CAROLIN GIESE

Dissertation

Biocatalytic Oxidation Reactions for Sustainable Syntheses of Bulk and Fine Chemicals

Universität Bielefeld

Biocatalytic Oxidation Reactions for Sustainable Syntheses of Bulk and Fine Chemicals

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CAROLIN GIESE
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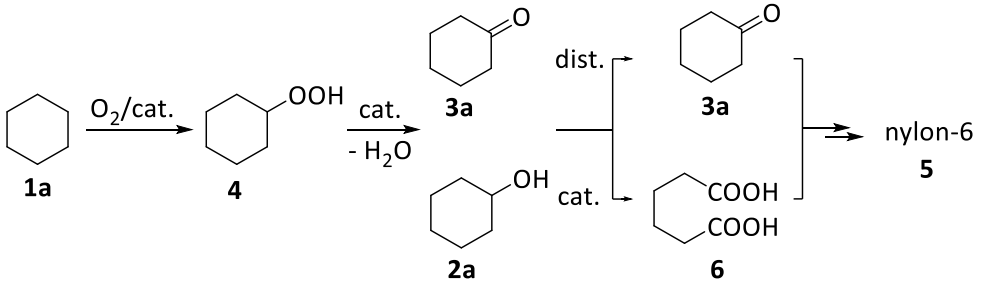
Contents

1 Introduction

The topic ‚oxidation reaction' encompasses a vast number of chemical and biological reactions that take place not only in the round bottom flasks of lab chemists. In cooking pots, table sugar is oxidized to delicious smelling caramel[1] while many vitamins lose their biologic effect as consequence of oxidation when heated.[2] At the hairdresser, requests like perm-fixing and bleached blonde hair are fulfilled *via* oxidation[3] and in the liver, drugs are made excretable by enzyme catalyzed oxidation reactions[4,5] Even taking a hot shower in the morning and going to work by car quite comfortable was unthinkable for a long time without oxidation of hydrocarbons. In the heating system of houses or in the tank of cars, fuels - composed of alkanes, cycloalkanes and other aromatic hydrocarbons - undergo complete combustion with atmospheric oxygen as oxidant, yielding H_2O and CO_2. Thereby, chemical energy is transformed in propulsive power and heat. Altogether, it is not possible to imagine life as we know it without oxidation of alkanes.[6]

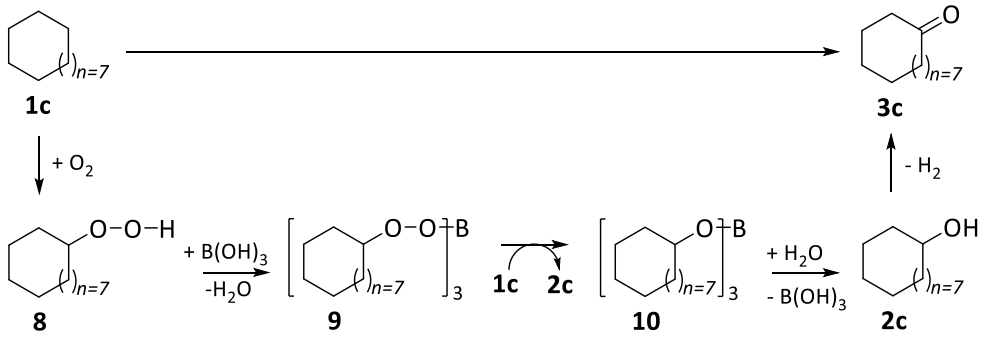
For the controlled partial oxidation of unfunctionalized hydrocarbons in particular, a certain extent of skill and care is required since these unactivated compounds are quite inert and harsh conditions are typically necessary to realize oxidation in the lab. Particulary, the oxidation of cycloalkanes **1** from petroleum to a mixture of corresponding cycloalkanols **2** and cycloalkanones **3** is of economic importance since these compounds serve as industrial feedstock for the synthesis of bulk products. For example, the synthesis of nylon-6 (**5**) can be realized *via* cyclohexanone (**3a**) as well as *via* adipic acid (**6**, Scheme 1).[7]



Scheme 1. Catalytic oxidation of cyclohexane (**1a**) with molecular oxygen for the synthesis of intermediates for nylon-6-production (**5**)

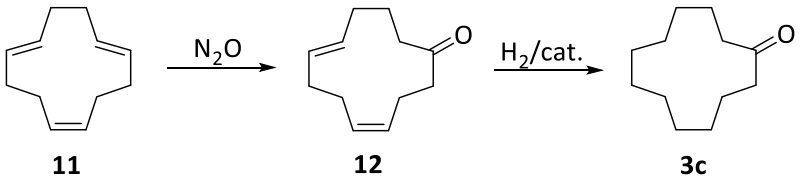
Another current route starting from the corresponding cycloalkane **1**, which is preferred for the synthesis of higher homologue cycloalkanones **3** from a size of ten carbon atoms (cyclodecanone (**3b**)) on a large scale is conducted in the presence of boric acid and molecular oxygen. A very important product of this BASHKIROV process with subsequent catalytic dehydration is cyclododecanone (**3c**), which is the feedstock of the synthesis of nylon-12 (**7**) (Scheme 2).[7-12]

In the presence of molecular oxygen cyclododecane (**1c**) is oxidized and the unstable cyclododecyl hydroperoxide (**8**) is formed. With boric acid, **8** is trapped to form the cyclododecyl perborate ester **9** in a condensation reaction. Additional reagent **1c** is oxidized by the perborate ester **9** to cyclododecanol (**2c**), forming the relatively stable borate ester **10**. A subsequent hydrolyzation gives access to the desired alkohol **2c**, and by catalytic dehydration the important intermediate **3c** is obtained (Scheme 2).[10]



Scheme 2. BASHKIROV process with subsequent catalytic dehydration is applied for the synthesis of cyclododecanone (**3c**), adapted and modified from MUSSER[10]

Drawbacks of this protracted procedure are the application of stoichiometric amounts of boric acid meaning an expended effort in the waste water treatment, the low selectivity of the oxidation and the extensive separation of unreacted reagent **1c** due to low conversions. Recently, in 2009 an innovative procedure was established by BASF, solving two problems simultaneously. Nitrous oxide (N_2O), an unpleasant side product that incurs during the industrial synthesis of adipic acid (**6**), usually has to be disposed of elaborately. The innovative commercial application of this noxious greenhouse gas as oxidant in the synthesis of cyclododecanone (**3c**) shortens the multistep procedure shown in Scheme 2 and additionally obviates the disposal problem of N_2O (Scheme 3).[11-14]



Scheme 3. Nitrous oxide (N_2O) is applied for the synthesis of cyclododecanone (**3c**) in which only one catalyst is necessary[11]

Furthermore, the oxidation of alcohols represents one of the most important and fundamental reactions in organic chemistry and therefore a vast number of oxidation methods is reported and summarized.[15] However, these reactions are often realized by the application of stoichiometric amounts of toxic and carcinogenic chromates. A range of industrial processes can be found in literature in which chromium-based oxidation steps are applied for the synthesis of, for example, active pharmaceutical ingredients (APIs), like cortisone (**13**), levofloxacine (**14**), dapsone (**15**)[16] or talampanol (**16**).[17]

A timely change in the environmental awareness in the more recent past initiated the call for substitution of these antiquated oxidation procedures, traditionally using metal catalysts or stoichiometric quantities of chromates by more contemporary procedures. Environmentally benign oxidants such as O_2 or H_2O_2, whereby only water accrues as side product, represent a green alternative.[8]

Besides these alternatives broached above, biocatalysis is a further, complementary growing branch for environmentally friendly oxidation reactions. Biocatalyzed oxidation reactions are carried out by a class of enzymes, called oxidoreductases, representing one of the six classes of enzymes, subdivided by an enzyme commission. The application of the different enzyme classes for organic synthesis is summarized in Figure 1 (left).[18] Oxidoreductase-catalyzed reactions make one fourth of these processes and about one half out of it accounts for oxidation reactions. On the right side of Figure 1, reaction types of biocatalytic oxidation reactions in industry are listed.[19]

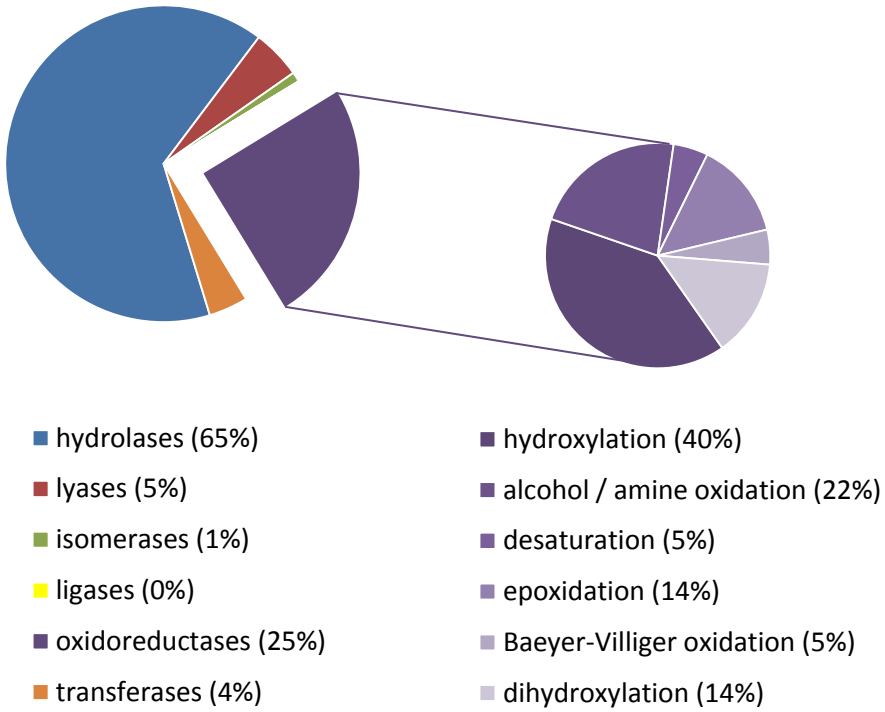


Figure 1. Application of the different enzyme classes for organic synthesis (left)[18] and types of biocatalytic oxidation reaction in industry (right)[19]

As apparent from Figure 1 (right), oxyfunctionalizations account for the major part and hold a great potential for a further green alternative.

2 Motivation and Aim

As elaborated in the previous chapter, a new environmental awareness leads to the claim of substitution of traditionally applied, obsolete oxidation procedures by more contemporary and green procedures. Metal catalysts or stoichiometric quantities of chromates are widely used as oxidation agents in oxidation reactions what makes these processes highly questionable, considering the sanitary consequences. In the particular case of large industrial-scale production of higher homologue cycloalkanones **3** with eight or more carbon atoms, the BASHKIROV process with subsequent catalytic dehydration is applied using molecular oxygen as the oxidant. However, several drawbacks of this procedure are the application of stoichiometric amounts of boric acid, the low selectivity of the oxidation and the extensive separation of unreacted reagent **1c** due to low conversions.[10]

Therefore the overall objective of this thesis is to develop a modern and green procedure for the direct functionalization of cycloalkanes **1** to cycloalkanones **3** for the main part. Based on the previous work by BURDA and STAUDT which is described further down in this chapter, the desired cycloalkanone **3** shall be synthesized directly by a one-pot process in water starting from the corresponding cycloalkane **1** and using atmospheric oxygen as oxidizing agent.[20-22] At different stages of the process design, all aspects - from efficiency of the working up and robustness of the analytical method in the beginning through to enzyme activities with variable substrates and finally the preparative oxidation of the substrates and whole cell biotransformation - should be considered. Prior to a preparative experiment or process, preliminary studies should ensure that by performing a certain procedure (reaction, working up, analytics) all circumstances that contribute to a distortion of the results will be recognized and regarded. First off all, a mass loss analysis has to be done to find out if the starting materials have a strong disposition to evaporate, to undergo decomposition or to adsorb to surfaces.

Therefore a simulation of reaction conditions and working up is carried out for substrate, intermediate and product. In an ideal case, the efficiency of the working up process should be considered separately first. Then the reaction time while also taking into account the method of working up is to be analyzed, and finally the reaction time while also taking into account the method of working up in the presence of biomass has to be investigated. Furthermore, a robust and exact analytical method for the absolute determination of all components (the respective substrate, intermediate and the product) should be established, ideally *via* gas chromatography in a concentration range relevant for the planned experiments. The definition of the methods detection limit (MDL) is an important step to get reliable results in the quantitative analysis of subsequent biotransformations. Aim of this section is to assure that neither a loss of material, nor an error-prone working up or an inappropriate analytical method leads to an

unrecognized distortion of the results. These sources of error that can appear in a preparative experiment are summarized in Figure 2.

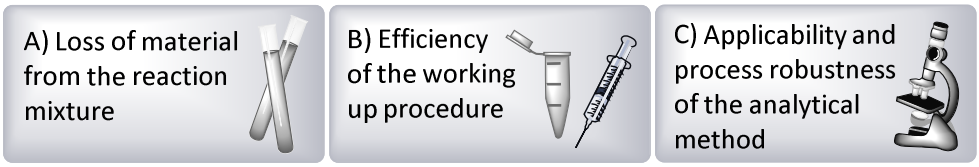
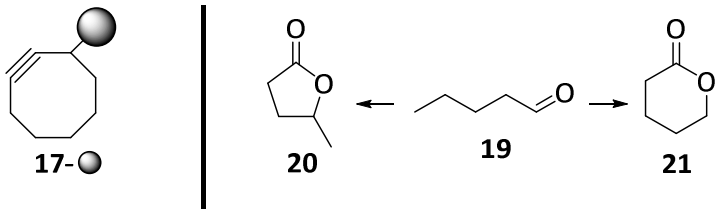


Figure 2. Sources of error in a preparative experiment

A preselection and evaluation of substrates - for various reasons attractive to be hydroxylated by cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) that were made available by the research group Prof. Dr. U. Schwaneberg (RWTH Aachen) - is to be investigated *via* UV/VIS-spectroscopy to find suitable substrate-enzyme combinations for subsequent preparative experiments.

From the category monounsaturated cyclic hydrocarbons, cyclooctyne (**17**) and cyclododecene (**18**) are chosen as possible substrates. Cyclooctynes are highly reactive and therefore established as tools in bioconjugations, probing biomolecules in living systems (Scheme 4, left).[23-24] Cyclododecene (**18**), is a versatile chemical intermediate for organic synthesis in general.[25]

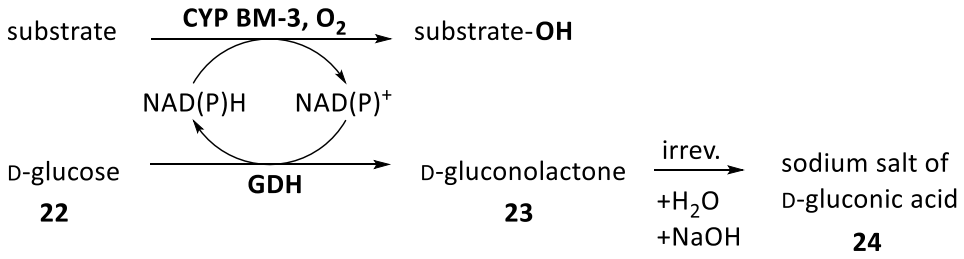
Furthermore the aldehyde pentanal (**19**) is supposed to be an excellent starting compound for an enzyme mediated synthesis of γ-valerolactone (**20**) and δ-valerolactone (**21**) (Scheme 4, right). Due to its olfactory qualities, γ-valerolactone (**20**) is used in the flavour and fragrance industry.[26] δ-Valerolactone (**21**) in contrast is applied in the synthesis of polymers (polyesters).[27]



Scheme 4. Cyclooctyne (**17**), bound to a reporter molecule ● (left) and the desired products γ-valerolactone (**20**) and δ-valerolactone (**21**), starting from pentanal (**19**) (right)

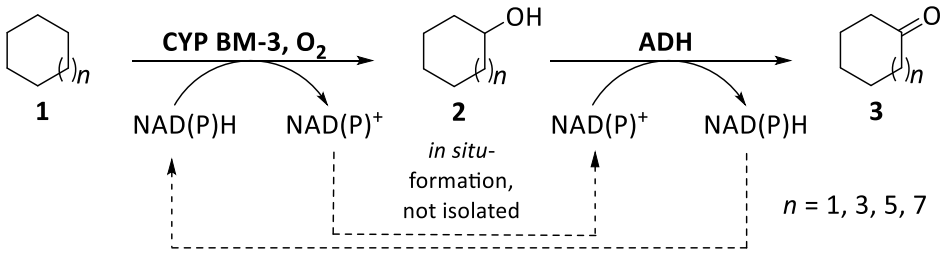
The third category to be tested is the class of cyclic alkanes **1**. Due to its extraordinary role as feedstock for the polyamide production elaborated in the previous chapter, a successful oxidation of these substrates would be of high importance and should be analyzed excessively. The most promising substrates that result from the preselection and evaluation should then be analyzed in preparative experiments testing the hydroxylation with CYP BM-3 to evaluate if a double oxidation is generally possible. Since without a second reaction step no intrasequential cofactor regeneration is possible, a common regeneration system using glucose dehydrogenase (GDH) should be applied for these experiments:

D-glucose (**22**) is oxidized by a GDH and D-gluconolactone (**23**) is formed which is spontaneously hydrolyzed whereupon D-gluconic acid (**24**) is formed. A subsequent neutralization with e.g. sodium hydroxyde forms the corresponding sodium salt of the D-gluconic acid (**24**) and the equilibrium is shifted towards the product. As a consequence the back reaction cannot take place (Scheme 5).[28]



Scheme 5. Cofactor recycling by the coupled enzyme method with glucose dehydrogenase (GDH)[28]

The substrates that show conversion in the first oxidation step, catalyzed by a CYP BM-3 with molecular oxygen yielding the corresponding alcohol, should then be applied in a double oxidation one-pot process, that can be described as a "dream reaction", a term that represents sustainable synthetic methods with high atom economy and efficiency.[8,29] In literature an enzymatic two-step one-pot process is already described by BURDA that basically can be applied for the synthesis of cyclic ketones **3**. The concept of this direct synthesis of cycloalkanones **3** from the corresponding cycloalkanes **1** is based on the cooperation of two enzymes: The first step of this process is the CYP BM-3 catalyzed hydroxylation reaction, analyzed before. For this reaction, the reduced form of the cofactor NAD(P)H is required and oxidized to $NAD(P)^+$. The second step is an alcohol dehydrogenase catalyzed oxidation of the *in situ* formed alkanol **2** in order to give the corresponding alkanone **3**. In this subsequent step, the oxidized form of the cofactor, which is generated in the CYP BM-3 catalyzed hydroxylation reaction, is required and retransformed into the reduced form NAD(P)H by the alcohol dehydrogenase. In this way the application of a further cosubstrate becomes redundant.[20] The general reaction scheme is shown below.



Scheme 6. General reaction concept of the biocatalyzed two-step one-pot process adapted from BURDA[20]

Aiming to identify active mutants of the cytochrome P450 monooxygenase (CYP BM-3) from *Bacillus megaterium* for the hydroxylation of cyclic alkanes **1**, BURDA

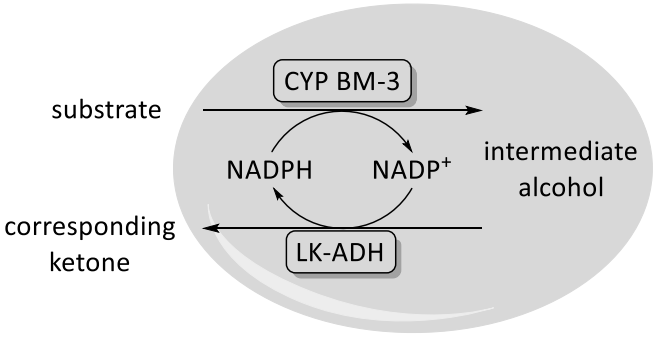
screened saturation mutagenesis libraries. The most promising mutants were cultivated, isolated, purified and lyophilized, then the concentration of the respective enzyme was determined *via* CO-difference spectroscopy. Furthermore the crude extracts were analyzed with regard to their ability to synthesize cyclic alcohols **2** from cyclic alkanes **1**. Sequencing of the best mutant of the cytochrome P450 monooxygenase revealed valine at position 87 and therefore CYP BM-3 F87V was exclusively used for preparative experiments. To realize the second oxidation step, BURDA applied the (*R*)-enantioselektive alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH). [20]

Moreover, STAUDT extensively analyzed the substrate-product-mixture cyclooctane (**1d**)/cyclooctanone (**3d**) in an aqueous reaction medium.[21,22] Despite the fact that these compounds have comparatively high boiling points (**1d**: 150-152°C[25]; **3d**: 195-197°C[30]), remarkable decreased amounts of the initially applied quantity were isolated according to ^{1}H-NMR-spectroscopy as a result of evaporation. Due to this evaporation, determination of conversion in a classical sense would be defective and instead the productivity (g/L) of the reaction was stated.[21] The well-established working up procedure developed by STAUDT comprises the removal of the solvent *in vacuo* (900 mbar) and the determination of the absolute amount of the respective cycloalkanone **3**. Therefore, the crude product was analyzed *via* ^{1}H-NMR-spectroscopy in the presence of pyridine (**25**) as external standard.[21,22]

Both BURDA and STAUDT registered an increase in productivity when catalytic amounts of 2-propanol were used as additive. This procedure allows the application of the more cost-effective cofactor in its oxidized form ($NADP^+$) since the required reduced form of the cofactor (NADPH) is generated in the course of the ADH-catalyzed oxidation of 2-propanol yielding acetone.[20-22] Aside from the wildtype and the above-mentioned mutant CYP BM-3 F87V, STAUDT investigated the mutant CYP BM-3 19A12 with regard to its efficiency to catalyze the aforementioned reaction (Scheme 6). The best result, namely a productivity of 0.80 g/L cyclooctanone (**3d**), was obtained by using the mutant CYP BM-3 19A12 in combination with the above-mentioned LK-ADH for the conversion of 100 mM cyclooctane (**1d**) in phosphate buffer.[22]

Hence the challenge is to analyze more active mutants of the cytochrome P450 monooxygenase (CYP BM-3) from *Bacillus megaterium* for the hydroxylation of cyclic alkanes **1** and further convenient substrates. The biocatalyzed double oxidation which was extensively analyzed for cyclooctane (**1d**) by STAUDT, should be equally addressed applying the substrates cyclohexane (**1a**), cyclodecane (**1b**) and cyclododecane (**1c**). To avoid the application of pyridine (**25**) as external standard as well as the analysis *via* ^{1}H-NMR-spectroscopy, a more economic and reliable GC-based analysis is planned to be established. In this way the removal of the solvent, which requires exact 900 mbar, can be circumvented. Applying the most stable system it should be analyzed if the immobilization of the cooperating enzymes is basically possible.

