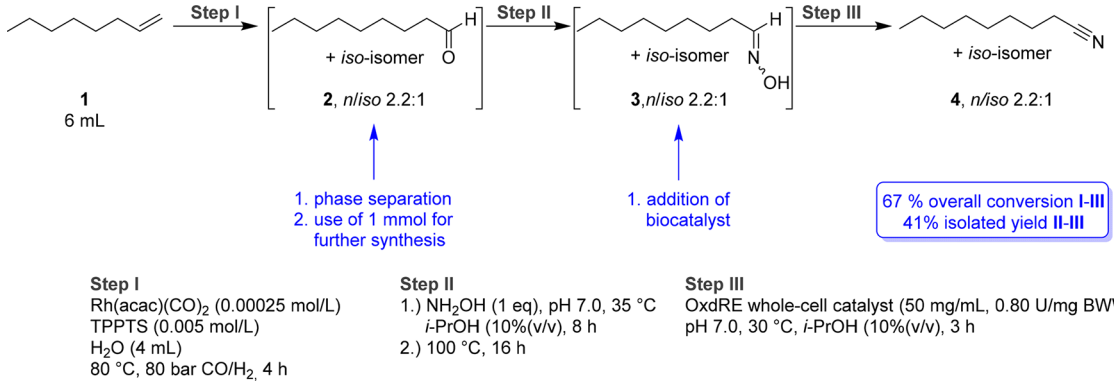
Scheme 6. Complete Reaction Sequence for the Synthesis of *n*/*iso*-Nonanenitrile Starting from *n*-Octene



0.8 U mg^{-1} BWW. The resulting mixture was stirred for 3 h at 30 °C, thus reaching a conversion of aldoxime mixture into the desired nitrile mixture of 90%. Subsequently, the nitrile mixture was purified by column chromatography resulting in an isolated yield of 41% calculated from the used aldehyde amount in the second step. Furthermore, an overall conversion of 67% starting from 1-octene was reached in our three-step, one-pot experiment (Scheme 6).

In conclusion, a route for aliphatic nitriles which corresponds to a formal hydrocyanation of alkenes without using hydrogen cyanide has been developed. This chemoenzymatic nitrile synthesis is based on a combination of metal-catalyzed hydroformylation with enzymatic aldoxime dehydration. In addition, as a missing link, an aldoxime formation with subsequent *in situ* removal of excess of hydroxylamine as a strongly enzyme-deactivating component was found, which then enabled the merging of these three steps, hydroformylation, aldoxime formation, and enzymatic dehydration, toward a nitrile synthesis without the need to purify the intermediates.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge on the ACS Publications Web site. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.8b05062.

General experimental information, standard protocols, activity measurements, kinetic measurements, aldoxime dehydratase (Oxd) sequences, plasmids and expression, and experimental information about the sequential one-pot processes and synthesis of reference compounds (PDF)

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

Approaching Bulk Chemical Nitriles from Alkenes: A Hydrogen Cyanide-Free Approach through Combination of Hydroformylation and Biocatalysis

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1 Experimental information

1-Octene (99+%) was purchased from Acros Organics. Water was demineralized and distilled twice (pH 5.5). The catalyst precursor bis(1,5-cyclooctadiene)dirhodium(I) dichloride ($[Rh(cod)Cl]_2$) (99.95%) was kindly provided by Umicore. The ligand 3,3',3"-phosphanetriyltris(benzenesulfonic acid) trisodium trisodium salt (TPPTS) (28% in distilled water) was kindly donated by OXEA GmbH. Substrate gases CO (≥ 98%) and H_2 (99.999%) as well as the inert gases N_2 and Ar were obtained from Messer. Chemicals for aldoxime and nitrile synthesis were purchased from VWR Chemicals, Carl Roth and Fluka Chemicals and used without further purification, respectively.

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^1H) or 125 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal ($CDCl_3$).

ICP-OES measurements: An Iris Intrepid ICP (Thermo Elemental) was used to determine of the rhodium and phosphorous content in both phases. For this, 0.23 g of a sample were measured out in a Teflon cup and 2.5 mL nitric acid (65%) and 4 mL sulfuric acid (96%) were added. The digestion process was conducted in a MWS µPrep start-system microwave (MLS). Upon completion of the digestion process, the samples were treated with 2 ml double-distilled water and 1 mL of H_2O_2 (Fisher Scientific, Optima grade, phosphorous free). The prepared samples were allowed to rest for twelve hours before measurement.

To examine samples of the biphasic aqueous hydroformylation in via gas chromatography, a Hewlett Packard 6890 Series GC System gas chromatograph was used, which is equipped with a thermal conductivity detector, a flame ionization detector and a Hewlett Packard Innowax column (30 m x 0.25 mm x 0.25 µm). The injection volume of the organic phase was 1 µL, the split ratio is 1:200. Helium was used as carrier gas with a flow of 1.0 mL/min. The starting temperature of the oven was 40 °C. After one minute, the temperature was increased to 140 °C with a rate of 7 °C/min. The temperature was then increased to 250 °C at a rate of 20 °C/min. This temperature was maintained for five minutes. The external standard quantitation method was used.

Table S1: GC retention times of aldehydes (**2a**,**b**), oximes (**3a**,**b**) and nitriles (**4a**,**b**).

Compound	Retention time /min
1	5.7
other octene isomers	5.4, 5.5, 5.9, 6.1
2a	9.7
2b	9.4

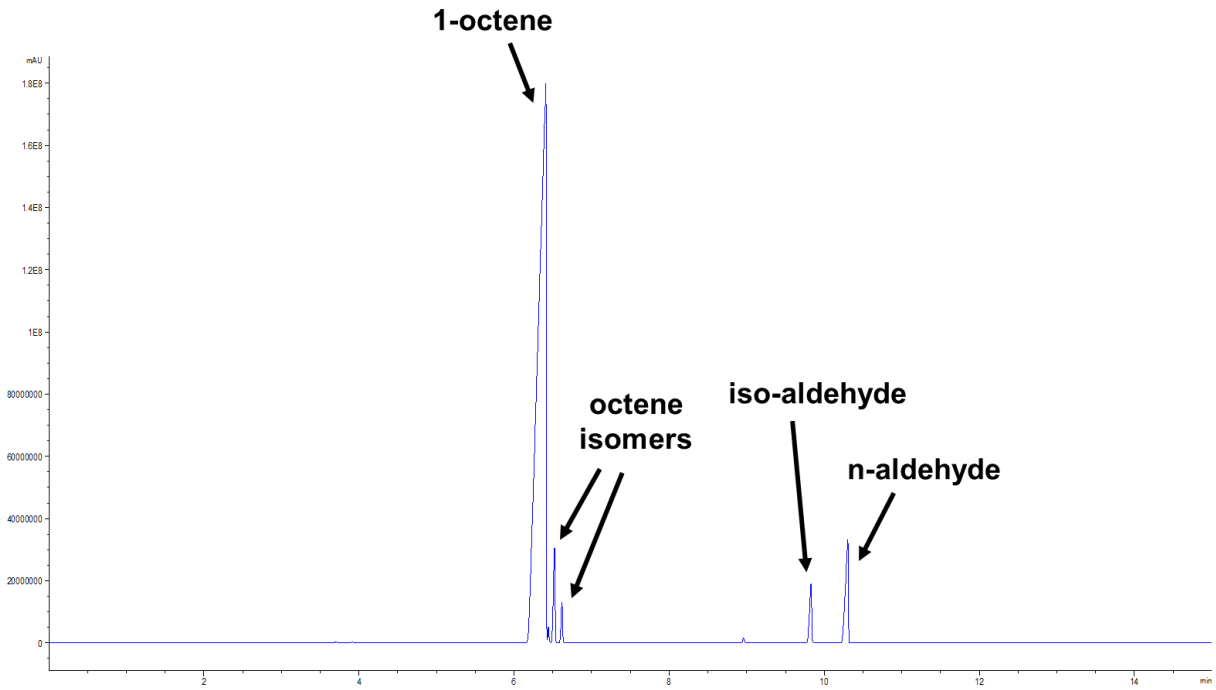


Figure S1: sample chromatogram for hydroformylation in 3600 mL reactor.

The measurement of the hydroformylation in 10 mL scale via gas chromatography (Shimadzu GC 2010) was conducted on a BGB-174 coloumn (30 m x 0.25 mm x 0.25 µm). The organic phase was dissolved in ethyl acetate (EtOAc, 1:100) prior to injection of 10 µL at 250 °C with a split ratio of 1:10. As carrier gas a N_2/synthetic air mixture was used in a pressure flow control mode with a column flow of 0.96 mL/min and a total flow of 13.5 mL/min. The following temperature gradient was used in this method: 50 °C start temperature, in 1 °C/min to 60 °C, in 20 °C/min to 100 °C, in 5 °C/min to 135 °C and in 40 °C/min to 200 °C (2.4 min). The flame detector temperature was 350 °C. Conversion was calculated using a calibration curve. The retention times for the compounds of interest are as follows:

Table S2: GC retention times of aldehydes (**2a**,**b**), oximes (**3a**,**b**) and nitriles (**4a**,**b**).

Compound	Retention time /min
1	5.7
other octene isomers	5.3, 5.5, 6.0, 6.6
2a	17.9
2b	17.0

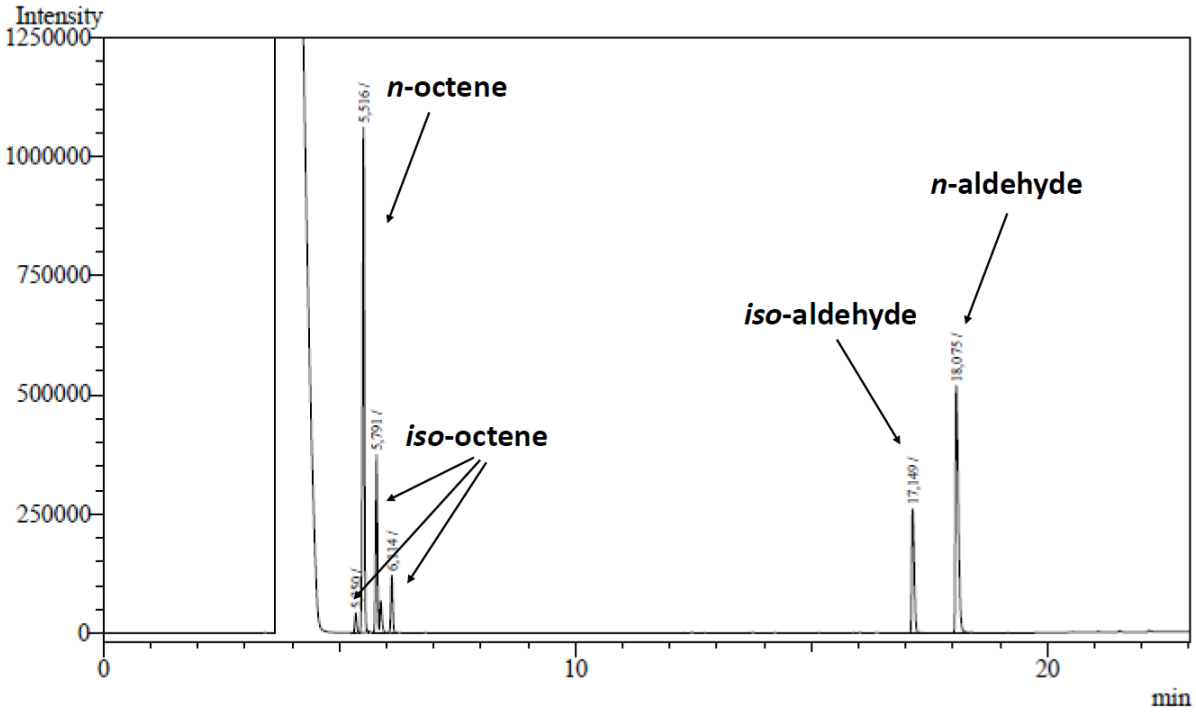


Figure S2: Sample chromatogram for hydroformylation in 75 mL reactor.

Conversion of the biotransformations was determined by GC measurements (Shimadzu GC 2010) in comparison to a calibration curve. Measurements were conducted on a Phenomenex ZB-5MSi (30 m x 0.25 mm x 0.25 µm). An injector temperature of 300 °C in a split injection mode (1:10) was used and a sample amount of 1 µL was injected in this method. As carrier gas a N_2/synthetic air mixture was used in a pressure flow control mode with a total flow of 13.9 mL/min and a column flow of 0.99 mL/min. The following temperature gradient was used in this method: 110 °C start temperature, in 5 °C/min to 140 °C and in 40 °C/min to 240 °C. The flame detector temperature was 350 °C. The retention times for the compounds of interest are as follows:

Table S3: GC retention times of aldehydes (**2a**,**b**), oximes (**3a**,**b**) and nitriles (**4a**,**b**).

Compound	Retention time /min
2a	3.5
2b	3.2
3a	5.9
3b	5.0
4a	4.3
4b	3.7

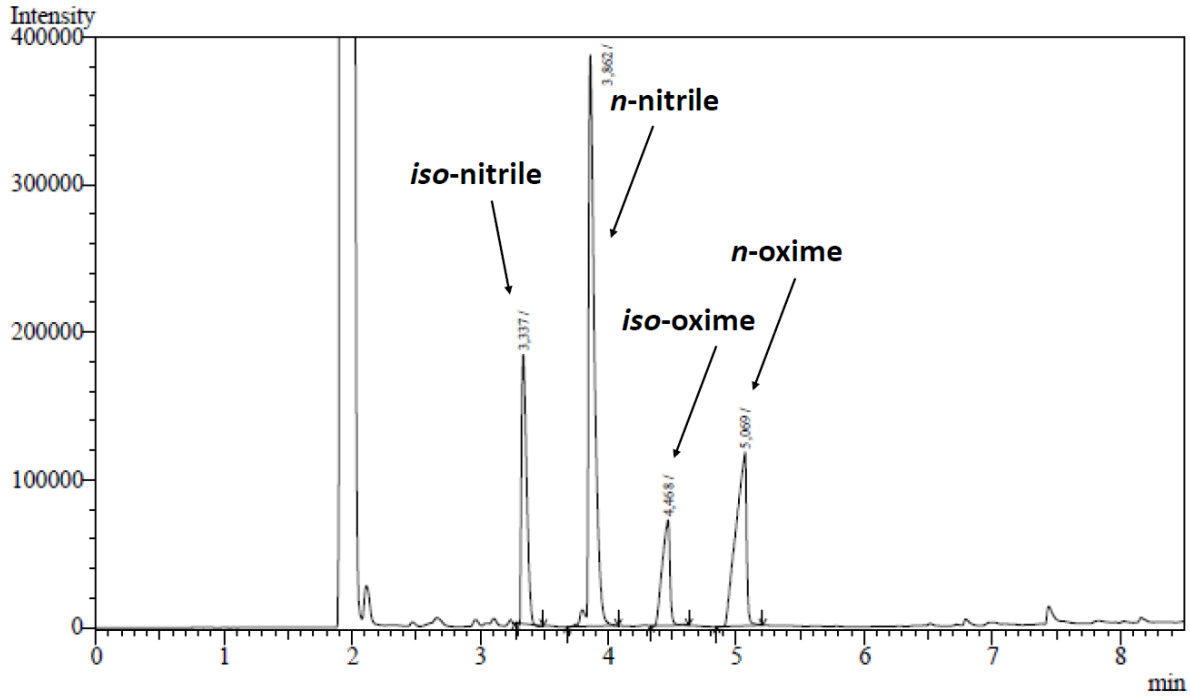


Figure S3: Sample chromatogram for biotransformation of **3**.

E.coli BL21-CodonPlus(DE3)-RIL cells were transformed with the corresponding plasmid containing the gene for each of the aldoxime dehydratases and stored at -80 °C as cryo-culture in glycerol prior to use.

The gene for the aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) was located in a pUC18 vector.

2 Standard protocol for hydroformylation of 1-octene in aqueous media

2.1 Protocol for hydroformylation

The $[Rh[cod]Cl]_2$ precursor (0.00025 mol/L_{aq}, 0.05 mmol, 26.6 mg) was dissolved in 50 mL of double-distilled water together with the TPPTS ligand (0.005 mol/L_{aq}, 0.11 mmol, 613.89 mg). 1-Octene (540 g, 4.8 mol, 760.6 mL) and water (35 g, 1.9 mol, 35 mL) were weighed out to give a volumetric fraction of the organic phase of 0.9. The hydroformylation was carried out in a 3600 mL stainless steel stirred tank reactor with heating jacket. Before the insertion of the chemicals the reactor is evacuated and flushed with nitrogen three times. Then the reactor is preheated under vacuum to 100 °C and when the heating jacket reached the desired temperature the aqueous catalyst phase and the 1-octene were transferred into the reactor. Then the reactor was pressurized with 80 bar synthesis gas (CO/H_2 = 1/1) and stirred at 1600 rpm. Samples were taken during the reaction process. After the reaction, the reactor was degassed and flushed with nitrogen. The crude reaction mixture was collected at the bottom of the reactor over a valve. The phases were separated in a separating funnel and the phases were analyzed by GC-FID to give the yields of the reaction. Catalyst leaching was determined with inductively coupled plasma optical emission spectroscopy (ICP OES)**.**

3 Aldoxime dehydratase (Oxd) sequences, plasmids and expression

The genes for the aldoxime dehydratases from *Pseudomonas chlororaphis* (OxdA), *Fusarium graminearum* (OxdFG), *Rhodococcus erythropolis* (OxdRE) and *Rhodococcus globerulus* (OxdRG) were purchased by GeneArt (Thermo Scientific) in a for expression in *E. coli* codon optimized form, located in pET28a plasmids (restriction sites: OxdA: NcoI and XhoI; OxdFG, OxdRG, OxdRE: NdeI and XhoI (stop-codon before XhoI)) with a sixfold N-terminal His-Tag. The gene for the aldoxime dehydratase from *Bacillus* sp. OxB-1 was cloned into a pUC18 vector using HindIII and PstI restriction sites.

3.1 Oxd sequences and plasmids

3.1.1 Oxd from *Pseudomonas chlororaphilis* B23 (OxdA) with C-terminal His_6-Tag (Accession number: GenBank: AB093544.1)

Base sequence (codon-optimized for *E. coli*):

ATGGAAAGCGCAATTGATACCCATCTGAAATGTCCGCGTACCCTGAGCCGTCGTGTTCC
GGAAGAATATCAGCCTCCGTTTCCGATGTGGGTTGCACGTGCCGATGAACAGCTGCAG
CAGGTTGTTATGGGTTATCTGGGTGTTCAGTATCGTGGTGAAGCACAGCGTGAAGCAGC
ACTGCAGGCAATGCGTCATATTGTTAGCAGCTTTAGCCTGCCGGATGGTCCGCAGACC
CATGATCTGACCCATCATACCGATAGCAGCGGTTTTGATAATCTGATGGTTGTGGGTTAT
TGGAAAGATCCGGCAGCACATTGTCGTTGGCTGCGTAGTGCCGAAGTTAATGATTGGTG
GACCAGCCAGGATCGTCTGGGTGAAGGTCTGGGTTATTTTCGTGAAATTAGCGCACCG
CGTGCAGAACAGTTTGAAACCCTGTATGCATTTCAGGATAATCTGCCTGGTGTTGGTGC
AGTTATGGATAGCACCAGCGGTGAAATTGAAGAACATGGTTATTGGGGTAGCATGCGTG
ATCGTTTTCCGATTAGCCAGACCGATTGGATGAAACCGACCAATGAACTGCAGGTTGTT
GCCGGTGATCCGGCAAAAGGTGGTCGTGTTGTTATTATGGGTCATGATAACATTGCACT
GATTCGTAGCGGTCAGGATTGGGCAGATGCAGAAGCAGAAGAACGTAGCCTGTATCTG
GATGAAATTCTGCCGACCCTGCAGGATGGTATGGATTTTCTGCGTGATAATGGTCAGCC
GCTGGGTTGTTATAGCAATCGTTTTGTTCGTAATATCGATCTGGATGGCAATTTTCTGGA
TGTGAGCTATAACATTGGTCATTGGCGTAGCCTGGAAAAACTGGAACGTTGGGCAGAAA
GCCATCCGACCCATCTGCGTATTTTTGTTACCTTTTTTCGTGTTGCAGCCGGTCTGAAAA
AACTGCGTCTGTATCATGAAGTTAGCGTGAGTGATGCAAAAAGCCAGGTGTTTGAATAT
ATCAACTGTCATCCGCATACCGGCATGCTGCGTGATGCAGTTGTTGCACCGACCAAGCT
TGCGGCCGCACTCGAGCACCACCACCACCACCACTGACTCGAGCACCACCACCACCAC
CACTGAGATCCGGCTGCTAACAAAGCCCGAAAGAAGTTTTTT

Amino acid sequence:

MESAIDTHLKCPRTLSRRVPEEYQPPFPMWVARADEQLQQVVMGYLGVQYRGEAQREAAL
QAMRHIVSSFSLPDGPQTHDLTHHTDSSGFDNLMVVGYWKDPAAHCRWLRSAEVNDWWT
SQDRLGEGLGYFREISAPRAEQFETLYAFQDNLPGVGAVMDSTSGEIEEHGYWGSMRDRF
PISQTDWMKPTNELQVVAGDPAKGGRVVIMGHDNIALIRSGQDWADAEAEERSLYLDEILPT
LQDGMDFLRDNGQPLGCYSNRFVRNIDLDGNFLDVSYNIGHWRSLEKLERWAESHPTHLRI
FVTFFRVAAGLKKLRLYHEVSVSDAKSQVFEYINCHPHTGMLRDAVVAPTLEHHHHHH

3.1.2 Oxd from *Bacillus* sp. OxB-1 (OxdB) without Tag
(Accession number: GenBank: AP013294.1)

Base sequence (codon-optimized for *E. coli*):
ATGAAAAATATGCCGGAAAATCACAATCCACAAGCGAATGCCTGGACTGCCGAATTTCC
TCCTGAAATGAGCTATGTAGTATTTGCGCAGATTGGGATTCAAAGCAAGTCTTTGGATCA
CGCAGCGGAACATTTGGGAATGATGAAAAAGAGTTTCGATTTGCGGACAGGCCCCAAA
CATGTGGATCGAGCCTTGCATCAAGGAGCCGATGGATACCAAGATTCCATCTTTTTAGC
CTACTGGGATGAGCCTGAAACATTTAAATCATGGGTTGCGGATCCTGAAGTACAAAAGT
GGTGGTCGGGTAAAAAAATCGATGAAAATAGTCCAATCGGGTATTGGAGTGAGGTAACG
ACCATTCCGATTGATCACTTTGAGACTCTTCATTCCGGAGAAAATTACGATAATGGGGTT
TCACACTTTGTACCGATCAAGCATACAGAAGTCCATGAATATTGGGGAGCAATGCGCGA
CCGCATGCCGGTGTCTGCCAGTAGTGATTTGGAAAGCCCCCTTGGCCTTCAATTACCG
GAACCCATTGTCCGGGAGTCTTTCGGAAAACGGCTAAAAGTCACGGCGCCGGATAATAT
TTGCTTGATTCGAACCGCTCAAAATTGGTCTAAATGTGGTAGCGGGGAAAGGGAAACGT
ATATAGGACTAGTGGAACCGACCCTCATAAAAGCGAATACGTTTCTTCGTGAAAATGCTA
GTGAAACAGGCTGTATTAGTTCAAAATTAGTCTATGAACAGACCCATGACGGCGAAATA
GTAGATAAATCATGTGTCATCGGATATTATCTCTCCATGGGGCATCTTGAACGCTGGAC
GCATGATCATCCAACACATAAAGCGATCTACGGAACCTTTTATGAGATGTTGAAAAGGCA
TGATTTTAAGACCGAACTTGCTTTATGGCACGAGGTTTCGGTGCTTCAATCCAAAGATAT
CGAGCTTATCTATGTCAACTGCCATCCGAGTACTGGATTTCTTCCATTCTTTGAAGTGAC
AGAAATTCAAGAGCCTTTACTGAAAAGCCCTAGCGTCAGGATCCAGTGA

Amino acid sequence:
MKNMPENHNPQANAWTAEFPPEMSYVVFAQIGIQSKSLDHAAEHLGMMKKSFDLRTGPKH
VDRALHQGADGYQDSIFLAYWDEPETFKSWVADPEVQKWWSGKKIDENSPIGYWSEVTTI
PIDHFETLHSGENYDNGVSHFVPIKHTEVHEYWGAMRDRMPVSASSDLESPLGLQLPEPIV
RESFGKRLKVTAPDNICLIRTAQNWSKCGSGERETYIGLVEPTLIKANTFLRENASETGCISS
KLVYEQTHDGEIVDKSCVIGYYLSMGHLERWTHDHPTHKAIYGTFYEMLKRHDFKTELALW
HEVSVLQSKDIELIYVNCHPSTGFLPFFEVTEIQEPLLKSPSVRIQ

3.1.3 Oxd from *Fusarium graminearum* (OxdFG) with N-terminal His_6-Tag
(Accession number: GenBank: AB214653.1)

Base sequence (codon-optimized for *E. coli*):
ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGC
CATATGCTGCGTAGCCGTTTTCCGGCAAGCCATCATTTCACCGTTAGCGTTTTTGGTTGT
CAGTATCATAGCGAAGCACCGAGCGTTGAAAAAACCGAACTGATTGGTCGTTTCGATAA
ACTGATTGATAGCGCAGCAATTCATGTGGAACATCTGGAACAGAATGATGTGCCGAGCA
AAATTTGGATGAGCTATTGGGAAAGTCCGCAGAAATTCAAACAGTGGTGGGAAAAAGAT
GATACCGCAAGCTTTTGGGCAAGCCTGCCGGATGATGCAGGTTTTTGGCGTGAAACCTT
TAGCCTGCCTGCAACCCGTGCAATGTATGAAGGCACCGGTAAAGATGCCTATGGTTTTG
GTCATTGTGGTAGCCTGATTCCGCTGACCACCAAAACCGGCTATTGGGGTGCATATCGT
AGCCGTATGACACCGGATTTTGAAGGTGATACCTTTTCAAGCCCGATTCCGACCTATGC
AGATCAGAGCGTTCCGGCAGATAAAATTCGTCCGGGTCGTGTTCGTATTACCGATTTTC
CGGATAATCTGTGCATGGTTGTTGAAGGTCAGCATTATGCAGATATGGGTGAACGTGAA

CGCGAATATTGGAACGAAAATTTTGATGGTCTGACGAAACAGTGGGTTACCAATGTTGTT
ACCGCAGGTCATGAACAGGGTATGGTTATTGCACGTGCCTGTCATGGTTTTGCCGGTGA
AAAAAAACTGGGTGCAACCAATGGTCCGGTGAATGGTATTTTTCCGGGTCTGGATTATG
TTCATCAGGCACAGATTCTGATTTGGCAGGATATTAGCAAAATGGAACATATCGGTCGTT
ATGATCAGACCCATGTTAAACTGCGTCGCGATTTTATGAAAGCCTATGGTCCGGGTGGT
GAAATGGAAGGTGGTGATCTGCTGCTGTGGGTTGATCTGGGTATTCTGAAAAAAGACGA
AATCGATGCCGAATATGTGGGTTGCTATGAAAGTACCGGTTTTCTGAAACTGGATAAAG
GCCAGTTTTTCAAAGTTGAAAGCACCGCAGGTAGCAAACTGCCGAGCTTTTTTGATGAA
CCGATTGAAAGCAAACCGATCGAATGGTAA

Amino acid sequence:
MGSSHHHHHHSSGLVPRGSHMLRSRFPASHHFTVSVFGCQYHSEAPSVEKTELIGRFDKLI
DSAAIHVEHLEQNDVPSKIWMSYWESPQKFKQWWEKDDTASFWASLPDDAGFWRETFSL
PATRAMYEGTGKDAYGFGHCGSLIPLTTKTGYWGAYRSRMTPDFEGDTFSSPIPTYADQSV
PADKIRPGRVRITDFPDNLCMVVEGQHYADMGEREREYWNENFDGLTKQWVTNVVTAGH
EQGMVIARACHGFAGEKKLGATNGPVNGIFPGLDYVHQAQILIWQDISKMEHIGRYDQTHVK
LRRDFMKAYGPGGEMEGGDLLLWVDLGILKKDEIDAEYVGCYESTGFLKLDKGQFFKVEST
AGSKLPSFFDEPIESKPIEW

3.1.4 Oxd from *Rhodococcus erythropolis* (OxdRE) with N-terminal His_6-Tag
(Accession number: GenBank: AB094201.1)

Base sequence (codon-optimized for *E. coli*):

ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGC
CATATGGAAAGCGCAATTGGTGAACATCTGCAGTGTCCGCGTACCCTGACCCGTCGTGT
TCCGGATACCTATACCCCTCCGTTTCCGATGTGGGTTGGTCGTGCAGATGATGCACTGC
AGCAGGTTGTTATGGGTTATCTGGGTGTTCAGTTTCGTGATGAAGATCAGCGTCCGGCA
GCACTGCAGGCAATGCGTGATATTGTTGCAGGTTTTGATCTGCCGGATGGTCCGGCAC
ATCATGATCTGACCCATCATATTGATAATCAGGGCTATGAAAACCTGATTGTGGTGGGTT
ATTGGAAAGATGTTAGCAGCCAGCATCGTTGGAGCACCAGCACCCCGATTGCAAGTTG
GTGGGAAAGCGAAGATCGTCTGAGTGATGGTCTGGGTTTTTTTCGTGAAATTGTGGCAC
CGCGTGCAGAACAGTTTGAAACCCTGTATGCATTTCAAGAAGATCTGCCTGGCGTTGGT
GCAGTTATGGATGGTATTAGCGGTGAAATTAACGAACATGGTTATTGGGGTAGCATGCG
TGAACGTTTTCCGATTAGCCAGACCGATTGGATGCAGGCAAGCGGTGAACTGCGTGTTA
TTGCCGGTGATCCGGCAGTTGGTGGTCGTGTTGTTGTTCGTGGTCATGATAACATTGCA
CTGATTCGTAGCGGTCAGGATTGGGCAGATGCCGAAGCAGATGAACGTAGCCTGTATC
TGGATGAAATTCTGCCGACCCTGCAGAGCGGTATGGATTTTCTGCGTGATAATGGTCCT
GCAGTTGGTTGTTATAGCAATCGTTTTGTGCGCAACATTGATATCGATGGCAATTTTCTG
GATCTGAGCTATAACATTGGTCATTGGGCAAGCCTGGATCAGCTGGAACGTTGGAGCG
AAAGCCATCCGACCCATCTGCGTATTTTTACCACCTTTTTTCGCGTTGCAGCCGGTCTGA
GCAAACTGCGTCTGTATCATGAAGTTAGCGTTTTTGATGCAGCAGATCAGCTGTATGAAT
ACATTAATTGTCATCCGGGTACAGGTATGCTGCGTGATGCAGTTACCATTGCAGAACATT
AA

Amino acid sequences:
MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADDALQ
QVVMGYLGVQFRDEDQRPAALQAMRDIVAGFDLPDGPAHHDLTHHIDNQGYENLIVVGYW

KDVSSQHRWSTSTPIASWWESEDRLSDGLGFFREIVAPRAEQFETLYAFQEDLPGVGAVM
DGISGEINEHGYWGSMRERFPISQTDWMQASGELRVIAGDPAVGGRVVVRGHDNIALIRSG
QDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSNRFVRNIDIDGNFLDLSYNIGH
WASLDQLERWSESHPTHLRIFTTFFRVAAGLSKLRLYHEVSVFDAADQLYEYINCHPGTGML
RDAVTIAEH

3.1.5 Oxd from *Rhodococcss globerulus* (OxdRG) with N-terminal His_6-Tag
(Accession number: GenBank: AM946017.1)

Base sequence (codon-optimized for *E. coli*):
ATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGC
CATATGGAAAGCGCAATTGGTGAACATCTGCAGTGTCCGCGTACCCTGACCCGTCGTGT
TCCGGATACCTATACCCCTCCGTTTCCGATGTGGGTTGGTCGTGCAGATGATACCCTGC
ATCAGGTTGTTATGGGTTATCTGGGTGTTCAGTTTCGTGGTGAAGATCAGCGTCCGGCA
GCACTGCGTGCAATGCGTGATATTGTTGCAGGTTTTGATCTGCCGGATGGTCCGGCACA
TCATGATCTGACCCATCATATTGATAATCAGGGCTATGAAAACCTGATTGTGGTGGGTTA
TTGGAAAGATGTTAGCAGCCAGCATCGTTGGAGCACCAGCCCTCCGGTTAGCAGTTGG
TGGGAAAGCGAAGATCGTCTGAGTGATGGTCTGGGTTTTTTTCGTGAAATTGTGGCACC
GCGTGCAGAACAGTTTGAAACCCTGTATGCATTTCAGGATGATCTGCCTGGTGTTGGTG
CAGTTATGGATGGTGTTAGCGGTGAAATTAATGAACATGGTTATTGGGGTAGCATGCGT
GAACGTTTTCCGATTAGCCAGACCGATTGGATGCAGGCAAGCGGTGAACTGCGTGTTG
TTGCCGGTGATCCGGCAGTTGGCGGTCGTGTTGTGGTTCGTGGTCATGATAACATTGCA
CTGATTCGTAGCGGTCAGGATTGGGCAGATGCCGAAGCAGATGAACGTAGCCTGTATC
TGGATGAAATTCTGCCGACCCTGCAGAGCGGTATGGATTTTCTGCGTGATAATGGTCCT
GCAGTTGGTTGTTATAGCAATCGTTTTGTGCGCAACATTGATATCGATGGCAATTTTCTG
GATCTGAGCTATAACATTGGTCATTGGGCAAGCCTGGATCAGCTGGAACGTTGGAGCG
AAAGCCATCCGACCCATCTGCGTATTTTTACCACCTTTTTTCGCGTTGCAGAAGGTCTGA
GCAAACTGCGTCTGTATCATGAAGTTAGCGTTTTTGATGCAGCAGATCAGCTGTATGAAT
ACATTAATTGTCATCCGGGTACAGGTATGCTGCGTGATGCAGTTATTACCGCAGAACATT
AA

Amino acid sequence:
MGSSHHHHHHSSGLVPRGSHMESAIGEHLQCPRTLTRRVPDTYTPPFPMWVGRADDTLH
QVVMGYLGVQFRGEDQRPAALRAMRDIVAGFDLPDGPAHHDLTHHIDNQGYENLIVVGYW
KDVSSQHRWSTSPPVSSWWESEDRLSDGLGFFREIVAPRAEQFETLYAFQDDLPGVGAVM
DGVSGEINEHGYWGSMRERFPISQTDWMQASGELRVVAGDPAVGGRVVVRGHDNIALIRS
GQDWADAEADERSLYLDEILPTLQSGMDFLRDNGPAVGCYSNRFVRNIDIDGNFLDLSYNIG
HWASLDQLERWSESHPTHLRIFTTFFRVAEGLSKLRLYHEVSVFDAADQLYEYINCHPGTG
MLRDAVITAEH

3.2 Oxd expression in *E.coli* BL21-CodonPlus(DE3)-RIL

E.coli BL21-CodonPlus(DE3)-RIL cells harboring the plasmids with the Oxd-genes were stored as glycerol stocks at -80 °C.

A sample from the glycerol stocks for each Oxd was plated on LB-agar containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdA, OxdFG, OxdRE and OxdRG in pET28a) or 100 µg/mL carbenicillin and 34 µg/mL chloramphenicol (OxdB in pUC18) and incubated for 12 to 18 h at 37 °C.

Pre-cultures were prepared in 5 mL LB-medium containing 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdA, OxdFG, OxdRE and OxdRG in pET28a) or 100 µg/mL carbenicillin and 34 µg/mL chloramphenicol (OxdB in pUC18) using a single colony from the LB-agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm.

Main cultures for Oxd expression were performed using TB-autoinduction medium. Sterile 20 g/L lactose solution in MilliQ water (50 mL) and sterile 50 g/L D-glucose solution in MilliQ water (5 mL) was added to 445 mL sterile TB-medium (Carl Roth) in a 500 mL Erlenmeyer flask. 50 µg/mL kanamycin and 34 µg/mL chloramphenicol (OxdA, OxdFG, OxdRE and OxdRG in pET28a) or 100 µg/mL carbenicillin and 34 µg/mL chloramphenicol (OxdB in pUC18) were added to the medium. Main cultures were inoculated with 1% (5 mL) of the relating pre-cultures and incubated for 1 h at 37 °C and 120 rpm. After 1 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 120 rpm and OxdA-, OxdFG-, OxdRE- and OxdRG-cultures were cultivated at 15 °C for 72 h and 120 rpm.

Cell harvest was performed at 4,000 xg for 15 min at 4 °C. The supernatant was discarded and cells were washed three times with 50 mM potassium phosphate buffer (PPB, KP_i) at pH 7.0. The biomass was determined (bio wet weight (bww)) and cells were resuspended in 50 mM PPB (pH 7.0) to a final concentration of 333 mg/mL cells in buffer. Cell suspensions were stored at 4 °C or on ice before usage in biotransformations.

Table S4: Used vector constructs, origins of the Oxd-genes, provider of the vector constructs and marker-resistance of the constructs.

Entry	Origin of Oxd-gene	Vector construct	Oxd	Provider	Resistance
1	*Pseudomonas chlororaphilis* B23	pET28b_OxdA-C-His	OxdA	Asano group	Kanamycin
2	*Bacillus* sp. OxB-1	pUC18_OxdB	OxdB	Asano group	Carbenicillin
3	*Fusarium graminearum*	pET28a_N-His-OxdFG	OxdFG	Thermo Fisher Scientific	Kanamycin
4	*Rhodococcus erythropolis*	pET28a_N-His-OxdRE	OxdRE	Thermo Fisher Scientific	Kanamycin
5	*Rhodococcus globerulus*	pET28a_N-His-OxdRG	OxdRG	Thermo Fisher Scientific	Kanamycin

Overexpression of Oxds in *E. coli* Bl21-CodonPlus(DE3)-RIL was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after cell disruption and denaturation of the proteins in the crude extracts.
Crude extracts of 33%wt cell suspensions were obtained by sonication (5x 1 min, 10 – 15% Output, Bandelin Sonopuls®) and subsequent centrifugation at 21,500 xg for 45 min at 4 °C. The pellet including the cell debris was discarded. Protein concentrations in crude extracts were determined by Bradford assay using a bovine serum albumin (BSA)-standard curve (1.4 mg/mL, 0.7 mg/mL, 0.35 mg/mL, 0.175 mg/mL, 0.0875 mg/mL) as reference. Protein dilutions of 1 µg/µL whole cell protein concentration were obtained by dilution of the crude extracts in water and Laemmli-buffer. 10 µL of these samples were transferred to a 12% SDS-PAGE.

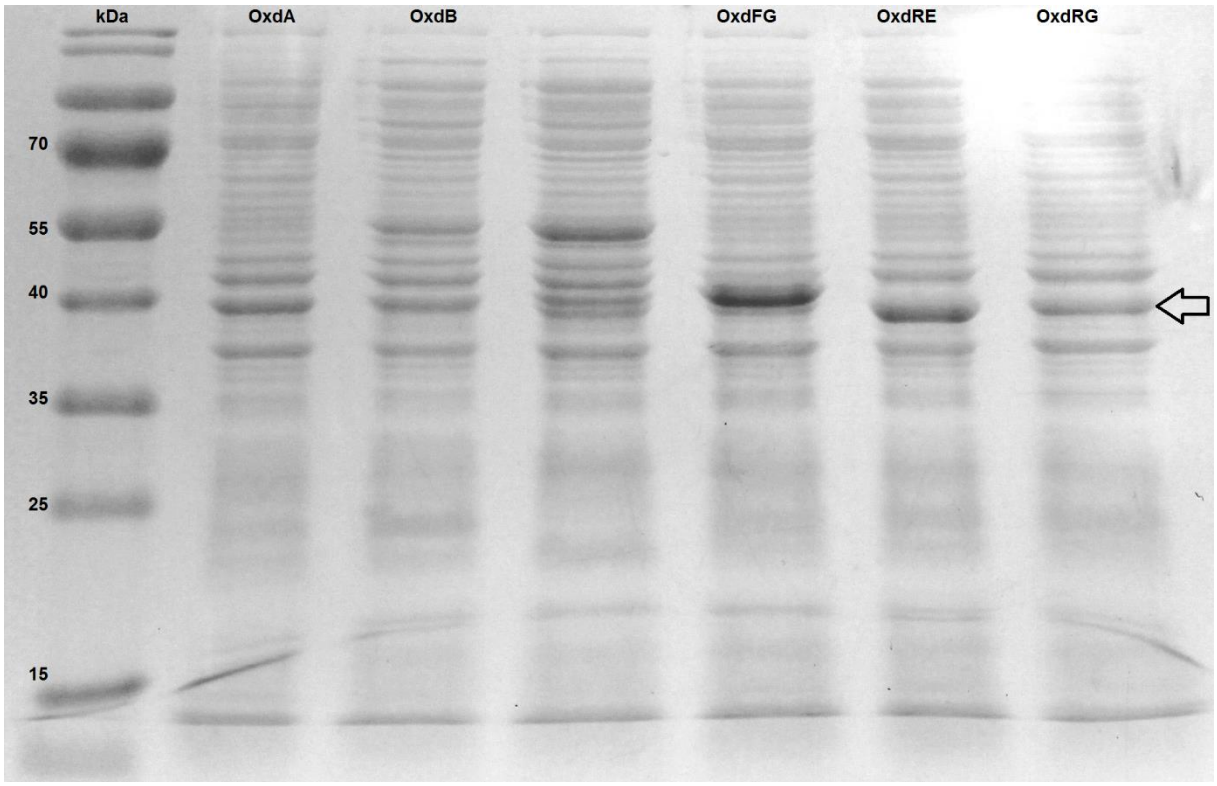


Figure S4: 12% SDS-PAGE of crude extracts of OxdA-, OxdB-, OxdFG-, OxdRE- and OxdRG-overexpressing cells. The molecular weights of the overexpressed Oxds correlates with the molecular weights determined from the amino acid sequences.

4 Standard activity measurements of the bioconversion of *n*- or *iso*-C_9-oxime using Oxds in whole cells

Standard activity assays of Oxds in whole cells using *n*-C_9-oxime **3a** were performed using 50 mg/mL whole cells (bww) in a total volume of 0.5 mL in 1.5 mL micro reaction tubes. The reaction was conducted in 50 mM PPB (pH 7.0) containing 10%(v/v) isopropanol (^{i}PrOH) as a co-solvent. Total substrate concentration of *n*-C_9-oxime **3a** of 100 mM was chosen and the activity assay performed at 30 °C for 15 min and 1,000 rpm in an Eppendorf ThermoMixer.

The reaction was stopped by addition of 500 µL EtOAc and extraction of substrate **3a** and product **4a** into the organic phase. The phase separation was simplified by centrifugation for 5 min at 14,000 xg at room temperature. The organic phase was analyzed by GC for conversion determination. Activity U / mg_{bww} was calculated as follows:

$$U \,/\, \mathrm{mg_{bww}} = \frac{500\ \mu\mathrm{mol} \cdot X}{15\ \mathrm{min} \cdot 25} \qquad (1)$$

Table S5: Standard activity of Oxds converting **3a**.

Oxd	*U* / mg_{bww} [µmol/min]
A	0.12
B	1.08
FG	0.12
RE	**0.80**
RG	1.10

For further investigations whole cell suspensions of 50 mg/mL (bww) reaching the standard activity were used.

5 Activity measurements of the bioconversion of *n*- or *iso*-C9-oxime using Oxds in whole cells

Standard activity assays of Oxds in whole cells using *n*-C_9-oxime **3a** or an *n*-/*iso*-C_9-oxime **3a**/**3b** mixture were performed using 50 mg/mL whole cells (bww) in a total volume of 0.5 mL in 1.5 mL micro reaction tubes. The reaction was conducted in 50 mM PPB (pH 7.0) containing 10%(*v*/*v*) isopropanol (^{i}PrOH) as a co-solvent. Total substrate concentration of *n*- or *n*-/*iso*-C_9-oxime **3a**/**3b** mixtures of 10 mM was chosen and the activity assay performed at 30 °C for 1 h and 1,000 rpm in an Eppendorf ThermoMixer.

The reaction was stopped by addition of 500 µL EtOAc and extraction of substrates **3a** and **3b** and products **4a** and **4b** into the organic phase. The phase separation was simplified by centrifugation for 5 min at 14,000 xg at room temperature. The organic phase was analyzed by GC for conversion determination.

6 Activity measurements of the bioconversion of *n*- or *iso*-C9-oxime using Oxds in whole cells under influence of additives

6.1 Activity under influence of *n*/*iso*-nonanal 2a/2b or hydroxylammonium chloride

The reactions were performed using 50 mg/mL whole cells (bww) in a total volume of 0.5 mL in 1.5 mL micro reaction tubes. The reaction was conducted in 50 mM PPB (pH 7.0) containing 10%(*v*/*v*) isopropanol (^{i}PrOH) as a co-solvent. Substrate concentration of *n*-/*iso*-C_9-oxime **3a**/**3b** mixtures of 10 mM (Oxd A) resp. 20 mM (OxdRE) was chosen and the desired additive concentration (0.05/0.1/0.5/1.0 mM) was adjusted using stock solutions of hydroxylamine chloride (in PPB buffer) or **2a**/**2b** (ratio 2:1, in ^{i}PrOH). After preincubation of the cells in buffer at 30 °C, the stock solutions of additive, the desired amount of co-solvent and subsequent of **3a**/**3b** mixtures in ^{i}PrOH were added. The activity assay was performed at 30 °C for 1 h and 1,000 rpm in an Eppendorf ThermoMixer.

The reaction was stopped by addition of 500 µL EtOAc and extraction of substrates **3a** and **3b** and products **4a** and **4b** into the organic phase. The phase separation was simplified by centrifugation for 5 min at 14,000 xg at room temperature. The organic phase was analyzed by GC for conversion determination.

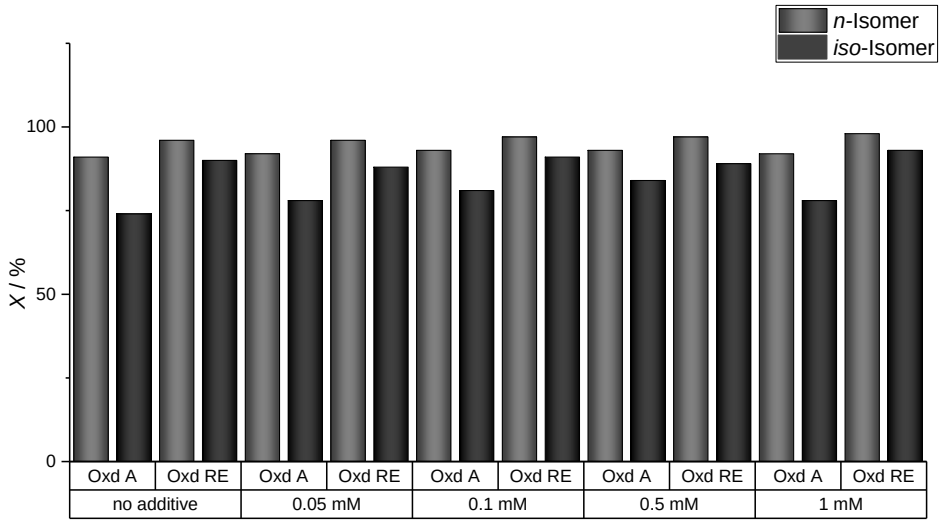


Figure S5: Absolute conversion of nonanaloxime mixture by Oxds under influence of nonanal addition.

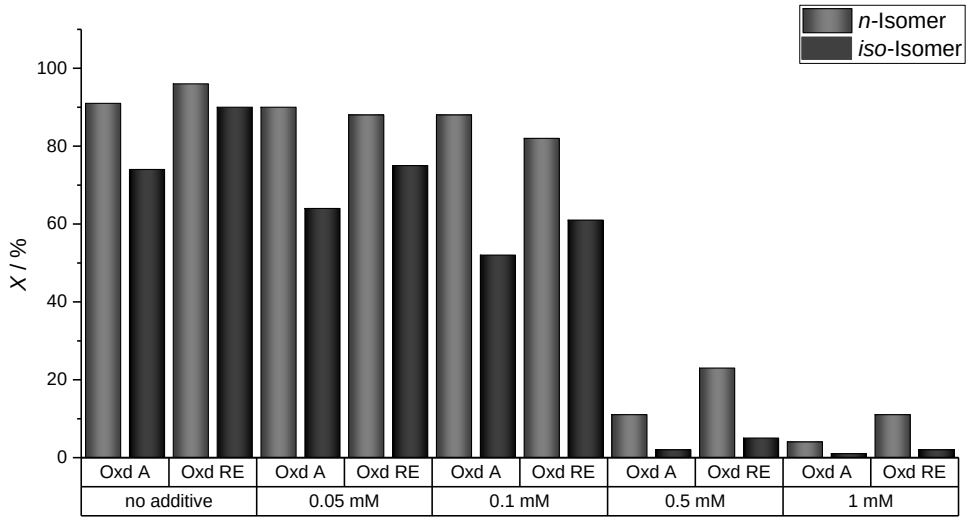


Figure S6: Absolute conversion of nonanaloxime mixture by Oxds under influence of hydroxylamine addition.

6.2 Influence of heating on the inhibiting effect of hydroxylamine

The reactions were performed using 50 mg/mL whole cells (bww) in a total volume of 0.5 mL in 1.5 mL micro reaction tubes. The reaction was conducted in 50 mM PPB (pH 7.0) containing 10%(v/v) isopropanol (iPrOH) as a co-solvent. Substrate concentration of *n*-/*iso*-C_9-oxime **3a**/**3b** mixtures of 20 mM was chosen and the desired additive concentration (0.05/0.1/0.5/1.0 mM) was adjusted using a stock solution of hydroxylamine chloride (20 mM in PPB buffer). Half the stock solution was heated for 16 h to reflux and used in the same amount as the non-treated solution, which was stored at 4 °C. After preincubation of the cells in buffer at 30 °C, the stock solutions of additive, the desired amount of co-solvent and subsequent of **3a**/**3b** mixtures in ^{i}PrOH were added. The activity assay was performed at 30 °C for 1 h and 1,000 rpm in an Eppendorf ThermoMixer.

The reaction was stopped by addition of 500 µL EtOAc and extraction of substrates **3a** and **3b** and products **4a** and **4b** into the organic phase. The phase separation was simplified by centrifugation for 5 min at 14,000 xg at room temperature. The organic phase was analyzed by GC for conversion determination.

7 Sequential one-pot process starting from *n*/*iso*-nonanal

Hydroxylamine hydrochloride (61 mg, 0.88 mmol) and sodium carbonate (47 mg, 0.44 mmol) were dissolved in PPB buffer (66 mL, 50 mM, pH = 7) at room temperature. Freshly destilled *n*-/*iso*-C_9-aldehyde **3a**/**3b** mixture (125 mg, 0.88 mmol, ratio 2:1) was dissolved in ^{i}PrOH (8.8 mL, 10% v/v). To 26 mL of the clear aqueous solution 3.5 mL of the oxime solution was added and stirred vigorously for 6 h at 35 °C. The mixture was heated to reflux for 16 h. After cooling, whole cell catalyst OxdRE (total 50 mg/mL bww) was added at 30 °C in PPB buffer (2.6 mL, 50 mM, pH = 7). After stirring for 5 h at 30 °C, the mixture was extracted 3 times with ethyl acetate. The combined organic layers were washed with water and dried over magnesium sulfate. The conversion was determined via GC (81%, *n*: 82%, *iso*: 7%, ratio 2.2:1). Evaporation of the solvent led to an oily mixture. The *n*-/*iso*-C_9-nitrile **4a**/**4b** mixture was purified via Preparative Thin Layer Chromatography (silica, ethyl acetate/cyclohexane, 7.1 cm) and obtained as a yellow oil (3 mg, 7%).

An identical experiment was conducted with same amount of solutions. After 6 h of stirring at 35 °C, the mixture was stored at -20 °C for 16 h. The solution was brought to 30 °C, until the whole cell catalyst was added. The mixture was stirred at 30 °C for 5 h. After work up the conversion was determined via GC (6%, *n*: 8%, *iso*: 3%, ratio 5.4:1).

In comparison, *n*-/*iso*-C_9-oxime **3a**/**3b** mixture (55 mg, 0.35 mmol) were dissolved in ^{i}PrOH (3.5 mL) and PPB buffer (26 mL, 50 mM, pH = 7) was added. The solution was brought to 30 °C, until the whole cell catalyst was added. The mixture was stirred at 30 °C for 5 h. After work up the conversion was determined via GC (99%, *n*: 99%, *iso*: 98%, ratio 2.0:1).

8 Complete sequence starting from *n*-octene

Hydroformylation was conducted in a 75 mL steel autoclave in a Parr Multi Reactor System using a magnetic stirrer (stick form, 30 mm). $Rh(acac)(CO)_2$ (0.00025 mol/L_{tot}, 3 mg, 0.01 mmol) and the TPPTS ligand (0.005 mol/L_{tot}, 142 mg, 0.25 mmol) were dissolved in water (20 mL, Milipore). 4 mL of this solution were filled into the autoclave and octane (6 mL, 170 mmol) was added. The reactor was evacuated and filled with argon three times prior to pressurize with 80 bar syngas (CO/H_2 = 1/1). The mixture was stirred at 80 °C and 1000 rpm for 4 h. After the reaction, the reactor was degassed and flushed with nitrogen. The phases were separated and the organic phase was analyzed by GC (overall conversion 85%, product related conversion 74%, *n*/*iso*-ratio 2.16:1).

An aliquot of the organic phase (193 mg, containing 1.0 mmol **2a**/**b**) was dissolved in ^{i}PrOH (10 mL, 10% *v*/*v*). PPB buffer (75 mL, 50 mM, pH = 7), Hydroxylamine hydrochloride (69 mg, 1.0 mmol) and sodium carbonate (53 mg, 0.5 mmol) were added at room temperature. The mixture was stirred vigorously for 6 h at 35 °C. The mixture was heated to reflux for 16 h. After

cooling, whole cell catalyst OxdRE (total 50 mg/mL bww) was added at 30 °C in PPB buffer (14 mL, 50 mM, pH = 7). After stirring for 3 h at 30 °C, the mixture was extracted 3 times with ethyl acetate. The combined organic layers were washed with water and dried over magnesium sulfate. The conversion was determined via GC (90%, *n*: 90%, *iso*: 90%, ratio 2.3:1, Figure S7). Evaporation of the solvent led to an oily mixture. The *n*-/*iso*-C_9-nitrile **4a**/**4b** was purified via flash column chromatography (silica, ethyl acetate/cyclohexane) and obtained as a yellow oil (57 mg, 41%, Figure S8).

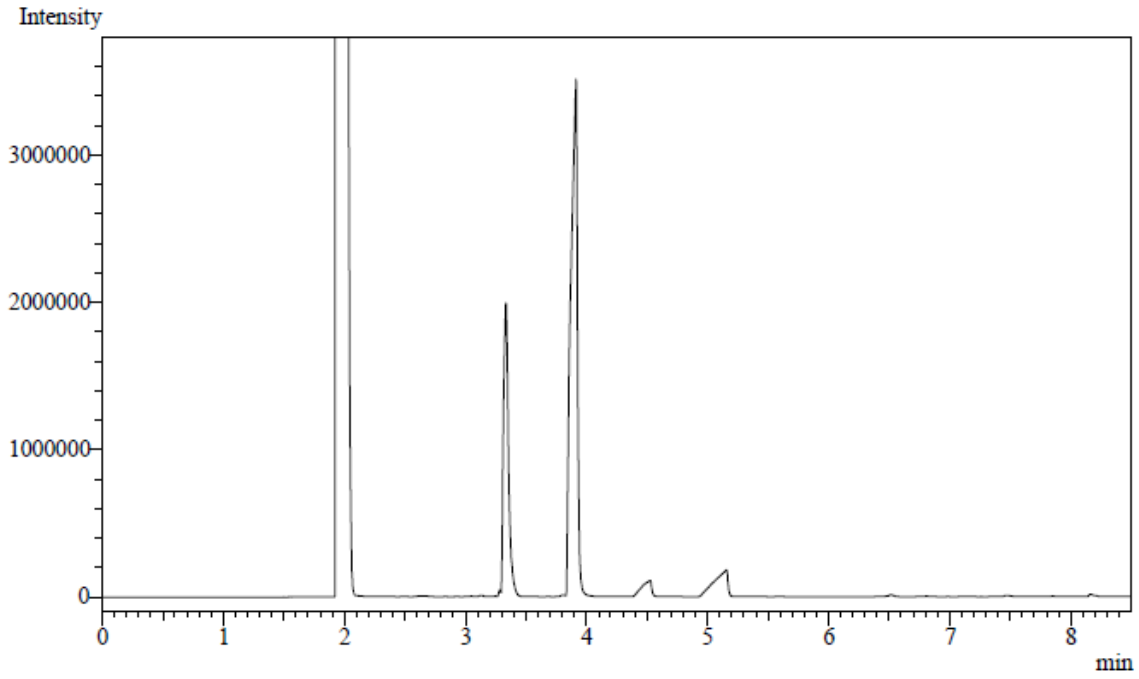


Figure S7: GC-chromatogram of crude product after biotransformation.

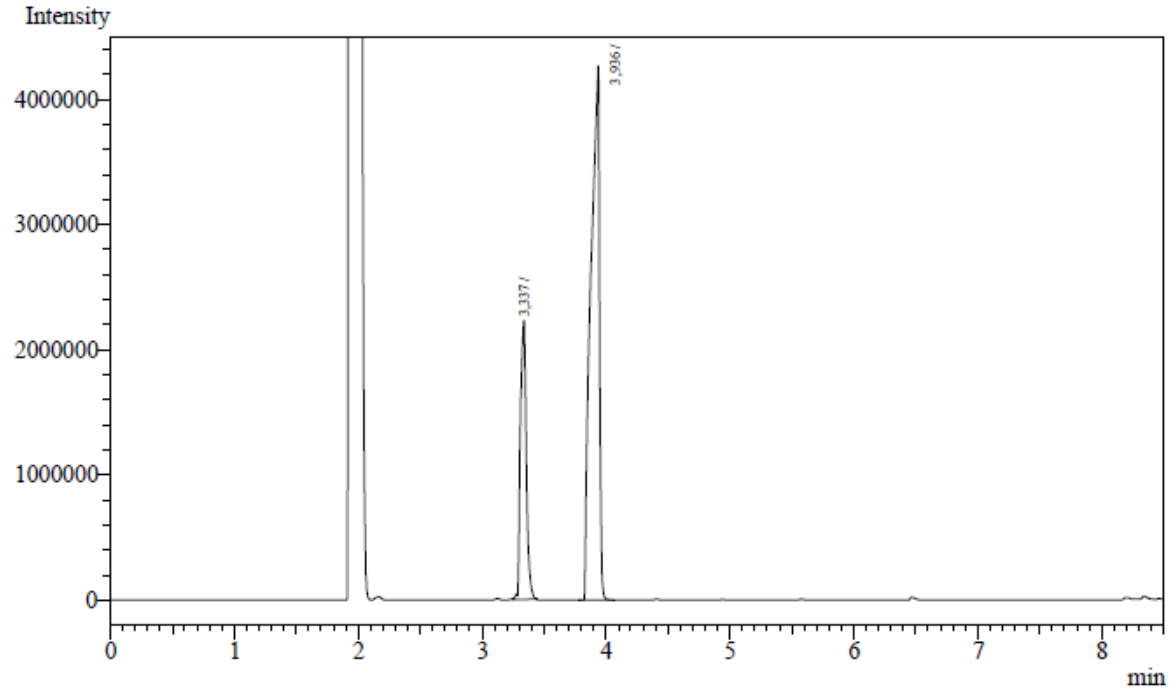


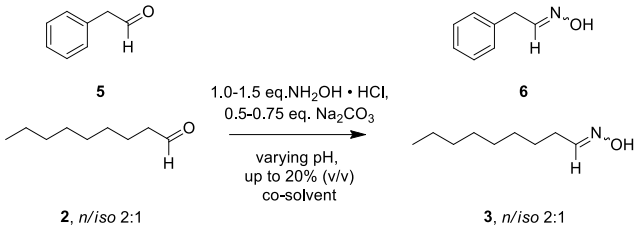
Figure S8: GC chromatogram of purified nitrile **4a**/**4b**.

9 Standard protocols for kinetic measurements of the condensation reactions leading to oximes

The reactions determining the reaction time of (*E/Z*)-phenylacetaldehyde oxime formation were performed in a 4.1 mmol scale (75–347 mM substrate concentration) resp. 2.1 mmol (25–50 mM substrate concentration). Total reaction volume was chosen according to the desired concentration (12–83 mL). Reactions performed without co-solvent were performed in water, others in 50 mM PPB (pH 7.0). Equivalents of hydroxylamine chloride were chosen as listed in Table 6. To release the hydroxylamine, 0.5 eq. sodium hydrogen carbonate per equivalent was applied. The solid salts were dissolved in water/buffer and cosolvent. Phenylacetaldehyde was added and the mixture was stirred at room temperature. After 10, 35, 60, 90, 120, 150 and 180 min aliquots of 1 mL were taken and extracted with ethyl acetate. After evaporation of the solvent the conversion was determined via ^{1}H-NMR.