Finally, the most promising substrates that showed adequate product formation in the double oxidation experiments should be applied in a whole cell biotransformation, since this concept was recently successfully described in literature with considerably high product formations (Scheme 7).[31,32]



Scheme 7. Reaction scheme of the whole cell double oxidation

3 Preliminary studies

3.1 Introduction, state of the art, and aim of this section

Prior to a preparative experiment - regardless of whether a classic chemistry experiment or a biotransformation is planned - some preliminary studies about analytical techniques are absolutely essential to be conducted. It has to be ensured that by performing a certain procedure (reaction, working up, analytics) all sources of error will be identified and regarded. First of all, a loss of material might occur if the applied starting compounds have a strong tendency to evaporate, to undergo decomposition or to adsorb to surfaces. To clarify this, reaction conditions have to be simulated and a mass loss analysis has to be conducted. Next, the efficiency of the working up process resp. of the isolation of the product is to be analyzed. Since the isolation of the oxidation products is realized by liquid-liquid extraction, some general considerations have to receive attention. A liquid-liquid extraction system consists of two immiscible solvents and a compound that shows a varying solubility is in these solvents. A linear correlation exists - in case of ideal solutions - between the concentrations on either sides of the phase boundary. This correlation can be described by the NERNST distribution law.[33]

$$\frac{\text{c (A in phase 1)}}{\text{c (A in phase 2)}} = K$$

The ratio of the concentration of compound A in solvent 1 and the concentration of A in solvent 2 are constant. *K* is the NERNST distribution coefficient and the NERNST distribution law is valid if only one compound is dissolved in both phases.[33] In reality, extraction mixtures often are non-ideal solutions which consist of more than one compound, making validations necessary. The efficiency of an extraction method is determined by the recovery, that describes the amount of a compound that is successfully extracted by a particular method.[34] Additionally, the recovery rate has to be analyzed with a convenient analytical method. Therefore a robust, fast and suitable analytical method is required and need to be tested in regard to its applicability. For example, there are different analysis methods such as HPLC, GC or NMR available and either an internal or external standard can be employed. By using an internal standard, the sample is mixed with a reference compound before extraction. This is an accurately quantified compound that is similar to the substance of interest but can be separated by analytical methods. Ideally, the signal ratio of the two compounds can be determined precisely with the chosen analytical method.[34] Is the concentration of the internal standard changed it is expected that the concentration of the substance of interest changed in the same way. Another method is to use an external standard that is analyzed independently from the substance of interest. The results are two reports, one of the standard and one of the substance of interest, where the integrals of the peak areas can be compared.[34]

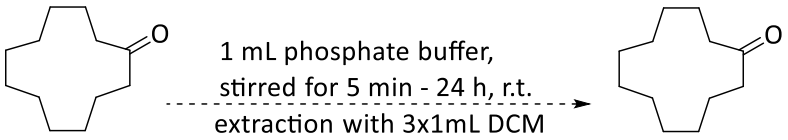
For a multipoint calibration, standard solutions of different concentrations are prepared and chromatographed under the same, predetermined conditions that are used to analyze the sample afterwards. Additional peaks have no influence on the result using this method.[35] The aim of this section is to assure that neither a loss of material, nor an error-prone working up or an inappropriate analytical method leads to an unrecognized distortion of the results.

3.2 Results and discussion

3.2.1 Simulation of reaction conditions and working up shown for cyclododecane (1c), cyclododecanol (2c) and cyclododecanone (3c)

A simulation of reaction conditions and working up was necessary and exemplarily realized for the desired product cyclododecanone (**3c**) first. Gas chromatography was used as analytical method and for the absolute determination of all components (the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product cyclododecanone (**3c**)), a straight calibration line was prepared. The detailed analytic procedure is described in the following chapter 3.2.2. These experiments, shown in Table 1, were conducted considering a) the method of working up (entry 1 and 4), b) the reaction time while also taking into account the method of working up (entry 2 and 5) and c) the reaction time while also taking into account the method of working up in the presence of biomass (entry 3 and 6) (SOP 2). It is apparent from Table 1 below, that the extractive work up of the reaction mixture is only slightly defective in a concentration range of 100 mM. Since the recovery is constantly below 100% it can be argued that the analysis of the data of the preparative hydroxylation will not yield results that are incorrectly increased.

Table 1. Results of the simulation of reaction conditions and working up for cyclododecanone (**3c**)



3c
100 mM

3c

Entry	Time	Initial weight [mg]	Output weight [mg][b)]	Recovery [%]
1	5 min	18.40	17.29	94
2	24 h	18.13	17.29	95
3[a)]	24 h	18.40	--[c)]	--
4	5 min	18.54	17.04	92
5	24 h	18.15	17.28	95
6[a)]	24 h	18.44	17.32	94

[a)]20 mg denaturated CYP BM-3 19A12 are added; [b)] calculated *via* GC; [c)]lost during working up.

In summary, very high recovery rates were determined at concentrations of 100 mM cyclododecanone (**3c**). Thus, the recovery rate in absence of biomass is 95% and even when biomass is present, likewise high 94% of the applied compound **3c** are isolated. Such a high recovery rate, especially in the presence of biomass, is not something that can be taken for granted and requires an appropriate working up procedure. This finding became clear when working up early experiments (SOP 1), where a notably correlation, namely the decreasing of the recovery rate with increasing biomass applied in an experiment, was observed. Without going into detail on the concept of the biotransformation, since at this point the main focus should be on the comparison of the applied biomass exclusively, it is noticeable that the recovery of **1c** deviated distinctly althought same amounts of **1c** were applied for both experiments (Table 2).

Table 2. Results of the double oxidation of cyclododecane (**1c**)

Entry	CYP BM-3 (biomass)	Cyclododecanone (**3c**) [g/l]	Recovery of substrate **1c** [%]
1	CYP BM-3 19A12 (19.6 mg)	0	92
2	CYP BM-3 F87V (127.0 mg)	0	83

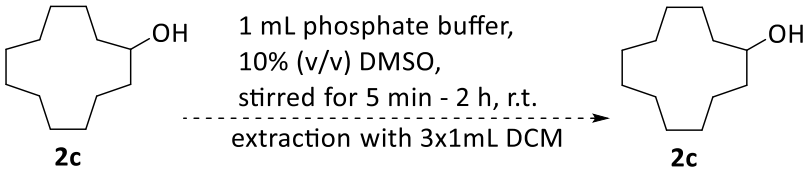
A possible explanation for this phenomenon can be found in the respective amount of biomass used for the experiment, regardless which mutant is applied. The initial step of the working up procedure is to denaturate the enzyme by adding dichloromethane. From this moment on different mutants of the cytochrome P450 monooxygenase can be considered as the same nonfunctional protein with identical molecular weight. Certainly, different lyophilized mutants have different gravimetric activities. To keep the parameter “enzyme activity” constant in preparative experiments, different amounts of biomass have to be applied. For example, for the biotransformation with the mutant CYP BM-3 19A12 only 19.6 mg protein were applied whereas with the less active mutant CYP BM-3 F87V a more than sixfold higher amount of biomass, namely 127.0 mg protein was neccessary to compensate the lower activity. This considerably increased amount of biomass manifests itself in the recovery rate of the substrate **1c**: while in the small enzyme pellet only traces of **1c** are absorbed, the more voluminous pellet is able to hold back higher amounts. A one-time extraction is therefore insufficient to isolate the compounds enriched in the biomass completely. (Figure 3).

Figure 3. Enzyme pellet from 19.6 mg biomass (CYP BM-3 19A12) (left) and enzyme pellet from 127.0 mg biomass (CYP BM-3 F87V) (right)

Taking this into account and aiming to further improve the recovery rate, the work up process of the reaction mixture was optimized. Likewise the aqueous phase, the enzyme pellet is also extracted three times. This optimized extractive work up of the reaction mixture ensures a high recovery rate, both in presence and absence of an enzyme pellet.

A simulation of reaction conditions and working up was additionally necessary for the intermediates **2** and the substrates **1**, as exemplarily established for cyclododecane (**1c**) and cyclododecanol (**2c**) (SOP 2). A further expansion of these preliminary studies to the application of lower substrate concentrations (1 and 10 mM instead of the previous used 100 mM) and considering that the main focus is later on cyclododecanol (**2c**) as product was neccessary (SOP 2). These experiments, shown in Table 3 and Table 4, were conducted considering a) the method of working up (always entry 1,4,7), b) the reaction time while also taking into account the method of working up (always entry 2,5,8) and c) the reaction time while also taking into account the method of working up in the presence of biomass (always entry 3,6,9). Gas chromatography was used as analytical method and for the absolute determination of the substrate **1c** and the intermediate **2c**, a straight calibration line was prepared. The detailed analytic procedure is described in the next chapter 3.2.2. Ideally, the recovery should be 100%, in the following the average deviation from this ideal value is listed (Table 3-4).

Table 3. Results of the simulation of reaction conditions and working up for cyclododecanol (**2c**)



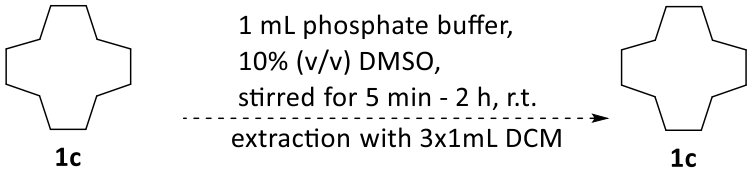
2c
1/10/100 mM

2c

Entry[a)]	**2c** [mmol]	Time	Initial weight [mg]	Output weight [mg][d)]	Deviation[e)] [%]
1	0.1	5 min	18.35	20.04	+9
2	0.1	2 h	18.72	20.16	+8
3[b)]	0.1	2 h	18.35	18.45	+1
4	0.01	5 min	2.21	2.27	+3
5	0.01	2 h	2.18	2.19	0
6[b)]	0.01	2 h	2.20	2.13	-3
7	0.001	5 min	0.184[c)]	0.151	-18
8	0.001	2 h	0.184[c)]	0.150	-19
9[b)]	0.001	2 h	0.184[c)]	0.136	-26

[a)]All measurements are conducted in a twofold determination; [b)]30 mg denaturated CYP BM-3 19A12 are added; [c)]weigh in *via* stock solution; [d)]calculated *via* GC; [e)]average deviation from a recovery of 100%.

Table 4. Results of the simulation of reaction conditions and working up for cyclododecane (**1c**)



1c
1/10/100 mM

1c

Entry[a)]	**1c** [mmol]	Time	Initial weight [mg]	Output weight [mg][d)]	Deviation[f)] [%]
1	0.1	5 min	16.89	18.26	+8
2	0.1	2 h	16.95	18.35	+8
3[b)]	0.1	2 h	16.97	18.03	+6
4	0.01	5 min	1.80	1.87	+4
5	0.01	2 h	1.75	1.79	+2
6[b)]	0.01	2 h	1.92	1.86[e)]	-3
7	0.001	5 min	0.168[c)]	0.162	-4
8	0.001	2 h	0.168[c)]	0.106	-37
9[b)]	0.001	2 h	0.168[c)]	0.039	-77

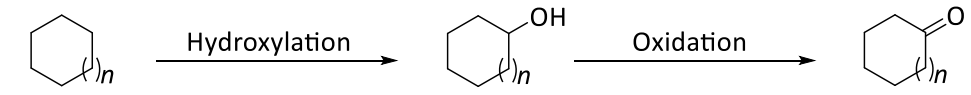
[a)]All measurements are conducted in a twofold determination; [b)]30 mg denaturated CYP BM-3 19A12 are added; [c)]weigh in *via* stock solution; [d)]calculated *via* GC; [e)]loss of one drop during working up; [f)]average deviation from a recovery of 100%.

It is apparent from Table 3 and Table 4 above, that the extractive work up of the reaction mixture is non-reliable for cyclododecane (**1c**) resp. cyclododecanol (**2c**) in the low concentration range of 1 mM. However, the recovery is constantly below 100%. In this regard, the analysis of the data of the preparative hydroxylation will not yield results that are incorrectly increased. In the concentration range of 10 to 100 mM, this method provides solid results with an average deviation from a recovery of 100%, that is at all times below 10%.

In summary, high recovery rates of 95% were determined at concentrations of 100 mM cyclododecanone (**3c**) and even in the presence of biomass, 94% of the applied compound **3c** can be isolated. Moreover, the extractive work up of a simulated reaction mixture for cyclododecane (**1c**) resp. cyclododecanol (**2c**) displays solid results with an average deviation from a recovery of 100%, that is at any time below 10%. This extensively realized simulation of reaction conditions and working up was exemplarily investigated for cyclododecane (**1c**), cyclododecanol (**2c**) and cyclododecanone (**3c**). The conclusions drawn from these experiments concerning the working up resp. the recovery rate of the respective compounds were then transferred to the further analyzed cycloalkanes **1**.

Due to the structural similarity of cyclodecane (**1b**), cyclodecanol (**2b**) and cyclodecanone (**3b**) compared with the analyzed cyclododecane (**1c**), cyclododecanol (**2c**) and cyclododecanone (**3c**), it is expected that the properties are comparable to a certain extent. To provide a better overview with regard to the volatility, the boiling points of all relevant cycloalkanes **1**, cycloalkanols **2** and cycloalkanones **3** are summarized in Table 5.

Table 5. Boiling points of all relevant cycloalkanes **1**, cycloalkanols **2** and cycloalkanones **3** at normal pressure



1 *n* = 1, 5, 7 **2** *n* = 1, 5, 7 **3** *n* = 1, 5, 7

Entry		*n* = 1: Cyclohex-	*n* = 5: Cyclodec-	*n* = 7: Cyclododec-
1	-ane **1**	81°C[36]	201°C[37]	244°C[39]
2	-anol **2**	161°C[10]	244°C[a)]	278°C[25]
3	-anone **3**	156°C[10]	197°C[b)] [38]	277°C[25]

[a)]Calculated with Advanced Chemistry Development ACD/Labs Software v11.02; [b)]The boiling point reported in literature is 106-107°C at 16 hPa[38]. Based on a boiling point depression of 15°C per halving of the pressure, the boiling point at normal pressure is calculated to be 197°C.[40]

The boiling points of the non-volatile C12-rings **1c**, **2c** and **3c** are in a range of 244-278°C[25,39] and also the C10-rings **1b**, **2b** and **3b** will not boil until temperatures of ca. 200°C and higher are reached.[37,38] Therefore it is assumed that the properties of the C12-rings **1c**, **2c** and **3c** analyzed within the simulation- and working up studies can be transferred to the C10-rings **1b**, **2b** and **3b**. The boiling points of the C6-rings **1a**, **2a** and **3a** however are considerably lowered compared to those of the C10- and C12-rings.[10,36] Especially the substrate **1a** has a low boiling point of 81°C and therefore a strong disposition to evaporate.[36] To meet this fact in particular and in order to include minor deviations in working up or recovery, an *absolute* quantification *via* gas chromatography secures that the final productivity (g/L) is not incorrectly increased.

3.2.2 Simultaneous quantification of cycloalkanes 1, cycloalkanols 2 and cycloalkanones 3 *via* gas chromatography

Quantification of cyclododecane (**1c**), cyclododecanol (**2c**), cyclododecanone (**3c**)

Gas chromatography is a well-established and extremely robust method for the absolute quantification of volatile compounds. It was already used in some early experiments and the absolute determination of all components (the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product

cyclododecanone (**3c**)) in a concentration range of 0.1 to 10 g/L is realized with a six-point calibration line for each compound (10 g/L, 5 g/L, 1 g/L, 0.5 g/L, 0.3 g/L, 0.1 g/L) (Method A for **1**-**3c**, SOP 3). Furthermore, an expansion of the above-mentioned method (Method A: 0.1-10 g/L) has been implemented to account for a considerably lower concentration range (Method B: 0.025-0.2 g/L). Moreover, the methods detection limit was defined to guarantee qualitatively and quantitatively correct results. Using this newly established methods for gas chromatography, an absolute determination of all components (the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product cyclododecanone (**3c**)) in a concentration range of 0.025 to 0.2 g/L (altogether four measured points: 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L) (Method B for **1**-**3c**, SOP 3) is possible down to a concentration of 0.005 g/L.

Definition of the methods detection limit (MDL)

The definition of the methods detection limit (MDL) is an important step to get valid results in the quantitative analysis of subsequent biotransformations. By means of the straight calibration line samples with different concentrations (0.04 g/L, 0.02 g/L, 0.01 g/L, 0.005 g/L, 0.002 g/L) are analyzed *via* gas chromatography. All measurements are conducted in a twofold determination (Table 6). In summary, concentrations down to 0.005 g/L can be measured reliably. The GC-determined percental deviation from the prepared concentrations is -13% for the substrate cyclododecane (**1c**), -2% for the intermediate cyclododecanol (**2c**) and -13% for the product cyclododecanone (**3c**). This detection limit is defined because very low concentrations (0.002 g/l, Table 6) are not detected reliably.

Table 6. GC-determined percental deviation from the prepared concentrations of cyclododecane (**1c**), cyclododecanol (**2c**) and cyclododecanone (**3c**)

Entry[a)]	Compound	Deviation[b)] at a concentration of…				
		…0.04 g/L	…0.02 g/L	…0.01 g/L	…0.005 g/L	…0.002 g/L
1	**1c**	+3%	-7%	-9%	-13%	-31%
2	**2c**	-1%	-6%	-6%	-2%	+6%
3	**3c**	-6%	-8%	-9%	-13%	-41%

[a)]All measurements are conducted in a twofold determination; [b)]average deviation from a recovery of 100%.

Quantification of cyclodecane (**1b**), cyclodecanol (**2b**), cyclodecanone (**3b**)

Gas chromatography was used to establish an extremely robust and exact analytical method for the absolute determination of all components (the substrate cyclodecane (**1b**), the intermediate cyclodecanol (**2b**) and the product cyclodecanone (**3b**) in a concentration range of 0.1 to 10 g/L (Method A) with altogether five measured points (10 g/L, 1 g/L, 0.5 g/L, 0.3 g/L, 0.1 g/L) (Method A for **1**-**3b**, SOP 3). In the context of the further expansion of this above-mentioned

method for detection (Method A: 0.1-10 g/L) to a considerably lower concentration range (Method B: 0.025 - 0.80 g/L, altogether six measured points: 0.8 g/L, 0.4 g/L, 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L) (Method B for **1**-**3b**, SOP 3), additionally the methods accuracy is investigated to ensure quantitatively correct results and to guarantee the reproducibility of the analysis *via* gas chromatography. Ideally, the recovery should be 100%, in the following the average deviation from this ideal value is listed (Table 7). It is apparent from Table 7 below that valid results are obtained even in microgram quantities.

Table 7. Results of the verification of the GC-method for **1b**, **2b** and **3b**

Entry[a)] (method A)	Compound	Deviation[c)] at a concentration of…		
		…7.0 g/L	…1.4 g/L	…0.2 g/L
1	**1b**	-1.7%	-3.6%	0.0%
2	**2b**	+2.9%	n.d.	-5.0%
3	**3b**	-1.4%	-2.7%	-8.6%
Entry[b)] (method B)	Compound	Deviation[c)] at a concentration of…		
		…0.8 g/L	…0.1 g/L	…0.025 g/L
1	**1b**	-1.4%	-2.7%	-10.0%
2	**2b**	-0.5%	+0.1%	+0.8%
3	**3b**	-1.5%	-2.1%	-5.0%

[a)]All measurements are conducted in a fourfold determination; [b)]All measurements are conducted in a twofold determination; [c)]average deviation from a recovery of 100%.

Quantification of cyclohexane (**1a**), cyclohexanol (**2a**), cyclohexanone (**3a**)

Gas chromatography was used to establish an extremely robust and exact analytical method for the absolute determination of all components (the substrate cyclohexane (**1a**), the intermediate cyclohexanol (**2a**) and the product cyclohexanone (**3a**) in a concentration range of 0.15 to 1.5 g/L (Method A) with altogether six measured points (1.5 g/L, 1.0 g/L, 0.75 g/L, 0.5 g/L, 0.3 g/L and 0.15 g/L) (Method A for **1**-**3a**, SOP 3). In the context of the further expansion of this above-mentioned method for detection (Method A: 0.15 - 1.5 g/L) to a considerably lower concentration range (Method B: 0.025 - 0.80 g/L, altogether six measured points: 0.80 g/L, 0.40 g/L, 0.20 g/L, 0.10 g/L, 0.05 g/L, 0.025 g/L) (Method B for **1**-**3a**, SOP 3), additionally the methods accuracy is investigated to ensure quantitatively correct results and to guarantee the reproducibility of the analysis *via* gas chromatography. Ideally, the recovery should be 100%, in the following the average deviation from this ideal value is listed (Table 8). It is apparent from Table 8 below that valid results are obtained even in microgram quantities.

Table 8. Results of the verification of the GC-method for **1a**, **2a** and **3a**

Entry[a)] (method A)	Compound	Deviation[c)] at a concentration of…		
		…1.3 g/L	…0.9 g/L	…0.2 g/L
1	**1a**	+7.8%	+12.2%	+13.9%
2	**2a**	-1.3%	-10.6%.	-4.5%
3	**3a**	-5.5%	-6.9%	-6.7%
Entry[b)] (method B)	Compound	Deviation[c)] at a concentration of…		
		…0.8 g/L	…0.1 g/L	…0.025 g/L
1	**1a**	+5.0%	+9.5%	+21.4%
2	**2a**	+4.5%	+1.9%	+2.1%
3	**3a**	-1.8%	-5.3%	-10.8%

[a)]All measurements are conducted in a fourfold determination; [b)]All measurements are conducted in a twofold determination; [c)]average deviation from a recovery of 100%.

Additionally, the direct analysis of the crude reaction mixture *via* headspace gas chromatography was investigated exemplarily for the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product cyclododecanone (**3c**), but not for cyclodecane (**1b**) or cyclohexane (**1a**) and their oxidation products. More importantly, this approach enables only a qualification of these compounds and is not appropriate for quantification in the concentration range required for this project.