Kinetic studies of the (*E*/*Z*)-nonanal oxime (*n*/*iso*) **3a**/**3b** formation were performed in 5 mL glass vials with a total reaction volume of 0.5 mL. Substrate concentration, hydroxylamine chloride equivalents and temperature were chosen as shown in table 1. 0.5 eq. sodium hydrogen carbonate per equivalent was applied. The reactions were performed in 50 mM PPB (pH 6.0–7.0) with 10%(v/v) ^{i}PrOH as a co-solvent. After 10, 30, 60, 90, 120, 180, 300 resp. 420 min the reaction mixtures were extracted with cyclohexane. After evaporation of the solvent the conversion was determined via ^{1}H-NMR.

Table S6: Synthesis of (*E/Z*)-phenylacetaldehyde oxime and (*E/Z)-n/iso*-nonanal oxime by condensation with hydroxylamine.

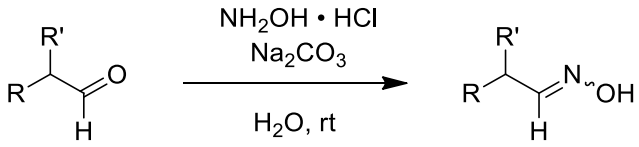


No.	Sub.	conc. [mM]	pH, co-solvent	eq. NH_2OH	T [°C]	time [min]	conv. [%][a]
1	5	347	8.9	1.2	rt	180	>99
2	5	347	8.7	1.0	rt	180	92
3[b]	5	50	7.0, 20% DMSO	1.2	rt	60	>99
4[b]	5	50	7.0, 20% DMSO	1.0	rt	60	99
5[c]	2 (*n*)	50	6.0, 10% 2-propanol	1.2	rt	420	>99
6[c]	2 (*n*/ *iso*)	50	6.0, 10% 2-propanol	1.2	rt	1440	99 (*n*), 94 (*iso*)
7[c]	2 (*n*/ *iso*)	50	6.0, 10% 2-propanol	1.2	35	90	>99
8[c]	2 (*n*/ *iso*)	50	6.0, 10% 2-propanol	1.0	35	300	>99
9[b]	2 (*n*/ *iso*)	20	7.0, 10% 2-propanol	1.0	35	300	>99

[a]Conversion determined by ^{1}H-NMR analysis. [b]KPB (50 mM, pH = 7.0) as aqueous medium. [c]KPB (50 mM, pH = 6.0) as aqueous medium.

10 Synthesis of reference compounds

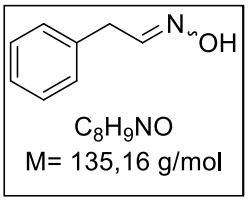
10.1 General procedure 1 (GP1): Synthesis of aldoximes by condensation of aldehydes with hydroxyl amine salts



R = Alkyl, Ph
R' = CH_3, H

Hydroxylamine hydrochloride (1.5 eq.) and sodium carbonate (0.75 eq.) were dissolved in aqueous medium at room temperature. Aldehyde (1 eq.) was added to this solution and stirred vigorously. The solution was extracted three times with ethyl acetate or cyclohexane and the combined organic phases were washed with H_2O. Drying over $MgSO_4$ and evaporation yielded the crude product, which was used without further purification.

10.1.1 Synthesis of (*E*/*Z*)-2-phenylacetaldehyde oxime



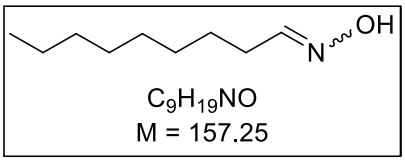
Hydroxylamine hydrochloride (2.16 g, 31.1 mmol) and sodium carbonate (1.65 g, 15.6 mmol) were dissolved in water (35 mL) at room temperature. To the clear solution Phenylacetaldehyde (2.50 g, 20.8 mmol) was added and stirred vigorously for 2.5 h upon which complete conversion was achieved according to TLC analysis (cyclohexane/ethyl acetate 3:1, v/v). The work up with ethyl acetate yielded the product as colorless solid, containing a mixture of *E*/*Z*-isomers.

Yield: 2.37 g, 84%.

^{1}H-NMR (500 MHz, $CDCl_3$): δ [ppm] = 7.58 (s, 1H, CH=NO*H*), 7.15 (s, 1H, CH=NO*H*), 7.55 (t, 1H, ^3J = 6.3 Hz, C*H*=NOH), 7.36-7.20 (m, 5H, Ph-*H*), 6.90 (t, 1H, ^3J = 5.3 Hz, CH=*N*OH), 3.74 (d, 2H, ^3J = 5.3 Hz, CH_2), 3.54 (d, 2H, ^3J = 6.3 Hz, CH_2).

The data corresponds with literature data[1].

10.1.2 Synthesis of (*E*/*Z*)-*n*-nonanal oxime



To *n*-nonanal (**2a**, 1.42 g, 10 mmol) hydroxylamine hydrochloride (1.04 g, 15 mmol) was added, the mixture suspended in water (20 mL) and mixed with sodium carbonate (0.80 g, 7.5 mmol). The emulsion was stirred vigorously for 16 h. Work up with cyclohexane and subsequent recrystallisation from cylcohexane led to colourless crystals.

Yield: 1.14 g, 72 %.

^{1}H-NMR (500 MHz, $CDCl_3$, *E*-Oxim) δ [ppm] = 7.42 (t, ^3J = 6.4 Hz, 1H, C*H*=NOH), 2.18 (q, ^3J = 7.3 Hz, 14.1 Hz, 2H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 10H), 0.88 (t, ^3J = 6 Hz, 3H, CH_3).

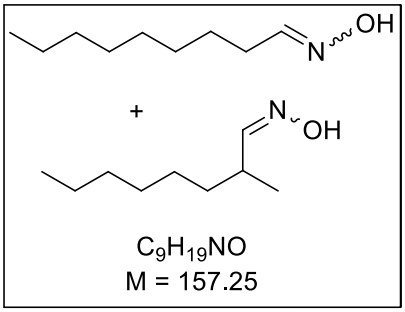
^{1}H-NMR (500 MHz, $CDCl_3$, *Z*-Oxim) δ [ppm] = 6.72 (t, ^3J = 6.0 Hz, 1H, C*H*=NOH), 2.37 (q, ^3J = 7.0 Hz, 13.6 Hz, 2H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 10H), 0.88 (t, ^3J = 6 Hz, 3H, CH_3).

^{13}C-NMR (126 MHz, $CDCl_3$) δ [ppm] = 153.05, 152.44, 77.16, 31.96, 29.62, 29.52, 29.41, 29.31, 29.29, 29.22, 26.69, 26.18, 25.13, 22.78, 14.22.

IR (neat): 3189, 3082, 3040, 2955, 2917, 2850, 1660.

HMRS (ESI): calculated for $C_9H_{19}NOH^+$ $[M+H]^+$: 158.1540, found: 158.1539.

10.1.3 Synthesis of a mixture of (*E*/*Z*)-*n*-nonanal oxime and (*E*/*Z*)-*iso*-nonanal oxime



Hydroxylamine hydrochloride (0.98 g, 14 mmol) and sodium carbonate (0.75 g, 7 mmol) were dissolved in PPB buffer (38 mL, 50 mM, pH = 7) at rt. *n*/*iso*-nonanal (1.50 g, 11 mmol, *n*/*iso*-ratio 2:1) and ^{i}Pr (10 mL) were added and the emulsion was stirred vigorously for 16 h at 35 °C. Work up with cyclohexane led to a wax-like colourless solid (*E*/*Z*-ratio 2.1:1).

Yield: 1.37 g, 82%.

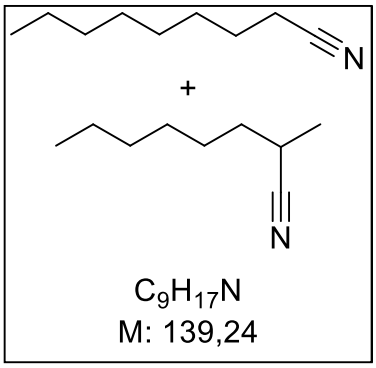
^{1}H-NMR (500 MHz, $CDCl_3$, *n*-*E*-Oxim) δ [ppm] = 7.42 (t, ^{3}J = 6.4 Hz, 1H, C*H*=NOH), 2.18 (m, 2H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 10H), 0.88 (t, ^{3}J = 6.0 Hz, 3H, CH_3).

^{1}H-NMR (500 MHz, $CDCl_3$, *n*-*Z*-Oxim) δ [ppm] = 6.72 (t, ^{3}J = 6.0 Hz, 1H, C*H*=NOH), 2.37 (m, 2H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 10H), 0.88 (t, ^{3}J = 6 Hz, 3H, CH_3).

^{1}H-NMR (500 MHz, $CDCl_3$, *iso*-*E*-Oxim) δ [ppm] = 7.30 (d, ^{3}J = 6.9 Hz, 1H, C*H*=NOH), 2.35 (m, 1H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 8H), 1.07 (d, ^{3}J = 6.9 Hz, 3H), 0.88 (t, ^{3}J = 6.0 Hz, 3H, CH_3).

^{1}H-NMR (500 MHz, $CDCl_3$, *iso*-*Z*-Oxim) δ [ppm] = 6.50 (t, J = 6.0 Hz, 1H, C*H*=NOH), 3.12 (m, 1H, CH_2-CH=NOH), 1.55-1.46 (m, 2H, CH_2-CH_2-CH=NOH), 1.2-1.4 (m, 8H), 1.04 (d, ^{3}J = 6.6 Hz, 3H), 0.88 (t, ^{3}J = 6.0 Hz, 3H, CH_3).

^{13}C-NMR (126 MHz, $CDCl_3$) δ [ppm] = 157.61, 156.67, 153.06, 152.39, 34.66, 34.40, 33.99, 31.82, 31.76, 31.74, 29.49, 29.39, 29.28, 29.26, 29.21, 29.18, 29.16, 29.10, 27.20, 26.96, 26.53, 26.06, 24.95, 24.88, 22.65, 22.62, 17.96, 17.48, 14.10, 14.08.



10.1.4 Synthesis of a mixture of *n*-nonanenitrile and *iso*-nonanenitrile

The syntheses were carried out in analogy to *Ma et al*. [2]. The mixture of (*E*/*Z*)-*n*-nonanal oxime and (*E*/*Z*)-*iso*-nonanal oxime (225 mg, 1.59 mmol, *n*/*iso*-ratio 85:15) was dissolved in acetonitrile (5 ml) at room temperature. Copper(II) acetate (15 mg, 0.08 mmol, 5 mol-%) was added and the solution was heated to reflux for 120 min. The crude product was filtered over a short plug of silica with cyclohexane to yield the product (20 mg, 9%, *n*/*iso*-ratio 90:10) as an oil.

^{1}H-NMR (500 MHz, $CDCl_3$, *n*-Nitrile) δ (ppm) = 2.33 (t, *J* = 7.5 Hz, 2H), 1.36-1.22 (m, 12H), 0.88 (t, *J* = 6.5 Hz, 3H).

^{1}H-NMR (500 MHz, $CDCl_3$, *iso*-Nitrile) δ (ppm) = 2.59 (m, 1H), 1.65 (m, 2H), 1.44 (d, J = 8.3 Hz, 3H) 1.36-1.22 (m, 8H), 0.88 (t, J = 6 Hz, 3H).

^{13}C NMR (126 MHz, CDCl3) δ (ppm) = 34.22, 31.96, 31.85, 31.69, 29.85, 29.11, 28.88, 28.82, 27.14, 25.52, 22.79, 22.74, 18.18, 17.31, 14.24, 14.21, 1.17.

11 References

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[2] Ma, X.-Y.; He, Y.; Lu, T.-T. Lu, M. Conversion of Aldoximes into Nitriles Catalyzed by Simple Transition Metal Salt of the Fourth Period in Acetonitrile. *Tetrahedron* 2013, *69*, 2560-2564.

6.6 Article 10 *submitted*

From biorenewables to industrial applicable bifunctional molecules using chemoenzymatic transformations of bioderived unsaturated fatty acids

A. Hinzmann, S. S. Druhmann, H. Gröger

Sustainable Chemistry **2020**, submitted manuscript

Manuscript-ID: suschem-900880

Author contribution

AH initiated the project and designed and performed the experiments with the help of SD (bachelor student supervised by AH). AH wrote the manuscript. AH and HG read and edited the manuscript.

sustainable chemistry

MDPI

Article

From biorenewables to industrial applicable bifunctional molecules using chemoenzymatic transformations of bioderived unsaturated fatty acids

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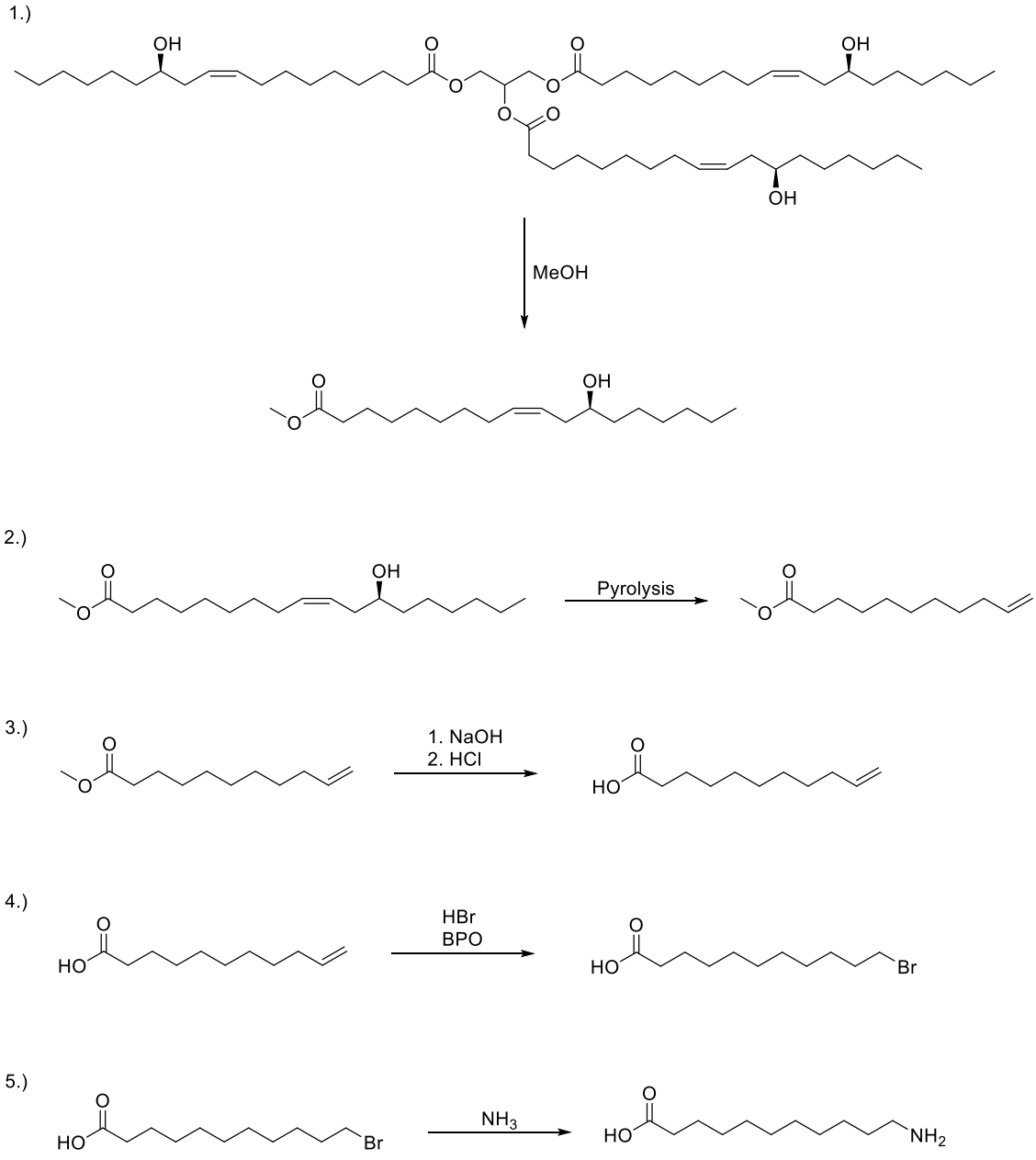
Abstract: Currently, investigations of polymer-building blocks made from biorenewable feedstocks such as, e.g., fatty acids are of high interest for the chemical industry. One of the main segments of chemical production are plastics being originally made from petrochemicals, such as polyethylene synthesized from ethene, polyterephthalic acid produced from the xylene fraction of crude oil and nylon made from diacids and diamines or lactams. Addressing the goal of the chemical industry to reduce the amount of crude oil, great research effort is currently spent to develop novel chemical processes being back-integrated to the use of biobased resources. In our work, we focused on the synthesis of monomers for polyamide synthesis starting from unsaturated fatty acids. We combine hydroformylation with a spontaneous condensation of the formed aldehydes with hydroxylamine to give aldoximes. Aldoximes can biocatalytically be dehydrated to nitriles in the presence of enzymes or by metal-catalysis with copper acetate. Nitriles are precursor for amines, which can be synthesized by hydrogenation. The fatty acid-derived amino acids being synthesized through the developed chemoenzymatic cascade could serve as precursor for various polyamide products.

Keywords: aldoxime dehydratases; amino acids; fatty acids; hydroformylation; oleic acid

1. Introduction

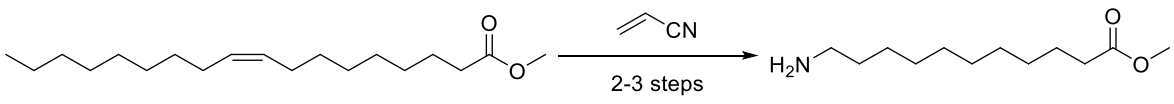
Polyamides (PAs) are very important polymers that are used in industry and in our everyday life. They are used for clothing, parachutes, hang-gliders, balloons, sails, technical fabrics, ropes and many technical parts such as screws. PAs can be produced by condensation of diacids like adipic acid with diamines, for example 1,6-hexanediamine, to give Nylon-6,6 or by condensation of amino acids or ring-opening polymerization of lactams. Especially, Nylon-6,6, Nylon-6 or Nylon-11 are often used polyamides and are produced in multi-ton scale in industry. Nylon-6,6 is synthesized by the polycondensation of adipic acid with 1,6-hexanediamine,[1] while Nylon-6 is produced by a ring-opening polymerization of ε-caprolactame.[2] Nylon-11 can be obtained by the polycondensation of 11-aminoundecanoic acid.[3] An already established process to produce 11-aminoundecanoic acid from biorenewable starts from castor oil. Castor oil consists to 90% of triglycerides of ricinoleic acid which converted within five steps to 11-aminoundecanoic acid.[4] In a first step, a transesterification of the triglycerides with methanol is performed. Secondly, a pyrolysis of methyl ricinoleate is performed which results in heptanal and methyl undecanoate. Methyl undecanoate is afterwards hydrolyzed to the free acid (10-undecanoic acid) and a hydrobromination of the double bond is performed. In a last step the bromine is substituted by an amino group using ammonia. A big disadvantage of this process is the pyrolysis step, which proceeds under very harsh gas phasic reaction conditions with temperatures of up to 600 °C. This route also requires the use of

hydrobromic acid for the synthesis of 11-bromoundecanoic acid, which then undergoes a substitution with ammonia to give the final product.



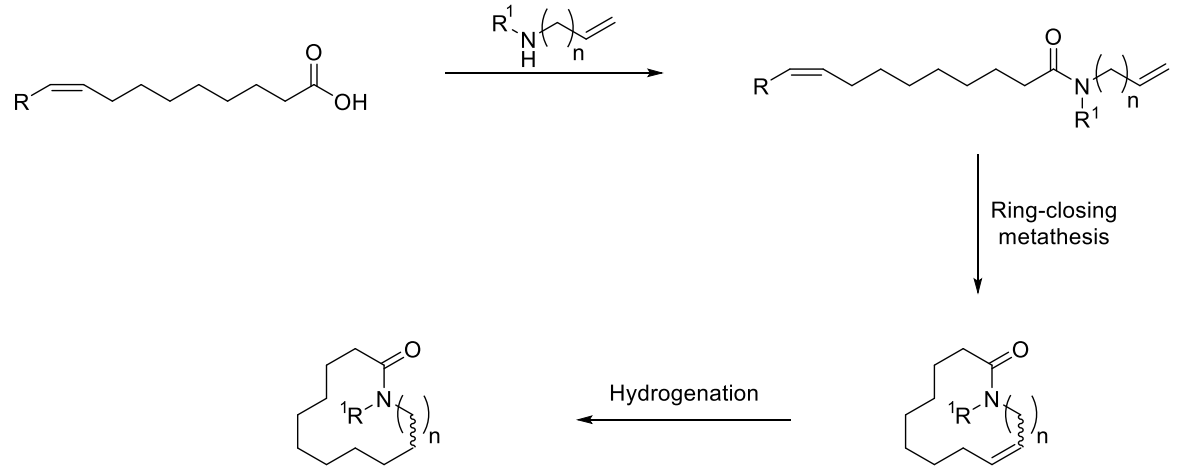
Scheme 1. Synthesis of 11-aminoundecanoic acid from castor oil.[4]

Another way of producing monomers for Nylon-11, -12 and -13 from oleic acid was found by Yamamoto *et al*. on the basis of previous patents[5,6] by ring-closing or cross-metathesis reactions using oleic acid as starting material.[7,8] A very elegant way of producing 11-aminoundecanoic acid (or methyl ester) described in this patent is the cross-metathesis of oleic acid (methyl ester) with acrylonitrile (Scheme 2).



Scheme 1. Cross-metathesis of oleic acid with acrylonitrile to produce 11-aminoundecanoic acid within 2-3 steps.[7][8]

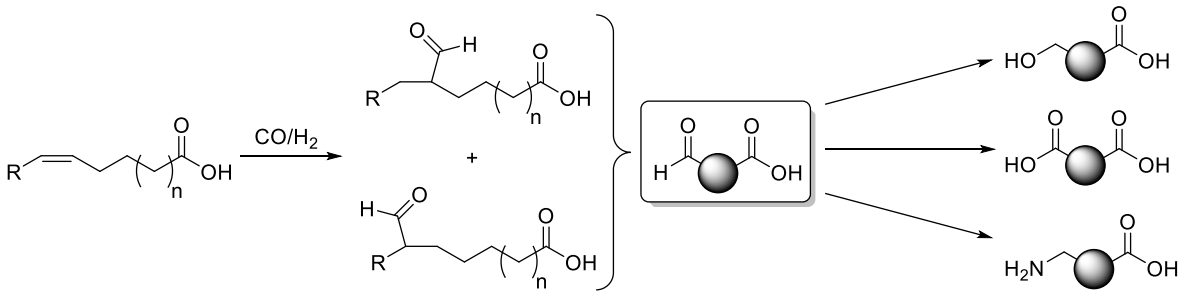
This process is very beneficial because acrylonitrile (which is enzymatically converted to acrylamide on large scale [9]) is readily available, and this method only requires two steps towards the final product by usage of the oleic acid. After the initial cross metathesis, the nitrile functionality is hydrogenated to the corresponding amine. For the ring-closing metathesis approach, they first require an amide, which they synthesize by amidation of an unsaturated acid with an unsaturated amine, which then can undergo ring-closing metathesis (Scheme 3).



Scheme 2. Ring-closing metathesis to produce polymerizable lactames with preciding amidation.[8]

A major disadvantage of this ring-closing metathesis concept is the amidation step, which requires harsh reaction conditions and/or coupling reactions or activated carboxylic acid derivatives such as acid chlorides.

In our work, we were focusing on the hydroformylation of unsaturated acids to the corresponding aldehydes. These aldehydes can be used as precursor for many different functional groups, such as alcohols, acids or amines, which all would result in bifunctional molecules being applicable as polymer precursors (Scheme 4).



Scheme 3. Synthesis of aldehydes from unsaturated acids leading to polymer building blocks.

We focused on the conversion of unsaturated fatty acids like oleic acid as biorenewable starting material towards polymerizable amino acids via hydroformylation to aldehydes, aldoxime intermediates and nitriles.

2. Materials and Methods

Hydroformylation of unsaturated acids using Rh-TPPTS in a biphasic reaction medium. Hydroformylation reactions were performed in a Parr Series 5000 Multiple Reactor System in which up to six different hydroformylation reactions were carried out in parallel. The maximum volume of the used autoclaves was 75 mL and the reaction mixtures were stirred at 1000 rpm. Before usage of the reactors, they were evaporated and filled with Argon twice. Catalyst stock solution consisting of

$Rh(acac)(CO)_2$ (0.00025 mol/L) and TPPTS (0.005 mol/L) were prepared in MilliQ distilled water and applied to the autoclaves first in Argon counterflow (4 mL). Afterwards the required unsaturated acid (hex-5-enoic acid (**6**), oct-7-enoic acid (**11**) or dec-9-enoic acid (**16**)) were added (6 mL) in Argon counterflow. The autoclaves were closed, washed with nitrogen (3x 20 bar, 1x 80 bar), washed with synthetic gas mixture (syn-gas) (H_2/CO 50 mol%/50 mol%) (3x 20 bar) and filled with syn-gas to 80 bar. The required reaction temperature was applied (hex-5-enoic acid (**6**) 80 °C, oct-7-enoic acid (**11**) and dec-9-enoic acid (**16**) 100 °C) and the reaction mixtures stirred at 1000 rpm for 4 h (hex-5-enoic acid (**6**)) or 6 h (oct-7-enoic acid (**11**) and dec-9-enoic acid (**16**)). After the reaction heating was stopped, syn-gas pressure was released and the autoclaves washed with nitrogen (3x 20 bar). A sample of the organic phase was used for ^{1}H- and ^{13}C-NMR analysis in $CDCl_3$. The crude reaction mixture was directly used for aldoxime synthesis.

Characterization of aldehydes is available in the Supporting Information.

Hydroformylation of oleic acid using Rh-TPP in a neat approach. Hydroformylation reactions were performed in a Parr Series 5000 Multiple Reactor System in which up to six different hydroformylation reactions were carried out in parallel. The maximum volume of the used autoclaves was 75 mL and the reaction mixtures were stirred at 1000 rpm. Before usage of the reactors, they were evaporated and filled with Argon twice. Catalyst stock solution consisting of $Rh(acac)(CO)_2$ (0.00025 mol/L) and TPP (0.005 mol/L) were prepared in oleic acid (**1**) and applied to the autoclaves first in Argon counterflow (6 mL). The autoclaves were closed, washed with nitrogen (3x 20 bar, 1x 80 bar), washed with synthetic gas mixture (syn-gas) (H_2/CO 50 mol%/50 mol%) (3x 20 bar) and filled with syn-gas to 80 bar. The required reaction temperature of 100 °C was applied and the reaction mixtures stirred at 1000 rpm for 6 h or 24 h. After the reaction heating was stopped, syn-gas pressure was released and the autoclaves washed with nitrogen (3x 20 bar). The crude reaction mixture was filtered over Celite directly into the reaction flask for aldoxime synthesis. A small sample was used for ^{1}H- and ^{13}C-NMR analysis in $CDCl_3$.

Characterization of aldehydes is available in the Supporting Information.

Condensation of aldehydes to aldoximes. The crude hydroformylation mixture was attached to a round bottom flask and hydroxylamine hydrochloride (1.5 eq based on used unsaturated acid for hydroformylation) and sodium carbonate (0.75 eq based on used unsaturated acid for hydroformylation) were added. In case of the Rh-TPP hydroformylation the salts ($H_2NOH{\cdot}HCl$ and Na_2CO_3) were dissolved in dest. H_2O (4 mL) beforehand and the hydroformylation product was added. The reaction mixture was stirred for 4 h. Precipitated colorless solid was filtered, washed with water (2x 15 mL) and dried in high vacuum. The filtrate was extracted with 2-Me-THF (3x 15 mL), combined organic phases were dried over $MgSO_4$, the solvent was removed *in vacuo* and the residue was dried in high vacuum. In case of oleic acid, no extraction was performed. Filtrated product and extracted product were analyzed by ^{1}H- and ^{13}C-NMR separately. Filtered product was found to be pure *n*-aldoxime *n*-**8**, *n*-**13** or *n*-**18**. Extracted product was a ~0.5:1 ratio of *n*-aldoxime *n*-**8**, *n*-**13** or *n*-**18** to *iso*-aldoxime *iso*-**8**, *iso*-**13** or *iso*-**18**.

Characterization of aldoximes is available in the Supporting Information.

Biotransformation of ω-aldoxime carboxylic acids using OxdB as whole cell catalyst and catalyst preparation. Chemical competent *E- coli* BL21-C odonPlus(DE3)-RIL cells (100 µL) were transformed with pUC18-plasmid DNA containing the gene encoding OxdB analogue to standard protocols.[10] Transformed *E. coli* cells were plated on LB-agar containing Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) and incubated overnight at 37 °C. Pre-cultures were prepared in LB-medium (20 mL) in 100 mL Erlenmeyer flasks containing Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) using a single colony from the LB-agar plate. The cultures were incubated overnight at 37 °C and 180 rpm. Main cultures for the expression of OxdB was performed using TB-autoinduction medium. Sterile 20 g/L d-Lactose solution in MilliQ water (160 mL) and sterile 50 g/L d-glucose solution in MilliQ water (16 mL) was added to 1424 mL sterile TB-medium

(Carl Roth) in a 2 L Erlenmeyer flask. Carbenicillin (100 µg/mL) and Chloramphenicol (34 µg/mL) were added to the medium. Main cultures were inoculated with 1% (16 mL) of the OxdB pre-culture and incubated for 2 h at 37 °C and 150 rpm. After 2 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 150 rpm. Cell harvest was performed at 5,000 g for 15 min and 4 °C. The supernatant was discarded and cells were washed three times with 50 mM potassium phosphate buffer (PPB, KPi) at pH 7.0. The biomass was determined (bio wet weight (bww)) and cells were resuspended in 50 mM PPB (pH 7.0) to a final concentration of 333 mg/mL cells in buffer. Cell suspensions were stored at 4 °C or on ice before usage.

Biotransformations were performed at a 5 mL scale using PPB (50 mM, pH 7) including 10% (*v*/*v*) ethanol as co-solvent at 30 °C and 24 h reaction time. The required amounts of aldoximes (see supporting information) were attached to a 10 mL glass vial, equipped with a magnetic stirrer. Ethanol (500 µL) and PPB (4 mL) were added. The mixture was stirred for 5 min at 30 °C before addition of whole cell suspension (500 µL). After 24 h reaction time the reaction mixture was transferred into a 50 mL Falcon tube and centrifuged for 5 min at 5000 g. The supernatant was transferred into a separating funnel and extracted with 2-methyl-TFH (5x 10 mL) including 0.1% trifluoroacidic acid. The cell pellet was extracted with 2-methyl-THF (5x 10 mL) by resuspension and centrifugation. The organic phases were combined, dried over $MgSO_4$ and the solvent was removed *in vacuo*. After drying of the product in high vacuum the products were analyzed by ^{1}H-NMR in DMSO-d6.