4 Hydroxylation with cytochrome P450 monooxygenases from *B. megaterium*

4.1 Introduction, state of the art, and aim of this section

In our diamagnetic world, the naturally occurring molecular oxygen is - under standard conditions - fairly unreactive and therefore the oxidation of the biotic and abiotic environment is extremely slow. As a consequence, the atmospheric oxygen needs to be activated to interact with compounds of the environment. Compared with the paramagnetic triplet oxygen, activated singlet oxygen is diamagnetic and therefore a very effective oxidant (Figure 4).[41,42]

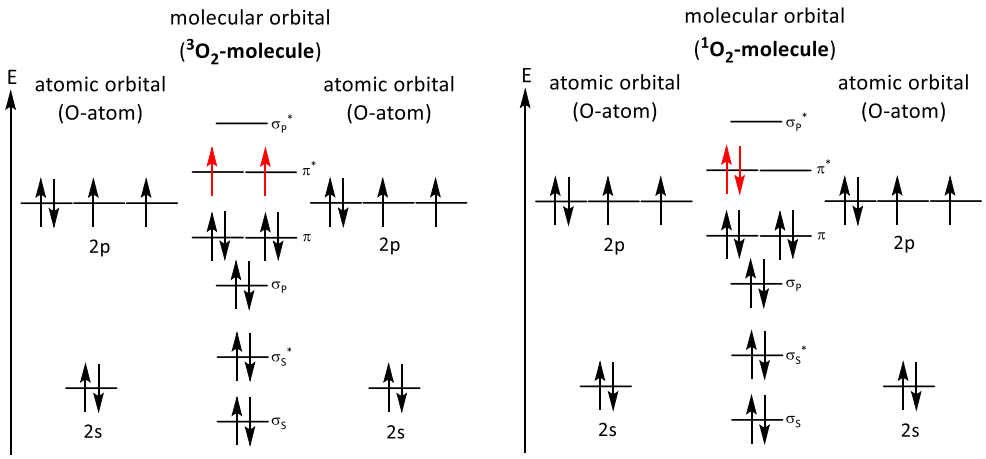


Figure 4. Molecular orbital diagram of triplet oxygen (ground state, left) and singlet oxygen (excited state, right)

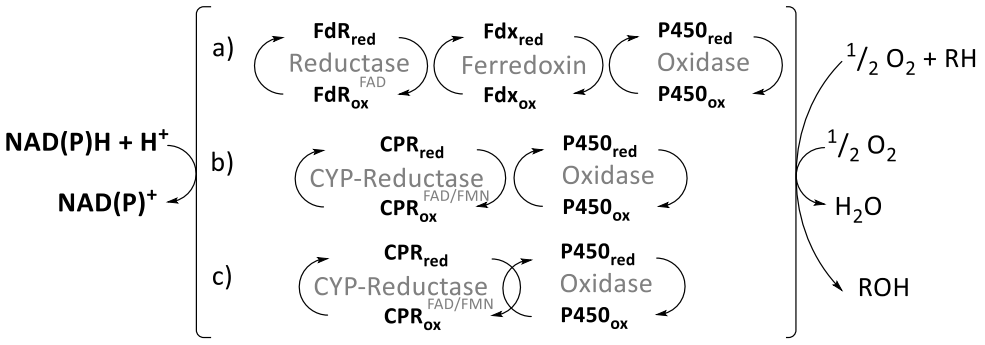
In the presence of oxygen, that makes up about 20% of the Earth`s atmosphere, the most energetically stable form of carbon is carbon dioxide (CO_2) and the thermodynamically favoured breakdown of organic materials resp. hydrocarbons principally yields carbon dioxide and water. This exotherm reaction, the oxidative combustion with oxygen, is releasing the energy which was previously fixed during photosynthesis.[43] Reasonably, the instant exhaustive breakdown of nearly all organic materials is limited for a specific activation energy must be overcome to induce a reaction: the transfer of electrons between states of different spin multiplicities is forbidden and since most molecules have spin-paired electrons and therefore exist in a singlet ground state, this spin barrier has to be overcome before a reaction with oxygen in the triplet ground state will take place. This was a brilliant achievement of evolution and a stroke of luck for the diversity of life as we know it.[43]

By means of biocatalysts these energy barriers are decreased and the activation energy is easy to overcome at moderate temperatures. In most cases these enzymes are metalloproteins and interaction of oxygen with the corresponding transition metal cation leads to activation of the oxygen. One example for oxygen-binding metalloproteins are cytochrome P450 monooxygenases (CYPs) and their contribution in oxidation reactions will be the main topic of this chapter.[43]

4.1.1 Cytochrome P450 monooxygenases (CYPs): Electron transfer, catalytic mechanism and coupling efficiency

In the year 1958 GARFINKEL and KLINGENBERG investigated independently of each other liver microsomes and found a pigment with a characteristic absorption maximum at 450 nm that was attributed to the binding of a carbon monoxide (CO) ligand to the heme. Later the term cytochrome P450 was used to describe these cellular pigments whereby "P" stands for pigment and "450" is derived from the UV absorption peak of the carbon monoxide complex. Thereby GARFINKEL and KLINGENBERG emerged as discoverers of the P450 superfamily which is nowadays one of the best known enzyme families.[44,45]

In the heme center of CYPs the activation of molecular oxygen proceeds and this leads to the insertion of a single atom of oxygen into a substrate while the other oxygen atom is reduced to water. This mechanism of monooxygenation depends on a coupled and stepwise supply of electrons which are provided from NAD(P)H and supplied by a redox partner.[46] CYPs are divided into classes according to the different types of the electron transfer system they use. Although there is a number of additional classes identified in the recent past and reviewed by HANNEMANN *et al.*[47] the focus should be on the three best-known systems (Scheme 8):



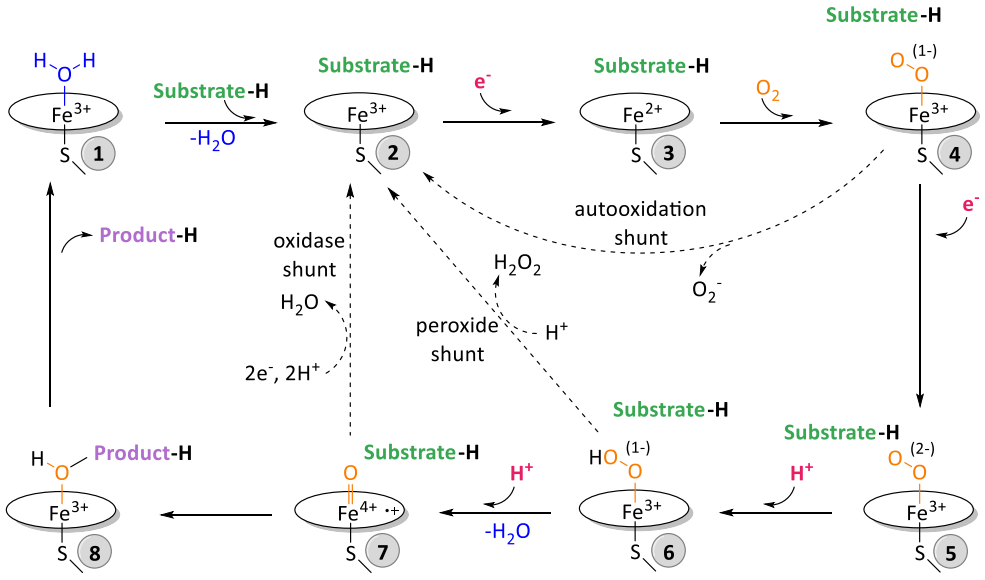
Scheme 8. Different types of electron transfer systems (taken from URLACHER *et al.*[48] and modified according to HANNEMANN *et al.*[47])

Class I-CYPs include most bacterial and (eukaryotic) mitochondrial systems and use three separate proteins: a FAD-containing ferredoxin reductase (FdR) transferring the reduction equivalents (electrons) from NAD(P)H to the second iron sulfur protein, a ferredoxin (Fdx), mostly of the [2Fe–2S] type. This ferredoxin in turn reduces the cytochrome P450 itself. The first bacterial P450 system

discovered, the camphor hydroxylase CYP CAM, is organized in that way: electrons are transferred from NADH *via* a putidaredoxin reductase (FdR) and an iron-sulfur putidaredoxin (Fdx) to CYP CAM (Scheme 8, path a).[47]

Class II-CYPs are mostly found in eukaryotes and show a high diversity in the catalyzed reactions. In mammals these enzymes play a key role in the oxidative metabolism of endo- and exogenous compounds.[47] The simplest form is located in the endoplasmatic reticulum of eukaryotes and is composed of two integral membrane proteins: a NADPH-cytochrome P450 reductase (CPR) within which the prosthetic group FAD accepts the electrons from NADPH and the FMN subunit transfers the redox equivalents to the cytochrome P450 itself (Scheme 8, path b).[47] In this way CPR is transferring the electrons from the two-electron donor NADPH to FAD and thence *via* FMN to the CYP and its one-electron acceptor containing heme. This ensures that electrons are delivered in two separated steps at the respective moments of the catalytic cycle. The third electron transfer pathway described in path c) of Scheme 8 illustrates the electron transfer of catalytically self-sufficient monooxygenases. These are CYPs which are fused to their reductase partner (CPR) in a single polypeptide chain. A very extensive and detailed studied member of this class is the cytosolic fatty acid hydroxylase flavocytochrome CYP102A1 (P450 or CYP BM-3, EC 1.14.14.1) of the soil bacterium *Bacillus megaterium*.[47]

The catalytic cycle of cytochrome P450 monooxygenases is most often described in literature for the very well studied substrate camphor (**26**). It is summarized in Scheme 9 and described below, adapted and modified from different publications.[49-53]



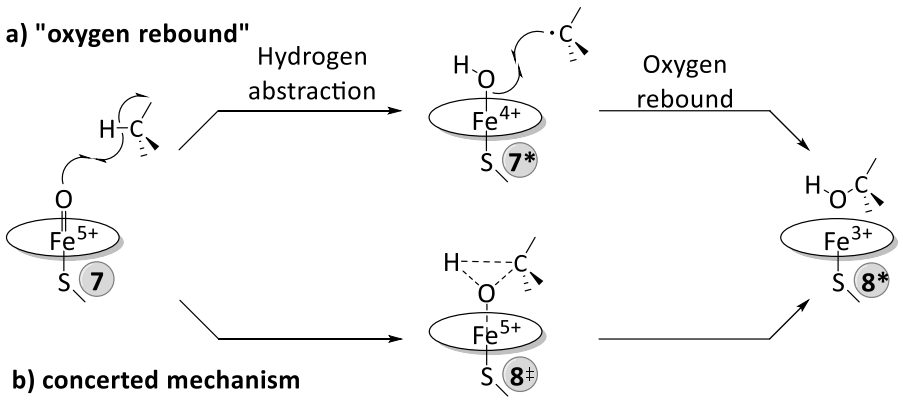
Scheme 9. Catalytic cycle of cytochrome P450 monooxygenases[49,50]

The form ① of the protein shows the substrate free resting state of the low spin (ls) Fe^{III} (d^5, S = ½) with sixfold coordinated metal (porphyrine, cysteinate, water).

The substrate binding to the active site of the enzyme displaces the labile axial water coordinated as the 6th ligand of the heme iron and the low spin state is changed to the high spin state (S = $^5/_2$). The high spin state is favored in the presence of substrate.[49-52]

The substrat binding can be described as an entropy-driven process: few water molecules are released from the active site resulting in a gain in entropy for the system.[51] It is known that in octahedral complexes the effective radius of the low spin Fe^{III} is smaller than in the high spin configurated Fe^{III} due to the lower repulsion of the ligands and therefore closer to the porphyrin plane.[42] In line with this, the transition of the six-coordinate low spin (ls) Fe^{III} ① to the five-coordinate high spin (hs) Fe^{III} ② effects a displacement of the iron from the plane of the porphyrin ring from 0.30 Å to 0.44 Å, known as out-of-plane structure.[51] The iron is therefore located slightly above the heme so that the coordination sphere of the metal is modified. This modification induces a change in the redox potential of the iron center by increasing it from -300 to -170 mV. With this increased reduction potential the heme is a better electron sink and the subsequent single electron transfer resulting in the corresponding Fe^{II} ③ is the initial step in the process of oxygen activation.[51] The Fe^{II}-complex binds then to molecular dioxygen and an initial dioxygen-adduct, the superoxo-complex ④ $Fe^{3+}OO^{-}$ is formed. This intermediate ④ is the last relatively stable compound in this cycle. The subsequent transfer of the second electron is relatively slow and therefore the rate determining step in many CYPs.[51] The reduction of ④ as a consequence thereof gives the ferric peroxo-anion $Fe^{3+}OO^{2-}$ ⑤ which is then quickly protonated to give the hydroperoxo-ferric intermediate ⑥. A second protonation at the distal oxygen-atom induces a subsequent elimination of water *via* the heterolytic cleavage of the O-O-bond to give the high valent iron species ⑦ which is thought to be the active entity in most CYP-catalyzed oxidations. This former "mysteric activated oxygen species"[49] was characterized by RITTLE and GREEN in 2010.[53] This high valent iron-(IV)-oxo species ⑦ is sometimes simplified to an iron-(V)-oxo species for the iron charge is +4 and additionally the porphyrine ring is a positively charged radical cation.[51]

The insertion of oxygen to the substrate - the alkane hydroxylation - was described by GROVES *et al.* in a so-called "rebound mechanism" which is shown in Scheme 10, path a.[54-56] After elimination of the monooxygenated substrate, the resting form is reconstructed by rebinding the sixth aqua ligand.[50] This consensus oxygen rebound mechanism is postulating that one hydrogen atom from the substrate is abstracted by ⑦ to form the radical substrate intermediate. Then the hydroxyl group is transferred to ⑦· and the catalytic cycle is completed by releasing the alcohol and restoring the resting state.[54-56] Although this mechanism was accepted for many years it is discussed controversially since NEWCOMB *et al.* took advantage of radical clocks to point out that a cationic reaction pathway is easily conceivable (Scheme 10, path b).[57-59]



Scheme 10. Insertion of one oxygen atom into the C-H-bond of the substrate in one (concerted)[57-59] or two (oxygen rebound)[54-56] steps (adapted from MEUNIER *et al*.[51])

Apart from the insertion of one oxygen atom into the C-H-bond of the substrate (*v.s.,* main pathway) there are three additional pathways described in literature and depicted in Scheme 9. All shuntways lead to the formation of the five-coordinate high spin (hs) Fe^{III} ②: the "peroxide shunt" describes the dissociation of the hydrogen peroxide ligand from the hydroperoxo-ferric intermediate ⑥ and is the most detailed investigated side pathway. In case of the high valent iron species ⑦ is using two additional electrons in order to eliminate water, this pathway is called "oxidation shunt". In a so-called "autooxidation shunt" superoxide is dissociated from the superoxo-complex ④ to form the five-coordinate high spin (hs) Fe^{III} ② by return.[50] All three additional pathways (shunts) have in common that the consumption of the cofactor is decoupled from the substrate oxidation as such, since the electrons are used elsewhere. One consequence of this uncoupling is that the precious cofactor is misspent. Another effect is that the formation of reactive species like hydrogen peroxide will lead to an (irreversible) inactivation of the enzyme.[60,61]

In numerous natural P450 monooxygenases like the camphor hydroxylase from *Pseudomonas putida* (CYP CAM) or the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3), the oxidation of the physiological substrate is highly coupled to the consumption of cofactor and excellent coupling efficiencies are obtained.[62] The coupling efficiency for the hydroxylation of camphor (**26**) by CYP CAM is stated in literature to be 95-100% under optimal conditions[62,63] while the hydroxylation of linear and branched C12−C20 fatty acids at subterminal (ω-1, ω-2, ω-3) positions - catalyzed by CYP BM-3 - is as well excellent coupled to the consumption of cofactor (88-98%).[62] However, this highly efficient but sensitive coupling is easily disrupted when amino acids are substituted or the enzyme is faced with non-natural substrates. This phenomenon is the consequence of a reduced interaction between substrate and enzyme since an enzymes binding pocket is optimized for the respective physiological substrate.[62] Almost complete coupling efficiencies of up to 100% are often decreased to less than 10%, when cytochrome P450 monooxygenases are applied for the biotransformation of non-physiological substrates.[64,65]

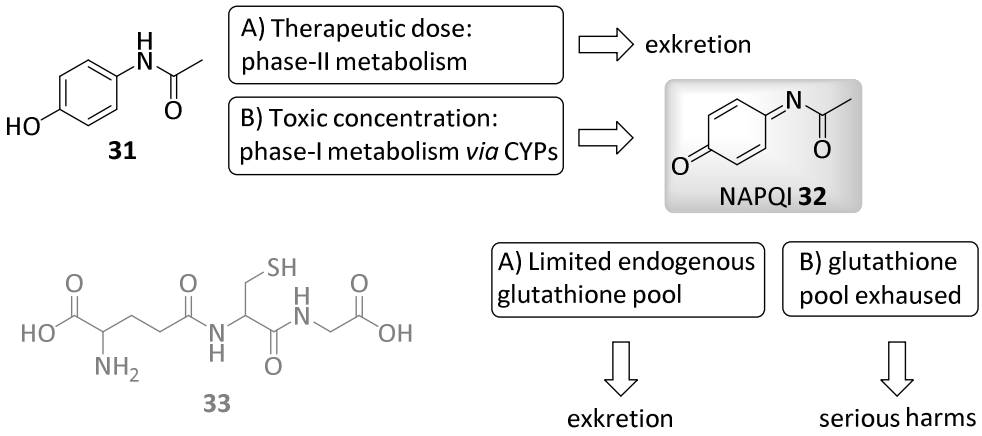
This problem can be addressed by protein engineering. For example, BELL *et al.* showed that the wildtype camphor hydroxylase from *Pseudomonas putida* (CYP CAM) converts butane (**27**) with a coupling efficiency of 4% while a quadruple mutant obtained 95%. The same enzyme oxidizes propane (**28**) with a coupling efficiency of 0.9% while a double mutant achieves 39%.[66] The wildtype cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) achieved only a coupling efficiency of 20% for the oxidation of *n*-heptane (**29**) while the double mutant R255P-P329H increases the coupling efficiency up to 45%.[32] Similar results were found for cyclic alkanes **1** by MAURER in 2005 when identifying mutants able to hydroxylate cyclohexane (**1a**). The CYP BM-3 double mutant R47L, Y51F revealed coupling efficiencies of 25% while only 6% were found for the wildtype CYP BM-3.[67] Another solution to the problem investigated by WATANABE *et al*. is the application of perfluorocarboxylic acids as decoy molecules which force long-alkyl-chain fatty acid hydroxylases to generate the active species and to catalyze oxidation of various non-natural substrates.[68,69] Using the decoy molecule perfluorooctanoic acid (**30**), a coupling efficiency of 45% was found for the hydroxylation of cyclohexane (**1a**).[68-69]

4.1.2 Cytochrome P450 monooxygenases (CYPs): Key role in the drug metabolism and structure of CYP BM-3

Cytochrome P450 monooxygenases play a key role in the drug metabolism and for this reason they are one of the best studied enzyme families. Exogenous unpolar compounds e.g. drugs as well as endogenous structures like hormones are made excretable by means of biotransformation. These biotransformations are mainly located in the liver and are subdivided into phase-I and phase-II-reactions: within a phase-I-reaction the structure of the drug is modified by different functionalization reactions (oxidation, reduction or hydrolysis). For most drugs, oxidation *via* cytochrome P450 monooxygenases plays the decisive role. The subsequent phase-II-reactions are conjugation reactions aiming to enhance the water solubility and therefore the renal elimination of the drug.[70,5] An everyday example of why drug metabolism studies with cytochrome P450 monooxygenases are essential and indispensable is revealed by the analgesic and antipyretic over-the-counter (OTC) drug paracetamol (**31**):[71-73]

Paracetamol (**31**) is almost exclusively metabolized in the liver. As the large part is converted into easily excretable compounds, a smaller part undergoes a cytochrome P450 monooxygenase catalyzed breakdown yielding *N*-acetyl-*p*-benzoquinone imine (NAPQI) (**32**). NAPQI **32** is highly reactive, able to link the hepatic proteins and therefore severe cellular damage is caused. This will lead to death within few days *via* complete liver failure, if untreated. Usually NAPQI **32** is rendered harmless by the tripeptide glutathione (**33**) and excreted renally. But excessive amounts of paracetamol (**31**) will lead to a rapid exhaustion of endogenous glutathione (**33**) and the reactive metabolite NAPQI **32** will accumulate in the liver to cause the harms outlined above.

The antidote of choice to treat paracetamol poisoning is *N*-acetylcystein (**34**). **34** replenishes the hepatic reservoir of glutathione (**33**) that is depleted in case of a paracetamol overdose by providing cysteine (**35**) which is essential for the formation of glutathione (**33**) (Scheme 11).[71-73]



Scheme 11. Pharmacokinetics of paracetamol (**31**), summarized according to various authors[71-73]

More than 90% of all oxidative drug reactions in humans are catalyzed by a quite small number of cytochrome P450 enzymes, like CYP1A2, CYP2C9, CYP2D6, CYP2E1 and CYP3A4 whereby the enzyme CYP3A4 is involved in the metabolism of more than 50% of all drugs.[74] But P450 enzymes can be found in all domains of life: besides the already mentioned human resp. mammalian CYPs these enzymes are as well found in e.g. bacteria, fungi, plants and insects.[75]

However, not only for metabolism studies in drug discovery processes cytochrome P450 monooxygenases are of enormous interest. The ability of these enzymes to oxyfunctionalize C-H-bonds under mild conditions and the numerous applications of these oxidation products arouse the interest of the scientific community and industry. Actually, the non-activated C-H-bond is quite strong (ca. 400 kJ mol^{-1}) and consequently graded as inert at mild temperatures and towards mild oxidants.[41,42,76]

Some examples of successful syntheses of fine chemicals using these enzymes are given below: due to the large variety of this field of research in oxyfunctionalization it is not possible to report on all P450 families and this work is in large parts restricted to the CYP102A1 monooxygenase used for the preparative experiments of this work. The cytochrome P450 monooxygenase from the soil bacterium *Bacillus megaterium* (CYP BM-3/CYP102A1/EC 1.14.14.1) resp. its activity as fatty acid hydroxylase was described for the first time in 1974 by MIURA and FULCO.[77-79] The common term “BM-3” is attributed to the fact that CYP BM-3 was the third monooxygenase isolated and characterized from this organism at all.[52,80] In the 1980s CYP BM-3 was found to show an unusual attribute:

This completely soluble enzyme with a molecular weight of 119 kDa is a fusion of the heme containing hydroxylase domain (55 kDa) and the reductase domain (65 kDa, containing FAD and FMN in equimolar ratio) connected *via* a short protein linker into one single polypeptide chain (Figure 5).[52,80,81]

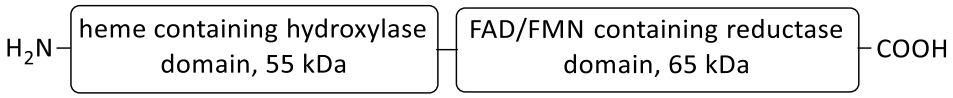


Figure 5. Schematic structure of CYP BM-3[52,80,81]

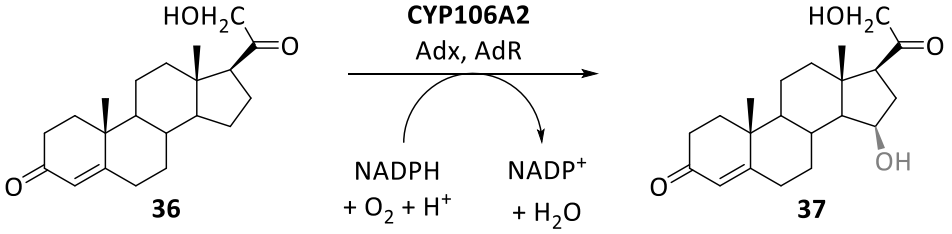
CYP BM-3 comprises an unequivocal *N*-terminal sequence and this is considered as a confirmatory evidence that not a very stable complex of several proteins but rather a single polypeptide chain exists.[80] In 1987 WEN and FULCO expressed the complete CYP BM-3 gene in *E. coli* and characterized it.[82] Sequence homology compared with eukaryotic monooxygenases makes it an excellent model system for this class of enzyme with the advantage that this soluble enzyme can be produced in recombinant form. While other CYPs require additional redox proteins, CYP BM-3 is catalytically self-sufficient and requires merely the reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen to catalyze the hydroxylation of long chain fatty acids.[82] Until today, the exact biological function of CYP BM-3 is unknown[83,84] but it hydroxylates primarily linear and branched medium- and long-chain fatty acids at the subterminal ω-1, ω-2 and ω-3 positions.[77-88]

Only the option of expression and purification paved the way for crystal structures of the heme containing hydrolase domain in substrate-free and substrate-bound form and therefore the way for rational protein design with this enzyme. On this basis, several amino acid hotspots with significant influence on binding resp. transformation of different substrates were identified. For example, a positively charged arginine residue (Arg47) is located at the entrance of the long, hydrophobic substrate access channel. The replacement of Arg47 by a negatively charged glutamic acid residue (Glu47) led to a catalytically inactive mutant. This result is probably referable to an interaction between Arg47 and the carboxyl group of the fatty acid neccessary for the conversion.[89] By mutation of certain amino acids the substrate spectrum of CYP BM-3 can be expanded enormously: phenylalanine at position 87 (Phe87) for example is located above the heme and therefore controlling the access of a substrate to the active site of the enzyme. This access can be facilitated by the mutation of Phe87 to the smaller amino acid valine (F87V). Another hotspot was found in position 328 which is predicted to interact with all substrates during the oxidation process.[48,89-92]

4.1.3 Cytochrome P450 monooxygenases (CYPs): Hydroxylation of different substrate classes

Besides its natural substrates, the fatty acids, bacterial CYPs are used to hydroxylate different substrate classes: the biotransformation of steroids, for

example, is well studied for human CYPs[93] and moreover industrially applied for fungal CYPs.[94-97] Due to biotechnological advantages of bacterial CYPs, these enzymes arouse the interest of academia and industry.[98-103] One example of a bacterial P450 monooxygenase which is able to hydroxylate steroids is CYP106A2 from *B. megaterium* ATCC 13368[98-101], that moreover can be coexpressed together with bovine AdR and Adx in *E. coli* to improve activity and stability (Scheme 12).[102]



Scheme 12. 15β-hydroxylation of the steroid 11-deoxycorticosterone (**36**) to 15β-hydroxy-11-deoxycorticosterone (**37**) by CYP106A2[102]

Another hydroxylating bacterium, *Bacillus stearothermophilus*, has been found to produce 20α-, 6β- and 6α-hydroxyprogesterone from progesterone (**38**).[103]

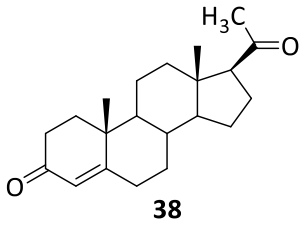


Figure 6. Structure of progesterone (**38**)

Furthermore hydrocarbons as terpenes and their oxyfunctionalized derivatives, the terpenoids[104] can be hydroxylated and make this enzymatic access to the higher-valued, oxidized compounds attractive. The scope of applications for terpenoids shows a very high diversity for it is used as flavour and fragrance but also as a builing block for pharmaceuticals. Using quite affordable bulk terpenes as limonene (**39**) or pinene (**40**) as precursors (Figure 7), most of the required terpenoids are synthesized chemically.[105] The isolation of these compounds resp. the essential oils from natural resources is unattractive from an economic point of view: the yield referred to the starting plant material is at best only few percent.[106]

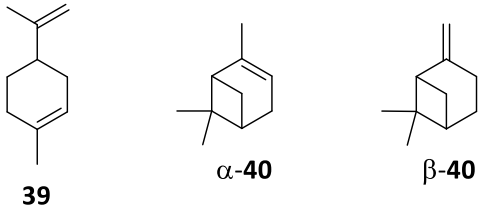


Figure 7. The terpenes limonene (**39**) or pinene (**40**) are used as precursors for the chemical synthesis of the required terpenoids[105]

The oxidation of alkanes is of particular importance for this work. And even though the CYP BM-3 catalyzed oxidation of medium to long chained linear and branched alkanes is well described in literature[77-88], this chapter will restrict its focus to the biotransformation of cyclic alkanes **1**. Only few enzymes are known that catalyze the biotransformation of cyclohexane (**1a**) and the breakdown of longer chained cyclic alkanes is yet scarcely analyzed.[107,108] While in the middle of the 1990s it was stated that CYP BM-3 is showing no activity referring to cyclohexane (**1a**)[109] more recent findings have led to a revision of this opinion:

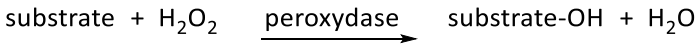
ADAM *et al.* pioneered in 2000, that the cyclic alkanes cyclohexane (**1a**) and cyclooctane (**1d**) can be hydroxylated by living cells of *Bacillus megaterium* giving the corresponding cycloalcohols cyclohexanol (**2a**) and cyclooctanol (**2d**). In this study it was also found that overoxidation to the corresponding ketones **3a** and **3d** occurs. The influence of the ring size on the oxidative activity was conspicuous, since 65% cyclooctanone (**3d**) were yielded while only 21% cyclohexanone (**3a**) were formed.[110]

In 2002 the ARNOLD group found a CYP BM-3 mutant (139-3) hydroxylating cyclohexane (**1a**) with a maximum turnover rate (mol substrate/min/mol enzyme) of 3910 for the mutant CYP BM-3 139-3, which was excellent compared to the wild type rate of 151.[111] Even though cyclohexanol (**2a**) was reported to be the sole product of the hydroxylation of cyclohexane (**1a**) the enzyme activity towards the oxidation product cyclohexanol (**2a**) was analyzed with regard to multiple oxidations when oxygen is not limiting. It was found to be 7% relative to cyclohexane (**1a**).[111] In a subsequent work by MAURER et *al.* in 2005 it was as well reported that cyclohexanol (**2a**) was the single product of all measured mutants in a biphasic reaction system, consisting of cyclohexane (**1a** as substrate and solvent) and buffer. In this study a set of CYP 102A1 mutants were screened for activity towards cyclohexane (**1a**) and the highest TTN of 12850 was detected for the CYP102A1 mutant R47L Y51F [67] It was as well in the year 2005, when KUBOTA *et al.* published the gene P450balk from the *n*-alkane-degrading bacterium *Alcanivorax borkumensis* SK2. This gene was fused to the reductase domain of the self-sufficient P450 monooxygenase (P450RhF) at the C-terminus and expressed in *Escherichia coli* with the aid of the vector pRED. Living cells of *E. coli* carrying P450balk produced 453 µg/mL cyclohexanol (**2a**) from cyclohexane (**1a**).[112] In 2011, DRONE and co-workers reported for the first time an efficiently coupled alkane ω-hydroxylase: CYP153A13a from *Alcanivorax borkumensis* SK2 artificially fused to RhFred and to a histidine affinity tag. In this way the P450 unit is equipped with a catalytic efficiency comparable to the construction of CYP BM-3. This self-sufficient and soluble enzyme A13red was cloned, expressed in *E. coli*, purified and characterized and then applied among others for the oxidation of cyclohexane (**1a**) with a TTN of 20.[113] In the URLACHER group notable oxidation rates (nmol product/nmol P450/min) were obtained with selected CYP BM-3 mutants determined *via* GC/MS after 15 min:[108]

The double mutant F87A A328V was shown to accept cyclooctane (**1d**) (oxidation rate: 87; conversion: 80%) and the mutant F87V A328F converted cyclooctane (**1d**) as well (oxidation rate: 230; conversion: 75%) as the single mutant A328V (oxidation rate: 200; conversion: 87%). Furthermore, for the first time also longer chained cyclic alkanes **1** were accepted by these mutants: cyclodecane (**1b**) was oxydized by applying the mutant A328V (oxidation rate: 106; conversion: 53%) and even cyclododecane (**1c**) was converted by the mutant F87A A328V (oxidation rate: 18; conversion: 46%) a reaction that is not catalyzed by the wild type CYP BM-3 at all.[48,108]

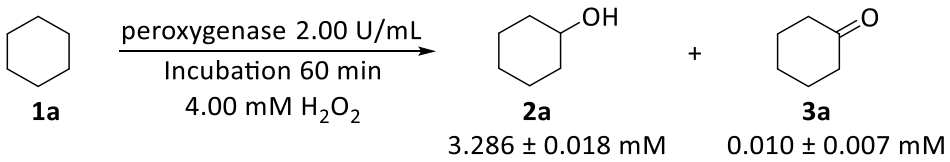
4.1.4 Alternative biocatalysts for the oxyfunctionalization of (cyclic) alkanes

Another group of biocatalyst that is able to oxyfunctionalize alkanes are heme peroxydases. One oxygen from H_2O_2 is used for the oxidation of the substrate and water is formed during the reaction (Scheme 13). Most of the peroxydases perform a one-electron-transfer instead of a two-electron-transfer and the active site is sterically hindered.[104] For that reason only few peroxydases are relevant for the oxyfunctionalization of the inert C-H-bond of alkanes but should nevertheless not be ignored in a chapter focussing on the biocatalytic oxidation of (cyclic) alkanes:[104]



Scheme 13. General reaction scheme of heme peroxydases[104]

For example, an enzyme reviewed by BORDEAUX *et al.* in 2012 as „the closest to ideal biocatalyst for (sub)terminal hydroxylation of short- and medium-chain alkanes under mild conditions to date“[114] was published one year before: [115] In 2011, PETER *et al.* reported an extracellular fungal peroxygenase secreted by *Agrocybe aegerita* (*Aae*UPO) that catalyzes the H_2O_2-dependent monooxygenation of short chained cyclic alkanes **1** (C5-C8) into the corresponding hydroxylated products with a total turnover number of 4501 (Scheme 14).[115]

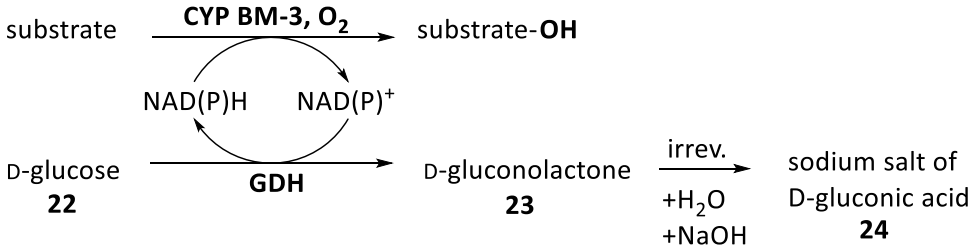


Scheme 14. Hydroxylation catalyzed by the peroxygenase from *A. aegerita*[115]

On the one hand this result is markedly below the results found by MAURER *et al.* in 2005 (In this study a set of CYP 102A1 mutants was screened for activity towards cyclohexane (**1a**) and the highest TTN of 12850 was detected for the CYP102A1 mutant R47L Y51F[67]). On the other hand the enzyme is incapable of hydroxylating the longer chained cyclodecane (**1b**) - a reaction catalyzed easily by different mutants of the CYP BM-3.[108]

4.1.5 Application of cofactors regeneration systems

With regard to industrial applications it is important to mention, that monooxygenases indeed depend on expensive cofactors like NAD(P)H as electron donors which are consumed during the reaction. To avoid the uneconomic and stoichiometric application of these cofactors, regeneration systems are employed to enable an enzymatic transformation with catalytic amounts of the cofactor. The application of a formate dehydrogenase (FDH) represents one of the best and most widely used enzyme coupled regeneration systems. Besides this another attractive and well known system is the regeneration of the cofactor *via* a glucose dehydrogenase coupled regeneration system (Scheme 15).[28]



Scheme 15. Cofactor recycling by the coupled enzyme method with glucose dehydrogenase (GDH)[28]

D-glucose (**22**) is oxidized by the GDH and D-gluconolactone (**23**) is formed which is spontaneously hydrolyzed whereupon gluconic acid (**24**) is formed. A subsequent neutralization with e.g. sodium hydroxyde forms the corresponding sodium salt of the D-gluconic acid (**24**) and the equilibrium is shifted irreversibly towards the product. As a consequence the back reaction can not take place.[28]

4.1.6 Spectrophotometric enzyme activity assays with substrates of interest

Prior to a preparative biotransformation it is recommendable to determine the enzyme activities of the available enzymes with regard to the substrates of interest. Based on an established procedure the enzyme activities are determined *via* an UV/VIS-spectrophotometric assay for NAD(P)H is used as cofactor.[116] Herein the consumption of the cofactor NAD(P)H through oxidation to $NAD(P)^+$ during the course of the reaction is recorded. The time dependent measurements are performed at a wavelength of 340 nm allowing to detect NAD(P)H only. The decline in absorption in the presence of the tested enzyme and the substrate of interest is plotted as a function of time. By means of the initial slope of the absorption curve and the following formula the volumetric enzyme activity can be determined.

$$\frac{U}{mL} = \frac{\Delta E_{340nm}\, V_t\, f}{\varepsilon\, V_s\, t\, d}$$

With U/mL = volumetric enzyme activity; $\Delta E_{340nm}/t$ = initial slope of the absorption curve, V_t = total volume [mL]; f = dilution factor; ε = molar extinction coefficient for NAD(P)H [6.3 mL*µmol^{-1}*cm^{-1}]; V_S = sample volume [mL]; d = path length of the cuvette [cm]

This method of measurement is also useful to investigate the kinetics according to MICHAELIS-MENTEN of a biotransformation. By means of the MICHAELIS-MENTEN-equation it is specified, to what extent the reaction rate depends on the substrate concentration. Therefore the initial slope v_0 of the absorption curve is plotted against the various substrate concentrations [S] in mM. In Figure 8, an idealized MICHAELIS-MENTEN diagram is shown that can be subdivided into three areas.[117]

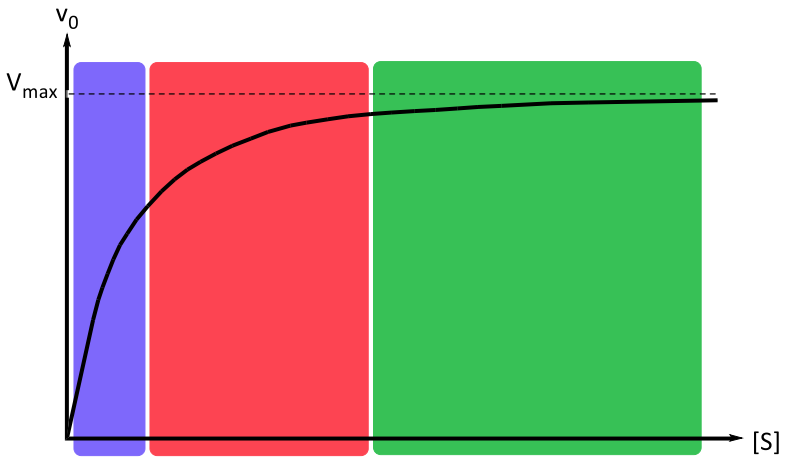


Figure 8. MICHAELIS-MENTEN diagram subdivided into three areas

■ low substrate concentration: Every substrate molecule can easily bind to an enzyme and the reaction rate increases (quasi) linearly by increasing the substrate concentration. The reaction rate depends on the substrate concentration.
■ medium substrate concentration: The substrate molecules start to compete for free enzyme.
■ high substrate concentration: Addition of more substrate has no effect on the reaction rate since the enzyme is saturated with substrate. The reaction rate depends on the enzyme concentration.

The information derived from the kinetics according to MICHAELIS-MENTEN is of particular importance if substrates are poorly soluble in the aqueous reaction medium. If, for example, substrate A is highly soluble, the maximum reaction rate is approximately reached since the enzyme is saturated with substrate. In contrast, a poor water soluble substrate B provides not enough substrate molecules in solution to saturate the available enzyme with substrate. The approximate maximum reaction rate remains inaccessible (Figure 9).[118]

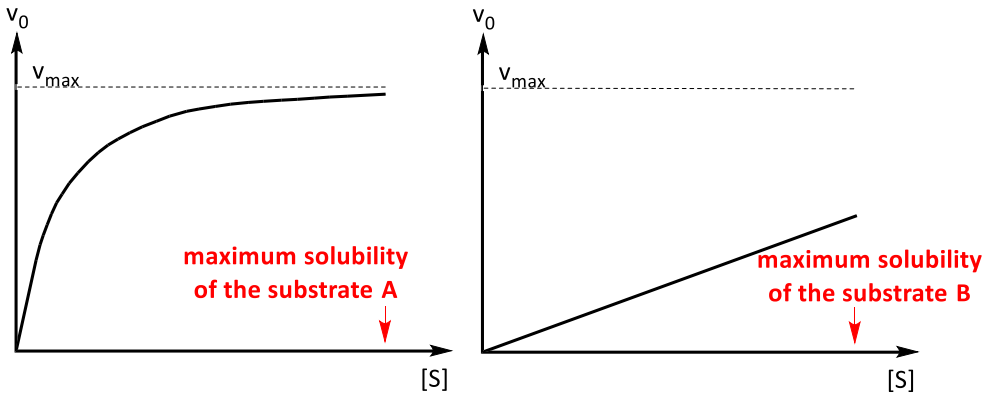
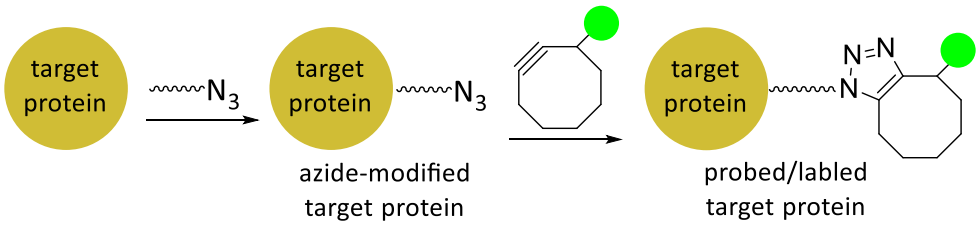


Figure 9. Exemplary MICHAELIS-MENTEN diagram of a highly soluble substrate A (left) and a poorly soluble substrate B (right)

UV/VIS-spectroscopy is a helpful tool to determine the enzyme activity for a certain substrate. Especially when substrates are applied that tend to be poorly water soluble, this analysis method can be used to analyze if the solubility of a certain substrate is high enough to set up an efficient biotransformation.

A preliminary selection of substrates - for various reasons attractive to be hydroxylated by cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) - is investigated *via* UV/VIS-spectroscopy to find suitable substrate-enzyme combinations for subsequent preparative experiments. This preliminary selection is explained in the following. From the category monounsaturated cyclic hydrocarbons, cyclooctyne (**17**) and cyclododecene (**18**) are choosen as possible substrates. Cyclooctyne (**17**) is the smallest cyclic alkyne that is stable at room temperature. Since the triple bond shows a severe deformation from the linear geometry, cyclooctynes are highly reactive and therefore established as tools in bioconjugations. This potential was found by BERTOZZI and coworkers who used functionalized cycloalkynes in the strain-promoted alkyne azide cycloaddition (SPAAC, the so-called "copper-free click chemistry").[23,24] Various cyclooctyne reagents have been designed that react selectively with azide-modified biomolecules, consequently probing biomolecules in living systems.[23,24] The general principle is shown in Scheme 16.

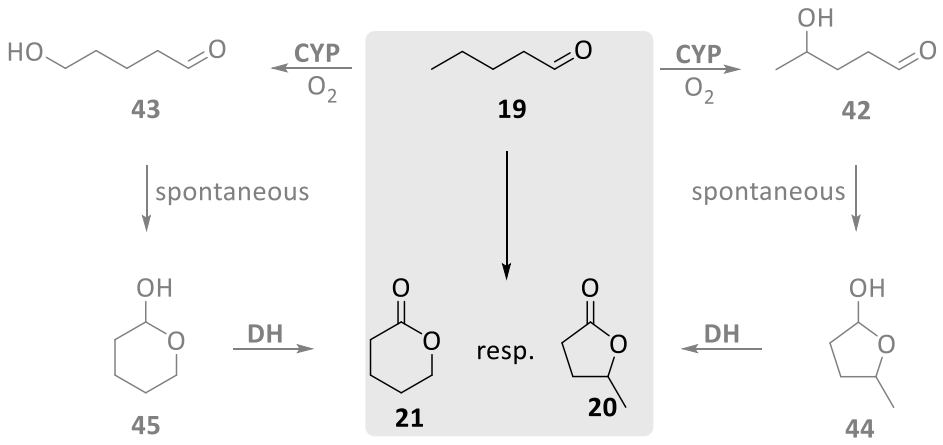


Scheme 16. Reaction of a target protein ● (azide modified) with a cyclooctyne derivative (linked to a reporter molecule ●) to lable the target protein

The oxyfunctionalization of cyclooctyne (**17**) is therefore preferable. Since the biocatalytic oxidation of short chained linear alkynes (C6 and C8) has already been reported in literature with productive outcomes,[119] it can be supposed that cyclic

alkynes are as well accepted as substrates. Cyclododecene (**18**), the second chosen monounsaturated cyclic hydrocarbon is a versatile chemical intermediate for organic synthesis in general and for that reason it is aligned in the array of products at EVONIK.[120] Compounds with a wood-like scent are synthesized from **18**, making this intermediate interesting for the fragrance industry. Quite often the desired target molecules are obtained by the oxidation of **18** making a biocatalytic oxidation to an attractive alternative synthesis route.[120]

Furthermore, different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) are analyzed with regard to their capability to convert pentanal (**19**). The aldehyde **19** can be synthesized from easily available 1-butene (**41**) *via* hydroformylation and therefore is an easily accessible starting material for the synthesis of valuable specialty chemicals.[121] In this work, the hydroxylation of pentanal (**19**) is supposed to be realized by a monooxygenase-catalyzed functionalization using molecular oxygen as oxidant to give the corresponding hydroxypentanals **42** resp. **43**, that should readily cyclize yielding the corresponding lactols **44** resp. **45**. The subsequent oxidation of the lactols **44** and **45** to synthesize the desired lactones, γ-valerolactone (**20**) and δ-valerolactone (**21**) should be performed by a dehydrogenase since this type of reaction is already reported in literature.[122,123] The planned synthesis of γ-valerolactone (**20**) and δ-valerolactone (**21**) from pentanal (**19**) is shown in Scheme 17.

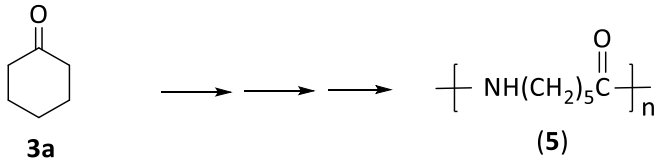


Scheme 17. Planned synthesis of γ-valerolactone (**20**) and δ-valerolactone (**21**) from pentanal (**19**)

Due to its olfactory qualities, γ-valerolactone (**20**) is used in the flavour and fragrance industry.[26] δ-Valerolactone (**21**) in contrast is applied in the synthesis of polymers (polyesters) and therefore for the production of lacquers and colours.[27] Altogether it is worthwhile to investigate pentanal (**19**) with respect to a biotransformation to gain access to valuable precursors.

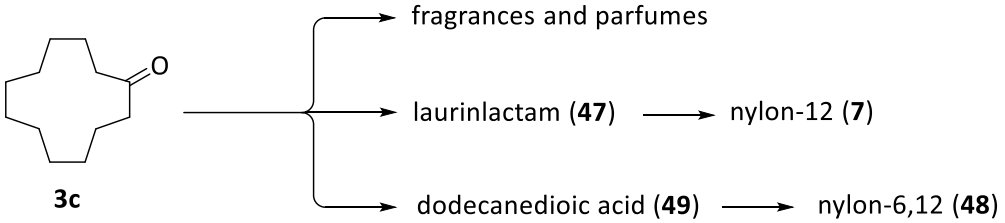
The last category to be tested is the class of cyclic alkanes **1** which play an extraordinary role as feedstock for the production of polyamides. Cycloalkanes **1** from petroleum are oxidized to a mixture of corresponding cycloalkanols **2** and cycloalkanones **3**.

This procedure is of particular economic importance since these compounds serve as industrial feedstock for the synthesis of bulk products. Cyclohexanone (**3a**) for example serves as starting compound for the synthesis of ε-caprolactam (**46**) that is applied for the synthesis of the polyamide nylon-6 (**5**) (Scheme 18). In 2010, the worldwide production of ε-caprolactam (**46**) was 4 million metric tons per year (Mt/a),[124] making the enzymatic synthesis of cyclohexanone (**3a**) attractive with regard to alternative synthesis routes.