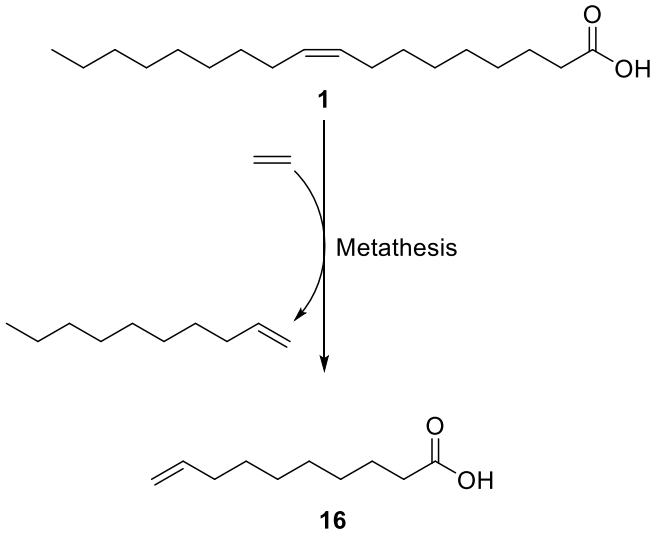
Table with concentrations and conversions is available in the Supporting Information.

Cu^{II} acetate catalyzed nitrile synthesis. Cu^{II} acetate (5 mol%) was attached to a round bottom flask and dissolved in acetonitrile (50 mL). The aldoxime was added and the mixture was refluxed for 16 h before cooling to room temperature, removal of solvent and silica filtration using 2-methyl THF (including 0.1% trifluoroacidic acid) as solvent. After removal of the solvent, the product was dried in high vacuum and analyzed by ^{1}H- and ^{13}C-NMR in $CDCl_3$ or DMSO-d6.

Characterization of nitriles is available in the Supporting Information.

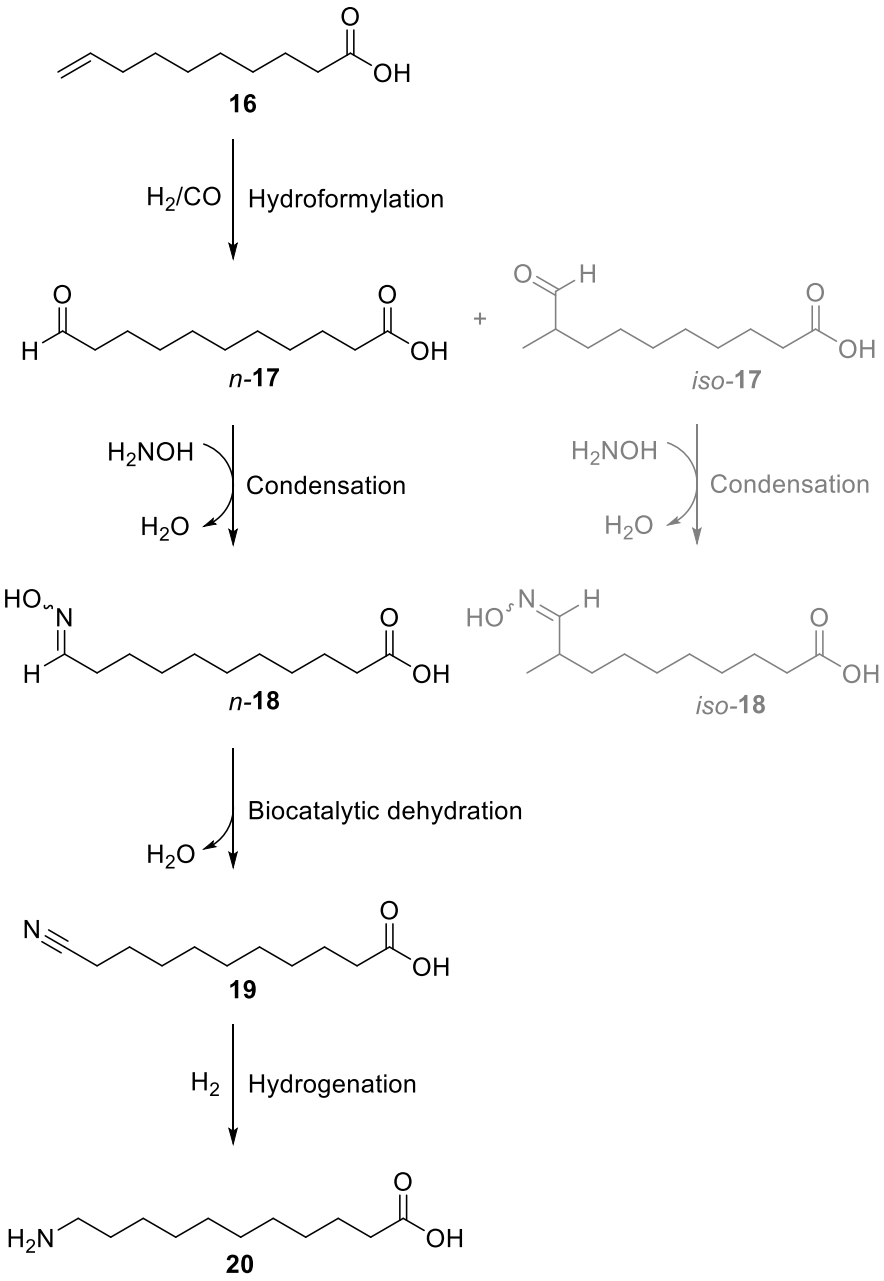
3. Results and discussion

We started with the terminal unsaturated acids as starting material, which can be obtained from natural unsaturated fatty acids like oleic acid by cross-metathesis with ethylene (Scheme 5).



Scheme 4. Possible access to 9-decenoic acid (**16**) starting from oleic acid as a renewable raw material by metathesis.

Besides 9-decenoic acid (**16**), we used 5-hexenoic acid (**6**) and 7-octenoic acid (**11**) as model substrates to synthesize polymer building blocks, which consist of a terminal acid group and a terminal amino functionality. During this project, we investigated the hydroformylation of these unsaturated acids to the corresponding aldehydes, the condensation of the aldehydes to aldoximes, dehydration to the nitriles biocatalytically by aldoxime dehydratases (Oxds) and the hydrogenation of the nitriles to the primary amines (Scheme 6).

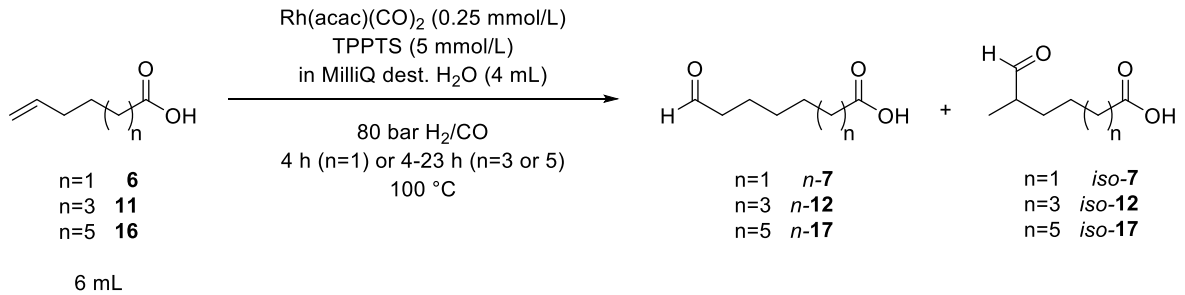


Scheme 5. Access to amino acids as polymer building blocks exemplified for 11-aminoundecanoic acid (**20**).

An alternative route towards the amine starting from hydroformylated acids could be a reductive amination for example. In this case, the aldoxime and nitrile intermediates would not be necessary and a conversion of aldehydes directly to the amine would be offered. Unfortunately, reductive amination usually lead to strong selectivity problems and/or very expensive catalysts are needed.[11,12] In a patent from 1996 Ayorinde *et al.* presented another alternative starting from

aldoximes (obtained from the corresponding aldehyde) in which the aldoxime first undergoes a Beckmann-rearrangement.[13] The carbamylcarboxylic acid is afterwards converted by a Hofmann degradation, which after hydrolysis lead to the final amino acid as product. This route also has some disadvantages, for example the reaction conditions required for Beckmann-rearrangement or the halogen which is used for the Hofmann degradation. Since we were interested in the establishment of a method for hydroformylation of unsaturated carboxylic acids and further conversion of the aldehydes towards nitriles (which then serve as precursor for amines), we investigated the chemoenzymatic cascade shown in Scheme 6 with aldoxime and nitrile intermediates.

The initial step of this cascade is the hydroformylation of the unsaturated acids to obtain the aldehydes. For our purpose we used as a catalyst $Rh(acac)(CO)_2$ in combination with the water-soluble TPPTS ligand, which is a comparably simple ligand for hydroformylation and results often a mixture of linear (*n*) and brunched (*iso*) aldehydes.[14] In Figure 1 the reaction conditions and results are shown.



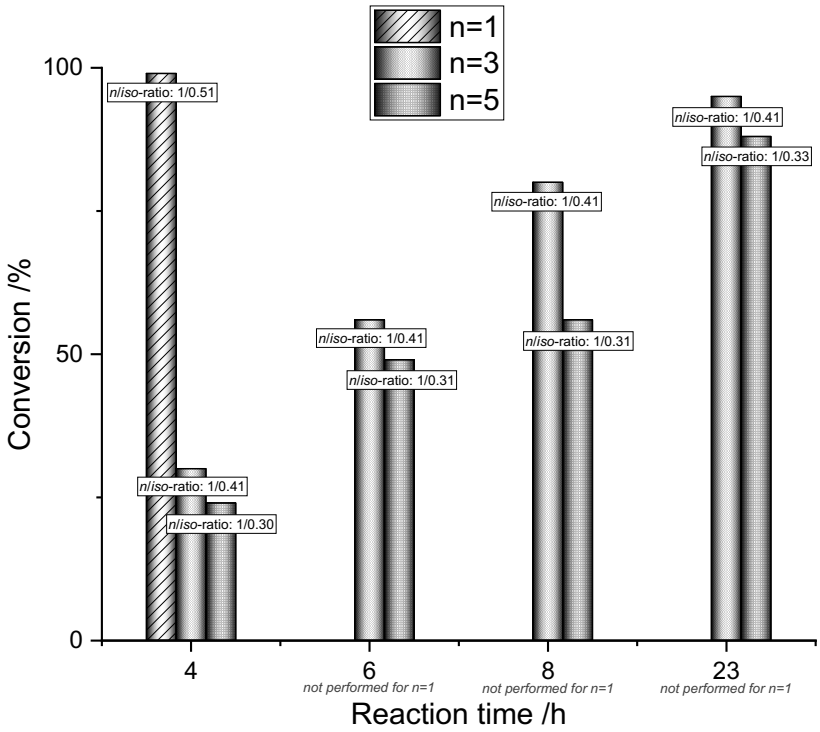
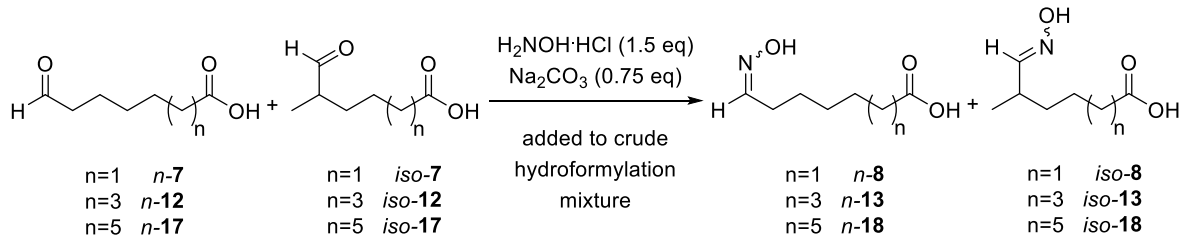


Figure 1. Results of hydroformylation experiments after different reaction times. Conversions were determined by ^{1}H-NMR spectroscopy.

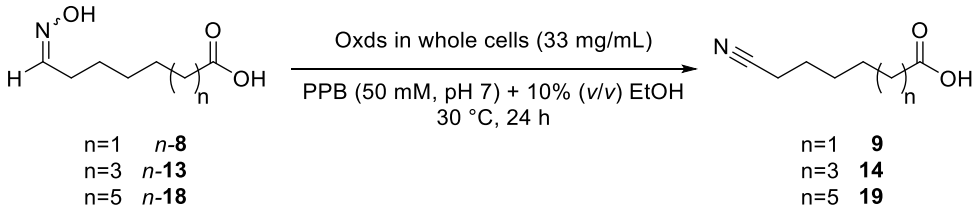
We found high conversions towards the aldehyde products with *n*/*iso*-ratios of 1:0.5 to 1:0.3. Especially for the shorter chain length with n=1 **6** after 4 h reaction time full conversion of the alkene **6** to the aldehyde mixture (*n*/*iso*)-**7** was observed. In case of the longer chain length, longer reaction times are needed to obtain high conversion of the alkenes **11** and **16** to the corresponding aldehyde mixtures (*n*/*iso*)-**12** and (*n*/*iso*)-**17**. Since aldehydes are usually very oxidation sensitive, we directly

used the crude aldehyde mixture for the next step of the cascade. To the hydroformylation mixture hydroxylamine hydrochloride and sodium carbonate were added.



Scheme 6. Condensation of aldehydes to aldoximes.

Aldoximes precipitated from the aqueous reaction mixture and were filtered. Fortunately, in the ^{1}H-NMR spectrum only the *n*-aldoximes (*n*-**8**, *n*-**13** or *n*-**18**) were found. Extraction of the filtrate with 2-methyl tetrahydrofuran (2-Me THF) provided a mixture of *n*- and *iso* aldoximes. Due to the simple isolation of the *n*-products we used the pure *n*-aldoxime for the next steps of the cascade. The aldoximes were converted by Oxds, which are enzymes that are efficient for the dehydration of aldoximes to nitriles.[10,15,16] We conducted the biotransformations in potassium phosphate buffer (PPB) at 30 °C reaction temperature (Figure 2).



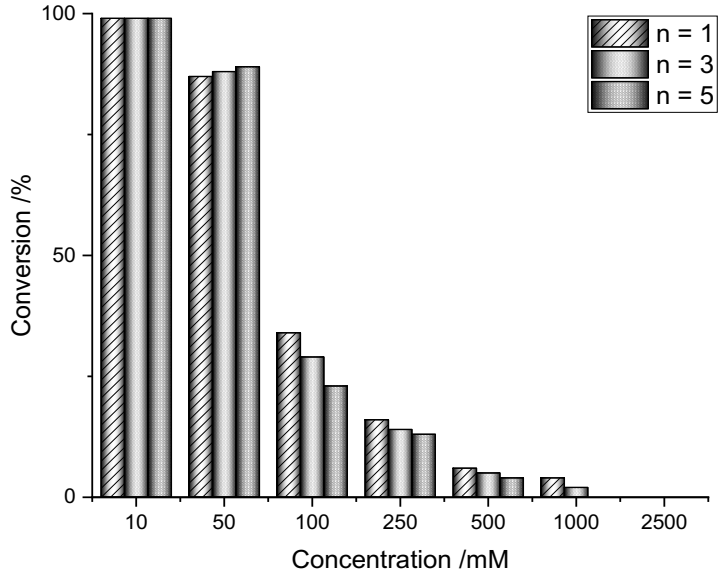
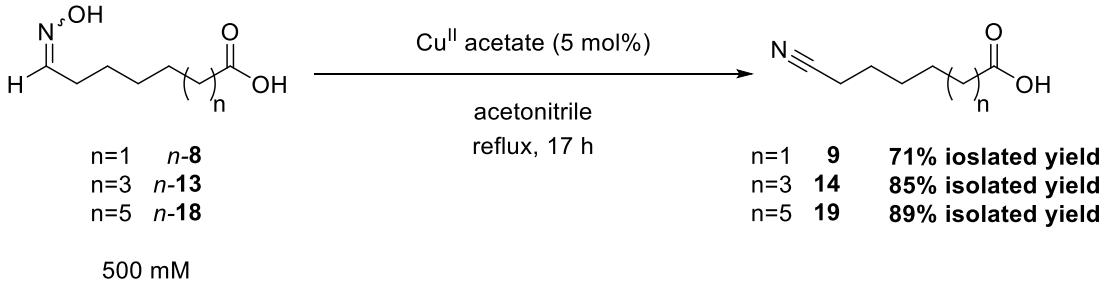


Figure 2. Biotransformations of aldoximes to nitriles using Oxds in 5 mL scale.

At a substrate concentration of 10 mM, all substrates were quantitatively converted to the corresponding nitriles using an aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB). An increase of the substrate concentration to 50 mM led to conversion of approx. 90%, while 100 mM or even higher substrate loading led to conversion lower than 50%. To prevent a purification of the nitrile products, only 10 mM substrate concentrations were found to be suitable for this biotransformation of

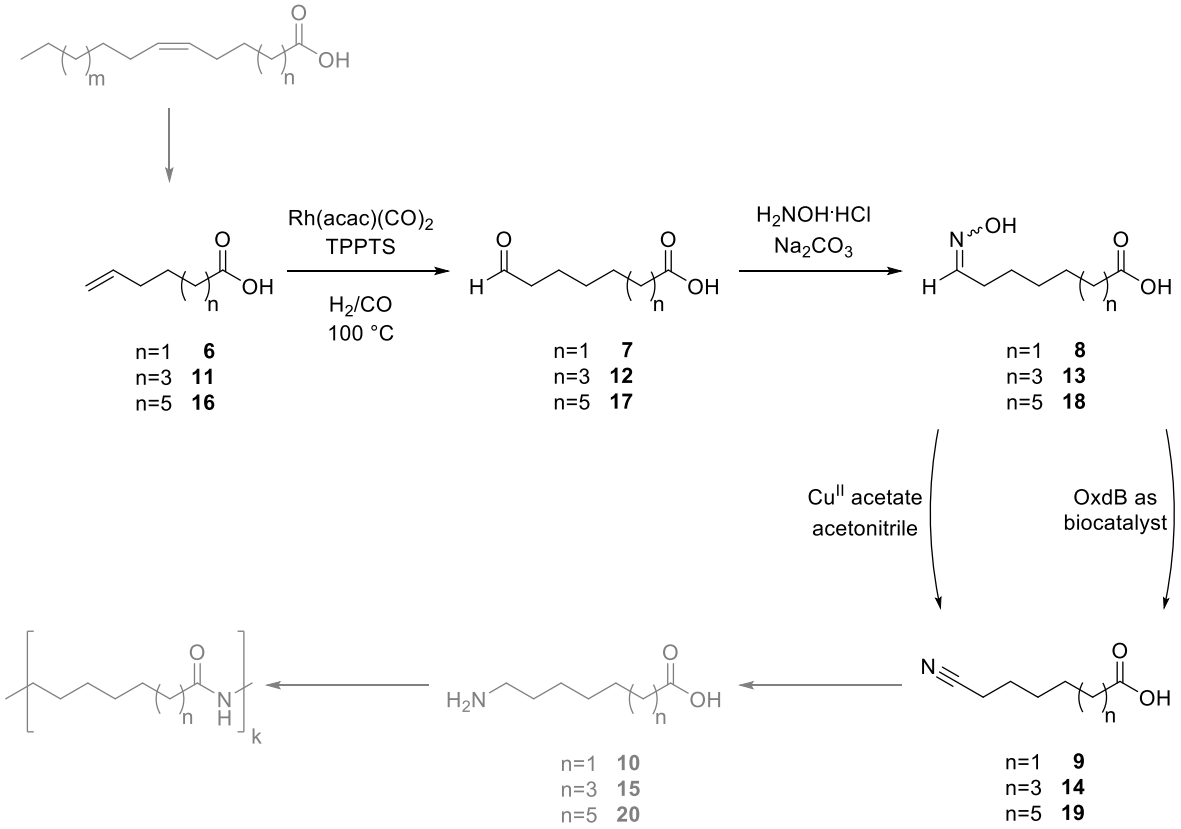
aldoximes to nitriles using OxdB as whole cell catalyst. An alternative to OxdB as catalyst is Cu^{II} acetate which is capable of converting aldoximes into nitriles by usage of an nitrile as co-substrate which is converted to an amide. We conducted our nitrile synthesis using 5 mol% Cu^{II} acetate as a catalyst and acetonitrile as both, solvent and co-substrate (Scheme 8).



Scheme 7. Nitrile synthesis using Cu^{II} acetate as a catalyst in acetonitrile.

Using this method, the nitriles were obtained with isolated yields of 71 -89% after column filtration to remove the Cu^{II} from the reaction medium. With this method a higher substrate loading of 500 mM can be applied and no residual aldoxime was found after the reaction.

All in all, we were able to perform a cascade starting from unsaturated acids, which can be obtained from fatty acids using metathesis, to nitrile-functionalized acids. Nitriles can be hydrogenated to amines, which would lead to polymer building blocks for polyamides. In Scheme 9 our cascade is summarized.

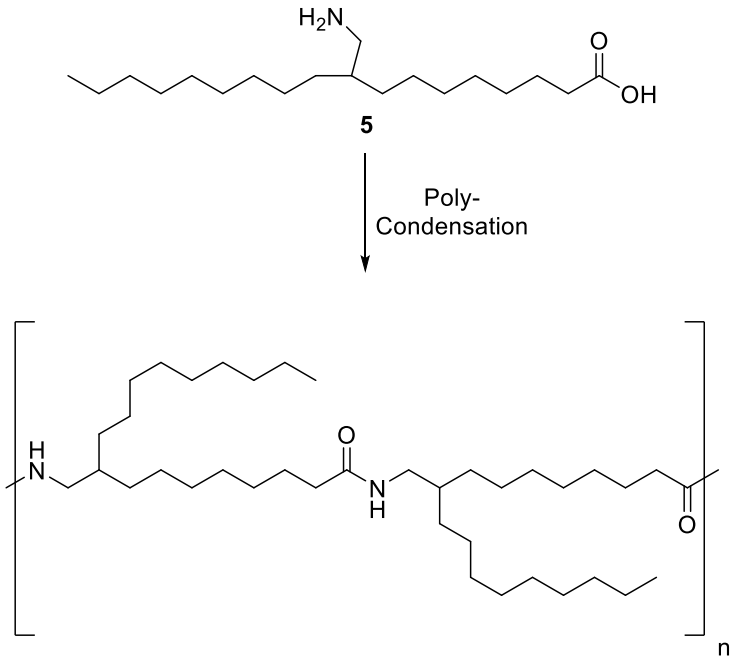


Scheme 8. Synthesis of bifunctional molecules as monomers for polyamides starting from fatty acids as biorenewable resource.

Synthesis of branched bifunctional molecules starting from oleic acid

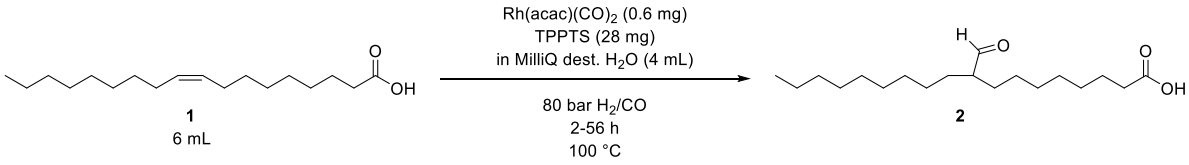
Hydroformylation of oleic acid (**1**), especially of different esters were already studies by many groups. [17–24] Our goal within this project was to apply a very simple method like the Rh-TPPTS system in a biphasic reaction approach or other very simple ligands, such as triphenylphosphine (TPP) as ligand and study the kinetics of this reaction.

A very selective hydroformylation system using Rh-TPP as catalyst is known since a long time[25] and is also an attractive alternative to the biphasic approach. Unsaturated fatty acids as biorenewable resources, for example oleic acid (**1**) can also directly be used for the synthesis of polymer building blocks. In this case, branched monomers are formed when hydroformylating oleic acid (**1**). Oleic acid (**1**) can also be polymerized with itself without derivatization beforehand,[26–29] often leading to oligomers of oleic acid which can be used as lubricants for example. After a hydroformylation of oleic acid (**1**), these molecules could potentially offer new polymer properties, due to the aliphatic chain as branch inside the molecule (Scheme 10).



Scheme 9. Polymerization of amino-functionalized oleic acid (**5**).

We first investigated the hydroformylation of oleic acid (**1**) using the Rh-TPPTS approach in a biphasic reaction medium consisting of water (in which the catalyst is dissolved) and oleic acid (**1**) as organic phase (Figure 3) since we successfully applied this method to 5-hexenoic acid (**6**), 7-octenoic acid (**11**) and 9-decenoic acid (**16**).



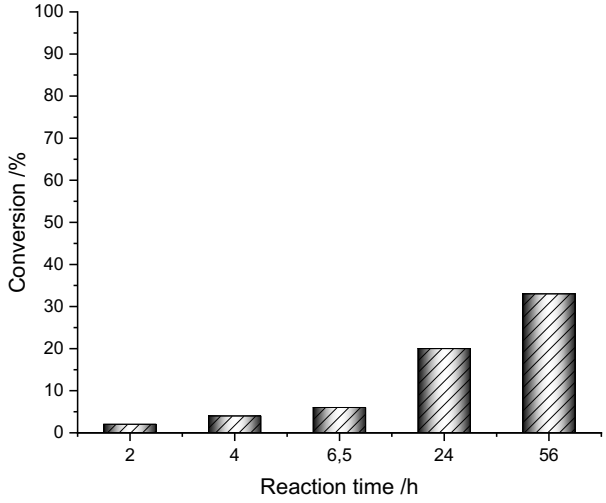
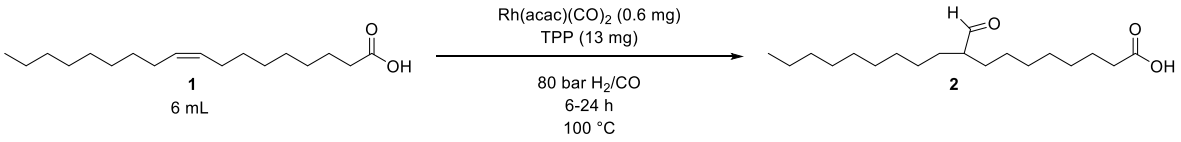


Figure 3. Hydroformylation of oleic acid (**1**) using Rh-TPPTS in a biphasic reaction medium.

Unfortunately, even after 56 h reaction time only a conversion of approx. 30% to the aldehyde **2** was found. The velocity of the hydroformylation of an inner double bond was found in this case to be much slower. Since this biphasic approach did not lead to high conversions, we further investigated the hydroformylation step. In a next step, we used a neat reaction approach in which the catalyst, in this case consisting of $Rh(acac)(CO)_2$ and TPP, is dissolved in oleic acid (**1**) as both solvent and substrate (Figure 4).



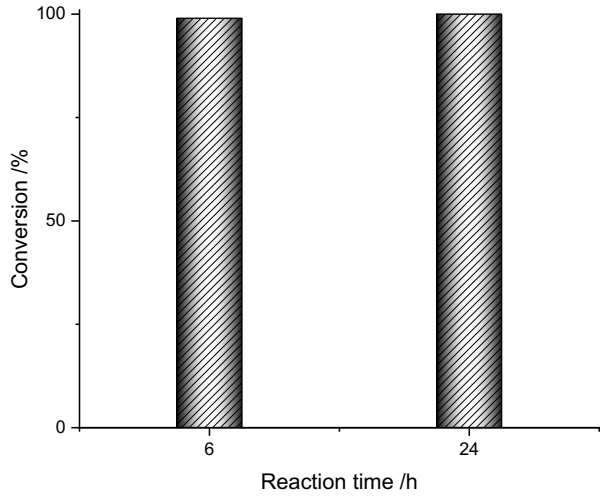
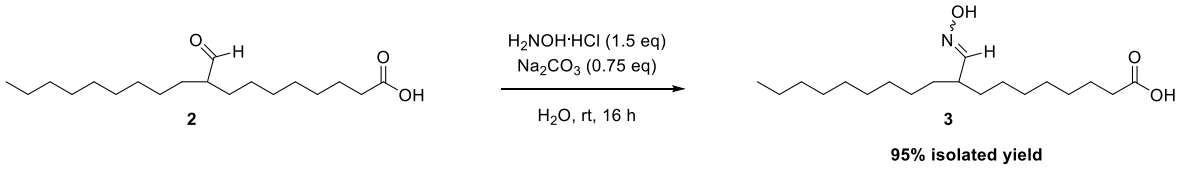


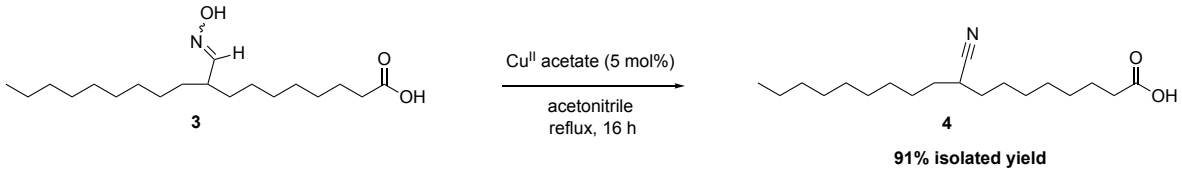
Figure 4. Hydroformylation of oleic acid using Rh-TPPTS in a biphasic reaction medium.

Using this approach, we already obtained 99% conversion after 6 h reaction time and >99% within 24 h. The catalyst can be removed by a filtration over celite and aldehyde **2** is obtained in a very pure form. To prevent oxidation of the aldehyde **2** to the acid, it is directly converted to the corresponding aldoxime **3** by condensation with hydroxylamine (Scheme 11).



Scheme 10. Synthesis of aldoxime **3** from formylstearic acid **2**.

The aldoxime **3** precipitated from the reaction medium and was easily separated by filtration with an isolated yield of 95%. In a next step, the aldoxime **3** was dehydrated to the nitrile **4**. In this case, we did not use an aldoxime dehydratase because this substrate is relatively bulky and we estimated that we will not find conversion to the nitrile. Therefore, we used again the Cu^{II} acetate method in acetonitrile as solvent and co-substrate (Scheme 12).



Scheme 11. Synthesis of nitrile **4** from aldoxime **3**.

After column filtration to remove the catalyst from the reaction medium, 91% isolated yield of nitrile **4** was obtained. This nitrile functionality could also be hydrogenated to the corresponding amine **5**, which would lead to a branched polymerizable amino acid. Our method, starting from unsaturated fatty acids as biorenewable resource to synthesize polymer building blocks, could also be applied to other substrates which would lead to a platform of monomers serving as precursor for various polymers.

4. Conclusions

In conclusion, we investigated a method to synthesize monomers as precursor for various polymers starting from fatty acids as biorenewable material. In our study, we focused on oleic acid (**1**) as bioderived fatty acid, which could be used in a first step in a metathesis reaction with ethene to obtain linear bifunctional molecules or directly be hydroformylated to gain branched bifunctional molecules. In our cascades, we combined hydroformylation reactions for the conversion of alkenes into aldehydes with condensation with hydroxylamine to the corresponding aldoximes. These aldoximes are then converted by usage of Oxds as biocatalysts or copper acetate in acetonitrile, which in a next step could be hydrogenated to amines. With these various amino acids different polyamides could be produced.

Supplementary Materials: Supplementary materials related to general experimental information (chemicals, analytical methods), characterization of aldehydes, aldoximes and nitriles, sequences for the aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB), and the expression of the OxdB are available online at www.mdpi.com/xxx/s1.

Author Contributions: Conceptualization, A.H. and H.G.; methodology, A.H. and S.S.D.; formal analysis, A.H. and S.S.D..; investigation, A.H. and S.S.D.; resources, H.G..; data curation, A.H. and S.S.D.; writing—original draft preparation, A.H.; writing—review and editing, H.G.; supervision, A.H. and H.G.; project administration, H.G.; funding acquisition, H.G. All authors have read and agreed to the published version of the manuscript.

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Supporting Information

From biorenewables to industrial applicable bifunctional molecules using chemoenzymatic transformations of bioderived unsaturated fatty acids

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General experimental information

Chemicals used

Material for cell cultivation and aldoxime dehydratase (Oxd) expression was purchased by Carl Roth (Antibiotics, LB- and TB-premixed medium, D-glucose, D-lactose). Buffer salts (KH_2PO_4 and K_2HPO_4) and hydroxylamine hydrochloride and sodium carbonate were obtained from VWR chemicals. Unsaturated fatty acids were purchased from Sigma Aldrich, TCI Chemicals or Alfa Aesar, respectively. $Rh(acac)(CO)_2$ was purchased from Alfa Aesar and TPPTS and TPP were purchased from TCI Chemicals.

Analytical methods

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^1H) or 125 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal.

Accurate mass nano-ESI measurements are performed using a Q-IMS-TOF mass spectrometer Synapt G2Si (Waters GmbH, Manchester, UK) in resolution mode, interfaced to a nano-ESI ion source. Nitrogen serves both as the nebulizer gas and the dry gas for nano-ESI. Nitrogen is generated by a nitrogen generator NGM 11. Helium 5.0 is used as buffer gas in the IMS entry cell, nitrogen 5.0 is used for IMS separations. 1,3-Dicyanobenzene is used as an electron-transfer reagent in ETD experiments. Samples were dissolved in acetonitrile and introduced by static nano-ESI using in-house pulled glass emitters.

Characterization of aldehydes

Hydroformylation of 5-hexenoic acid. Hydroformylation was carried out at 100 °C for 4 h resulting in >99% conversion towards a *n*-/*iso*-mixture of 1:0.51 in a yield of ?%. 7-Oxoheptanoic acid: ^1H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.98 (s, 1H), 9.65 (t, 1H), 2.40 (m, 2H), 2.20 (m, 2H), 1.49 (m, 4H), 1.26 (m, 2H).

The ^1H-NMR spectrum is in accordance with the literature.[1]

Hydroformylation of 7-octenoic acid. Hydroformylation was carried out at 100 °C for different reaction times resulting in 95% conversion within 23 h towards a *n*-/*iso*-mixture of 1:0.75 in a yield of ?%. 9-Oxononanoic acid: ^1H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.98 (s, 1H), 9.65 (t, 1H), 2.40 (m, 2H), 2.20 (m, 2H), 1.49 (m, 4H), 1.26 (m, 2H).

The ^1H-NMR spectrum is in accordance with the literature.[2]

Hydroformylation of 9-decenoic acid. Hydroformylation was carried out at 100 °C for different reaction times resulting in 95% conversion within 23 h towards a *n*-/*iso*-mixture of 1:0.75 in a yield of ?%. 11-Oxoundecanoi acid: ^1H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.98 (s, 1H), 9.76 (t, 1H), 2.42 (m, 2H), 2.35 (m, 2H), 1.63 (m, 2H), 1.56 (m, 2H), 1.30 (m, 10H).

The ^1H-NMR spectrum is in accordance with the literature.[3]

Hydroformylation of oleic acid using Rh-TPP in a neat approach. 9-Formylstearic acid was obtained as a colourless oil with an isolated yield of ?%. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ/ppm: 11.40 (s, 1H), 9.54 (d, 1H), 2.35 (t, 2H), 2.24 (m, 1H), 1.65 (m, 2H), 1.45 (m, 2H), 1.28 (m, 22H), 0.88 (t, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ/ppm: 205.96, 180.13, 52.12, 34.17, 30.02, 29.85, 29.75, 29.58, 29.34, 29.28, 29.17, 29.08, 29.04, 27.23, 27.16, 24.74, 22.80, 14.24.

Characterization of aldoximes

7-*E*-Aldoxime heptanoic acid. The *E*-aldoxime was isolated by filtration as a colourless solid with an isolated yield of ?%. ^{1}H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.98 (s, 1H), 10.71 (s, 1H), 6.62 (t, 1H), 2.20 (m, 4H), 1.50 (p, 2H), 1.40 (p, 2H), 1.29 (m, 2H); ^{13}C-NMR (DMSO-d6, 125 MHz) δ/ppm: 174.43, 150.25, 33.54, 28.37, 25.34, 24.42, 24.23; HRMS (ESI, negative ions) *m/z*: $[M-H]^-$ calcd for $C_7H_{12}NO_3^-$ 158.0823, found 158.0827.

9-*E*-Aldoxime nonanoic acid. The *E*-aldoxime was isolated by filtration as a colourless solid with an isolated yield of ?%. ^{1}H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.97 (s, 1H), 10.70 (s, 1H), 6.62 (t, 1H), 2.20 (m, 4H), 1.48 (p, 2H), 1.39 (p, 2H), 1.26 (m, 6H); ^{13}C-NMR (DMSO-d6, 125 MHz) δ/ppm: 174.47, 150.34, 33.64, 28.72, 28.46, 28.44, 25.57, 24.52, 24.45; HRMS (ESI, negative ions) *m/z*: $[M-H]^-$ calcd for $C_9H_{16}NO_3^-$ 186.1136, found 168.1140.

11-*E*-Aldoxime undecanoic acid. The *E*-aldoxime was isolated by filtration as a colourless solid with an isolated yield of ?%. ^{1}H-NMR (DMSO-d6, 500 MHz) δ/ppm: 11.96 (s, 1H), 10.69 (s, 1H), 6.62 (t, 1H), 2.20 (m, 4H), 1.47 (p, 2H), 1.38 (p, 2H), 1.25 (m, 10H); ^{13}C-NMR (DMSO-d6, 125 MHz) δ/ppm: 174.49, 150.36, 33.66, 28.93, 28.84, 28.80, 28.70, 28.53, 25.61, 24.53, 24.49; HRMS (ESI, negative ions) *m/z*: $[M-H]^-$ calcd for $C_{11}H_{21}NO_3^-$ 216.1594, found 216.1594.

9-Formyloxime stearic acid. The *E*-/*Z*-aldoxime mixture (~1:1) was isolated by filtration as a colourless solid with an isolated yield of ?%. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ/ppm: 9.04 (s, 1H), 7.21 (d, 1H, *Z*-Aldoxime-H), 6.44 (d, 1H, *E*-Aldoxime-H), 3.08 (m, 1H, *Z*), 2.31 (t, 2H), 2.19 (m, 1H, *E*), 1.60 (m, 2H), 1.30 (m, 26H), 0.88 (t, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ/ppm: 179.58, 156.49 (*Z*), 155.79 (*E*) 39.96, 35.22, 34.49, 33.19, 33.02, 32.03, 29.83, 29.75, 29.60, 29.41, 29.17, 29.07, 27.18, 24.94, 22.80, 14.25; HRMS (ESI, positive ions) *m/z*: $[M-H]^+$ calcd for $C_{19}H_{37}NO_3H^+$ 328.2846, found 328.2839.

Characterization of nitriles

6-Cyanohexanoic acid. The nitrile was obtained as a yellowish oil from the Cu^{II}-acetate synthesis in an isolated yield of 71%. ^{1}H-NMR (DMSO-d6, 500 MHz) δ/ppm: 2.38 (m, 4H), 1.69 (m, 4H), 1.53 (m, 2H).

The ^{1}H-NMR spectrum is in accordance with the literature.[1]

8-Cyanooctanoic acid. The nitrile was obtained as a yellowish oil from the Cu^{II}-acetate synthesis in an isolated yield of 85%. ^{1}H-NMR (DMSO-d6, 500 MHz) *δ*/ppm: 2.35 (m, 4H), 1.66 (m, 4H), 1.46 (m, 2H), 1.36 (m, 4H).

The ^{1}H-NMR spectrum is in accordance with the literature.[4]

10-Cyanodecanoic acid. The nitrile was obtained as a yellowish oil from the Cu^{II}-acetate synthesis in an isolated yield of 89%. ^{1}H-NMR (DMSO-d6, 500 MHz) *δ*/ppm: 2.34 (m, 4H), 1.64 (m, 4H), 1.44 (m, 2H), 1.31 (m, 8H).

The ^{1}H-NMR spectrum is in accordance with the literature.[5]

9-Cyanostearic acid. The nitrile was obtained as a yellowish oil from the Cu^{II}-acetate synthesis in an isolated yield of 91%. ^{1}H-NMR (DMSO-d6, 500 MHz) *δ*/ppm: 11.71 (s, 1H), 2.48 (m, 1H), 2.40 (m, 2H), 1.58 (m, 8H), 1.29 (m, 20H) 0.87 (t, 3H); ^{13}C-NMR ($CDCl_3$, 125 MHz) *δ*/ppm: 180.94,130.44, 61.49, 34.24, 32.01, 31.93, 31.84, 29.45, 29.24, 29.09, 29.01, 28.87, 28.79, 27.15, 27.04, 24.54, 22.62, 21.03, 14.04; HRMS (ESI, positive ions) *m/z*: $[M-H]^+$ calcd for $C_{19}H_{35}NO_3H^+$ 310.2741, found 310.2737.

Oxd sequences

Aldoxime dehydratase from *Bacillus* sp. OxB-1 (OxdB) (Accession number: GenBank: AP013294.1)

ATGAAAAATATGCCGGAAAATCACAATCCACAAGCGAATGCCTGGACTGCCGAATTTCC
TCCTGAAATGAGCTATGTAGTATTTGCGCAGATTGGGATTCAAAGCAAGTCTTTGGATCA
CGCAGCGGAACATTTGGGAATGATGAAAAAGAGTTTCGATTTGCGGACAGGCCCCAAA
CATGTGGATCGAGCCTTGCATCAAGGAGCCGATGGATACCAAGATTCCATCTTTTTAGC
CTACTGGGATGAGCCTGAAACATTTAAATCATGGGTTGCGGATCCTGAAGTACAAAAGT
GGTGGTCGGGTAAAAAAATCGATGAAAATAGTCCAATCGGGTATTGGAGTGAGGTAACG
ACCATTCCGATTGATCACTTTGAGACTCTTCATTCCGGAGAAAATTACGATAATGGGGTT
TCACACTTTGTACCGATCAAGCATACAGAAGTCCATGAATATTGGGGAGCAATGCGCGA
CCGCATGCCGGTGTCTGCCAGTAGTGATTTGGAAAGCCCCCTTGGCCTTCAATTACCG
GAACCCATTGTCCGGGAGTCTTTCGGAAAACGGCTAAAAGTCACGGCGCCGGATAATAT
TTGCTTGATTCGAACCGCTCAAAATTGGTCTAAATGTGGTAGCGGGGAAAGGGAAACGT
ATATAGGACTAGTGGAACCGACCCTCATAAAAGCGAATACGTTTCTTCGTGAAAATGCTA
GTGAAACAGGCTGTATTAGTTCAAAATTAGTCTATGAACAGACCCATGACGGCGAAATA
GTAGATAAATCATGTGTCATCGGATATTATCTCTCCATGGGGCATCTTGAACGCTGGAC
GCATGATCATCCAACACATAAAGCGATCTACGGAACCTTTTATGAGATGTTGAAAAGGCA
TGATTTTAAGACCGAACTTGCTTTATGGCACGAGGTTTCGGTGCTTCAATCCAAAGATAT
CGAGCTTATCTATGTCAACTGCCATCCGAGTACTGGATTTCTTCCATTCTTTGAAGTGAC
AGAAATTCAAGAGCCTTTACTGAAAAGCCCTAGCGTCAGGATCCAGTGA
#

Oxd expression

E.coli BL21-CodonPlus(DE3)-RIL cells harboring the pUC18 plasmid including with the OxdB-gene were stored as glycerol stocks at -80 °C. A sample from the glycerol stocks for each Oxd was plated on LB-agar containing 34 µg/mL Chloramphenicol and 100 µg/mL Carbenicillin and incubated for 12 to 18 h at 37 °C. Pre-cultures were prepared in 5 mL LB-medium containing 50 µg/mL Kanamycin and 34 µg/mL Chloramphenicol (OxdRE) or 100 µg/mL Carbenicillin (OxdB) using a single colony from the LB-agar plate. The cultures were incubated for 12 to 18 h at 37 °C and 180 rpm. Main cultures for Oxd expression were performed using TB-autoinduction medium (40 mL 20 g/L lactose solution, 4 mL 50 g/L D-glucose solution and 356 mL TB-medium (purchased from Carl Roth) in a 500 mL Erlenmeyer flask). 34 µg/mL Chloramphenicol (OxdRE) and 100 µg/mL Carbenicillin were added to the medium. Main cultures were inoculated with 1 % (4 mL) of the relating pre-cultures and incubated for 2 h at 37 °C and 150 rpm. After 1 h incubation at 37 °C OxdB-cultures were cultivated at 30 °C for 72 h and 150 rpm. Cell harvest was performed at 4,000 xg for 15 min at 4 °C. Cells were washed three times with PPB (50 mM, pH

7) and afterwards resuspended in 2x mass of the bio wet weight (bww) of the cells PPB (50 mM, pH 7) and stored at 4 °C until usage. Expression success was analyzed by SDS-PAGE using a 12 % separation gel in comparison to a protein marker (*PageRuler Prestained* (Thermo Fisher)).

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Article 11

Selective Hydrogenation of Fatty Nitriles to Primary Fatty Amines: Catalyst Evaluation and Optimization Starting from Octanenitrile

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Author contribution

HG initiated the project. AH designed and performed the experiments. AH and HG wrote the manuscript. AH and HG read and edited the manuscript.

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Selective Hydrogenation of Fatty Nitriles to Primary Fatty Amines: Catalyst Evaluation and Optimization Starting from Octanenitrile

*Alessa Hinzmann and Harald Gröger**

In this contribution, an evaluation of the potential of various homogeneous and heterogeneous catalysts for a selective hydrogenation of fatty nitriles toward primary amines is reported exemplified for the conversion of octanenitrile into octane-1-amine as a model reaction. When using heterogeneous catalysts such as the ruthenium catalyst Ru/C, the palladium catalyst Pd/C, and the platinum catalyst Pt/Al_2O_3, low selectivities in the hydrogenation are observed, thus leading to a large portion of secondary and tertiary amine side-products. For example, when using Ru/C as a heterogeneous catalyst, high conversions of up to 99% are obtained but the selectivity remains low with a percentage of the primary amine being at 60% at the highest. The study further reveals a high potential of homogeneous ruthenium and manganese catalysts. When also taking into account economical considerations with respect to the metal price, in particular, manganese catalysts turn out to be attractive for the desired transformation and their application in the model reaction leads to the desired primary amine product with excellent conversion, selectivity, and high yield.

***Practical Applications*: This work describes an optimized hydrogenation process for transforming fatty nitriles to their corresponding primary amines. In general, fatty amines belong to the most applied fatty acid-derived compounds in the chemical industry with an annual product volume exceeding 800 000 tons per year in 2011 and are widely required in the chemical industry since such compounds are either directly used in home products such as fabric softeners, dishwashing liquids, car wash detergents, or carpet cleaners or in a broad range of industrial products, for example, lubricating additives, flotation agents, dispersants, emulsifiers, corrosion inhibitors, fungicides, and bactericides, showing additional major applications, e.g., in the detergents industry. Among them primary amines play an important industrial role. However, a major concern of current processes is the lack of selectivity and the formation of secondary and tertiary amines as side-products. By modifying a recently developed catalytic system based on manganese as economically attractive and environmentally benign metal component an efficient and selective access to fatty amines when starting from the corresponding nitriles is achieved. For example, hydrogenation of octanenitrile leads to a synthesis of octane-1-amine with >99% conversion and excellent selectivity with formation of secondary and tertiary amine side-products being suppressed to an amount of <1%.**

1. Introduction

Fatty amines play an important role in the chemical industry since such compounds are either directly used in home products such as fabric softeners, dishwashing liquids, car wash detergents, or carpet cleaners or in a broad range of industrial products, for example, lubricating additives, flotation agents, dispersants, emulsifiers, corrosion inhibitors, fungicides, and bactericides.[1–3] The worldwide demand of fatty amines was 800 000 tons in 2011,[3] thus representing a further large-scale option for the use of fats and oils as renewable building blocks and alternative raw material sources to petrochemicals. Among them, primary fatty amine are highly requested target products. When starting from fats and oils, today's established large-scale applied route is based on the hydrogenation of fatty nitriles (**Scheme 1**).

Such fatty nitriles needed as substrates are today industrially manufactured via transformation of vegetable oils through their acids into fatty amides, followed by subsequent dehydration into their corresponding fatty nitriles under high temperatures of 280–360 °C and frequently in the presence of a metal oxide catalyst (Scheme 1).[1] As an alternative to this route via the amide intermediate (which shows disadvantages such as the required high reaction temperatures),[1] such fatty nitriles can be also prepared by a chemoenzymatic pathway starting from fatty aldehydes as further type of fatty acid derivatives (Scheme 1),[4–6]

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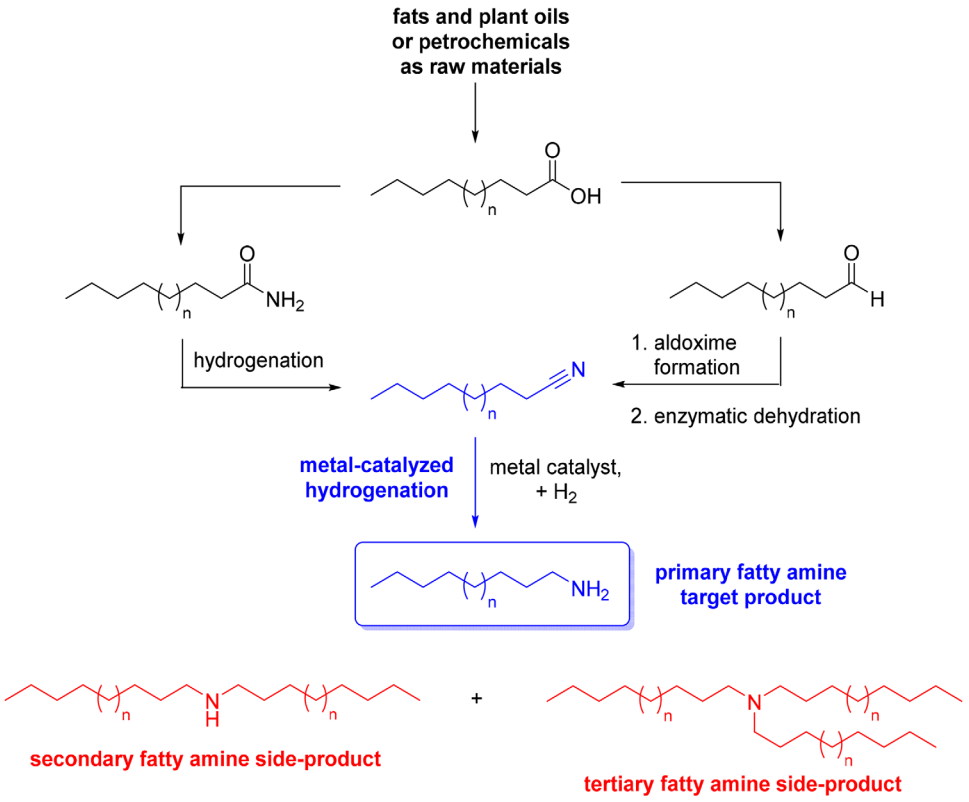

Scheme 1. Overview about the overall industrial production concept for primary fatty amines.

Recently, we demonstrated such a process being suitable to be operated at high substrate loading of up to 1.4 kg L^{-1} aqueous medium exemplified for octanenitrile.[5]

However, in spite of straightforward accesses to the fatty nitrile substrates and established hydrogenation technologies at large industrial scale, a hurdle that remained widely unsolved up to now in this field of fatty amines is the insufficient selectivity of the hydrogenation process (Scheme 1). Typically, with the currently applied heterogeneous metal catalysts the corresponding secondary and tertiary amines are also formed besides the desired primary amine products. For example, when using Pd/C as a heterogeneous catalyst (being frequently used for various types of hydrogenation reactions), the tertiary amine is formed as a major product in the hydrogenation of aliphatic nitriles.[7,8] Also in case of the heterogeneous catalysts Ru/C[9] and Raney nickel,[10,11] insufficient selectivities toward the primary amine have been observed. Although some modifications of hydrogenation conditions and additives were found, which positively influence the selectivity of the Ni-catalyst for primary amines in the hydrogenation of nitriles, there is still a demand for general procedures to hydrogenate various nitriles and in particular aliphatic nitriles selectively to primary amines.[12,13]

A perspective to overcome these hurdles can be seen in the use of homogeneous in spite of the today's applied heterogeneous catalysts. Due to the option to vary the ligand sphere, selectivity can be fine-tuned, thus shifting the selectivity into direction of the desired primary amines. However, again there are some challenges to solve: often homogeneous catalysts for this purpose are based on expensive heavy metals. Further issues to be solved are the tasks of high catalytic efficiently (and, thus, high turnover number) and bulk technically feasible strategies for separating and reusing the catalysts. Addressing the first two issues of using a low cost metal component being ideally environmental benign and achieving a high efficiency besides excellent selectivity, we became interested to explore manganese catalysts. Our interest in such catalysts was sparked by the recent achievements from the Kirchner group[14,15] who reported on hydrogenation of various nitriles using such manganese catalysts.

In the following, we report our results on the search of a suitable catalyst providing both high efficiency as well as selectivity for the hydrogenation of fatty nitriles exemplified for octanenitrile (**Scheme 2**). This study comprised an initial re-evaluation of various types of heterogeneous and homogeneous metal catalysts in terms of selectivity and a subsequent study and optimization of a manganese hydrogenation catalysts, which turned out to be promising for the desired reaction.

2. Results and Discussion

First, we defined a model reaction for our catalyst evaluation study and chose the hydrogenation of octanenitrile into octan-1-amine as representative reaction due to practical and analytical reasons. After having established a NMR-based -analytical method for the determination of the reaction course, we started with the screening of a range of heterogeneous catalysts as such

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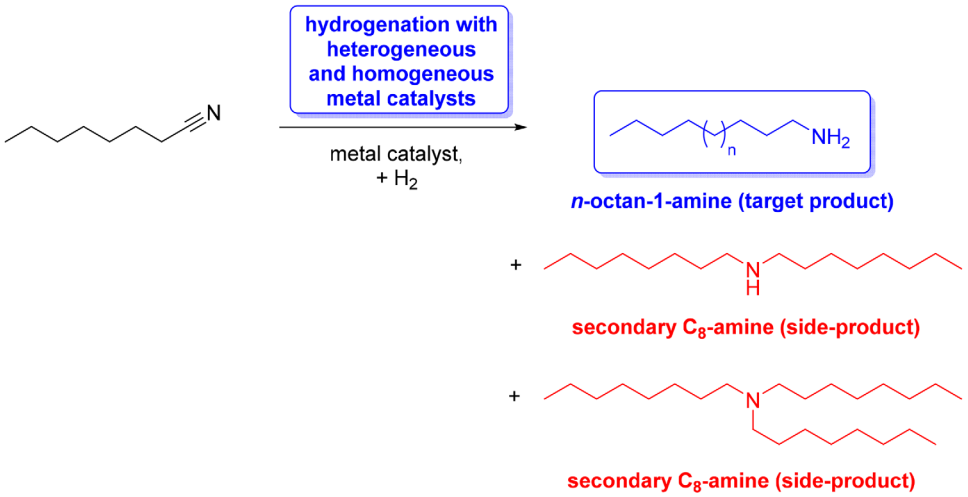
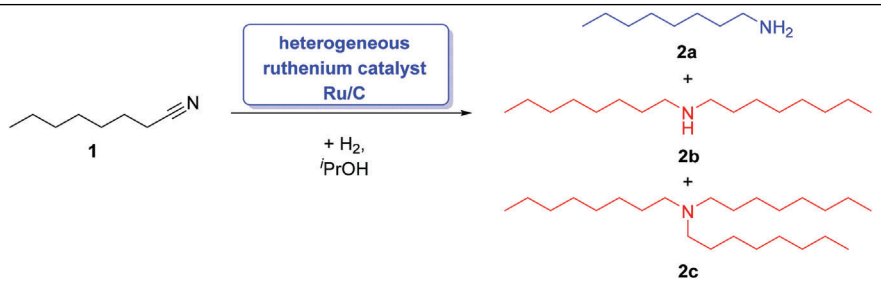


Scheme 2. Hydrogenation of octanenitrile as model reaction.

Table 1. Hydrogenation of octanenitrile (**1**) using the heterogeneous ruthenium catalyst Ru/C.



Catalyst	*T* [°C]	*p* [bar]	*t* [h]	Conversion [%]	2a/2b/2c
Ru/C (1 mol%)	27	14	20	–	n.d.
Ru/C (1 mol%)	27	22	20	–	n.d.
Ru/C (5 mol%)	27	13	20	–	n.d.
Ru/C (5 mol%)	27	22	20	–	n.d.
Ru/C (1 mol%)	27	1	19	–	n.d.
Ru/C (5 mol%)	27	1	19	–	n.d.
Ru/C (1 mol%)	100	10	20	72	45/53/2
Ru/C (1 mol%)	100	20	20	77	50/48/2
Ru/C (5 mol%)	100	10	20	71	45/54/1
Ru/C (5 mol%)	100	20	20	81	45/52/3
Ru/C (1 mol%)	80	1	19	–	n.d.
Ru/C (5 mol%)	80	1	19	–	n.d.
Ru/C (1 mol%)[a)]	100	20	20	99	60/37/3

[a)] Addition of 2 equivalents NH_3 to the hydrogenation reaction.

types of catalysts are today used in industry, thus representing the industrial benchmark. Furthermore, we were interested whether adjusting the reaction conditions to mild conditions would in principle enable a sufficient reactivity while achieving an improvement in terms of selectivity. To start with the heterogeneous ruthenium catalyst Ru/C,[9,16] we varied the temperature broadly between 27 and 100 °C in addition to the hydrogen pressure, which was adjusted between 1 and 22 bar (**Table 1**). The reaction time remains relatively constant at 19 or 20 h, respectively. It turned out that in general an elevated temperature of 100 °C is needed for this transformation, reaching conversions of 71% to 99% in these cases. For the latter case, addition of ammonia (2 equivalents) was needed. In general, ammonia is added to hydrogenation reactions of nitriles in order to prevent the formation of secondary and tertiary amines,[9] since ammonia then serves as an alternative to the amine in the reaction with

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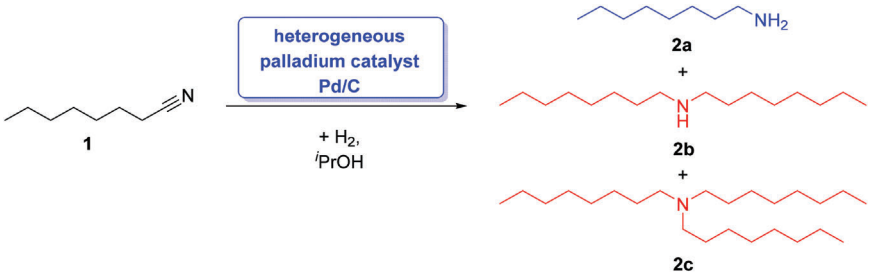
Table 2. Hydrogenation of octanenitrile (**1**) using the heterogeneous palladium catalyst Pd/C.



Catalyst	*T* [°C]	*p* [bar]	*t* [h]	Conversion [%]	2a/2b/2c
Pd/C (10 mol%)	25	1	21	>99	0/96/4
Pd/C (10 mol%)[a)]	25	10	21	>99	5/77/18
Pd/C (10 mol%)	25	10	21	>99	0/81/19
Pd/C (10 mol%)	25	20	21	>99	0/89/11
Pd/C (10 mol%)[a)]	25	20	21	>99	5/82/13
Pd/C (1 mol%)	25	10	21	88	0/53/47
Pd/C (0.1 mol%)	25	10	21	65	0/65/35
Pd/C (0.05 mol%)	25	10	21	27	11/70/19
Pd/C (1 mol%)	25	1	21	>99	0/62/38
Pd/C (0.1 mol%)	25	1	21	<1	n.d.
Pd/C (1 mol%)[a)]	25	20	20	>99	4/45/51

[a)] Addition of 2 equivalents NH_3 to the hydrogenation reaction.

the imine intermediate. An elevated hydrogen pressure (20 bar in comparison to 10 bar) had a slightly positive impact on the conversion of nitriles, although the selectivity with respect to the formation of primary, secondary, or tertiary amines is not significantly influenced (Table 1). However, it is noteworthy that independent of the reaction conditions (and even when adding ammonia as a strategy to suppress formation of secondary and tertiary amines), the selectivity remained low with a percentage of the desired primary amine being at 60% at the highest. Thus, formation of secondary and tertiary amines remained as severe side-reactions having a significant impact in general when using Ru/C as a heterogeneous catalyst.

The formation of such side products are a result of the derivatization of the formed primary amine products, which are converted into imines and subsequently reduced to secondary amines.[16] An analogous derivatization of the secondary amine then gives the tertiary amine.

A further heterogeneous catalyst being commonly used for metal-catalyzed hydrogenation is Pd/C.[7,8,16–18] When operating under smooth reaction conditions such as 25 °C as reaction temperature and 1–20 bar hydrogen pressure, a minimum amount of 1 mol% of the catalyst Pd/C was found to be needed for achieving a high conversion within a reaction time of 20 or 21 h (**Table 2**). For example, >99% conversion was obtained at just 1 bar of hydrogen pressure and at a low reaction temperature of 25 °C. However, the amount of the desired primary fatty amine was very low with 0–4% under such conditions. Even when varying the catalyst loading between 0.05 and 10 mol%, the highest percentage of the primary amine product compared to the secondary and tertiary amine products was only 11% (Table 2). Varying the hydrogen pressure and catalyst loading does not have a strong impact on the selectivity of the hydrogenation reaction. When using this catalyst, the major product was always found to be the secondary amine even when ammonia was added as an additive (the only exception is the reaction with 1 mol% Pd/C and 20 bar of hydrogen, which gave the tertiary amine as major product). However, as expected, the conversion of the nitrile drops with the decrease of the catalyst loading.