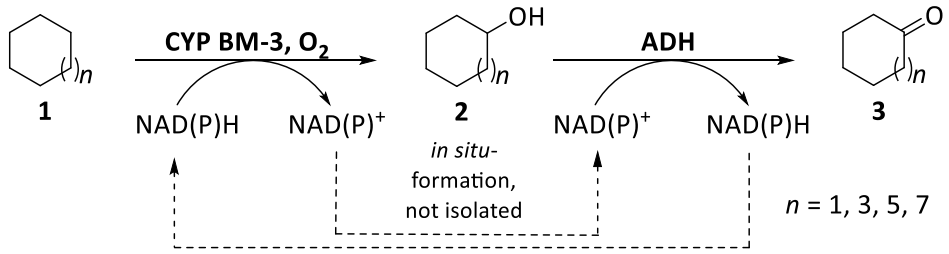
Scheme 18. Synthesis of the polyamide nylon-6 (**5**) from cyclohexanone (**3a**)

Higher homologue cycloalkanes **1** are oxidized in the presence of boric acid and molecular oxygen - a process called BASHKIROV process - and a subsequent catalytic dehydration provides access to the corresponding cycloalkanones **3**.[7-12] An industrial important cycloalkanone which is widely used in industry is cyclododecanone (**3c**), that is applied for the synthesis of nylon-12 (**7**) *via* laurinlactam (**47**) and the synthesis of nylon-6,12 (**48**) *via* dodecanedioic acid (**49**). Besides its role as feedstock for the polyamide production, cyclododecanone (**3c**) is applied in syntheses of musk fragrances (Scheme 19).[106,120]



Scheme 19. Cyclododecanone (**3c**) is a versatile starting material for the synthesis of valuable products

It is generally known that cyclic alkanes **1** are accepted and converted by cytochrome P450 monooxygenases yielding the corresponding oxidation products.[67,107-113] Recently, in the interesting field of cycloalkane oxidation a pioneering enzymatic two-step one-pot process was described by BURDA that basically can be applied for the synthesis of cyclic ketones **3**. The direct synthesis of cycloalkanones **3** from the corresponding cycloalkanes **1** is based on the cooperation of two enzymes: The introductory step of this process is the CYP BM 3 catalyzed hydroxylation reaction followed by an alcohol dehydrogenase catalyzed oxidation of the *in situ* formed alkanol **2** giving the corresponding alkanone **3**. The general reaction scheme is shown below.[20]



Scheme 20. General reaction scheme of the biocatalyzed double oxidation

Furthermore STAUDT analyzed mixtures of the substrate cyclooctane (**1d**) and the product cyclooctanone (**3d**) in the aqueous reaction medium and established a working up procedure based on the removal of the solvent *in vacuo* (900 mbar) and the determination of the absolute amount of the cycloalkanone **3** as productivity (g/L) of the reaction. Pyridine (**25**) was applied as external standard to analyze the crude product *via* ^{1}H-NMR-spectroscopy.[21,22] Both, BURDA and STAUDT used catalytic amounts of 2-propanol as additive to increase the productivity and enable the application of the more cost-effective cofactor in its oxidized form ($NADP^{+}$) since the required reduced form of the cofactor (NADPH) is generated in the course of the ADH-catalyzed oxidation of 2-propanol yielding acetone.[20-22] A productivity of 0.80 g/L cyclooctanone (**3d**) was obtained by using the mutant CYP BM-3 19A12 in combination with the above-mentioned ADH from *Lactobacillus kefir* (LK-ADH) for the conversion of 100 mM cyclooctane (**1d**) in phosphate buffer.[22]

Due to the qualities as building blocks for the synthesis of variable products, the oxyfunctionalization of further cycloalkanes **1** is highly preferable. Since this biocatalyzed double oxidation was analyzed for cyclooctane (**1d**) by STAUDT, the transfer of this procedure should be realized applying the substrates cyclohexane (**1a**), cyclodecane (**1b**) and cyclododecane (**1c**). Moreover, the challenge is to investigate more potentially active mutants of the cytochrome P450 monooxygenase (CYP BM-3) from *Bacillus megaterium* for this worthwhile hydroxylation of cyclic alkanes **1**. In order to avoid the application of pyridine (**25**) as external standard, the removal of the solvent at exact 900 mbar and the analysis *via* ^{1}H-NMR-spectroscopy, a GC-based analysis is planned to be established since this method is more reliable and even more economic.

4.2 Results and discussion

4.2.1 Spectrophotometric enzyme activity studies

Spectrophotometric assays were conducted since this is a current method to determine the specific activity in U/g lyophilized crude extract for the respective enzyme-substrate combinations. However, in the case of cytochrome P450 monooxygenases this practice provides indications but can not be used to derive absolute data concerning the product formation.

The reason is that these enzymes have a reputation for showing deviation between the consumption of the cofactor NAD(P)H and formation of the product or more precisely, consuming cofactor without oxidizing the substrate.[62-65] Altogether three additional shunt pathways are known to occur at different moments of the catalytic cycle and all three additional pathways (shunts) have in common that the consumption of the cofactor is decoupled from the substrate oxidation as such, since the electrons are used elsewhere. This phenomenon of uncoupling was described in detail earlier (4.1.1).

The measurements to determine the enzyme activities with an UV/VIS-spectrophotometer were conducted always at least in a twofold determination with the result that for every measurement the average of a twofold dataset is calculated. The content of CYP [nmol resp. µmol/g lyophilized crude extract] was determined *via* CO-difference spectroscopy by the research group Prof. Dr. U. Schwaneberg (RWTH Aachen) and is summarized in Table 9.

Table 9. Content of CYP BM-3 determined via CO-difference spectroscopy

Entry	Enzyme lot of the cytochrome P450 monooxygenase from *Bacillus megaterium*[a)]	Content of CYP [µmol/g lyophilized crude extract]
1	CYP BM-3 19A12 (0)	0.123
2	CYP BM-3 19A12 (1)	0.060
3	CYP BM-3 19A12 (2)	0.120
4	CYP BM-3 19A12 (3)	0.115
5	CYP BM-3 F87A A328V (1)	0.480
6	CYP BM-3 F87A A328V (2)	0.989
7	CYP BM-3 F87P (1)	0.235
8	CYP BM-3 F87P (2)	0.223
9	CYP BM-3 R255P-P329H	0.220
10	CYP BM-3 F87V	0.525
11	CYP BM-3 WT	1.260

[a)] In parentheses the different enzyme formulations are given.

On the basis of this data, the content of CYP [µmol/g lyophilized crude extract] and the subsequent determined specific activity [U/g lyophilized crude extract], the turnover frequency (TOF) [min^{-1}] is calculated according to the following formula.

$$\text{Turnover Frequency (TOF) } [min^{-1}] = \frac{\text{specific activity [U/g lyophilized crude extract]}}{\text{content of CYP [µmol/g lyophilized crude extract]}}$$

Cyclododecene (**18**) was analyzed as a possible substrate from the category monounsaturated cyclic hydrocarbons in an enzyme activity test. The following mutants of the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) were tested: CYP BM-3 19A12 (1), CYP BM-3 F87P (2), CYP BM-3 F87V, CYP BM-3 F87A A328V (2), CYP BM-3 WT and CYP BM-3 R255P-P329H (SOP 4). In Figure 10, the specific activity in U/g lyophilized crude extract is plotted against the respective mutant.

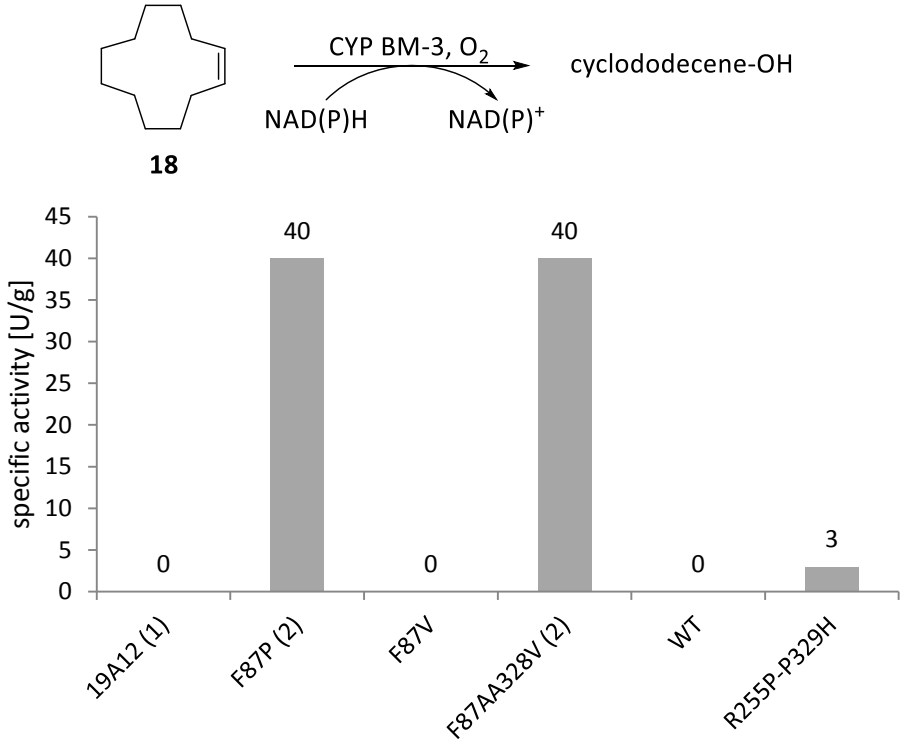


Figure 10. Results of the enzyme activity test with cyclododecene (**18**) as substrate

As apparent from Figure 10, the mutants CYP BM-3 F87P (2) and CYP BM-3 F87A A328V (2) turned out to be most promising for preparative biotransformations. For the mutant CYP BM-3 F87P a specific activity of 40 U/g was found and with the CYP-content of 0.223 µmol/g in the lyophilized crude extract, a TOF of 179 min^{-1} was calculated. For CYP BM-3 F87A A328V, as well a specific activity of 40 U/g was found and with the CYP-content of 0.989 µmol/g in the lyophilized crude extract, a TOF of 40 min^{-1} was obtained.

The next substrate, cyclooctyne (**17**) was first synthesized from cyclooctene (**50**) according to a two-stage procedure reported in literature[125] *via* the intermediate 1-bromocyclooctene (**51**).[125-126] Then it was analyzed as a second possible substrate from the category monounsaturated cyclic hydrocarbons in an enzyme activity test with the mutants CYP BM-3 F87P (2) and CYP BM-3 F87A A328V (2) of the cytochrome P450 monooxygenase from *Bacillus megaterium* (SOP 4). Both mutants showed no activity and therefore the intention to apply cyclooctyne (**17**) in a preparative biotransformation was dropped.

Furthermore, different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) were analyzed with regard to their capability to convert pentanal (**19**). Since the tendency of aldehydes to undergo oxidation by simply stirring their aqueous emulsions in air to give the corresponding carboxylic acids in high yields recently was described in literature[127] the stability of pentanal (**19**) in water was investigated over the time. Therefore a mixture of pentanal (**19**) in D_2O was stirred for 0 - 24 h and analyzed *via* ^{1}H-NMR-spectroscopy at different reaction times. The formation of the corresponding carboxylic acid was found to be very small at an extent of 5.4% on average, identified on the basis of the characteristic triplet caused by the two C_α-protons at 2.2 ppm. The following mutants of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) were tested: CYP BM-3 F87A A328V (2), CYP BM-3 F87P (2), CYP BM-3 19A12 (2), CYP BM-3 F87V, CYP BM-3 R255P P329H and CYP BM-3 WT (SOP 4). In Figure 11, the specific activity in U/g lyophilized crude extract is plotted against the respective mutant.

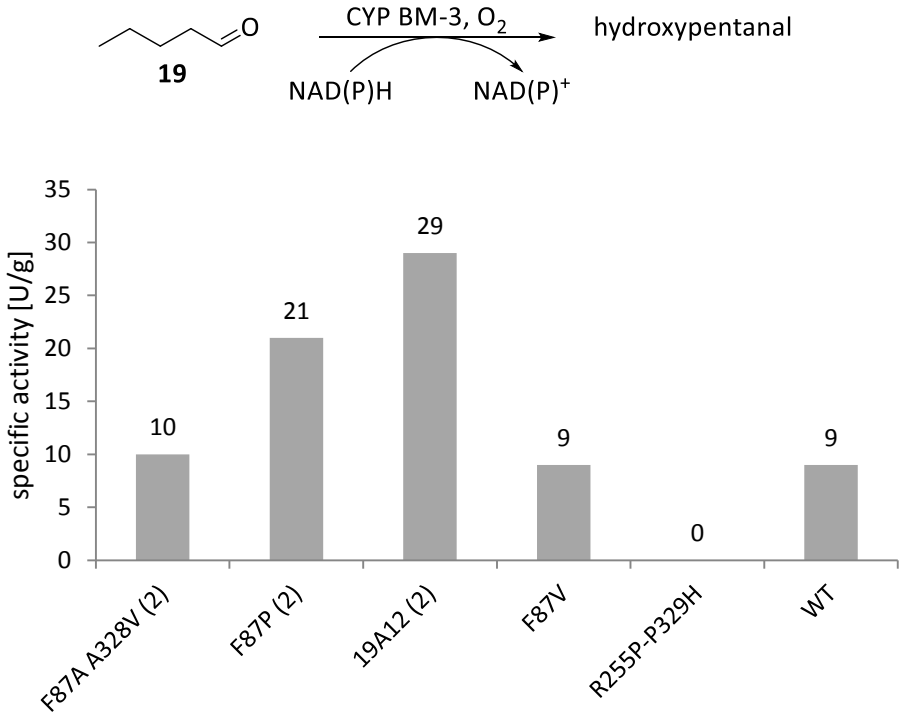


Figure 11. Results of the enzyme activity test with CYP BM-3 and pentanal (**19**)

As apparent from Figure 11, the mutants CYP BM-3 F87P (2) and CYP BM-3 19A12 (2) are most promising for a preparative biotransformation. For the mutant CYP BM-3 F87P a specific activity of 21 U/g was found and with the CYP-content of 0.223 µmol/g in the lyophilized crude extract, a TOF of 94 min^{-1} was calculated. For CYP BM-3 19A12 (2), a specific activity of 29 U/g was found and with the CYP-content of 0.120 µmol/g in the lyophilized crude extract, a TOF of 242 min^{-1} was calculated.

Different mutants of the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12 (formulation 0-3), CYP BM-3 F87P (1-2), CYP BM-3 F87V, CYP BM-3 WT, CYP BM-3 R255P-P329H, CYP BM-3 F87A A328V (1-2)) were analyzed at different times to transform cyclododecane (**1c**) into the corresponding cyclododecanol (**2c**) (SOP 4). In Figure 12, the specific activity in U/g lyophilized crude extract is plotted against the respective mutant.

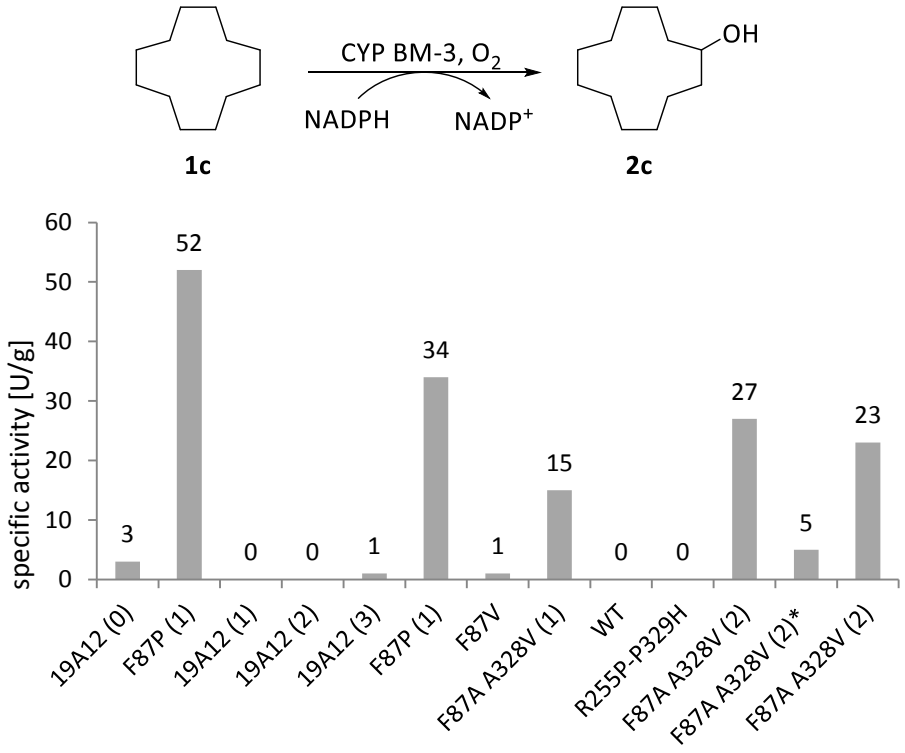


Figure 12. Results of the enzyme activity test with cyclododecane (**1c**) as substrate. Repetitions are referred to multiple measurements of individual enzyme lots during the project term. In that case, the more recent the measured value, the further right it is placed on the x-axis. A decrease in activity over the course of time can be ascribed to a loss in stability of the enzyme lot during long-term storage.

It is noticable from Figure 12, that only the mutants CYP BM-3 F87P and CYP BM-3 F87A A328V showed an activity in converting the substrate **1c**, while all other mutants showed no or only poor activity. Therefore these mutants are most promising for a preparative biotransformation. For the mutant CYP BM-3 F87P a specific activity of 52 U/g and later 34 U/g was found and with the CYP-content of 0.235 µmol/g in the lyophilized crude extract, a TOF of 221 resp. 145 min^{-1} was calculated. For CYP BM-3 F87A A328V (1), a specific activity of 15 U/g was found and with the CYP-content of 0.480 µmol/g in the lyophilized crude extract, a TOF of 31 min^{-1} was calculated. With a new formulation of CYP BM-3 F87A A328V (2), 27 U/g and a TOF of 27 min^{-1} was obtained. However, in the presence of higher concentrations of DMSO (F87A A328V (2)* in Figure 12) the specific activity and therefore the turn over frequency (TOF) is lowered noticable.

Different mutants of the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12 (formulation 0-3), CYP BM-3 F87P (1), CYP BM-3 F87V, CYP BM-3 WT, CYP BM-3 R255P-P329H, CYP BM-3 F87A A328V (1)) and empty vector *E.coli* BL21 as blank were analyzed at different times in the course of this project with respect to their ability to transform cyclodecane (**1b**) into the corresponding cyclodecanol (**2b**) (SOP 4). In Figure 13, the specific activity in U/g lyophilized crude extract is plotted against the respective mutant.

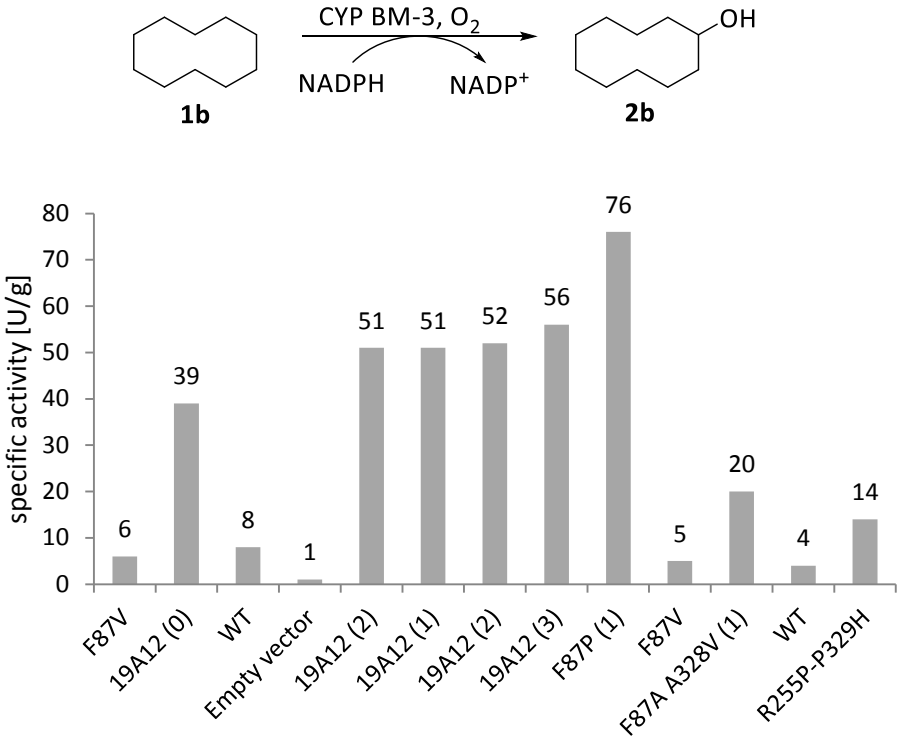


Figure 13. Results of the enzyme activity test with cyclodecane (**1b**) as substrate. Repetitions are referred to multiple measurements of individual enzyme lots during the project term. In that case, the more recent the measured value, the further right it is placed on the x-axis. A decrease in activity over the course of time can be ascribed to a loss in stability of the enzyme lot during long-term storage.

It is noticable from Figure 13, that all available formulations of the mutant CYP BM-3 19A12 showed the best specific activity for cyclodecane (**1b**) by far (39-56 U/g; TOFs of 317-850 min^{-1}) and therefore this mutant was preferred for preparative experiments with this substrate. One of the later performed tests manifests the mutant CYP BM-3 F87P (1) as well as a promising mutant for the conversion of cyclodecane (**1b**). A specific activity of 76 U/g was found but with the CYP-content of 0.235 µmol/g in the lyophilized crude extract, a turn over frequency of 323 min^{-1} was calculated.

Different mutants of the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12 (formulation 0-3), CYP BM-3 F87P (1), CYP BM-3 F87V, CYP BM-3 WT, CYP BM-3 R255P-P329H, CYP BM-3 F87A A328V (1)) and empty vector *E.coli* BL21 as blank were analyzed at different times in the course of

this project with respect to their ability to transform cyclohexane (**1a**) into the corresponding cyclohexanol (**1b**). In Figure 14, the specific activity in U/g lyophilized crude extract is plotted against the respective mutant.