Besides ruthenium and palladium, platinum is a further metal component being frequently used for hydrogenation purpose.[19–21] When utilizing Pt/Al_2O_3 as a catalyst at a catalytic loading of 1 mol%, a high conversion of up to 92% was achieved when operating at a hydrogen pressure of 20 bar and at a reaction temperature of 100 °C (**Table 3**). However, again the selectivity was limited and the percentage of the desired primary amine did not exceed 44%. In contrast to the hydrogenation experiments with the Ru/C catalyst, we could not find any or only a very low positive effect of the addition of ammonia on the selectivity of the hydrogenation when using Pd/C or Pt/Al_2O_3 as a catalyst. While in case of Ru/C, the product ratio changed from 45/52/3 to 60/37/3, which shows that a significantly larger amount of primary amine was formed during the reaction in the presence of added ammonia, in case of Pt/Al_2O_3, the ratio did not improve in terms of formation of the primary amine, and in case of Pd/C only a slightly increased amount of primary amine was formed.

In general, these results are in accordance to those in literature for the hydrogenation of nitriles to amines in the presence of heterogeneous catalysts.[7–13] For example, Sajiki et al. showed

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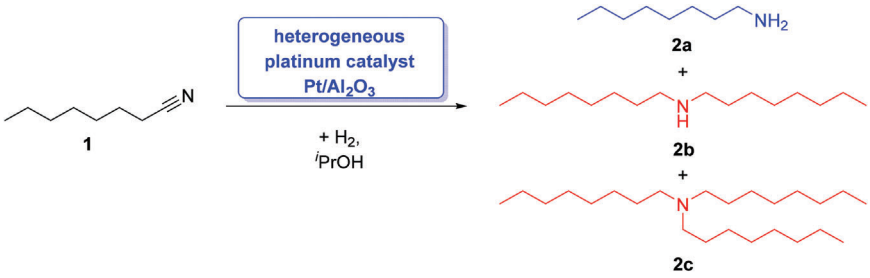
Table 3. Hydrogenation of octanenitrile (**1**) using the heterogeneous platinum catalyst Pt/Al_2O_3.



Catalyst	T [°C]	p [bar]	t [h]	Conversion [%]	2a/2b/2c
Pt/Al_2O_3 1 mol%)	100	20	20	85	44/53/3
Pt/Al_2O_3 1 mol%)[a)]	100	20	20	92	41/56/3

[a)] Addition of 2 equivalents NH_3 to the hydrogenation reaction.

that utilizing Pd/C as heterogeneous catalyst at 25°C and H_2 atmosphere for the hydrogenation of decanenitrile in methanol mainly leads to the secondary and tertiary amine.[7] This result is in principle in good agreement with our findings when using this catalyst in isopropanol for the hydrogenation of octanenitrile (Table 2). In our case, at atmospheric pressure of hydrogen at room temperature mainly the secondary amine was formed as a product. Compared to our study in literature often other nitrile substrates were used such as 3-methoxy propionitrile,[9] benzonitrile,[18] decanenitrile,[7] or pentanenitrile,[8] which makes a direct comparison of conversions, yields, and selectivities difficult. However, in particular in case of pentanenitrile[7] and decanenitrile,[8] which are structurally close to our model substrate octanenitrile, the results in literature[7,8] and from our study appear to be comparable.

Taking into account the low selectivities and high amount of unfavored side-products when using heterogeneous catalysts, we turned our interest to the evaluation of homogeneous catalysts for the model reaction. Homogeneous catalysis in our cases always means that the catalyst is dissolved in the reaction mixture during the hydrogenation reaction. Furthermore, even after the hydrogenation reaction the reaction mixture was found to homogeneous without precipitation of catalyst or additives. Based on the work of Beller et al. with ruthenium catalysts,[22] we applied various species for the hydrogenation of octanenitrile in different solvents (**Table 4**). The best results were obtained when using the commercial Ru-MACHO-BH catalyst [23] in isopropanol or alternatively the Ru-Gusev catalyst,[24,25] which turned to be suitable in various solvents such as isopropanol, diisopropylethyer, and cyclohexane. In these hydrogenations, high conversions in the range of 81–99% were obtained at a catalyst loading of 0.5 mol%. Furthermore, excellent selectivities were achieved in such hydrogenations with an amount of desired product of at least 98%. For example, when using the Takasago catalyst Ru-MACHO-BH,[18] 98% conversion and a product ratio of >99:0:0 were observed in our model reaction.

The increased selectivity of hydrogenation reactions for primary amines when using a homogeneous catalyst in comparison to heterogeneous catalysts might be rationalized by the option of homogeneous catalysis to fine-tune the catalytic complex by means of ligands, which, among other effects, then could result in a different catalytic activity for hydrogenating the various conceivable imine intermediates as precursors for the primary, secondary, and tertiary amine products. In this connection, it should be added that a detailed calculation for the heterogeneous Pd-catalyzed hydrogenation of acetonitrile and the reaction steps toward the corresponding primary, secondary, and tertiary amines have been performed and discussed by Adamczyk.[26]

Next, we focused on the impact of catalyst loading which appears to be even more crucial in homogeneous catalysis due to the more difficult catalyst recovery and re-use compared to heterogeneous catalysis. However, when reducing the catalyst loading to 0.1 mol%, conversion drops significantly and did not exceed 18% (Table 4). Thus, improving catalyst activity as well as stability and developing options for its re-use are a task for future research in this field. At the same time we were seeking for a more economical alternative with respect to the metal component due to the high price of ruthenium being currently in the range of 8.150 \$ kg^{-1}.[27] An economically attractive alternative would be iron or manganese as center ion in homogeneous metal catalysts. Inspired by a successful manganese catalyst reported recently from the Kirchner group[14,15] for being suitable in hydrogenation of various functional groups including nitriles (leading to 93% yield for octanenitrile),[15] we focused on this type of catalysts also for our model reaction. Under slightly different reaction conditions and catalyst structure to those described in literature with a prolonged reaction time from 18 to 19 h and using ethyl instead of *n*-propyl substituents at the phosphine ligand of the manganese complex for practical reasons, we achieved a high conversion of >99% in the presence of 2 mol% of the manganese catalyst **6** (**Table 5**). The reaction was conducted at 100 °C in toluene at a substrate concentration of 144 mM and a hydrogen pressure of 50 bar. After work-up, we obtained octan-1-amine in 68% yield, indicating a loss of product during downstream-processing. In order to further improve stability of this catalyst, we speculated if more rigid derivatives bearing cyclohexyl instead of ethyl substituents also could represent active catalysts with potential for higher stability. Thus, we prepared the new catalyst **7** and to our delight this catalyst led to both, excellent conversion of >99% and yield of 96%.

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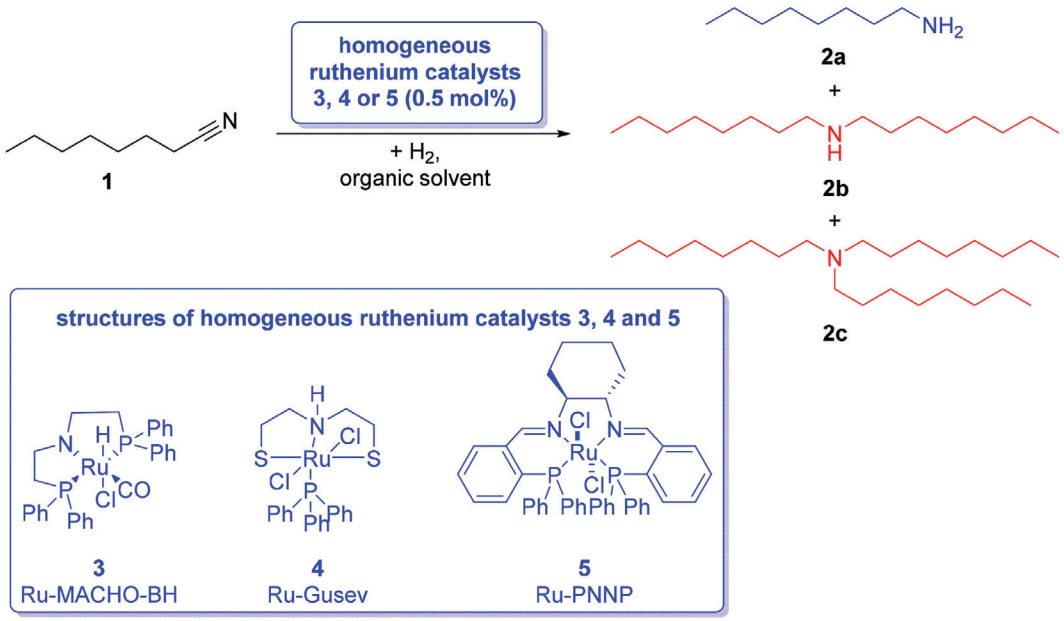
Table 4. Hydrogenation of octanenitrile (**1**) using the homogeneous ruthenium catalysts 3–5.



Catalyst	Solvent	T [°C]	p [bar]	t [h]	Conversion [%]	2a/2b/2c
3	^{i}PrOH	70	20	3	98	>99/0/0
3[a)]	^{i}PrOH	70	20	3	18	>99/0/0
3	THF	70	20	3	12	>99/0/0
3	$^{i}Pr_2O$	70	20	3	6	86/8/6
3	Cyclohexane	70	20	3	2	>99/0/0
4	^{i}PrOH	70	50	3	99	98/2/2
4	THF	70	50	3	71	65/31/4
4	$^{i}Pr_2O$	70	50	3	98	1/97/2
4	Cyclohexane	70	50	3	81	99/0/1
5	^{i}PrOH	70	60	3	6	0/83/17
5	$^{i}Pr_2O$	70	60	3	9	0/75/25
5	Cyclohexane	70	60	3	<1	0/0/100

[a)] In this experiment, 0.1 mol% catalyst loading was used instead of 0.5 mol% (which was the catalyst loading in case of all other experiments shown in Table 4)

Encouraged by these results, we further investigated the hydrogenation of octanenitrile using the Mn^{I}-catalyst **7** focusing on reduction of the catalyst loading as a next step (**Table 6**). When reducing the catalyst loading from 2 mol%, which was used in the initial experiment, to 1, 0.5, and 0.1 mol%, we could obtain a quantitative conversion towards octane-1-amine without identifying secondary or tertiary amines as by-product in case of 1 mol%. By lowering the catalyst loading to 0.5 mol%, however, a decreased conversion of 51% toward the primary amine was found.

In addition, we investigated the impact of different solvents on this hydrogenation reaction. Besides toluene as solvent used in the initial experiments with the Mn-catalysts **6** and **7**, we tested tetrahydrofurane (THF), 2-methyl tetrahydrofurane (2-Me-THF), ethanol, and isopropanol (Table 6). When using a catalyst loading of 2 mol% of catalyst **7**, full conversion toward the primary amine was reached in all cases without formation of by-products. However, at a decreased catalyst loading of 1 mol% of catalyst **7**, only in case of toluene and 2-Me-THF a full conversion was obtained, while the use of ethanol, isopropanol, and THF led to lower conversions.

After optimization of the hydrogenation conditions for the synthesis of octane-1-amine, we conducted a hydrogenation experiment under such optimized conditions using dodecanenitrile (**8**) as a substrate (**Scheme 3**).

When using this fatty nitrile **8** as a starting material and the manganese complex **7** as a catalyst at a catalytic loading of 1 mol%, also full conversion to the primary amine **9a** without formation of secondary or tertiary amines was obtained. This experiment indicates that this hydrogenation method is applicable for the synthesis of various fatty primary amines of different chain-lengths starting from the corresponding aliphatic nitriles.

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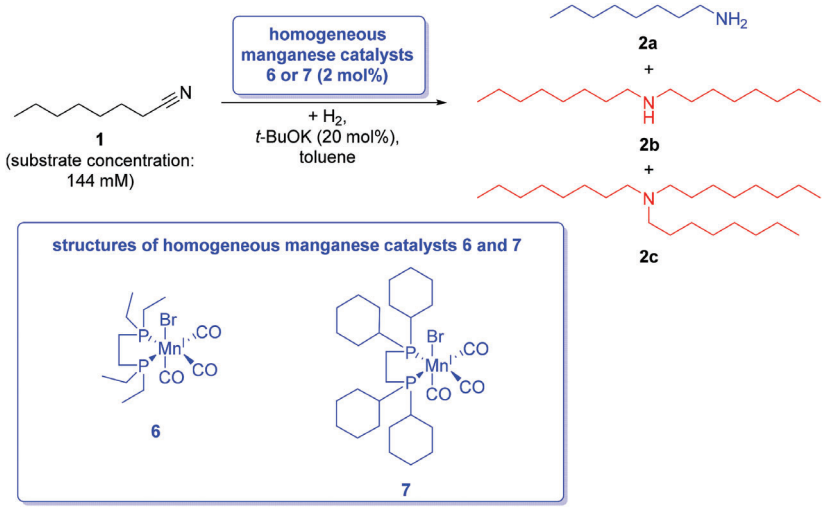
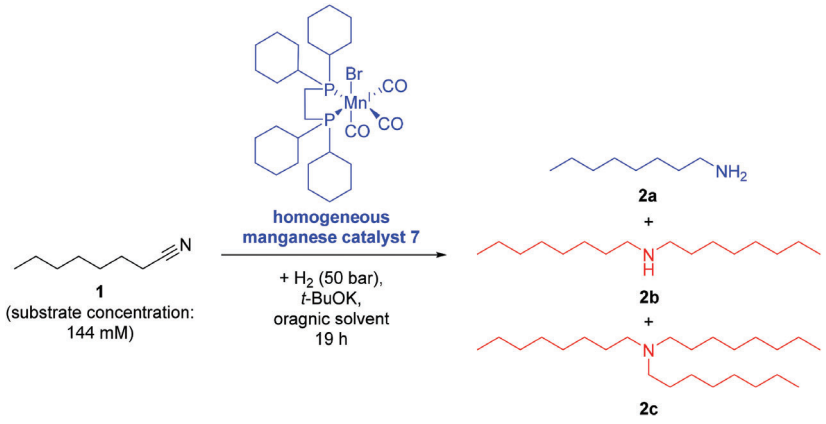
Table 5. Hydrogenation of octanenitrile (**1**) using the homogeneous manganese catalysts **6** and **7**.



Catalyst	T [°C]	p [bar]	t [h]	Conversion [%]	Yield [%]	2a/2b/2c
6	100	50	19	>99	68	>99/0/0
7	100	50	19	>99	96	>99/0/0

Table 6. Hydrogenation of *n*-octanenitrile (**1**) using the homogeneous manganese catalyst **7**—optimization experiments in terms of catalyst loading and solvent.



Catalyst loading	*t*-BuOK loading	Organic solvent	T [°C]	Conversion [%]	2a/2b/2c
2 mol%[a)]	20 mol%	Toluene	100	>99	>99/0/0
1 mol%	10 mol%	Toluene	100	>99	>99/0/0
0.5 mol%	5 mol%	Toluene	100	51	51/0/0
0.1 mol%	1 mol%	Toluene	100	<1	n.d.
2 mol%	20 mol%	THF	100	>99	>99/0/0
2 mol%	20 mol%	2-Me-THF	100	>99	>99/0/0
2 mol%	20 mol%	Ethanol	100	>99	>99/0/0
2 mol%	20 mol%	Isopropanol	100	>99	>99/0/0
1 mol%	10 mol%	THF	100	71	>99/0/0
1 mol%	10 mol%	2-Me-THF	100	>99	>99/0/0
1 mol%	10 mol%	Ethanol	100	53	>99/0/0
1 mol%	10 mol%	Isopropanol	100	58	>99/0/0

[a)] This experiment is also shown in Table 5.

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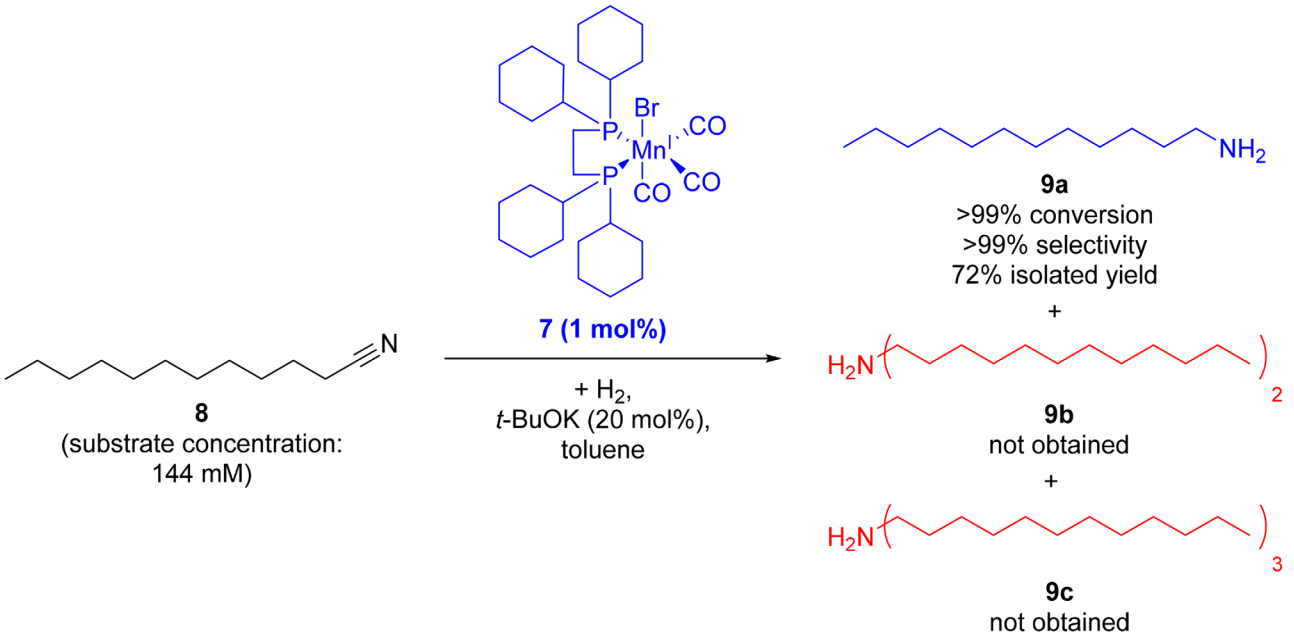

Scheme 3. Hydrogenation of dodecanenitrile using Mn-catalyst **7**.

3. Conclusions

In conclusion, an evaluation of the potential of various homogeneous and heterogeneous catalysts for a selective hydrogenation of fatty nitriles toward primary amines was done exemplified for the conversion of octanenitrile into octan-1-amine as a model reaction. When using heterogeneous catalysts, low selectivities were observed, thus leading to a large portion of secondary and tertiary amine side-products. The study further revealed a high potential of homogeneous ruthenium and manganese catalysts. When also taking into account economical considerations with respect to the metal price, in particular manganese catalysts turned out to be attractive for the desired transformation and their application in the model reaction led to the desired primary amine product with excellent conversion and selectivity and in high yield.

4. Experimental Section

Hydrogenation reactions were performed in a Parr Series 5000 Multiple Reactor System in which up to six different hydrogenations were conducted in parallel. The maximum volume of the used autoclaves was 75 mL and the reaction mixtures were stirred at 1000 rpm. In case of heterogeneous catalysts, a Teflon inlet was used to prevent adsorption of the catalyst to the stainless-steel autoclave material. Before usage of the reactors, they were evaporated and filled with Argon twice. Afterward the nitrile (250 mM in all cases except for hydrogenations using Mn^{I}-catalysts **6** and **7**) and solvent (10 mL total volume) were added in an Argon counter flow. Finally, the catalyst and potentially used additives were added under inert conditions. In case of hetereogeneous catalysts sometimes ammonia was used as an additive. Ammonia (2 eq based on nitrile concentration) was added by adding ammonia in methanol (7 M) to the reaction mixture. Manganese catalysts **6** and **7** were used in combination with *t*-BuOK (10x catalyst concentration, e.g. 10 mol% *t*-BuOK when 1 mol% **6** or **7** was used) as additive. The autoclaves were placed into the Parr Series 5000 Multiple Reactor System, which among others consists of a burst protection for high pressure reactions. The reactors were washed with nitrogen (3 × ≈20 bar) to prevent formation of oxyhydrogen gas formation and afterward with hydrogen (2 × ≈20 bar, 1× required pressure) before adjusting the hydrogen-pressure to required pressure in every reactor. After stirring for the desired reaction time, the autoclaves were washed with nitrogen (3 × ≈20 bar) before cooling to room temperature and work-up. Heterogeneous catalysts were filtered and homogeneous catalysts were removed by filtration over a ≈2 cm celite (Celite 545, Merck) layer. The filter cake or celite layer was washed with solvent (same as used in hydrogenation experiment) twice (each ≈5 mL). The solvent was removed in vacuo and the product was analyzed by ^{1}H-NMR spectroscopy and ESI–MS. NMR spectra were recorded on a Bruker Avance III 500 at a frequency of 500 MHz (^{1}H) or 125 MHz (^{13}C). Chemical shift δ was given in ppm and referenced to the corresponding solvent signal ($CDCl_3$). Nano-ESI mass spectra were recorded using an Esquire 3000 ion trap mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) equipped with a nano-ESI source. Samples were dissolved in methanol or acetonitrile and introduced by static nano-ESI using in-house pulled glass emitters. Nitrogen served both as nebulizer gas and dry gas. Nitrogen was generated by a Bruker nitrogen generator NGM 11. Helium served as cooling gas for the ion trap and collision gas for MS^n experiments. The mass axis was externally calibrated with ESI-L Tuning Mix (Agilent Technologies, Santa Clara, CA, USA) as calibration standard.

Octan-1-amine (**2a**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm = 2.66 (t, 2H, NH_2-CH_2-$(CH_2)_6$-CH_3), 1.42-1.29 (m, 12H, NH_2-CH_2-$(CH_2)_6$-CH_3), 0.86 (t, 3H, -CH_3).

ESI-MS (positive ions) $[C_8H_{19}N+H]^+$ *m/z* (calc.) = 130.15, *m/z* (exp.) = 130.10.

Dodecan-1-amine (**9a**): ^{1}H-NMR (500 MHz, $CDCl_3$) δ/ppm = 2.69 (t, 2H, NH_2-CH_2-$(CH_2)_6$-CH_3), 1.43-1.25 (m, 20H, NH_2-CH_2-$(CH_2)_6$-CH_3), 0.87 (t, 3H, -CH_3).

ESI-MS (positive ions) $[C_{12}H_{27}N+H]^+$ *m/z* (calc.) = 186.21, *m/z* (exp.) = 186.12.

Abbreviation

equiv, equivalents

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Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

fatty amines, fatty nitriles, hydrogenation, manganese catalysts, primary amines

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Supporting Information

Selective Hydrogenation of Fatty Nitriles to Primary Fatty Amines: Catalyst Evaluation and Optimization Starting from Octanenitrile

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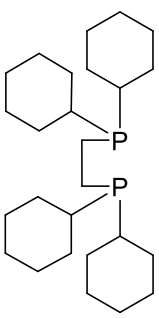
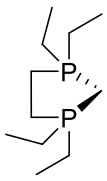
Synthesis of manganese hydrogenation catalysts



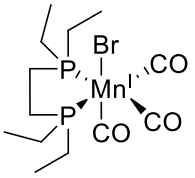
L2



L1

Complex synthesis of manganese salt with ligands were conduction in analogy to *Kirchner et al.*.[1] Ligand **L1** or **L2** (185.6 mg or 380.35 mg, 0.9 mmol) was dissolved in dry toluene (10 mL) in a dry Schlenk flask under Argon atmosphere. $Mn(CO)_5Br$ (247.4 mg, 0.9 mmol) was added in one portion in Argon counterflow. The solution was heated to reflux for 10 min and the solvent was removed to approx. 0.3 mL and *n*-pentane (10 mL) was added. A yellow solid precipitated and the solvent was decanted from the solid, the solid was washed with cold *n*-pentane (3x 5 mL) and afterwards dried *in vacuo*. The resulting complexes **6** or **7** were analyzed by NMR-spectroscopy, IR and accurate mass spectrometry.

Fac-1,2-bis(diethyl)ethane tricarbonyl manganese (I) bromide 6 (*fac*-$[Mn(Et_2PCH_2CH_2PEt_2)(CO)_3Br]$)



6

Chemical Formula: $C_{13}H_{24}BrMnO_3P_2$
Molecular Weight: 425,122

^{1}H-NMR (500 MHz, $CDCl_3$):

δ/ppm = 2.0 (m, 12H), 1.2 (m, 12H).

^{13}C-NMR (500 MHz, toluene-d8):

δ/ppm = 202.3, 195.54, 23.3, 19.4, 16.3, 8.2, 7.9.

^{31}P-NMR (500 MHz, toluene-d8):

δ/ppm = 72.0.

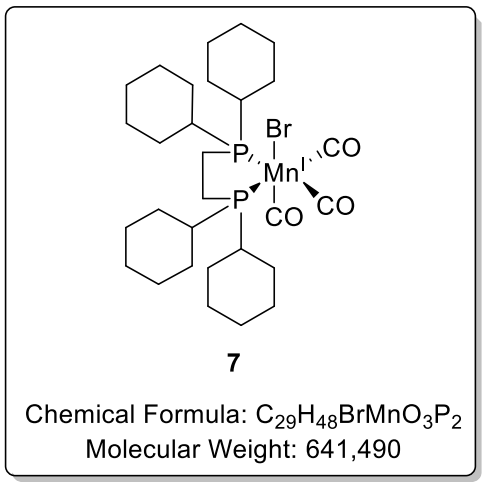
Mass:

HRMS (ESI, positive ions)*: m/z*:[M+Na]+ calcd for C13H2O3P2BrMnNa+ 446.96567, found 446.9649.

ATR-IR (solid):

ν/cm^{-1} = 2001 (ν_{CO}), 1934 (ν_{CO}), 1910 (ν_{CO}).

Fac-1,2-bis(dicyclohexyl)ethane tricarbonyl manganese (I) bromide 7 (*fac*-[Mn(Cyhex$_2$PCH$_2$CH$_2$PCyhex$_2$)(CO)$_3$Br])



7

Chemical Formula: $C_{29}H_{48}BrMnO_3P_2$
Molecular Weight: 641,490

^{1}H-NMR (500 MHz, toluene-d8):

δ/ppm = 2.61-2.4 (m, 4H) 2.0-1.09 (m, 44H).

^{13}C-NMR (500 MHz, toluene-d8):

δ/ppm = 223.4, 220.3, 38.8, 36.0, 30.3, 29.6, 27.93, 26.51, 21.7.

^{31}P-NMR (500 MHz, toluene-d8):

δ/ppm = 72.7.

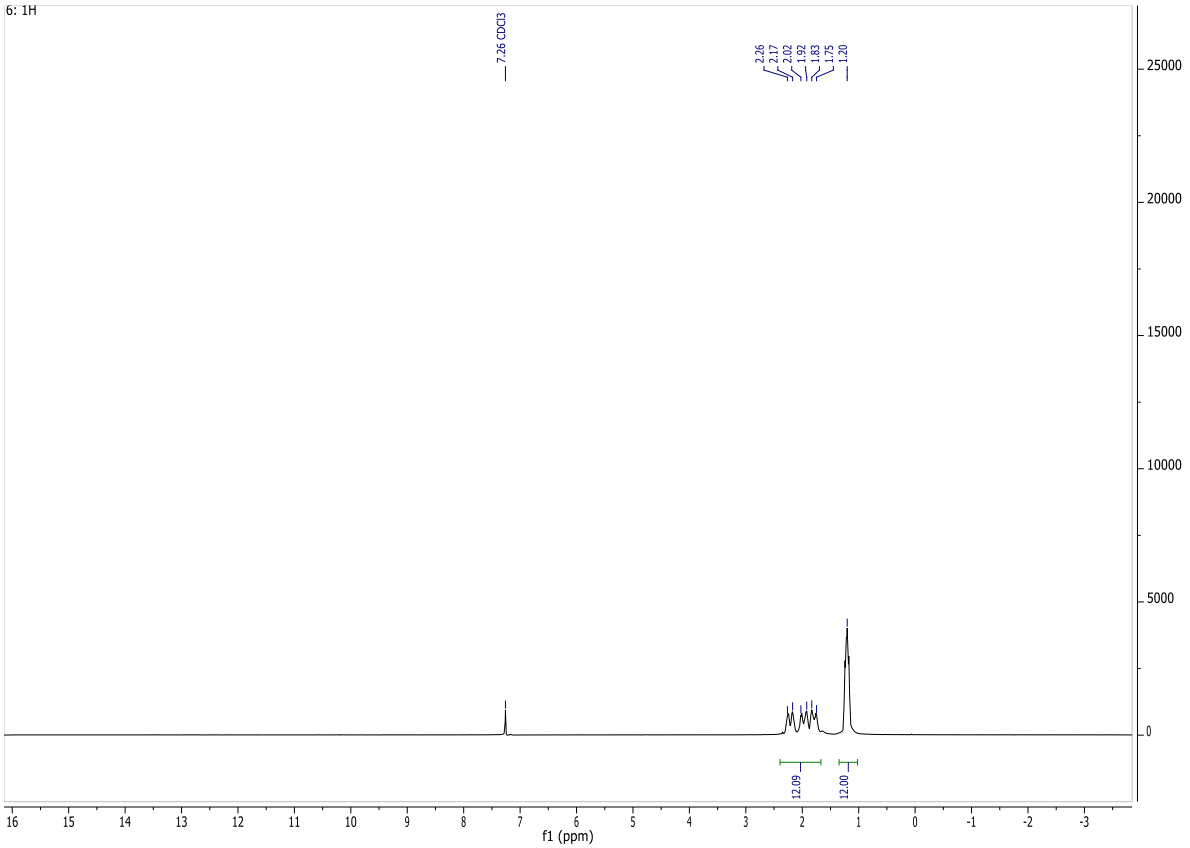
Mass:

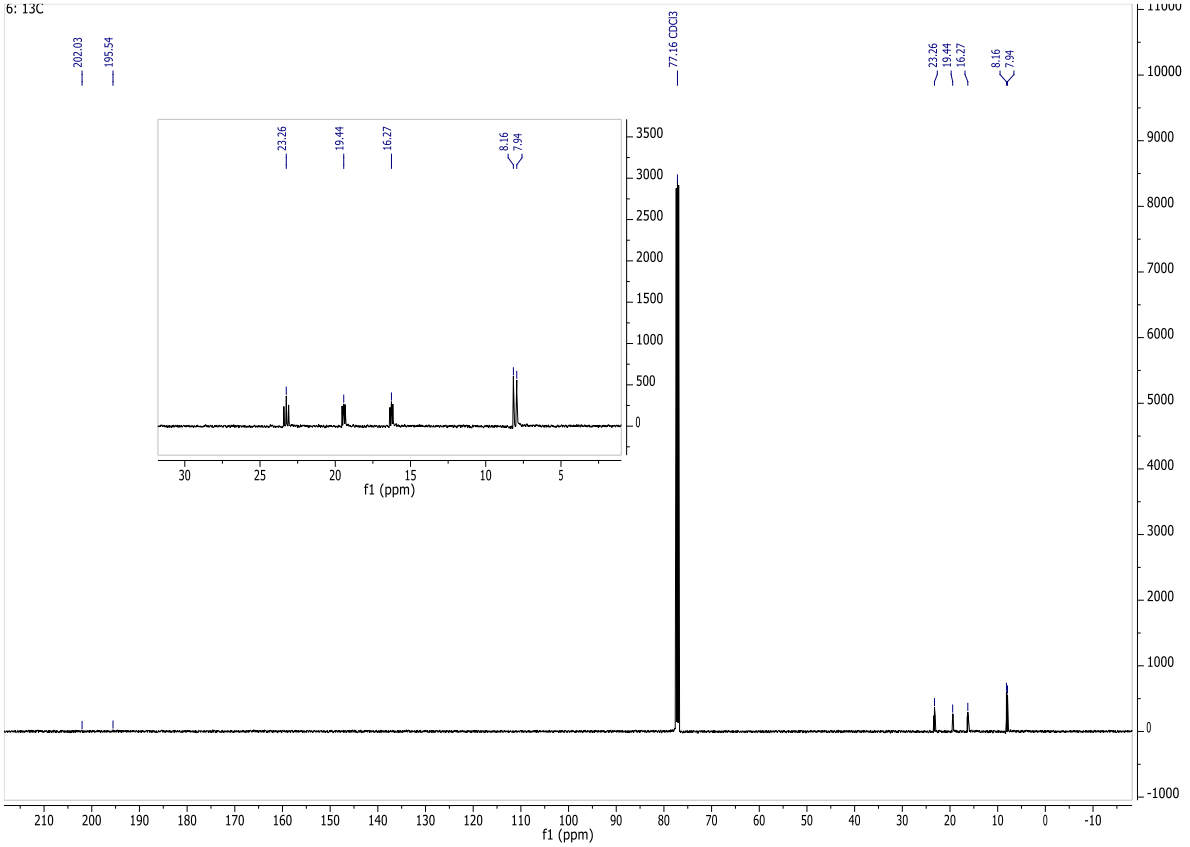
HRMS (ESI, positive ions): *m/z*:[M+Na]+ calcd for C29H4O3P2BrMnNa+ 663.15347, found 663.1536.