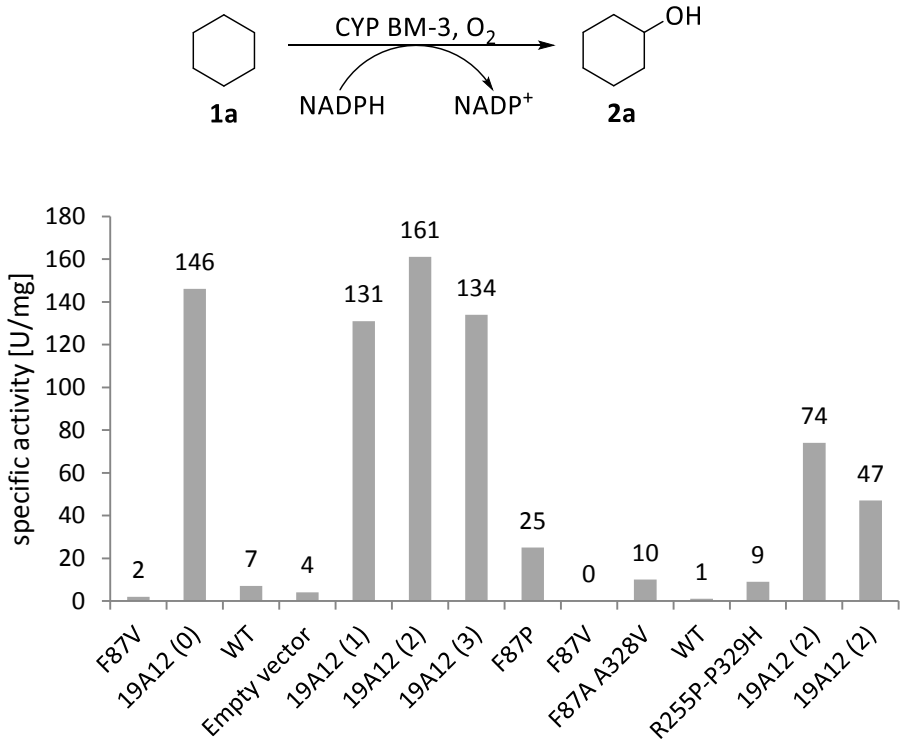
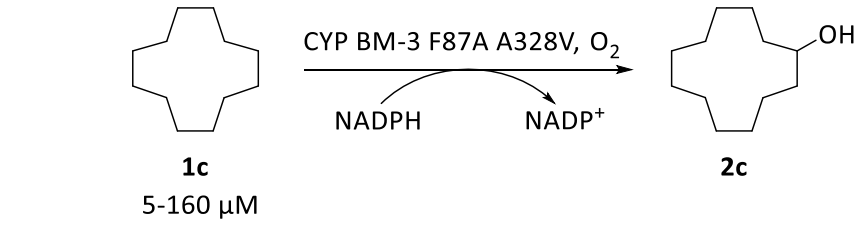


Figure 14. Results of the enzyme activity test with cyclohexane (**1a**) as substrate. Repetitions are referred to multiple measurements of individual enzyme lots during the project term. In that case, the more recent the measured value, the further right it is placed on the x-axis. A decrease in activity over the course of time can be ascribed to a loss in stability of the enzyme lot during long-term storage.

As already with the substrate cyclodecane (**1b**), the mutant CYP BM-3 19A12 was by far the best enzyme to catalyze the oxidation of cyclohexane (**1a**). Moreover, this mutant emerged to be the one of choice for the whole project. Without exception, all other tested enzymes lag far behind this remarkable activity. For the formulation CYP BM-3 19A12 (0), a specific activity of 146 U/g was found and with the CYP-content of 0.123 µmol/g in the lyophilized crude extract, a TOF of 1187 min^{-1} was calculated. The formulations CYP BM-3 19A12 (1) (131 U/g; TOF = 2183 min^{-1}), CYP BM-3 19A12 (2) (161 U/g; TOF = 1342 min^{-1}) and CYP BM-3 19A12 (3) (134 U/g; TOF = 1165 min^{-1}) were at the same promising range and therefore CYP BM-3 19A12 was used extensively for preparative experiments.

Moreover, UV/VIS-spectroscopy was used to investigate the kinetics according to MICHAELIS-MENTEN of a biotransformation and by the MICHAELIS-MENTEN-equation it was specified, to what extent the reaction rate depends on the substrate concentration. This information is of particular importance if substrates are poorly soluble in the aqueous reaction medium.

Since the log P value of cyclododecane (**1c**) is 6.6[128] - exemplarily for the higher homologue cycloalkanes - this compound is known to show a very poor solubility in water. Therefore it has to be analyzed if the effective solubility of a certain substrate, e.g. cyclododecane (**1c**) is high enough for an efficient biotransformation: if not enough substrate molecules are in solution to saturate the available enzyme with substrate, the approximate maximum reaction rate remains inaccessible. To analyze this parameter, a serial dilution of **1c** in DMSO was prepared from a stock solution (0.5-16 mM). The concentration of the saturated solution (~16 mM) was identified *via* ^{1}H-NMR-spectroscopy using pivalic acid (**52**) as standard. A set of serial dilution (16 mM, 12 mM, 8 mM, 4 mM, 2 mM, 1 mM, 0.5 mM) was prepared to measure the following absolute concentrations in a photometric assay: 160 µM, 120 µM, 80 µM, 40 µM, 20 µM, 10 µM and 5 µM. A 0.6 mM solution of NAD(P)H was used for the measurements (SOP 5). In this way the kinetics according to MICHAELIS-MENTEN of the cyclododecanol (**2c**)-formation were determined *via* an enzyme activity test with the cytochrome P450 monooxygenase mutant from *Bacillus megaterium* (CYP BM-3 F87A A328V) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) and cyclododecane (**1c**) as substrate (Figure 15).



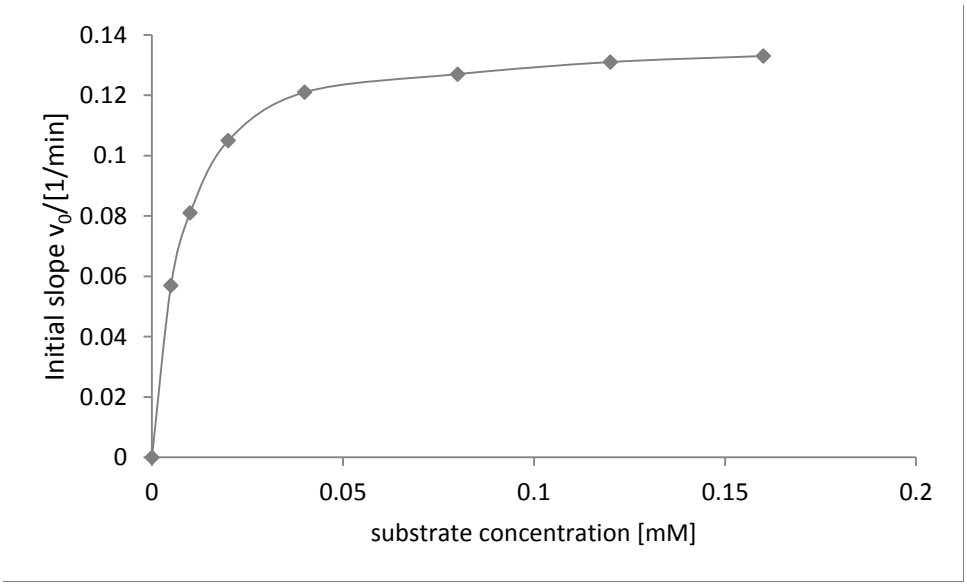


Figure 15. Results of the enzyme activity test with cyclododecane (**1c**) as substrate combined in a MICHAELIS-MENTEN diagram of the biocatalyzed hydroxylation

As it is apparent from Figure 15, the substrate molecules in solution are entirely sufficient so that the enzyme is working at full capacity. Adding more substrate would not affect the reaction rate since the enzyme is already saturated with substrate at the given concentration.

To make a statement about the stability of CYP BM-3 F87A A328V, the enzyme activity of CYP BM-3 F87A A328V to convert the substrate **1c** to the corresponding product **2c** is investigated as a function of time. Therefore the cytochrome P450 monooxygenase mutant from *Bacillus megaterium* (CYP BM-3 F87A A328V) was stirred in the presence of 0.02 mmol D-glucose (**22**) and DMSO (10% v/v) in phosphate buffer (pH 7.0, 50 mM) and samples are taken at (regular) intervals over a period of 28h (SOP 6). These samples were analyzed *via* UV/VIS-spectroscopy and the initial slope of the absorption curve v_0 [1/min] was plotted against the time [h] (Figure 16).

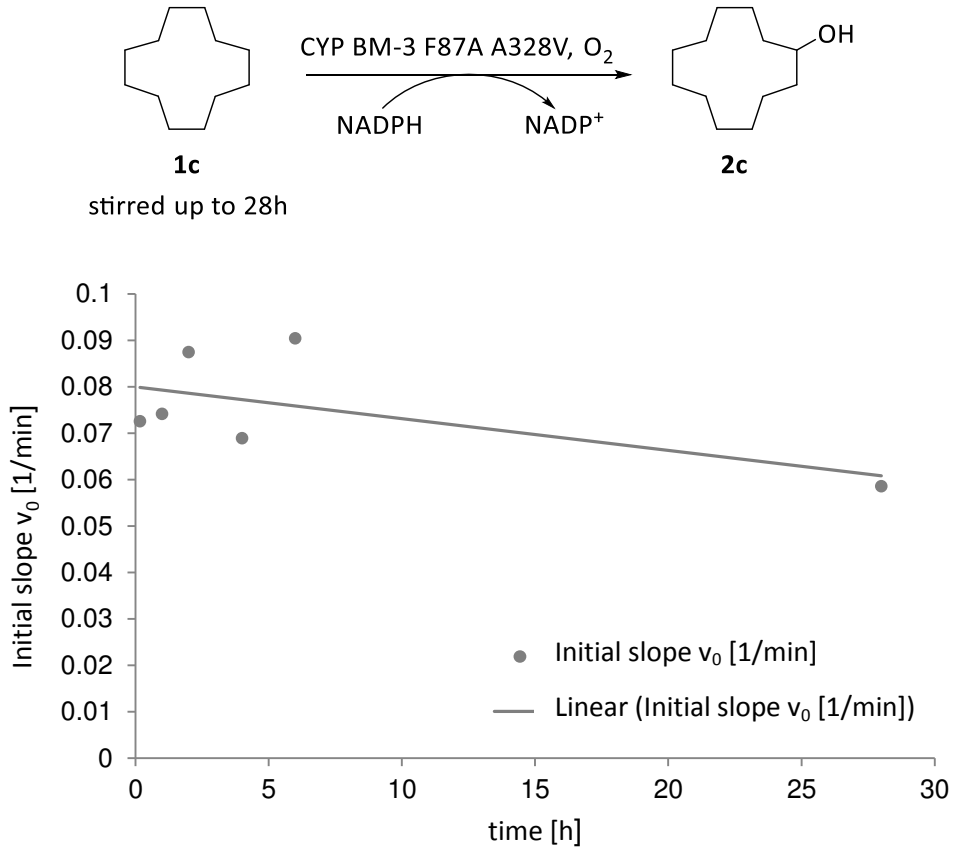


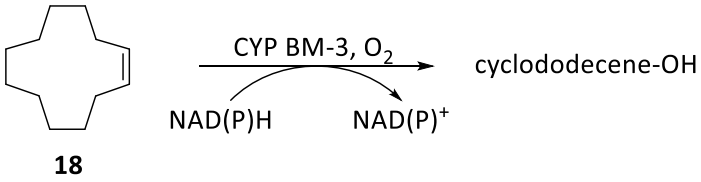
Figure 16. Stability of CYP BM-3 F87A A328V as a function of time

It is apparent from Figure 16 that a reaction time of 28h and even the presence of DMSO (10% v/v) in the reaction mixture have no severe negative effect on the stability and therefore the enzyme activity of mutant CYP BM-3 F87A A328V. Of course a theoretical inhibition of the enzyme by product formation or denaturation of the enzyme through long contact with the substrate or product is not considered with this test.

4.2.2 Biocatalyzed oxidation of monounsaturated cyclic hydrocarbons with different CYP BM-3 and glucose dehydrogenase (GDH)

Cyclododecene (**18**)

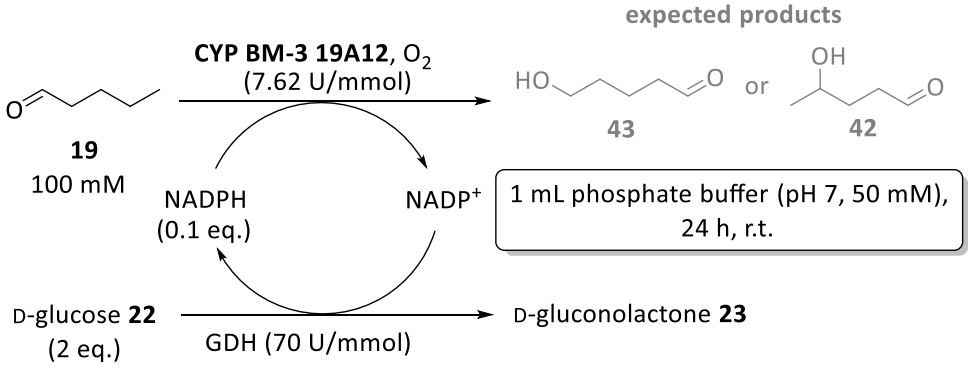
C-C multiple bonds in unsaturated hydrocarbons lag far behind the single bonds in saturated hydrocarbons regarding stability and are therefore more easily attacked.[76] Cyclododecene (**18**), a valuable intermediate for e.g. the synthesis of parfumes and fragrances,[25] was tested as agent of this class of substrates with different mutants of the cytochrome P450 monooxygenases from *Bacillus megaterium*.



Scheme 21. Anticipated enzymatic oxidation of Cyclododecene (**18**)

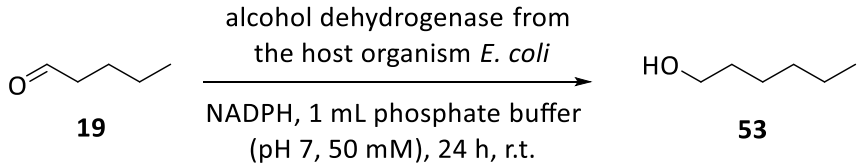
The promising mutants which were detected in the enzyme activity test with the unsaturated cyclic hydrocarbon cyclododecene (**18**) as substrate were the mutants CYP BM-3 F87P (2) and CYP BM-3 F87A A328V (2) (SOP 4). The specific activities of CYP BM-3 F87P (40 U/g) and CYP BM-3 F87A A328V (40 U/g) are in a range worthy to test and therefore those two mutants are applied in a preparative biocatalyzed oxidation. The formation of an oxidation product was not detected *via* ^{1}H-NMR-spectroscopy, neither with CYP BM-3 F87P nor with CYP BM-3 F87A A328V (by comparison with the ^{1}H-NMR-spectra of **18**), that means that cyclododedene (**18**) was neither converted by CYP BM-3 F87P nor by CYP BM-3 F87A A328V. The activity detected in the enzyme activity test can be attributed to an undefined background reaction consuming the cofactor NADPH resp. uncoupling in general.

4.2.3 Biocatalyzed oxidation of pentanal (19) with different CYP BM-3 and glucose dehydrogenase (GDH)



Scheme 22. Anticipated enzymatic oxidation of pentanal (**19**)

The cytochrome P450 monooxygenase from *Bacillus megaterium* CYP BM-3 19A12 (2) was expected to be suitable for an enzymatic oxidation of pentanal (**19**) because its specific activity (29 U/g) was the highest activity found in these enzyme activity tests (SOP 4). Therefore this mutant is applied in a preparative enzymatic transformation (Scheme 22).



Scheme 23. Biocatalyzed reduction of pentanal (**19**) yielding 1-pentanol (**53**)

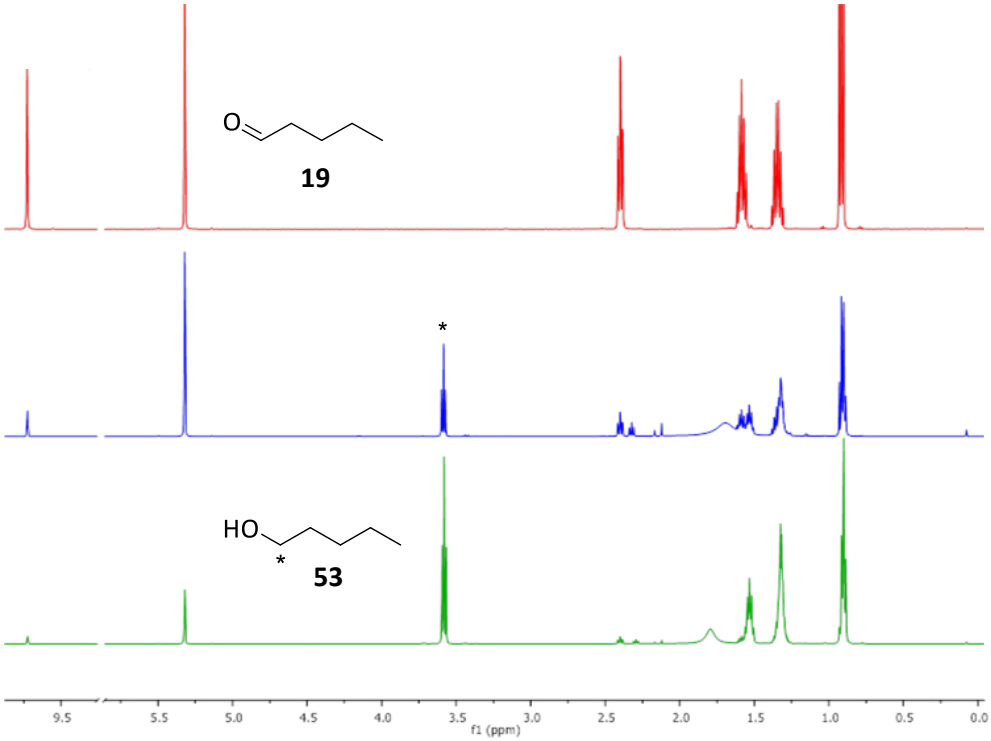


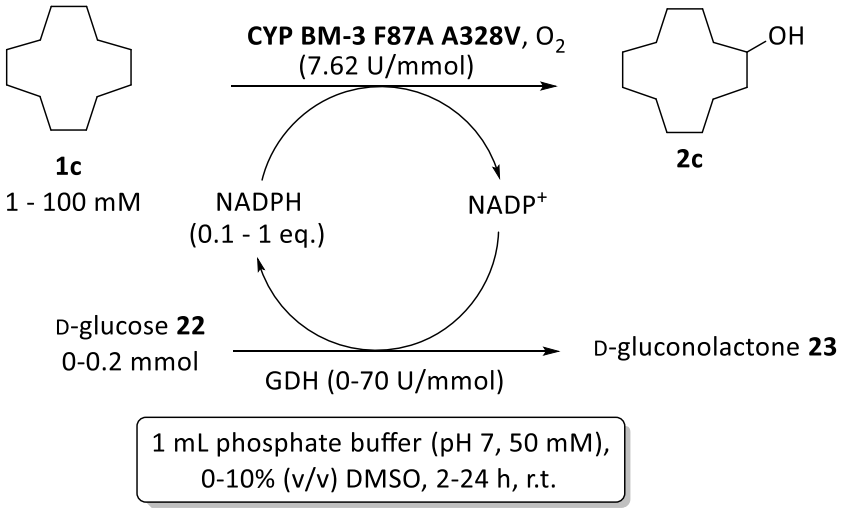
Figure 17. Results of the enzymatic oxidation of pentanal (**19**) (red, above), biotransformation of pentanal (**19**) (blue, middle) and biotransformation sample after addition of the assumed product 1-pentanol (**53**)

Compared to the ^{1}H-NMR spectrum of pentanal (**19**) (red, above), the ^{1}H-NMR spectrum of the biocatalytic transformation with CYP BM-3 19A12 (blue, middle) showed a clear modification (Figure 17). Especially the triplet that appears at 3.58 ppm indicates the formation of a new compound. The addition of the assumed product 1-pentanol (**53**) to the biotransformation sample (^{1}H-NMR spectrum in green, below) confirms the assumption, that not the oxidation reaction takes place but the reduction of pentanal (**19**) to the corresponding alcohol **53** occurs (Figure 17). The most obvious explanation for this phenomenon is an undesired background activity caused by an alcohol dehydrogenase from the host organism *E. coli*, operating in the reductive direction (Scheme 23).

4.2.4 Biocatalyzed hydroxylation of higher homologous cycloalkanes with different CYP BM-3 and glucose dehydrogenase (GDH)

Cyclododecane (**1c**)

The mutants CYP BM-3 F87P and CYP BM-3 F87A A328V seemed to be very promising for the conversion of the substrate cyclododecane (**1c**) (TOF of 145 resp. 31 min^{-1}) in an initial enzyme activity test (SOP 4). Therefore the preparative hydroxylation of **1c** with 0.762 U of the mutant CYP BM-3 F87A A328V was carried out first. All experiments were conducted by varying the following parameters: Concentration of the substrate **1c**, concentration of the cofactor, volume of the cosolvent DMSO and reaction time. In addition, the absence of the cofactor regeneration system was analyzed (Scheme 24). Despite a scientifically supported confidence especially for the double mutant CYP BM-3 F87A A328V, which is known to convert even the long chained cyclic alkane cyclododecane (**1c**),[108] in most experiments the conversion of cyclododecane (**1c**) to cyclododecanol (**2c**) was below 1% and the results could not be reproduced (SOP 9). Probably one of the most obvious reasons why WEBER *et al.* found conversion in their experiments is the comparatively low substrate concentration of 200 µM while in the experiments shown below substrate concentrations up to 0.1 M were applied.[108]



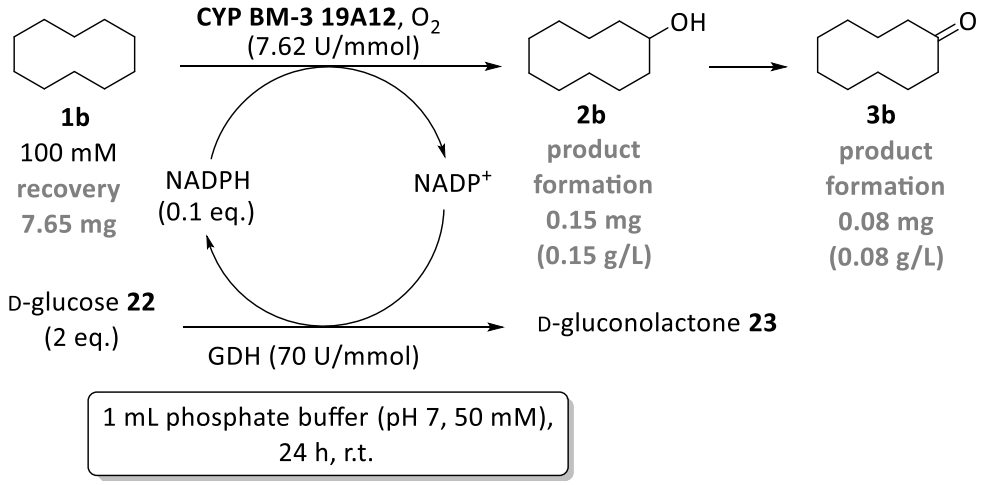
Scheme 24. Biocatalyzed hydroxylation of cyclododecane (**1c**)

A series of experiments with different CYP BM-3, mainly CYP BM-3 F87P was conducted with the result that the formation of cyclododecanol **2c** was below 5 mg/L and conversion could not be determined. For the sake of completeness experiments with all spectrophotometrically inactive mutants were carried out with exemplarily 50 mg of the respective mutant to ensure that no active mutant is missed. But in all experiments the formation of cyclododecanol **2c** was below 5 mg/L and conversion was always below 1% (SOP 9).

In summary it can be stated that there was a very low acceptance of all tested mutants towards the substrate cyclododecane (**1c**) in the preparative experiments although activity was shown in an initial enzyme activity test. This activity may be caused from another NADPH-consuming background reaction because crude extract is used for both, the enzyme activity test and the preparative experiments. The fact that cyclododecane (**1c**) is hardly soluble in the aqueous phase could be another reason for the biotransformation did not work. Compared to other analyzed liquid substrates the additional lattice energy of the molecular crystal has to be overcome in that case and it is not surprising that the solubility of the unpolar **1c** in the aqueous phase is quite poor.

The corresponding alcohols **2** formed during the biotransformation are notoriously not as stable and inert as the substrate alkanes **1**[76] and for this reason the further oxidation of the alcohol **2** can not be excluded. For example, it is supposed that the sesquiterpene (+)-valencene (**54**) has multiple binding orientations in CYP BM-3 and therefore - besides the favoured (+)-nootkatone (**55**) - several further oxidation products are formed.[129] In 2000, ADAM *et al.* showed, that cyclohexane (**1a**) and cyclooctane (**1d**) can be hydroxylated by living cells of *Bacillus megaterium* giving the corresponding cycloalcohols cyclohexanol (**2a**) and cyclooctanol (**2d**). In this study it was additionally found that overoxidation to the corresponding ketones **3a** and **3d** takes place. The influence of the ring size on the oxidative activity was conspicuous, since 65% cyclooctanone (**3d**) were yielded while only 21% cyclohexanone (**3a**) were formed.[110] Therefore the hydroxylation of cyclohexane (**1a**) and cyclodecane (**1b**) is realized and for that reason analyzed with regard to a possible further oxidation.

Cyclodecane (**1b**)



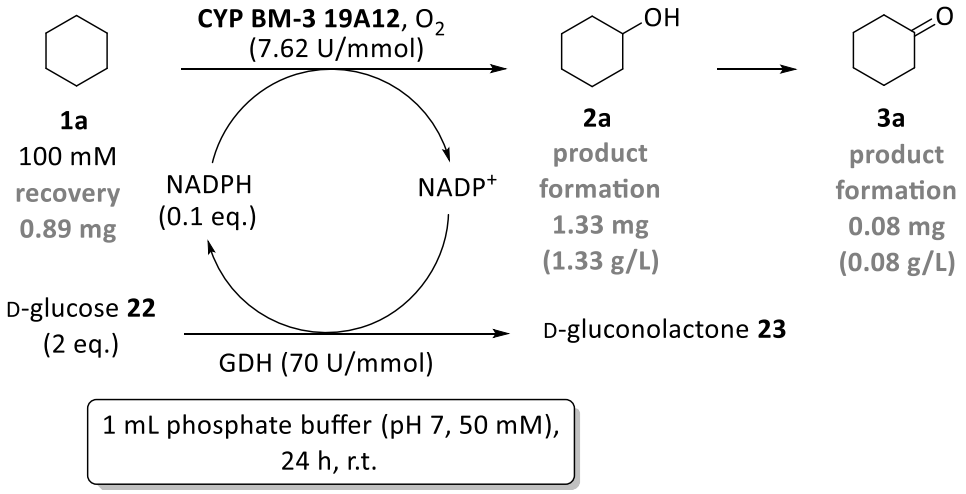
Scheme 25. Results of the oxidation of cyclodecane (**1b**) with CYP BM-3 19A12 (SOP 10)

The cytochrome P450 monooxygenase from *Bacillus megaterium* CYP BM-3 19A12 is expected to be suitable for an enzymatic oxidation of **1b**. The specific activity of CYP BM-3 19A12 was significantly higher than all other mutants (39-56 U/g; TOFs of 317-850 min^{-1}, SOP 4) and therefore this mutant is applied in a preparative enzymatic transformation (Scheme 25). The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract.

In the experiment shown in Scheme 25, 7.65 mg of the substrate **1b** were recovered and as a product of further oxidation cyclodecanone (**3b**) was found (0.08 mg). The product formation of **2b** was 0.15 mg.

Cyclohexane (**1a**)

The cytochrome P450 monooxygenase from *Bacillus megaterium* CYP BM-3 19A12 was by far the best enzyme to catalyze the oxidation of cyclohexane (**1a**) and specific activities up to 161 U/g were found. Therefore this mutant is applied in a preparative enzymatic transformation (Scheme 26). The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract.



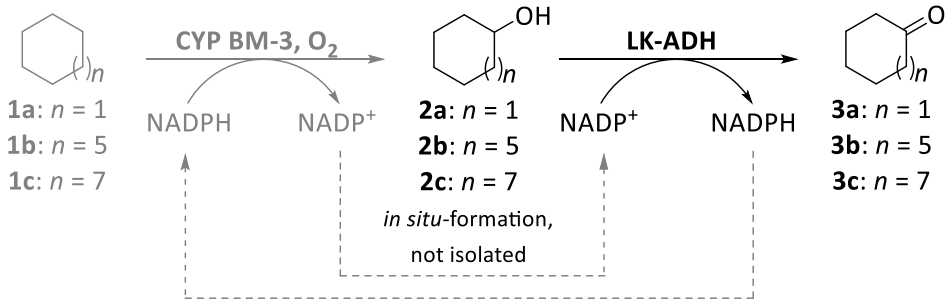
Scheme 26. Results of the oxidation of cyclohexane (**1a**) with CYP BM-3 19A12 (SOP 10)

In the experiment shown in Scheme 26, 0.89 mg substrate **1a** were recovered and as a product of further oxidation cyclohexanone (**3a**) was found (0.08 mg). In all, 1.33 mg of the product **2a** were formed. It can be summarized that the double oxidation induced by CYP BM-3 can be detected for both substrates, cyclohexane (**1a**) and cyclodecane (**1b**). However, it plays a major role for cyclodecane(**1b**).

5 Double oxidation combining a cytochrome P450 monooxygenase from *B. megaterium* and an alcohol dehydrogenase from *L. kefir* in an one-pot process

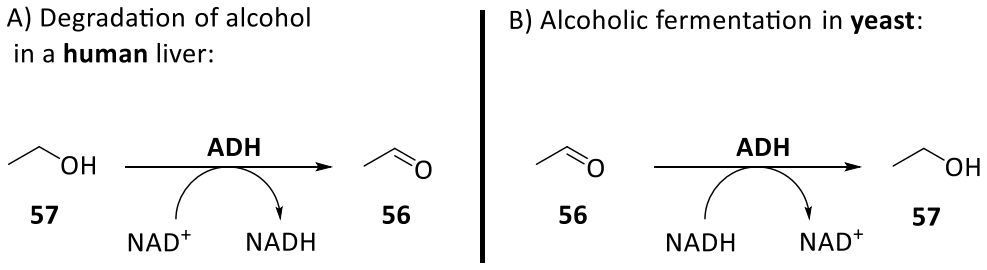
5.1 Introduction, state of the art, and aim of this section

Since a whole chapter is already focused on the first step of the planned double oxidation, namely the cytochrome P450 monooxygenase catalyzed hydroxylation (chapter 4), the key aspect of this chapter is the oxidation of the alcohol **2** to the corres-ponding ketone **3**, catalyzed by an alcohol dehydrogenase from *Lactobacillus kefir*, which is highlighted in Scheme 27.



Scheme 27. Double oxidation of cycloalkanes **1** with ADH-catalyzed step

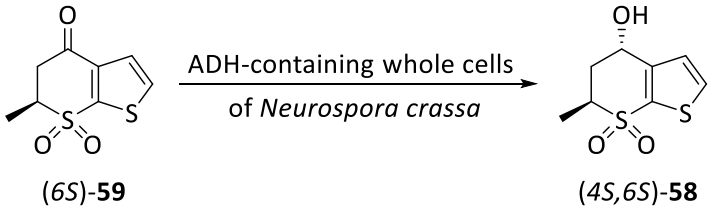
Alcohol dehydrogenases (ADHs) occur in all domains of life.[19] In humans, for example, ADHs are found in high concentrations in liver and stomach, catalyzing the oxidative degradation of toxic alcohols (Scheme 28, left).[130] In yeast, the last step of the alcoholic fermentation is the ADH-mediated transformation of acetaldehyd (**56**) yielding ethanol (**57**) (Scheme 28, right).[131]



Scheme 28. Details of A) the degradation of alcohol in humans[130] and B) the alcoholic fermentation in yeast cells[131]

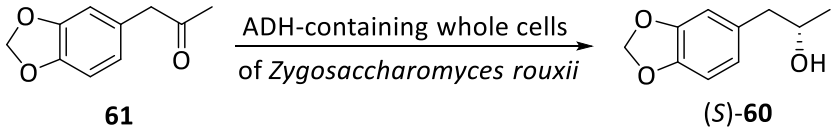
In the field of organic chemistry, the ketoreductase function of alcohol dehydrogenases is often used to generate a stereogenic center and therefore a valuable structural motif. Three impressive examples for the application of ADHs on an industrial scale are shown below:[132]

The first example is the synthesis of the (4*S*,6*S*)-hydroxysulfone ((4*S*,6*S*)-**58**) performed at AstraZeneca which serves as an intermediate in the synthesis of Trusopt™, a drug for the treatment of glaucoma. Suspended whole cells are applied in aqueous medium to produce (*4S,6S*)-**58** from the methyl ketosulfone ((6*S*)-**59**) on a multi ton scale with a yield of >85% and >98% ee (Scheme 29).[132]



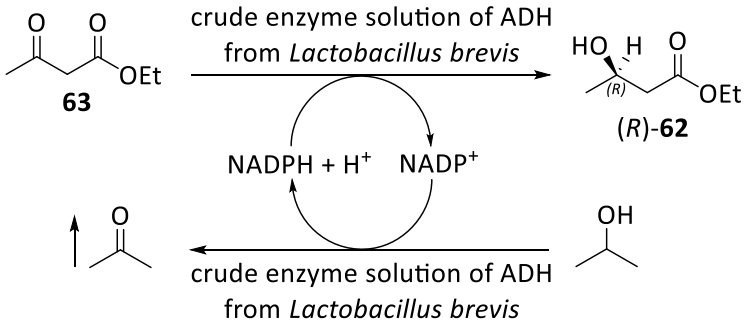
Scheme 29. Selected step of the synthesis route for the drug Trusopt™

A further example is a selected step in the synthesis of LY 300164, an orally administered benzodiazepine produced by Eli Lilly and Company. Suspended whole cells are applied in aqueous medium to produce the precursor (*S*)-**60** from 3,4-methylenedioxyphenylacetone (**61**) on a kilogram scale with a yield of 96% and >99.9% ee (Scheme 30).[132]



Scheme 30. Selected step of the synthesis route for the drug LY 300164

The next biocatalytic synthesis is performed at Wacker Chemie. A crude enzyme solution of alcohol dehydrogenase from *Lactobacillus brevis* is applied in aqueous medium to produce (*R*)-ethyl-3-hydroxybutyrate ((*R*)-**62**) from ethyl acetoacetate (**63**) on a scale of 35 t/a with a yield of 96% and >99.8% ee. The coproduct acetone which is formed during the regeneration of the cofactor is continuously stripped to shift the reactions equilibrium to the product site. (Scheme 31).[132]

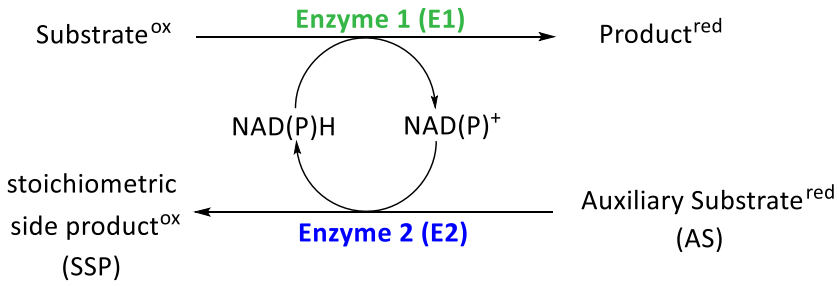


Scheme 31. Selected step of the synthesis route of (*R*)-**62**

As apparent from the example shown in Scheme 31, one single enzyme (alcohol dehydrogenase from *Lactobacillus brevis*) catalyzes both, the desired biotrans-

formation as well as the biotransformation of the auxiliary substrate 2-propanol. This coupled substrate process is one possibility for the recycling of the high priced cofactor to its reduced form.

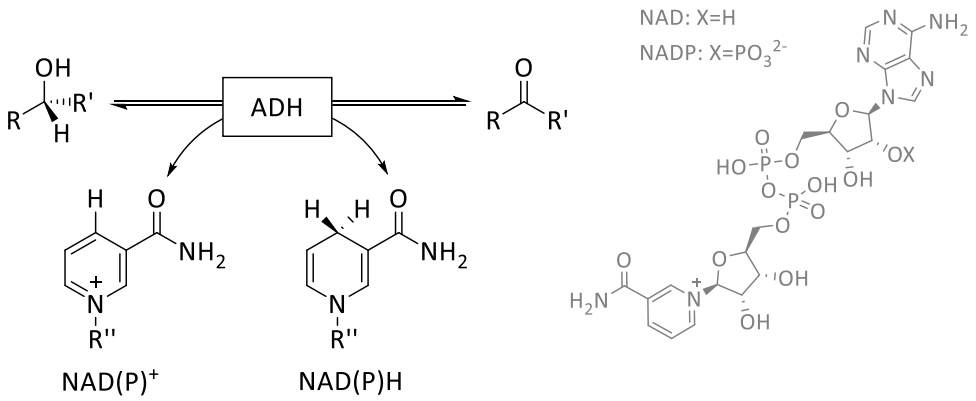
Another approach is a coupled enzyme method where two different enzymes (E1 and E2) are applied, one (E1) for the formation of the product of interest and another (E2) for the recycling of the cofactor (Scheme 32).[28]



Scheme 32. Coupled-enzyme method for cofactor recycling

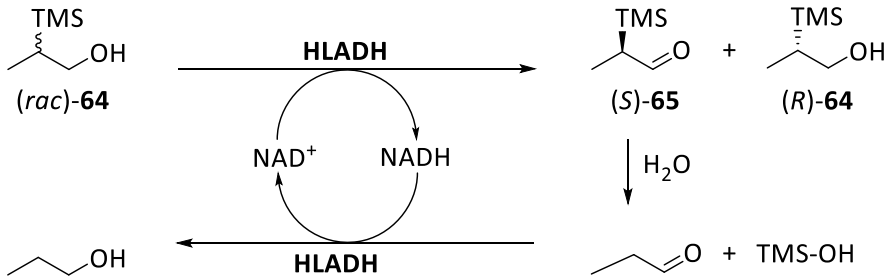
The application of ADHs is described in literature for the transformation of the main product[133] but also for its function in cofactor recycling: besides other well-known systems for recycling of the cofactor (E2 = Formate dehydrogenase (FDH); AS = formate; SSP = CO_2 resp. E2 = Glucose dehydrogenase (GDH); AS = glucose; SSP = gluconic acid), alcohol dehydrogenases are as well established in the coupled method for cofactor recycling.[28] These applications make clear that the oxidative direction of ADHs is as well important for designing enzyme reactions and should not be disregarded. Nevertheless, the capability of these enzymes to catalyze oxidation reactions as well is often outshined by the corresponding and above-mentioned reduction reactions.[28,104]

Some generally accepted reasons for the fact that dehydrogenase-mediated oxidation reactions attach less importance than the corresponding reduction reactions are outlined below. In the course of oxidation of an unsymmetric secondary alcohol a chiral center - generally valuable for synthetic applications - is destroyed.[28] Furthermore, aldehydes and ketones tend to bind more tightly to the hydrophobic active site of the dehydrogenase than the employed alcohols and product inhibition might be a result hereof.[28] In addition, oxidation reactions of primary and secondary alcohols that depend on the cofactor $NAD(P)^+$ are thermodynamically unfavoured (ethanol/acetaldehyde: -199 mV; 2-propanol/acetone: -286 mV) since the redox couple NAD(P)H/$NAD(P)^+$ has a redox potential of -320 mV.[28,104] Despite these drawbacks, alcohol dehydrogenases are the most popular biocatalysts for the oxidation of alcohol substrates (Scheme 33).[19,104]



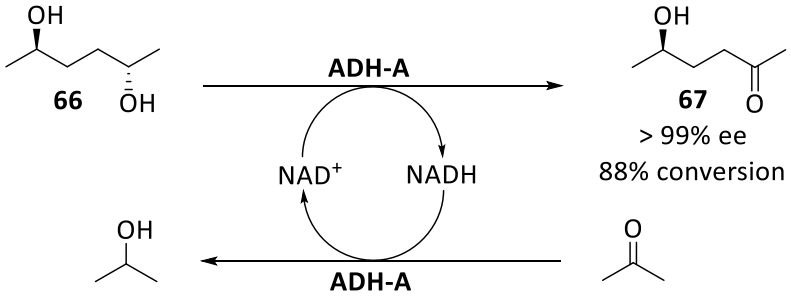
Scheme 33. General reaction equation of the ADH-catalyzed oxidation of alcohols[19,104]

The alcohol dehydrogenase isolated from horse liver (HLADH) for example is one of the first enzymes applied in organic syntheses and therefore very well characterized.[134] HLADH shows an (*S*)-enantioselectivity and is successfully applied in oxidation reactions for enzymatic kinetic resolution of alcohols. A prominent example is the HLADH-catalyzed enantioselective dehydrogenation of racemic β-hydroxysilanes (*rac*)-**64**. One enantiomer (*R*)-**64** remains untouched while the other is converted into the corresponding β-ketosilane (*S*)-**65**, that hydrolyzes spontaneously and promotes the regeneration of the cofactor (Scheme 34).[135]



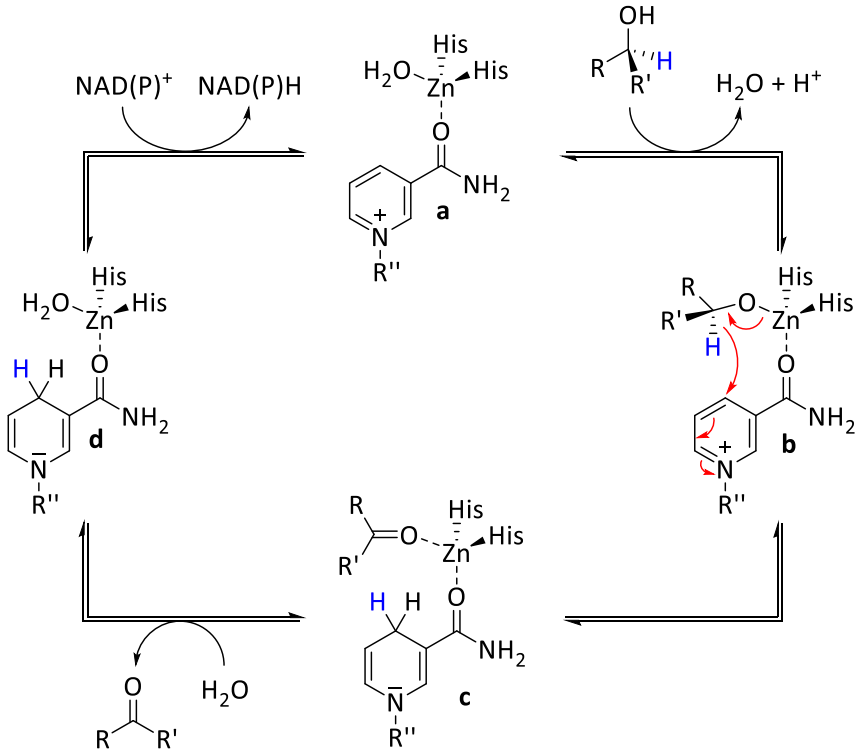
Scheme 34. Kinetic resolution of racemic β-hydroxysilane (*rac*)-**64** [135]

A further example was reported in 2006 when a secondary ADH from *Rhodococcus ruber* DSM 44541 (ADH-A) was found to catalyze the desymmetrisation of *meso*-2,5-hexanediol (**66**) yielding (*R*)-5-hydroxy-2-hexanone ((*R*)-**67**) (Scheme 35).[19,136]



Scheme 35. ADH-A catalyzed desymmetrisation of *meso*-2,5-hexanediol (**66**) yielding (*R*)-5-hydroxy-2-hexanone ((*R*)-**67**)[19,136]

The simplified mechanism of oxidations catalyzed by NADP(P)$^+$-dependent ADHs is depicted in Scheme 36.[104,130] The cofactor NAD(P)$^+$ binds to the protein by coordinating zinc at the ADHs active site. After the dissociation of water, the alcohol substrate is as well coordinated to the zinc of the ADH-NAD(P)$^+$-complex. A conformation change effects, that the catalytic domain moves closer to the cofactor to easify the hydride transfer. The first step in the redox reaction is the deprotonation of the alcohol. The resulting alkoxide ion is complexed by the active-site zinc to stabilize it and prevent extraction of an adjacent proton forming the alcohol substrate again (**b**). The second step is the hydride transfer from the alkoxide ion to NAD(P)$^+$. Simultaneously the double bond is formed (**c**). The release of the oxidized ketone is forced by the - at this point favored - recoordination of water to zinc (**d**). Finally the reduced cofactor NAD(P)H dissociates and the catalytic cycle is ready to restart from (**a**)(Scheme 36)[104,130]



Scheme 36. Simplified mechanism of the ADH-catalyzed oxidation[104]

Aim of this section is to realize the second, ADH-catalyzed oxidation step simultaneously to the first CYP BM-3 catalyzed oxidation step in a one pot process to synthesize the cyclic ketones **3** directly from the corresponding cycloalkanes **1**.

In the previous chapter 4, the first hydroxylation step, which is catalyzed by a cytochrome P450 monooxygenase (CYP BM-3), was investigated in detail. In order to complete the enzymatic two-step one-pot process that is based on the cooperation of two enzymes, a second alcohol dehydrogenase catalyzed oxidation step has to be implemented. In this way the oxidized form of the cofactor $NADP^+$, which is generated in the first CYP BM-3 catalyzed hydroxylation step, is retransformed into the reduced form NAD(P)H by the alcohol dehydrogenase. By this means the application of a further cosubstrate becomes redundant. Using this process, cyclic ketones **3** can be synthesized directly from the corresponding cycloalkanes **1**. BURDA applied the (*R*)-enantioselective alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) for the direct oxidation of cycloalkanes **1** to cycloalkanones **3** with oxygen in water and STAUDT extensively analyzed the synthesis of cyclooctanone (**3d**) in an aqueous reaction medium while using this system.[20-22] On the basis of these results, the application of this (*R*)-enantioselective alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) for the second oxidation step was adopted to complete the double oxidation sequence as well for the synthesis of further homologue cycloalkanones **3**.

With intent to make enzymatic applications even more attractive, the conversion in relation to the catalyst can be enormously enhanced by using the catalyst more often than once. In literature, several methods of enzyme immobilization are reported aiming to overcome general limitations of enzyme applications like the difficult recovery of the biocatalyst or low stability in organic syntheses.[137] In this way, the recycling of the (to some extent) costly biocatalysts is possible. According to SHELDON, the immobilization of enzymes can be distinguished into three groups, abridged in Figure 18.