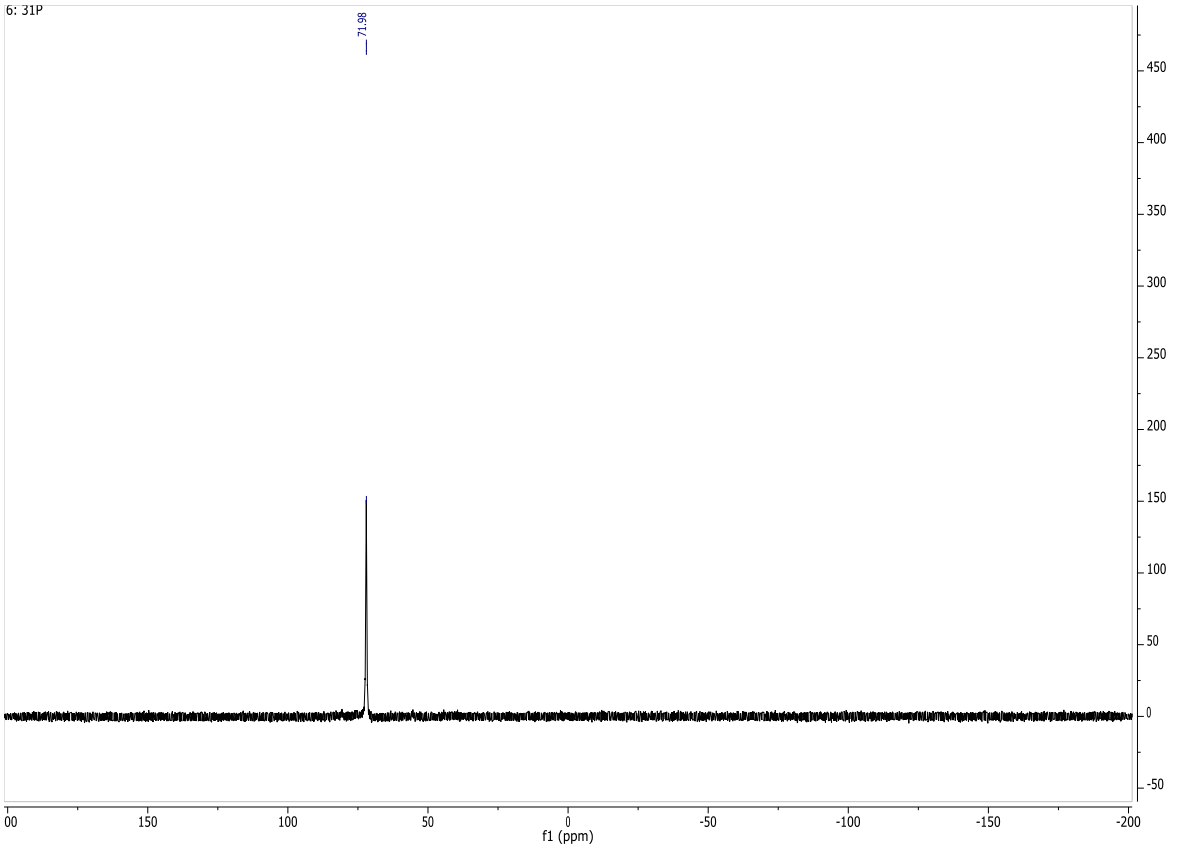
ATR-IR (solid):

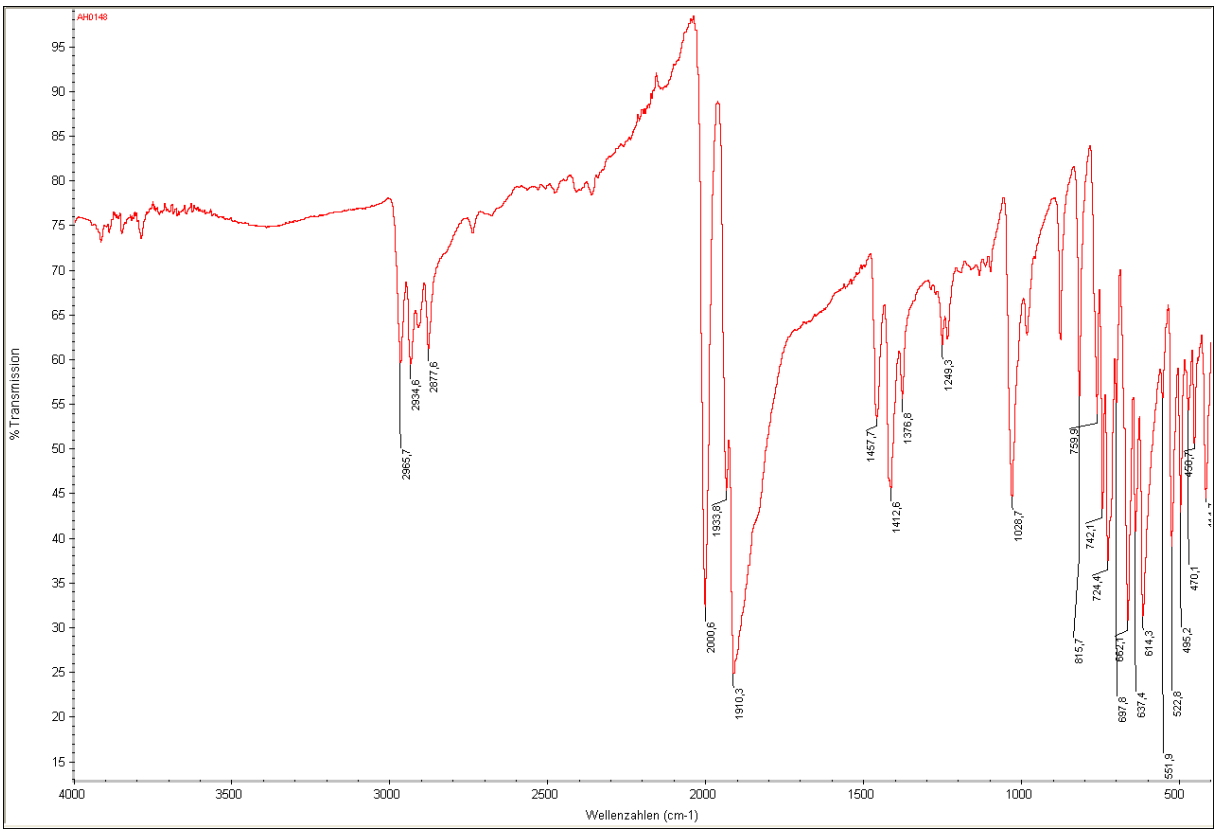
ν/cm^{-1} = 2000 (ν_{CO}), 1925 (ν_{CO}), 1897 (ν_{CO}).

NMR-spectra and IR-spectrum of *fac*-1,2-bis(diethyl)ethane tricarbonyl manganese (I) bromide 6 (*fac*-[Mn($Et_2PCH_2CH_2PEt_2$)$(CO)_3Br$])

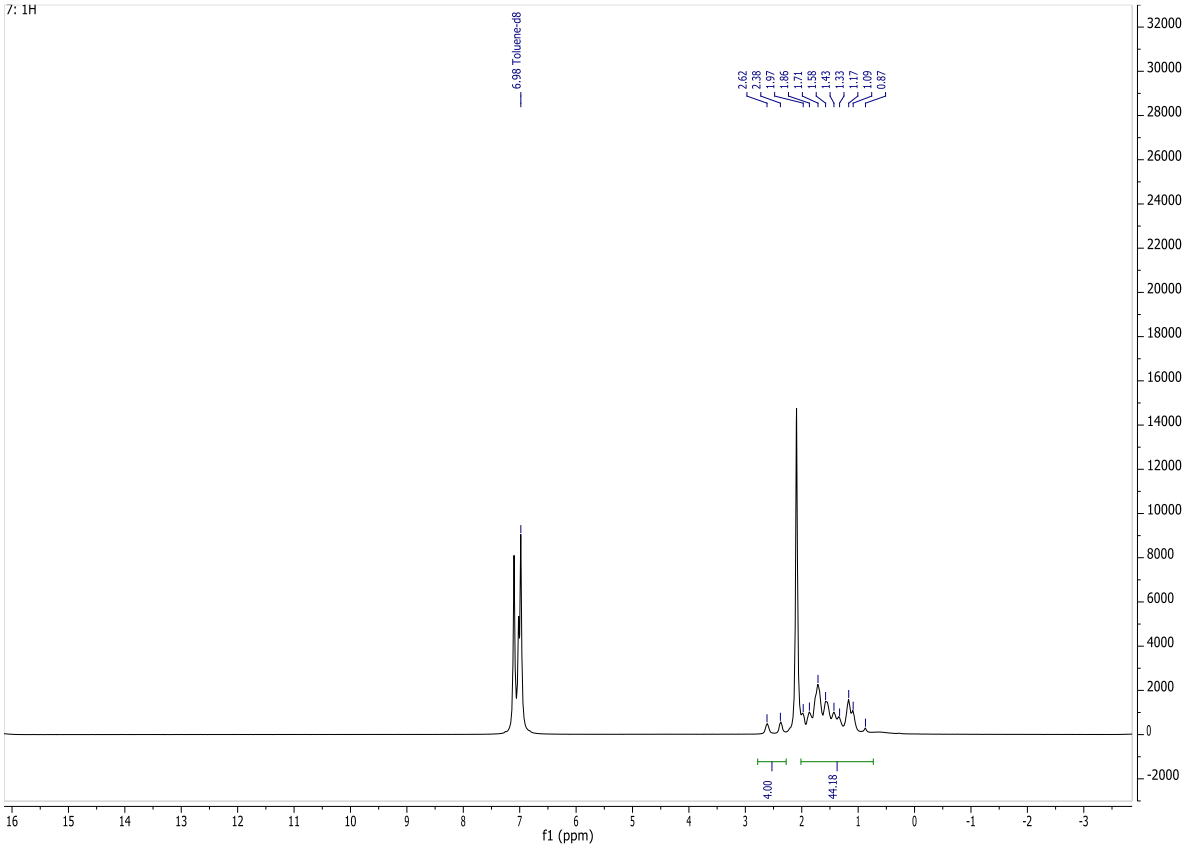


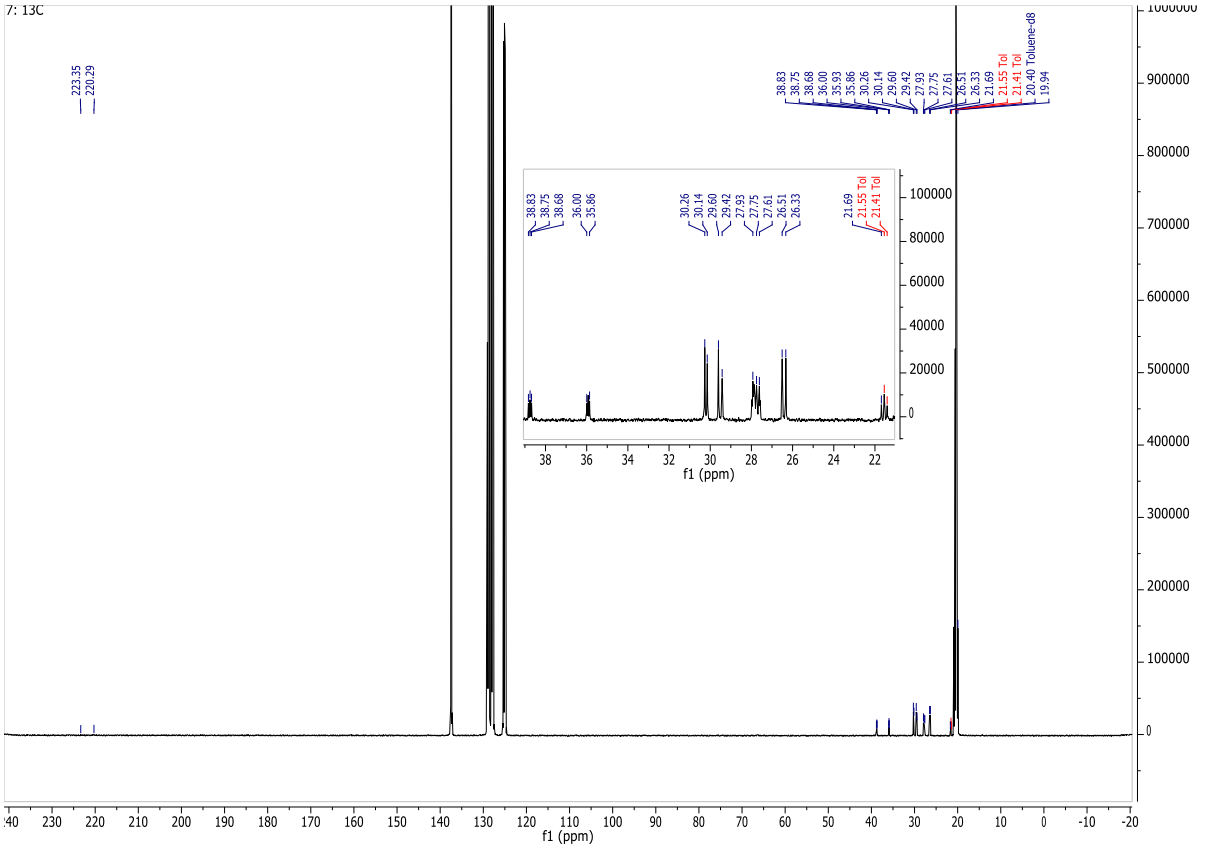


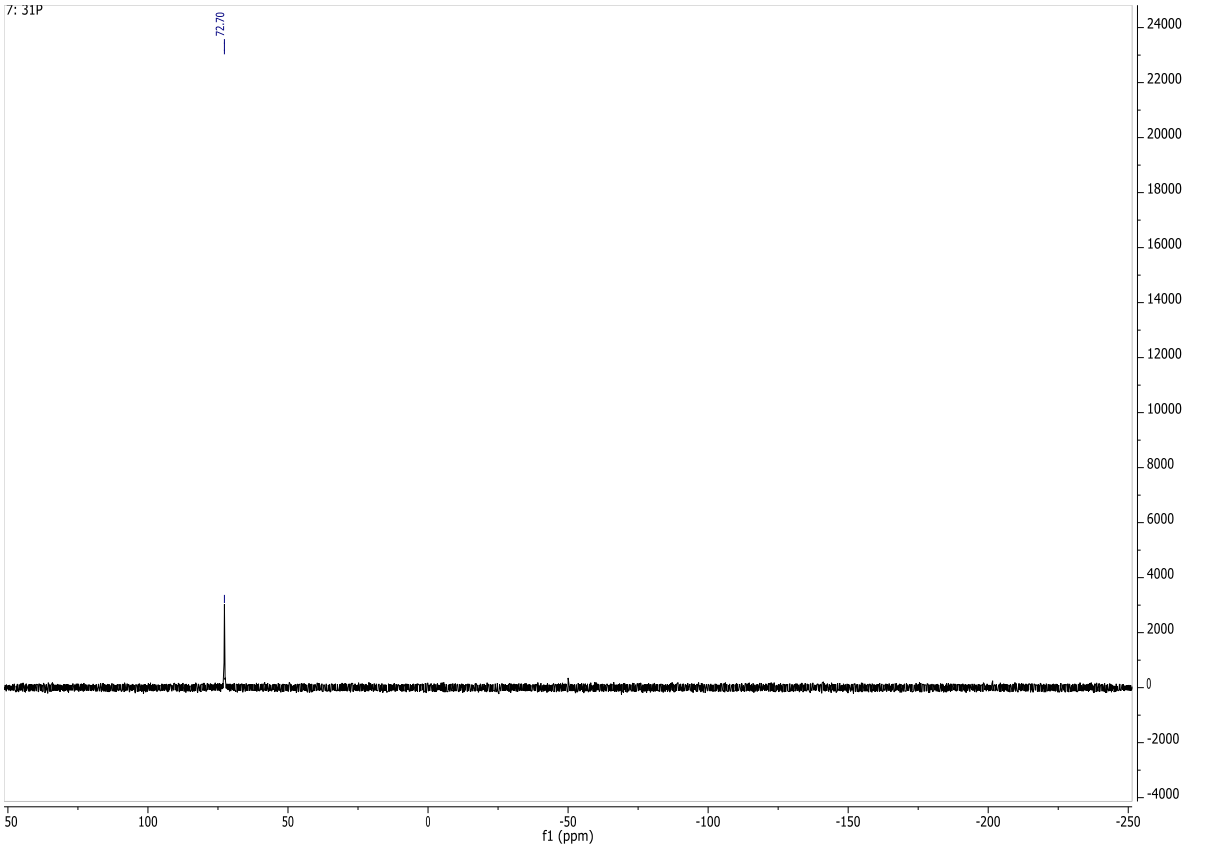

6: 31P
71,98
f1 (ppm)

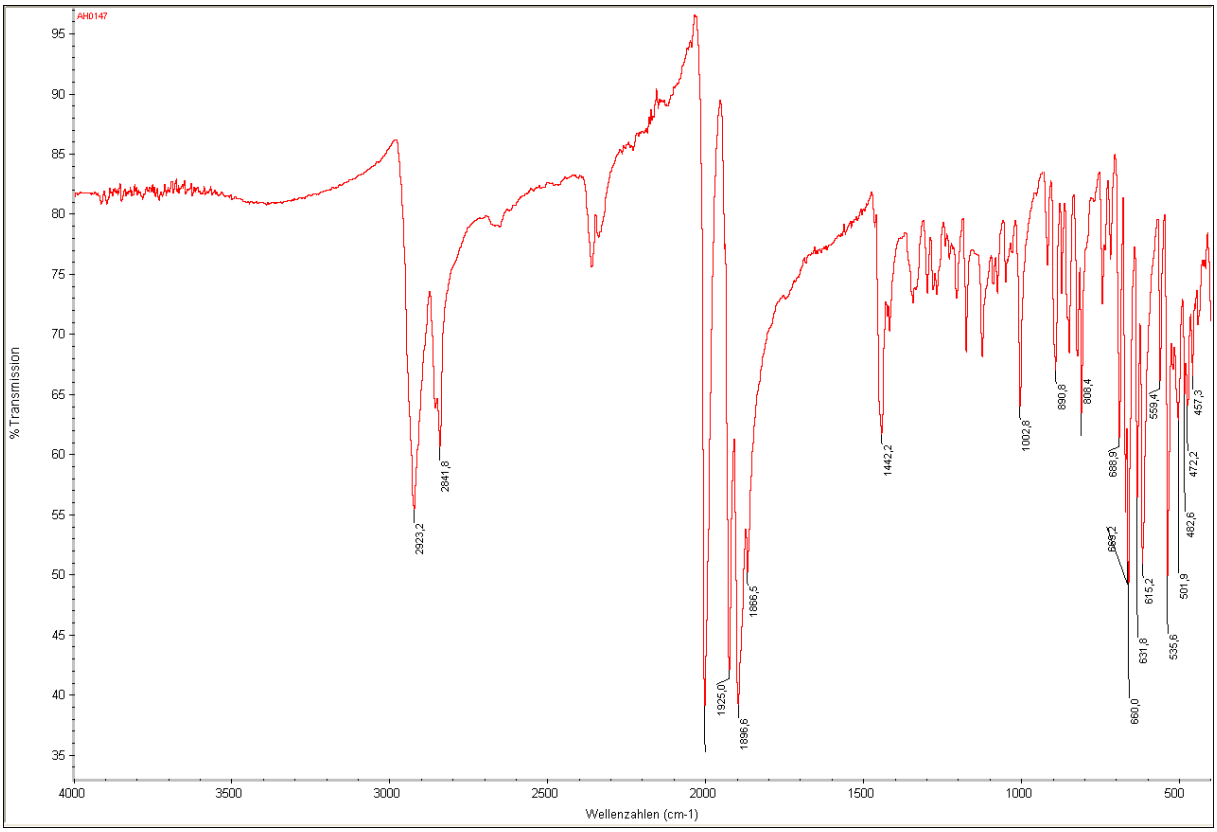

AH0148
% Transmission
Wellenzahlen (cm-1)
2965,7
2934,6
2877,8
2000,6
1933,8
1910,3
1457,7
1412,6
1376,8
1249,3
1028,7
759,8
742,1
724,4
697,8
662,1
637,4
614,3
551,9
522,8
495,2
470,1

NMR-spectra and IR-spectrum of *fac*-1,2-bis(dicyclohexyl)ethane tricarbonyl manganese (I) bromide 7 (*fac*-[Mn(Cyhex$_2$PCH$_2$CH$_2$PCyhex$_2$)(CO)$_3$Br])




7: 13C
223.35
220.29
38.83
38.75
38.68
36.00
35.86
30.26
30.14
29.60
29.42
27.93
27.75
27.61
26.51
26.33
21.69
21.55 Tol
21.41 Tol
20.40 Toluene-d8
19.94
f1 (ppm)


7: 31P
72.70
f1 (ppm)


AH0147
% Transmission
Wellenzahlen (cm-1)
95
90
85
80
75
70
65
60
55
50
45
40
35
4000
3500
3000
2500
2000
1500
1000
500
2923,2
2841,8
1925,0
1896,6
1866,5
1442,2
1002,8
890,8
808,4
688,9
660,0
631,8
615,2
559,4
535,6
501,9
482,6
472,2
457,3

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6.7 Article 12 *submitted*

Hydrogenation Reactions with a Heterogeneous Palladium-Substituted Mixed Cerium–Tin Oxide Cross-Coupling Catalyst

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Chemistry **2020**, submitted manuscript

Manuscript-ID: chemistry-853763

Author contribution

KH and HG initiated the project. AH designed and performed the experiments. KH prepared the catalyst. AH wrote the manuscript. AH, KH, H. G.-W. and HG read and edited the manuscript.

chemistry

MDPI

Communication

Hydrogenation Reactions with a Heterogeneous Palladium-Substituted Mixed Cerium–Tin Oxide Cross-Coupling Catalyst

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Abstract: A heterogeneous and readily accessible palladium-substituted mixed cerium–tin oxide catalyst, namely $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$, which is already established as suitable catalyst for cross-coupling reactions has been studied for hydrogenation reactions. Heterogeneous catalysts are valuable tools for organic chemistry and especially for hydrogenation reactions. Typical advantages are their recyclability and stability. However, in case of many catalysts leaching of the active metal is a major problem. Addressing this limitation, the heterogeneous catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ has already proven its stability and negligible leaching of the metals in Suzuki cross-coupling reactions, thus making it also to a potential alternative to conventional hydrogenation catalysts. Within this proof of concept study now, this catalyst was investigated for the hydrogenation of different functional groups and revealed a high selectivity towards the hydrogenation of double bonds, while other functional groups such as esters remain stable. Thus, these catalytic properties enable the application of this catalyst for selective hydrogenations of molecules with more than one functional group, which all can be converted under other hydrogenation conditions. Furthermore, this study shows that besides cross-coupling reactions this heterogeneous palladium-substituted mixed cerium–tin oxide catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ is also applicable for hydrogenations, thus making it a multi-functional catalyst for different types of reaction processes.

Keywords: Alkenes; C=C double bond reduction; heterogeneous catalysis; hydrogenation; palladium catalysts

1. Introduction

Palladium-substituted cerium and mixed cerium–tin oxides emerged as suitable catalysts for cross-coupling reactions such as Heck reaction and Suzuki-Miyaura reactions[1–6]. They can easily be prepared by the solution combustion technique, described by Singh *et al.* in 2010[7]. Using their method, palladium-substituted cerium and mixed cerium–tin oxides are synthesized at gram scale via a practical route and starting from cheap precursors, resulting in highly crystalline and pure catalysts[7–9]. These palladium-substituted cerium or cerium-tin oxide catalysts have initially been developed as catalysts for gas-phase oxidation reactions[10]. However, recent research also showed high activities of these heterogeneous catalysts for cross-coupling reactions[1–6]. Their efficiency has been demonstrated in various studies as well as their applicability for continuous processes, for which in particular the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst was used[5,6]. It is note-worthy that such heterogeneous catalysts exhibit high functional group tolerance and minimal leaching behavior being in the ppb range[2,4]. The latter property represents a major advantage over other heterogeneous catalysts such as palladium on charcoal, which is known to show a high leaching rate of palladium

from the support material[11,12]. These reasons make palladium-substituted cerium and mixed cerium–tin oxides suitable catalysts for applications in organic synthesis. However, in spite of their remarkable properties, these catalysts have been rarely studied for other types of reactions apart from gas-phase oxidation and cross-coupling reactions. Thus, such an evaluation raised our interest with a particular focus on hydrogenation reactions due to the importance of these types of transformations in organic chemistry and since in these reactions heterogeneous catalysts are preferably. Such hydrogenation processes are often catalyzed by expensive noble metals such as platinum, palladium or ruthenium and their heterogenization is conducted also to make them recyclable[13,14]. As for heterogeneous palladium catalysts such as palladium on charcoal leaching is a problem[11,12], the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst might be a suitable alternative. In the following we report our results on the investigation of the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ with a proven suitability for cross-coupling reactions[2–6] for its use in hydrogenation processes.

2. Materials and Methods

Hydrogenation reactions were performed in a Parr Series 5000 Multiple Reactor System, in which up to 6 reactions can be performed in parallel. The maximum volume of the autoclaves was 75 mL and the reaction mixtures were stirred at 1000 rpm using a magnet stirrer. Before usage of the reactors, they were evaporated and filled with Argon twice. Afterwards the substrate of interest, the base and the catalyst were added. The autoclaves were placed into the Parr Series 5000 Multiple Reactor System, which among others consists of a burst protection for high pressure reactions. The reactors were washed with nitrogen (2 × ~20 bar) to prevent formation of oxyhydrogen gas formation and afterward with hydrogen (2 × ~20 bar, 1 × required pressure) before adjusting the hydrogen-pressure to required pressure in every reactor. After stirring for the desired reaction time at the desired reaction temperature, the autoclaves were washed with nitrogen (3 × ~20 bar) before cooling to room temperature and work-up. The catalyst was removed by filtration over Celite (Celite 545, Merck) and evaporation of the solvent. The product was analyzed by ^{1}H-NMR-spectroscopy and compared with commercially available samples. NMR-spectra of the used compounds and products are found in spectra collections[15]. 3-Phenylpropanoic acid, 1-decanol and 4-bromo-toluene were purchased from Sigma Aldrich and used without further purification. NMR spectra were recorded on a Bruker Avance III 500 at a frequency of 500 MHz and chemical shift δ were given in ppm referenced to the corresponding solvent signal ($CDCl_3$).

Cinnamic acid (370 mg, 2.5 mmol) was dissolved in isopropanol (10 mL), the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (70 mg, 18% (*w*/*w*)) was added and the reaction mixture was treated with hydrogen pressure (100 bar) for 17 h at 100 °C. After removal of the catalyst and solvent, the starting material was recovered (350 mg, 95%).

Cinnamic acid (370 mg, 2.5 mmol) was dissolved in isopropanol (10 mL), KOMe (175 mg, 2.5 mmol, 1 equiv.) and the catalyst Ce0.20Sn0.79Pd0.01O2-δ (70 mg, 18% (w/w)) were added and the reaction mixture was treated with hydrogen pressure (100 bar) for 17 h at 100 °C. After removal of the catalyst, base and solvent 3-phenylpropanoic acid was isolated (345 mg, 92%, 90% purity).

Cinnamic acid (370 mg, 2.5 mmol) was dissolved in an organic solvent (isopropanol, ethanol, tetrahydrofurane (THF), 2-methyl tetrahydrofurane (2-Me-THF), cyclohexane or toluene) (10 mL), KOMe (175 mg, 2.5 mmol, 1 equiv.) and the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (70 mg, 18% (*w*/*w*)) were added. The reaction mixture was treated with hydrogen pressure (100 bar) for 17 h at 100 °C. After removal of the catalyst, base and solvent 3-phenylpropanoic acid was isolated (isopropanol: 349 mg, 93%, 95% purity; ethanol: 352 mg, 94%; 97% purity; THF: 345 mg, 92%, 90% purity; 2-Me-THF: 342 mg, 91%, 95% purity; cyclohexane: 348 mg, 93%, 89% purity; toluene: 340 mg, 91%, 76% purity).

Ethyl decanoate (501 mg, 2.5 mmol) was dissolved in isopropanol (10 mL), KOMe (175 mg, 2.5 mmol, 1 equiv.) and the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (90 mg, 18% (*w*/*w*)) were added and the reaction mixture was treated with hydrogen pressure (100 bar) for 18 h at 100 °C. After removal of the catalyst, base and solvent 3-phenylpropanoic acid was isolated (489 mg, 98%).

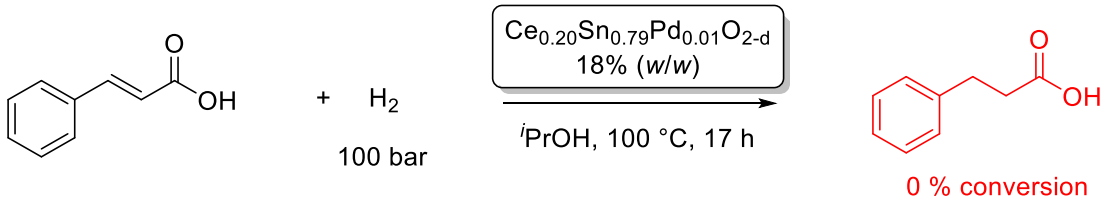
Ethyl cinnamate (441 mg, 2.5 mmol) was dissolved in isopropanol (10 mL), KOMe (175 mg, 2.5 mmol, 1 equiv.) and the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (79 mg, 18% (*w*/*w*)) were added and the reaction

mixture was treated with hydrogen pressure (100 bar) for 17 h at 100 °C. After removal of the catalyst, base and solvent ethyl 3-phenylpropanoate was isolated (430 mg, 97%).

4-Bromotoluene (428 mg, 2.5 mmol) was dissolved in isopropanol (10 mL), KOMe (175 mg, 2.5 mmol, 1 equiv.) and the catalyst Ce0.20Sn0.79Pd0.01O2-δ (77 mg, 18% (w/w)) were added and the reaction mixture was treated with hydrogen pressure (100 bar) for 17 h at 100 °C. After removal of the catalyst, base and solvent the starting material was recovered (419 mg, 98%).

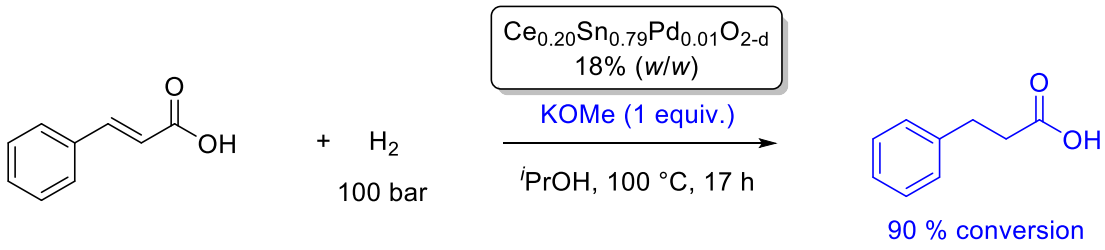
3. Results

In the initial step, we studied the hydrogenation of C=C double bonds with this catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$, exemplified for the conversion of cinnamic acid as a substrate. The catalyst loading has been adjusted to relatively high 18% (w/w) since the purpose of this investigation was to demonstrate a proof of concept for a hydrogenation reaction rather than a process optimization. Unfortunately, however, in this initial experiment we could not find any conversion to the desired product 3-phenylpropanoic acid and the starting material was recovered (Scheme 1).



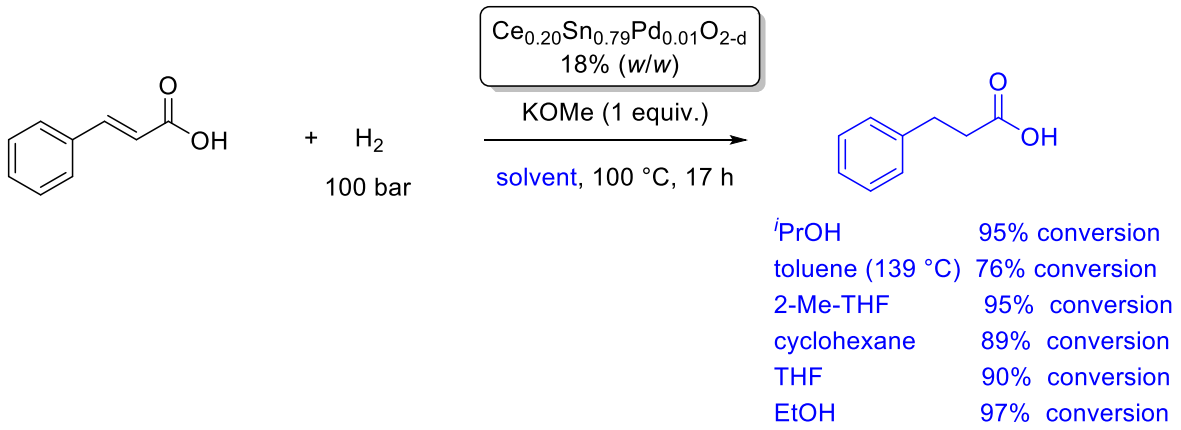
Scheme 1. Hydrogenation of cinnamic acid using the palladium-containing mixed cerium-tin oxide $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ as a heterogeneous catalyst.

Since for many hydrogenation catalysts additives such as a base are used to activate the catalyst or the substrate, in a further attempt the base KOMe was utilized as an additive for the hydrogenation of cinnamic acid in the presence of the palladium-containing mixed cerium-tin oxide $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ as a heterogenous catalyst. By means of this process modification, we were pleased to find that under these conditions now the desired hydrogenation of cinnamic acid proceeds successfully, leading to the product 3-phenylpropanoic acid with 90% conversion (Scheme 2).



Scheme 2. Hydrogenation of cinnamic acid using the heterogenous catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ with the base KOMe as an additive.

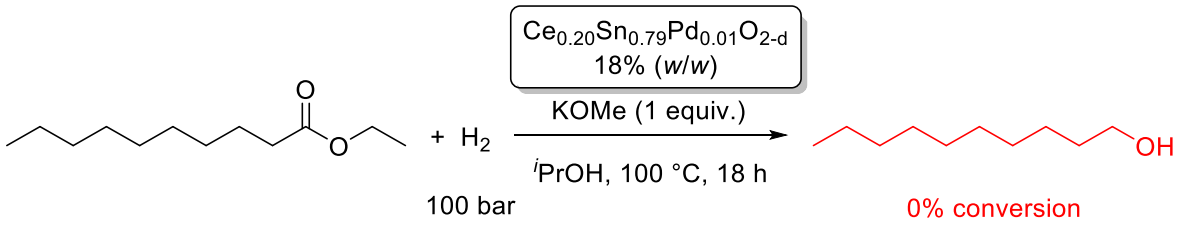
In order to confirm that under these reaction conditions with a hydrogen pressure of 100 bar and a reaction temperature of 100 °C the hydrogenation is related to the applied heterogeneous catalyst, a control experiment without catalyst was performed. As expected, in this control reaction no conversion was obtained, thus indicating that the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst catalyzes the hydrogenation of the double bond. In these initial experiments, isopropanol was used as organic solvent for the hydrogenation reaction. In a next step, the impact of the organic solvent component on the course of the hydrogenation reaction was investigated by utilizing six different organic solvents as reaction medium for the hydrogenation of cinnamic acid in the presence of the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ (Scheme 3).



Scheme 3. Hydrogenation of cinnamic acid using the heterogeneous catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ in different organic solvents as reaction media.

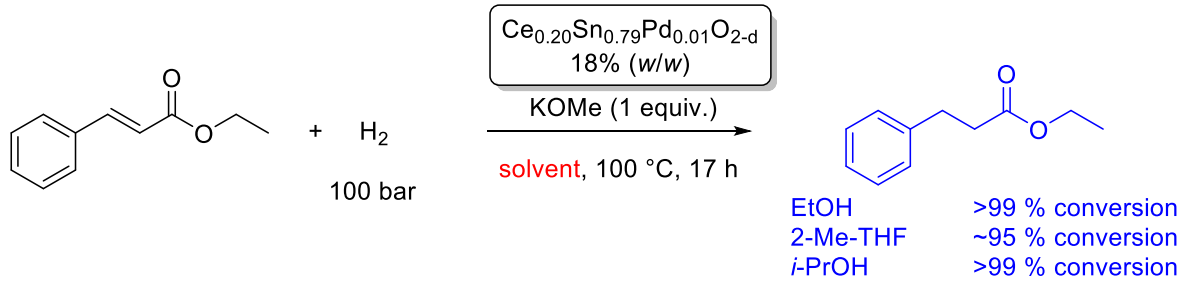
In all tested organic solvents, the hydrogenation reaction proceeds, thus showing also a generality of this catalyst with respect to its applicability in various reaction media. However, in toluene only 76% conversion within 17 h was found, while in both tested alcohols (isopropanol and ethanol) and cyclic ethers such as THF and 2-methyl-tetrahydrofurane (2-Me-THF) high conversions of at least 90% were obtained. For this reason, the further experiments were performed in isopropanol as prioritized organic solvent and reaction medium.

Encouraged by the suitability of this catalyst for hydrogenation reactions, next we investigated which types of functional group hydrogenations can be performed with this catalyst. As besides the hydrogenation of double bond in enoates also hydrogenation of esters is known to be performed with Pd-catalysts, we studied such a hydrogenation with the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst exemplified for ethyl decanoate as a substrate (Scheme 4). The reaction was performed in isopropanol under the same conditions as mentioned above for the hydrogenation of cinnamic acid with 100 bar hydrogen pressure and 100 °C reaction temperature. However, in this experiment no conversion to the desired product 1-decanol was obtained, which indicates that the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst does not catalyze ester hydrogenations under the chosen reaction conditions.



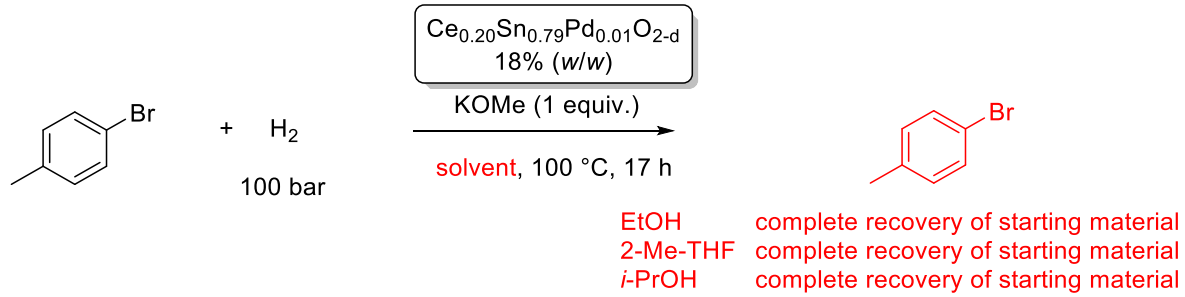
Scheme 4. Hydrogenation of an aliphatic ester using the heterogenous catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$.

It should be added that this lack of conversion in the ester hydrogenation can be beneficial as it indicates a chemoselectivity for C=C double bond hydrogenation over ester hydrogenation. Consequently, such a chemoselectivity should then allow the selective hydrogenation of C=C double bonds in molecules, in which also an ester group is present (which then will remain untouched). To demonstrate such an application, we chose ethyl cinnamate as a molecule with a double bond as well as an ester functionality, which both could be hydrogenated in principle (Scheme 5). When using this molecule for the hydrogenation with $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ as a catalyst under the same conditions, selective hydrogenation of the double bond of ethyl cinnamate was achieved while keeping the ester functionality stable. An excellent conversion of >99% to the desired product ethyl 3-phenylpropanoate was obtained when using isopropanol and ethanol as solvent, while ~95% was obtained with 2-Me-THF.



Scheme 5. Hydrogenation of ethyl cinnamate using the heterogeneous catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$.

To prove the stability of further functional groups, which might be present in a target molecule for a selective hydrogenation of a double bond, we investigated the stability of a bromo-substituent in toluene (Scheme 6). Under the chosen hydrogenation conditions of 100 bar hydrogen pressure and 100 °C in different solvents, the bromo-substituent remained stable and 4-bromo toluene was recovered from the reaction medium. This result might be beneficial for those cases, in which selectively a double bond should be hydrogenated while keeping a carbon-halogen bond and an ester functionality being present in the substrate molecule, unchanged.



Scheme 6. Stability study of the C-Br bond of 4-bromo toluene under hydrogenation conditions with the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$.

4. Discussion

In conclusion, the suitability of the catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$, being used so far, e.g., for cross-coupling reactions, has been demonstrated for hydrogenation reactions in this work. In detail, this study shows that the $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$-catalyst selectively catalyze the hydrogenation of C=C double bonds, while other functionalities remained untouched. Taking into account known advantages of this catalyst $Ce_{0.20}Sn_{0.79}Pd_{0.01}O_{2-\delta}$ in terms of suppression of leaching and its easy synthetic access, this catalyst represents a promising alternative for efficient hydrogenation processes to existing catalysts such as Pd/C as a frequently used hydrogenation catalyst. Among current research tasks are the expansion of the catalytic applicability towards a broader substrate range, the optimization of the catalyst loading (which has not been considered so far as the major focus of this study was to present a proof of concept) as well as studies of catalyst leaching will be also suppressed in analogy to previous work for these types of hydrogenation reactions.

Author Contributions: Conceptualization, A.H., K.H., H.G.-W. and H.G..; methodology, A.H.; validation, A.H.; formal analysis, A.H..; investigation, A.H. and K.H..; resources, H.G.-W. and H.G..; data curation, A.H..; writing—original draft preparation, A.H. and H.G.; writing—review and editing, A.H., K.H., H.G.-W. and H.G.; visualization, A.H.; supervision, H.G.-W. and H.G.; project administration, A.H.; funding acquisition, H.G.-W. and H.G. All authors have read and agreed to the published version of the manuscript.

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Ministry of Food and Agriculture (BMEL), respectively, within the funding programme on the utilization of biorenewables (grant number: 22001716).

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Conflicts of Interest: The authors declare no conflict of interest.

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Supplementary material

6.8 Analytical methods and devices

NMR spectra were recorded on a Bruker Avance III 500 at a frequence of 500 MHz (^{1}H) or 125 MHz (^{13}C). The chemical shift δ is given in ppm and referenced to the corresponding solvent signal.

GC-Analysis of hydroformylation mixtures were performed on a Shimadzu GC 2010 using a BGB-174 column (30 m x 0.25 mm x 0.25 µm). The organic phase was dissolved in ethyl acetate as stated prior to injection of 10 µL at 250 °C with a split ratio of 1:10. As carrier gas a N_2/synthetic air mixture was used in a pressure flow control mode with a column flow of 0.96 mL/min and a total flow of 13.5 mL/min. The following temperature gradient was used in this method: 50 °C start temperature, in 1 °C/min to 60 °C, in 20 °C/min to 100 °C, in 5 °C/min to 135 °C and in 40 °C/min to 200 °C (2.4 min). The flame detector temperature was 350 °C. Conversion was calculated using a calibration curve. The retention times for the compounds of interest are shown in Table 5.

Table 5. Retention times of compounds of interest.

Compound	**Retention time /min**
n-Oct-1-ene	5.7
Octen-isomers	5.3, 5.5, 6.0, 6.6
n-Nonanal (*n*-aldehyd)	17.9
2-methyl octanal (*iso*-aldehyde)	17.0

Absorbance spectra were recorded on a Jasco V-630 Spectrophotometer in 1 mL Quartz glass cuvettes.

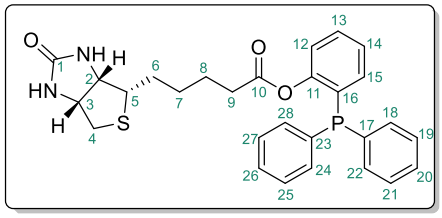
IR spectra were recorded on a Nicolet 380 of the Thermo Electron Corporation.

Hydroformylation experiments were performed in a Parr Multi Reactor System in which up to 6 experiments can be performed in parallel in 75 mL stainless steel autoclaves using a magnetic stirrer (stick form, 30 mm).

6.9 Artificial hydroformylase (Streptavidin-biotin approach)

6.9.1 Syntheses of biotinylated phosphine ligands

6.9.1.1 *ortho*-Biotinylated triphenylphosphine coupled via an ester



(+)-Biotin N-hydroxysuccinimide ester (133.38 mg, 0.4 mmol, 1 eq), (2-hydroxyphenyl)diphenylphosphine (133.84 mg, 0.5 mmol, 1.2 eq), triethylamine (0.25 mL, 1.8 mmol, 4.5 eq) in DMF (3 mL) was mixed under inert conditions and heated to reflux. Reaction control was performed by TLC (Cy/EtOAc, 4:1). After 45 h reaction time additional (2-hydroxyphenyl)diphenylphosphine (55.75 mg, 0.2 mmol, 0.5 eq) was added. The reaction was stopped after 69 h and the crude product was purified via automated column chromatography (Biotage Isolera™ One, SNAP Ultra 10 g) using a Cy/EtOAc-gradient from 5%-100% EtOAc in Cy. The *ortho*-biotinylated triphenylphosphine ligand (131.8 mg, 2.6 mmol, 65 %) was obtained as a colourless solid.

Isolated yield: 65%.

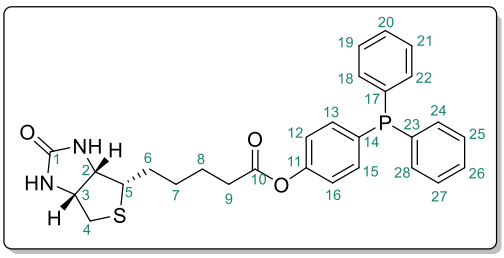
***R*$_f$ (Cy/EtOAc, 4:1):** 0.09.

^{1}H-NMR (500 MHz, DMSO-d$_6$): δ /ppm = 10.52 (s, 2H, N**H**), 7.70 – 7.45 (m, 12H, C^{12}**H**-C^{28}**H**), 6.96 (t, *J* = 7.5 Hz, 1H, C^{12}**H**-C^{15}**H**), 6.87 (dd, *J* = 8.2, 5.3 Hz, 1H, C^{12}**H**-C^{15}**H**), 4.00 – 3.91 (m, 1H, C^5**H**), 2.58 (d, *J* = 12.5 Hz, 2H, C^4**H$_2$**), 1.60 – 1.42 (m, 1H, C^2**H**), 1.32 – 1.20 (m, 6H, C^6**H$_2$**, C^8**H$_2$**, C^9**H$_2$**), 1.14 – 1.01 (m, 1H, C^3**H**), 0.88 – 0.76 (m, 2H, C^7**H$_2$**).

^{13}C{^{1}H}-NMR (126 MHz, DMSO-d6): δ /ppm = 172.78, 134.24, 131.73, 131.36, 131.28, 128.46, 128.36, 119.65, 25.22.

IR (neat): ν /cm^{-1} = 2921, 2852, 1713, 1436, 1216, 1167.

6.9.1.2 *para*-Biotinylated triphenylphosphine coupled via an ester



(+)-Biotin N-hydroxysuccinimide ester (134.13 mg, 0.4 mmol, 1 eq), (4-hydroxyphenyl)diphenylphosphine (167.33 mg, 0.6 mmol, 1.5 eq), triethylamine (0.25 mL, 1.8 mmol, 4.5 eq) in DMF (4 mL) was introduced under inert conditions and heated to reflux. Reaction control was performed by TLC (Cy/EtOAc, 4:1). The reaction was stopped after 69 h and the crude product was purified via automated column chromatography (Biotage Isolera™ One, SNAP Ultra 25 g using a cyclohexane (Cy)/EtOAc-gradient from 5%-100% EtOAc in Cy. The *para*-biotinylated triphenylphosphine ligand (81.0 mg, 0.16 mmol, 40 %) was obtained as a colourless solid.

Isolated yield: 40%.

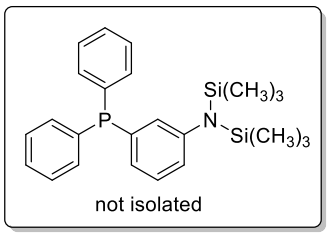
***R*$_f$ (Cy/EtOAc, 4:1):** 0.07.

^{1}H-NMR (500 MHz, DMSO-d$_6$): δ /ppm = 10.23 (s, 2H, N**H**), 7.59 – 7.40 (m, 12H, C^{12}**H**-C^{28}**H**), 6.91 (m, 2H, C^{12}**H**, C^{16}**H**), 3.96 (m, 1H, C^5**H**), 2.59 (m, 2H, C^4**H$_2$**), 1.50 (m, 1H, C^2**H**), 1.31 – 1.20 (m, 6H, C^6**H$_2$**, C^8**H$_2$**, C^9**H$_2$**), 1.10 (m, 1H, C^3**H**), 0.87 – 0.75 (m, 2H, -C^7**H$_2$**).

^{13}C{^{1}H}-NMR (126 MHz, DMSO-d6): δ /ppm = 172.95, 133.57, 133.48, 131.74, 131.45, 131.37, 128.65, 128.56, 115.70, 115.60, 28.89, 25.23, 11.19.

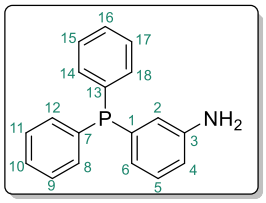
IR (neat): ν /cm^{-1} = 2924, 2854, 1720, 1430, 1215, 1167.

6.9.1.3 Synthesis of (3-aminophenyl)diphenylphosphine



Chlorodiphenylphosphine (2.4 mL, 12.5 mmol, 1 eq) was dissolved in tetrahydrofurane (THF) (20 mL) under inert conditions. 3-[Bis(trimethylsilyl)amino]phenylmagnesium chloride was added as solution in THF (12.5 mL of a 1 M solution, 12.5 mmol, 1 eq) at room temperature within 20 min. After 22 h reaction time the reaction was quenched by addition of dest. H_2O (40 mL). The solution was extracted with Et_2O (3 x 40 mL) and washed with a saturated NaCl solution (200 mL). Via TLC (Cy/EtOAc, 4:1) only the protected amine was detected. The solvent was removed and the crude product directly used for the following deprotection reaction.

***R*$_f$ (Cy/EtOAc, 4:1):** 0.70.



Under inert conditions the protected amine was heated in MeOH (50 mL) to reflux. The reaction progress was monitored by TLC (Cy/EtOAc, 4:1). After 24 h the reaction was stopped and the solvent removed *in vacuo*. The crude product was purified by automated column chromatography (Biotage Isolera^{TM} One, SNAP Ultra 50 g) using a Cy/EtOAc-gradient from 5%-100% EtOAc in Cy. (3-Aminophenyl)diphenylphosphine (2.65 g, 9.6 mmol, 77 %) was obtained as a colorless solid.

Isolated yield: 77%.

***R*$_f$ (Cy/EtOAc, 4:1):** 0.24.

^{1}H-NMR (500 MHz, $CDCl_3$): δ /ppm = 7.37 – 7.29 (m, 10H, C^8**H**- C^{18}**H**), 7.13 (t, J = 7.7 Hz, 1H, C^2**H**), 6.73 – 6.67 (m, 1H, C^4**H**), 6.67 – 6.57 (m, 2H, C^5**H**; C^6**H**), 3.65 (s, 2H, N**H$_2$**).

^{13}C{^{1}H}-NMR (126 MHz, $CDCl_3$): δ /ppm = 146.44, 137.09, 134.02, 133.87, 129.51, 128.83, 128.60, 128.55, 124.23, 120.16, 115.80.

IR (neat): ν /cm^{-1} = 33455, 3439, 2997, 1614, 1587.

6.9.2 Screening of hydroformylation conditions of *n*-oct-1-ene

n-Oct-1-ene (6 mL, 71 mmol) was placed into a 75 mL stainless steel autoclave and aqueous phase (4 mL) consisting of TPPTS (5 mmol/L) and $Rh(acac)(CO)_2$ (250 µmol/L) was added. Afterwards the autoclave was closed and washed with Argon twice and with nitrogen (2x 20-30 bar), before washing with nitrogen using the required pressure for hydroformylation. The autoclave was washed with syn-gas mixture (2x 20-30 bar) before adjusting the pressure to this which is required for the reaction. Afterwards the autoclave was closed, the reaction temperature set and stirred at 1000 rpm. After the desired reaction time, the pressure was released, the heating was stopped, and the autoclave washed with nitrogen (3x 20-30 bar). The phases were separated and the organic phase analyzed by GC (10 µL organic phase in 990 µL EtOAc) after filtration over silica. The conditions and results are shown in Table 6.

Table 6. Screening conditions and results after GC-analysis of *n*-oct-1-ene hydroformylation.

#	***T*** **/°C**	***p*** **/bar**	***t*** **/h**	**Conv. /%[a]**	**Not-defined impurities /%[b]**	***n*-Aldehyde /%[c]**	***iso*-Aldehyde /%[c]**	***n*-Alcohol /%[b]**	***iso*-Alcohol /%[b]**
1	80	80	4	67	1	45	21	<1	<1
2	80	100	4	85	6	46	27	4	3
3	70	80	4	38	<1	25	13	<1	<1
4	70	100	4	65	2	39	20	2	1
5	60	80	4	23	<1	14	8	<1	<1
6	60	100	4	39	1	24	13	<1	<1
7	60	100	4	6	<1	4	2	<1	<1
8	60	120	4	29	<1	18	11	<1	<1
9	60	11	20	93	6	42	28	10	7
10	60	120	20	96	6	44	31	9	6
11	50	120	4	9	<1	5	3	<1	<1
12	50	120	20	57	3	27	18	5	4

[a] *Conversions were determined by GC-analysis. GC-areas of octene were compared to all other areas of substances.*
[b] *Amounts were determined by comparison of the GC-areas of this compound to all other areas of substances in the chromatogram.*
[c] *Amounts of aldehyde were determined by comparison to standard-curves and to all other areas of substances in the chromatogram.*

6.10 Artificial hydroformylase (Metal-exchange approach of CYP119 monooxygenase)

6.10.1 Comparison of free Rh(acac)(CO)$_2$ to Rh(acac)(CO)$_2$ in combination with TPPTS or TPP

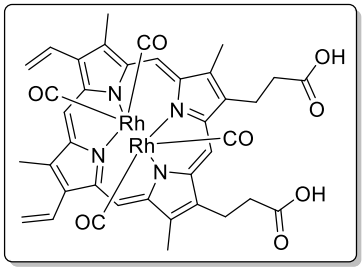
n-Oct-1-ene (6 mL, 71 mmol) was placed into a 75 mL stainless steel autoclave and aqueous phase (4 mL) consisting Rh(acac)(CO)$_2$ (250 µmol/L) (reaction **a**) or TPPTS (5 mmol/L) and Rh(acac)(CO)$_2$ (250 µmol/L) (reaction **b**) or TPPS (5 mmol/L), NEt$_3$ (10 mmol/L) and Rh(acac)(CO)$_2$ (250 µmol/L) (reaction **c**) were added. Afterwards the autoclave was closed and washed with Argon twice. The autoclave was washed with nitrogen (2x 20-30 bar) before washing with nitrogen using the required pressure for hydroformylation. The autoclave was washed with syn-gas mixture (2x 20-30 bar) before adjusting the syn-gas pressure to 80 bar. Afterwards the autoclave was closed, the reaction temperature set to 80 °C and the reaction mixture stirred for 4 h at 1000 rpm. The pressure was released, the heating was stopped, and the autoclave washed with nitrogen (3x 20-30 bar). The phases were separated and the organic phase analyzed by GC (10 µL organic phase in 990 µL EtOAc) (Table 7) after filtration over silica.

Table 7. GC-results of hydroformylation of *n*-oct-1-ene using different ligands.

Reaction	Conversion of *n*-Oct-1-ene /%[a]	Ratio of *n*- to *iso*-aldehyde[b]
a	75	1.8:1
b	46	2.4:1
c	84	1.6:1

[a] *Conversions were determined by GC-analysis. GC-areas of octene were compared to all other areas of substances.*
[b] *Amounts of aldehyde were determined by comparison to standard-curves and to all other areas of substances in the chromatogram.*

6.10.2 Synthesis of a Rh-porphyrin complex from protoporphyrin IX



$Rh(acac)(CO)_2$ (227.21 mg, 0.9 mmol, 2 eq.) were dissolved in dichloromethane (10 mL) and Ar-atmosphere was attached. Protoporphyrin IX (250.21 mg, 0.4 mmol) and NEt_3 (0.61 mL, 4.4 mmol, 10 eq.) were added while stirring at room temperature. The reaction mixture was heated to reflux for 2 h before the solvent was removed and the Rh-heme b (329.70 mg, 0.4, 85%) complex dried in high vacuum. The complex was dissolved in DMSO to 50 mM as stock solution for hydroformylation and reconstitution experiments with an apoprotein of CYP119 monooxygenase (performed by Michael Stricker). The absorbance spectrum of the complex was recorded in 0.025 mM concentration in dest. water including 5% DMSO.

Isolated yield: 85%

UV/VIS-spectrum (250-900 nm): Maxima at 360 nm, 460 nm (global maximum), 530 nm, 480 nm and 640 nm. This spectrum is in accordance to a comparable dinuclear Rh-porphyrin complex published by Osuka *et al*.[86]

6.10.3 Hydroformylation of *n*-oct-1-ene using Rh-heme b as catalyst

n-Oct-1-ene solution (6 mL, 71 mmol) including $Rh(acac)(CO)_2$ (250 µmol/L) (reaction **a**) or Rh heme b (125 µmol/L) (reaction **b**) was placed into a 75 mL stainless steel autoclave. Afterwards the autoclave was closed and washed with Argon twice. The autoclave was washed with nitrogen (2x 20-30 bar) before washing with nitrogen using the required pressure for hydroformylation. The autoclave was washed with syn-gas mixture (2x 20-30 bar) before adjusting the syn-gas pressure to 80 bar. Afterwards the autoclave was closed, the reaction temperature set to 80 °C and the reaction mixture stirred for 4 h at 1000 rpm. The pressure was released, the heating was stopped, and the autoclave washed with nitrogen (3x 20-30 bar). The phases were separated and the organic phase analyzed by GC (10 µL organic phase in 990 µL EtOAc) (Table 8) after filtration over silica.

Table 8. GC-results of hydroformylation of *n*-oct-1-ene using $Rh(acac)(CO)_2$ or Rh-heme b.

Reaction	**Conversion of *n*-Oct-1-ene /%[a]**	**Ration of *n*- to *iso*-aldehyde[b]**
a	28	2.0:1
b	77	2.1:1

[a] *Conversions were determined by GC-analysis. GC-areas of octene were compared to all other areas of substances.*
[b] *Amounts of aldehyde were determined by comparison to standard-curves and to all other areas of substances in the chromatogram.*