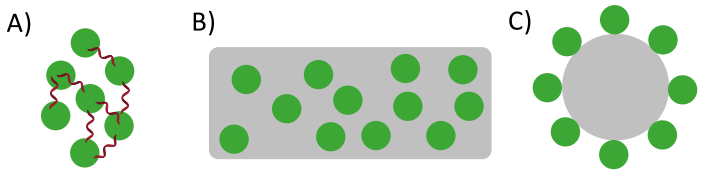
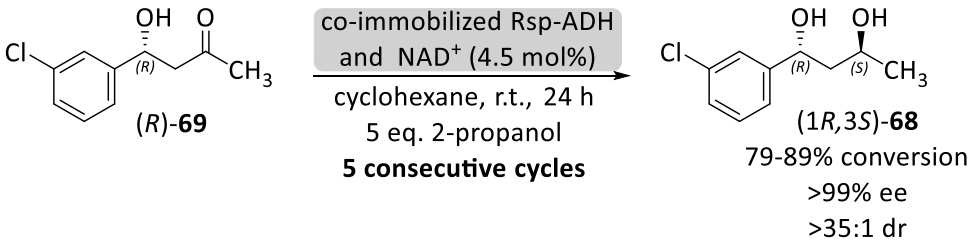


Figure 18. Three methods to immobilize enzymes (enzyme: ●; carrier: ●): A) Cross-Linking B) Entrapment C) Binding to a support [137]

While cross-linking (method A) is a carrier-free approach, encapsulation in a support (method B) resp. binding on a support (method C) is a carrier-based procedure. If covalent binding is used to attach the enzyme to a support, enzyme leaching from the surface of the carrier is prevented.[138] However it is a drawback of this generally robust method that the cofactor is not co-immobilized[139] and if the enzyme is deactivated, the whole system (biocatalyst and support) is useless.[137]

In 2009 it was reported by JEROMIN, that an entrapment of an ADH in a hydrogel matrix (superabsorbent polymer Favor SXM 9155) was accomplished, in which the required cofactor was co-immobilized. The application of this system in aqueous medium enabled a recyclization of the immobilized enzyme over five cycles, providing constantly high substrate conversion. The main disadvantage of this approach is the very high excess enzyme loading, that renders the application unattractive.[139] Recently RULLI *et al.* presented a further development of this encapsulation system for the synthesis of different 1,3-diols with drastically reduced enzyme loading (LK- resp. Rsp.-ADH). However, under these conditions no conversion was detected after the third cycle. These results emphasized that leaching in aqueous media is a problem to consider.[140] This problem was adressed by a most recent publication, describing the application of a co-immobilized, superabsorbed ADH in organic media (Scheme 37). In this way, the enzyme can be removed from its natural aqueous environment into a biphasic system with the result that leaching is suppressed and extraction of the product is redundant.[141]



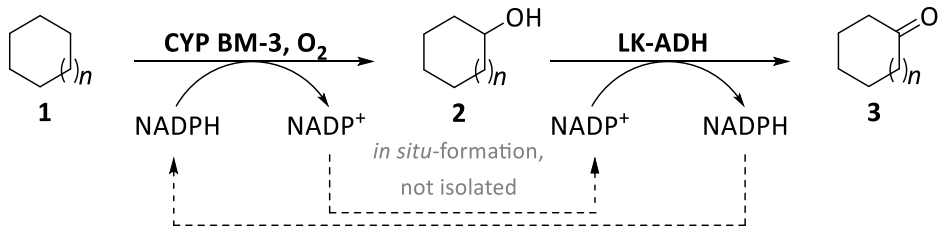
Scheme 37. Synthesis of the 1,3-diol (1*R*,3*S*)-**68** from (*R*)-**69** with high conversion and ee over five cycles[141]

The aim of this section is to analyze, if the recently presented achievement of HEIDLINDEMANN *et al*. can be generally transferred to the above described double oxidation system. For this purpose a co-immobilisate of enzymes, hereinafter referred to as 'superabsorbed enzyme tandem', consisting of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) and an alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) together with the cofactor NADPH is prepared and applied for the enzymatic two-step one-pot process.

5.2 Results and discussion

5.2.1 Photometric assay to determine the enzyme activity of the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH)

The recombinant ADH from *Lactobacillus kefir* (LK-ADH) turned out to be a suitable catalyst for the oxidation of cyclohexanol (**2a**) yielding cyclohexanone (**3a**). Very recently, in 2013, STAUDT *et al.* presented a proof of concept for the synthesis of the bulk chemical ε-caprolactone (**70**) in a biocatalytic one-pot process. This process is based on a double oxidation starting from cyclohexanol (**2a**), which is oxidized by an LK-ADH to provide cyclohexanone (**3a**). This key intermediate **3a** is then directly transformed into the desired product ε-caprolactone (**70**) with up to 97% conversion in a second oxidation step, catalyzed by a Baeyer-Villiger monooxygenase.[142] Based on these positive findings this ADH from *Lactobacillus kefir* (LK-ADH) was chosen for the second step of the double oxidation of cycloalkanes **1** shown in Scheme 38.

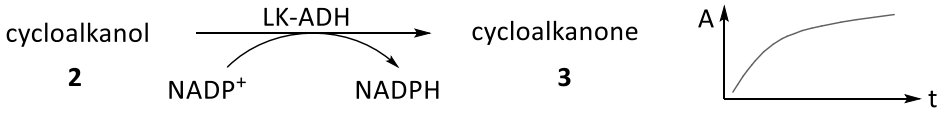


Scheme 38. Double oxidation of cycloalkanes **1** (*n* = 1, 3, 5)

Prior to a preparative biotransformation it is essential to determine the enzyme activities with regard to the selected substrates. The enzyme activities can be determined with an UV/VIS-spectrophotometer for $NADP^+$ is used as cofactor. The formation of NADPH through reduction of $NADP^+$ is measured spectrophotometrically at a wavelength of 340 nm in the presence of the LK-ADH and the respective substrate. This increase in extinction is recorded time-dependent by a spectrophotometer and is schematically shown in Scheme 39 on the right side. By means of the initial slope of the absorption curve and the following formula, the volumetric enzyme activity can be determined.

$$\frac{U}{mL} = \frac{\Delta E_{340nm}\, V_t\, f}{\varepsilon\, V_s\, t\, d}$$

With U/mL = volumetric enzyme activity; $\Delta E_{340nm}/t$ = initial slope of the absorption curve, V_t = total volume [mL]; f = dilution factor; ε = molar extinction coefficient for NAD(P)H [6.3 mL*µmol^{-1}*cm^{-1}]; V_S = sample volume [mL]; d = path length of the cuvette [cm]



Scheme 39. General reaction scheme of the photometric assay applying LK-ADH (left) and schematically plotted increase of NADPH over the course of the enzyme activity test (right)

Additionally to the respective substrates a reference compound is measured and its activity is defined as 100% in order to make a relative statement on the enzyme activities. The specific activities of the substrates are related to this value. Over the course of time, the reference compound 1-phenylethanol (**71**) as well as the substrates cyclohexanol (**2a**) and cyclodecanol (**2b**) were repeatedly measured and the results of the measurements were of the same order of magnitude (SOP 11). It is noticable that the substrate cyclodecanol (**2b**) is accepted 3 to 6 times better by the LK-ADH than the substrate cyclohexanol (**2a**), which is shown clearly in Figure 19.

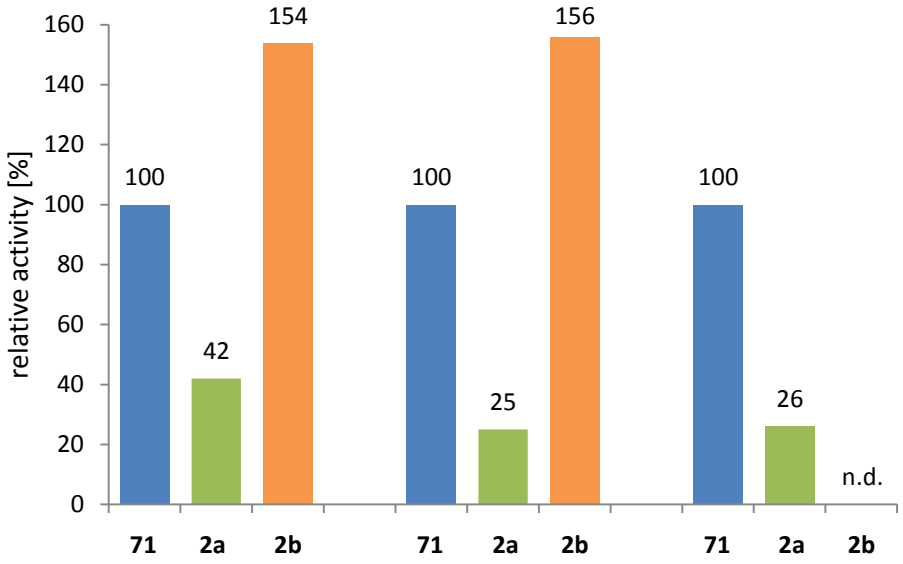


Figure 19. Relative activities of reference compound 1-phenylethanol (**71**) and the substrates cyclohexanol (**2a**) and cyclodecanol (**2b**) with LK-ADH, measured at different times (n.d. not determined)

An explanation can be found in the log-P-values of the substrate alcohols **2a** and **2b**. The octanol-water partition coefficient is a non-dimensional partition coefficient (log P) that describes the ratio of the concentrations of a compound in a biphasic system, containing of water and 1-octanol. By means of log P, the hydrophobicity of a compound can be deduced. While a high log P-value indicates for a hydrophobic compound, a low log P-value describes a more hydrophilic substance.[143,128] As apparent from Figure 20, cyclodecanol (**2b**) is composed of a larger cyclic alkyl chain than cyclohexanol (**2a**), making this substrate **2b** considerably more hydrophobic.

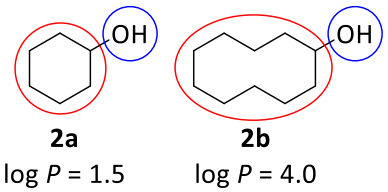
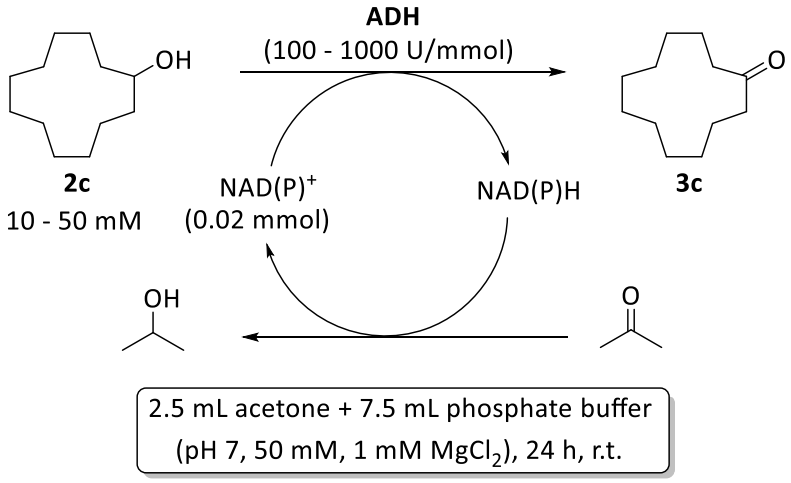


Figure 20. Cyclohexanol (**2a**) and cyclodecanol (**2b**) with differently sized hydrophobic areas (◯: hydrophobic, ◯: hydrophilic)[128]

A substrate has to pass a hydrophobic tunnel-like region[130] to reach the as well hydrophobic active site.[130,28] Therefore it seems to be obvious that the more hydrophilic substrate **2a** is accepted to a lesser extent.

5.2.2 Biocatalyzed oxidation of cyclododecanol (2c) with different alcohol dehydrogenases

Since the main focus of this thesis was on the first oxidation step catalyzed by a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (chapter 4), comparatively few experiments were conducted to oxidize cyclododecanol (**2c**) to cyclododecanone (**3c**) with alcohol dehydrogenases (Scheme 40).



Scheme 40. ADH-catalyzed oxidation of cyclododecanol (**2c**)

Despite knowing that only the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) is a suitable catalyst for the double oxidation as it is NADPH-dependent, two more NADH-dependent alcohol dehydrogenases, namely an alcohol dehydrogenase from *Rhodococcus species* (Rsp.-ADH) and the ADH evo-1.1.200 were tested in preparative experiments (SOP 12). The conversion was determined by comparison of the GC-areas of the substrate **2c** and the product **3c**. In order to verify this method, the simulated conversion is related to the actually measured conversion. The simulated conversion is obtained by weighting in the substrate **2c** and the product **3c** and is calculated to be 50.22%. The conversion determined by means of gas chromatography, 49.99%, is obtained by comparing the substance-specific surface integrals of **2c** and **3c** (SOP 12). The results are summarized in Table 10.

Table 10. Results of the ADH-catalyzed oxidation of cyclododecanol (**2c**) according to Scheme 40

Entry	ADH/µL or mL resp. mg	Activity[b)] [U/mmol]	Cofactor	Substrate [mmol]	Conversion [%]
1	LK-ADH/32.4 µL[a)]	100	$NADP^+$	0.5	2
2	Rsp.-ADH/2.5 mL[a)]	100	NAD^+	0.5	1
3	ADH evo-1.1.200/11.1 mg	100	NAD^+	0.5	2
4	LK-ADH/64.8 µL[a)]	1000	$NADP^+$	0.1	10
5	ADH evo-1.1.200/22.2 mg	1000	NAD^+	0.1	5

[a)]diluted 1:1 in glycerol; [b)]referred to the respective standard substrate (LK-ADH: acetophenone (**72**) (1544 U/mL[a)]); Rsp.-ADH: *p*-chloroacetophenone (**73**) (49 U/mL[a)]); ADH evo-1.1.200: ethyl acetoacetate (**63**) (4.5 U/mg)).

These preliminary experiments were performed in order to generally analyze if cyclododecanol (**2c**) is a substrate for the available alcohol dehydrogenases. However, the activities applied were referred to the oxidized form of the respective standard substrate (LK-ADH: acetophenone (**72**); Rsp.-ADH: *p*-chloro-acetophenone (**73**); ADH evo-1.1.200: ethyl acetoacetate (**63**) (Figure 21).

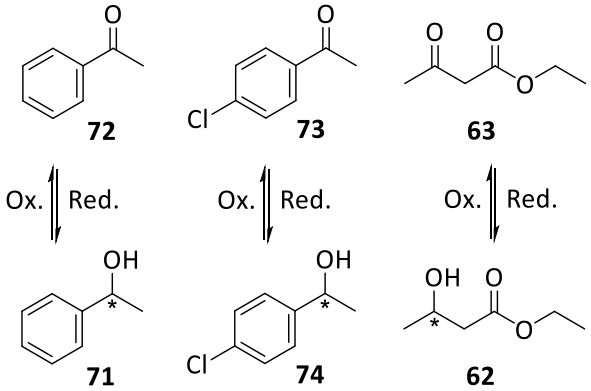


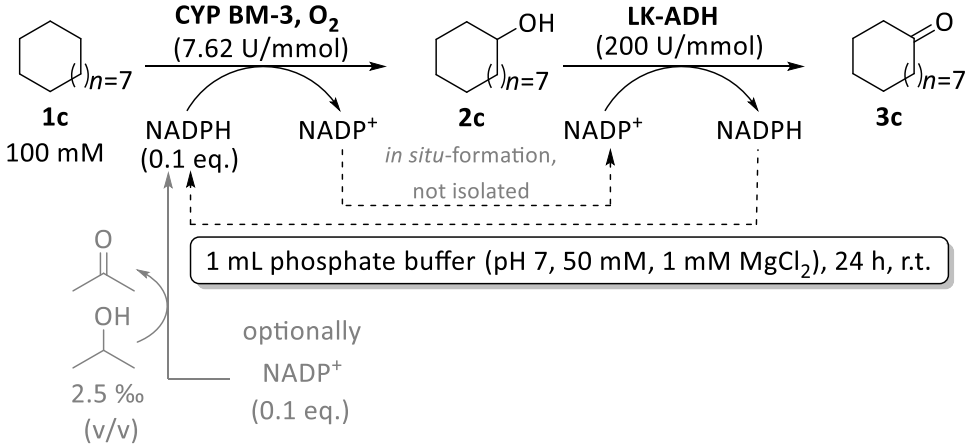
Figure 21. Structures of the standard substrates acetophenone (**72**) (LK-ADH), *p*-chloro-acetophenone (**73**) (Rsp.-ADH) and ethyl acetoacetate (**63**) (ADH evo-1.1.200) as well as the corresponding reduced forms 1-phenylethanol (**71**), *p*-chloro-1-phenylethanol (**74**) and ethyl 3-hydroxybutyrate (**62**)

For the performed oxidation reaction, which is not favoured compared to the reduction reaction, higher amounts of enzyme need to be applied, as an enzyme activity test has to be done with the reduced form of the standard substrates 1-phenylethanol (**71**), *p*-chloro-1-phenylethanol (**74**) and ethyl 3-hydroxy-butyrate (**62**) (Figure 21). Therefore, even better results can be expected if the experiments are repeated with redefined amounts of ADHs.

5.2.3 Biocatalyzed double oxidation of cyclododecane (1c) with CYP BM-3 and LK-ADH

The double oxidation with cyclododecane (**1c**) as substrate was performed twice and as no product formation was detected, the first oxidation step catalyzed by a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) was focused for the main part. Nevertheless an important discovery was made performing these experiments, based on the varying recovery of the substrate **1c** (SOP 13)(Table 11).

Table 11. Results of the double oxidation of cyclododecane (**1c**)



Entry	CYP BM-3 (biomass)	IPA	Cofactor	Cyclododecanone (**3c**) [g/l]]	Recovery of substrate **1c** [%]
1	19A12 (19.6 mg)[a)]	✓	$NADP^+$	0	92
2	F87V (127.0 mg)[b)]	-	NADPH	0	83

[a)]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b)]The content of CYP BM-3 F87V amounts to 0.525 µmol/g lyophilized crude extract.

As already described detailed in chapter 3, high recovery rates in the presence of biomass are not something that can be taken for granted and require an appropriate working up procedure. This correlation, namely the decreasing of the recovery rate with increasing biomass applied in an experiment, can be seen from both preparative double oxidations of cyclododecane (**1c**) summarized in Table 11. No product **3c** was detected and although same amounts of **1c** were applied, the recovery of **1c** deviated enormously. The explanation for this phenomenon is that for the biotransformation with the mutant CYP BM-3 19A12 only 19.6 mg protein contained the required activity (0.762 U referred to **1b**), whereas in contrast it was necessary to apply a more than sixfold higher amount of biomass, namely 127.0 mg protein by using the mutant CYP BM-3 F87V, in order to obtain the same activity.

This considerably increased amount of biomass manifests itself by the recovery rate of the substrate **1c**: while in the small enzyme pellet only traces of the substance **1c** are restrained, the more voluminous pellet is able to hold back higher amounts. A one-time extraction of the pellet (later mentioned as SOP A) is therefore insufficient to isolate the absorbed compounds completely (Figure 22).

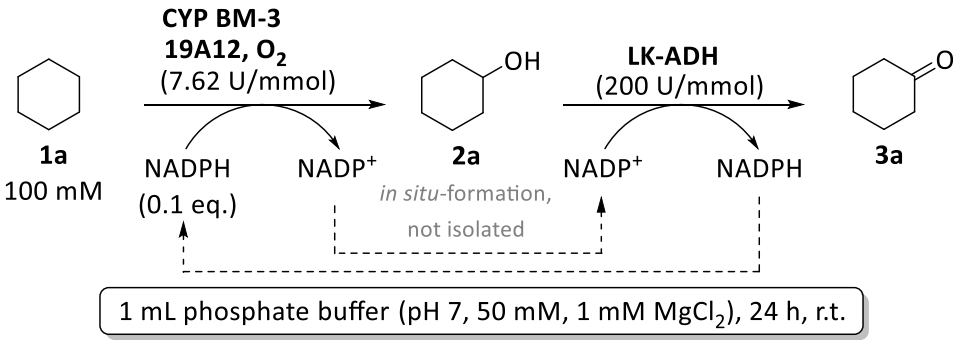
Figure 22. Enzyme pellet from 19.6 mg biomass (CYP BM-3 19A12) (left) and enzyme pellet from 127.0 mg biomass (CYP BM-3 F87V) (right)

Taking this into account and aiming to further improve the recovery rate, the working up process of the reaction mixture was optimized to the effect that - likewise the aqueous phase - the enzyme pellet is extracted three times (later mentioned as SOP B). This optimized extractive working up of the reaction mixture ensures a high recovery rate both, in presence and absence of an enzyme pellet.

5.2.4 Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM 3 and LK-ADH

The double oxidation of cyclohexane (**1a**) and cyclodecane (**1b**) was realized in the presence of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) in phosphate buffer. To start the reaction, cofactor NADPH is added (SOP 14). The results of the oxidation of **1a** are summarized in Table 12, whereby it is unmissable, that two experiments (entry 1-2) yield 0.41 g/L while two further experiments, that were conducted about one year later (entry 3-4) yield 0.98 g/L on an average.

Table 12. Results of the double oxidation of cyclohexane (**1a**) with CYP BM-3 19A12



En-try	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	NADPH [mol%]	Recovery/formation[d] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/5.2[a]	200/326	10	1.01	0.43	0.41
2	0.762/5.2[a]	200/326	10	1.05	n.d.[e]	0.40
3	0.762/10.3[b]	200/1057	10	0.61	0.53	0.96
4	0.762/10.3[b]	200/528[c]	10	1.61	0.16	0.99

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography; [e]n.d.: not determined, no clear assignment possible.

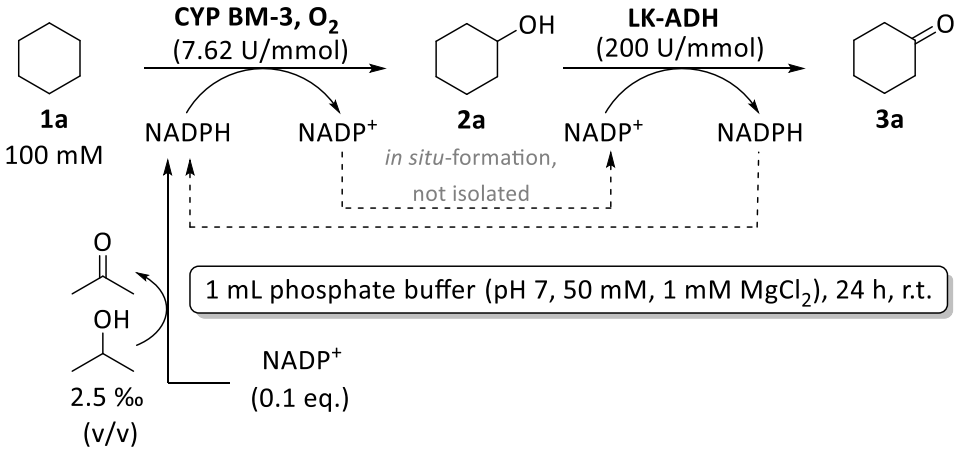
An explanation for this is the protein concentration of CYP BM-3 19A12, which was twice as high (entry 3-4: 10.3 mg) as in the previously conducted experiments (entry 1-2: 5.2 mg) and additionally, the amount of LK-ADH (528 µL crude extract resp. 1057 µL crude extract dil. 1:1 in glycerol instead of 326 µL crude extract dil. 1:1 in glycerol) was increased. This phenomenon is known as 'Macromolecular Crowding', describing the enhanced stability of an enzyme in the presence of higher total protein concentration.[144] For this purpose bovine serum albumin (BSA) is applied to stabilize proteins resp. enzymes and ranks among the most commonly used stabilizers in buffer solutions.[145]

The double oxidation of cyclodecane (**1b**) with CYP BM 3- F87V and LK-ADH yielded 0.20 g/L cyclodecanone (**1b**) but since slightly better results were reached for this substrate by adding an 2-propanol-starter, the biotransformation was generally conducted in the presence of 2-propanol.

5.2.5 Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH and addition of 2-propanol

By adding 2-propanol, the reduced cofactor form NADPH which is required for the initial hydroxylation step, is generated *in situ* from $NADP^+$. This approach is preferable for $NADP^+$ is more cost-effective than the reduced NADPH.[146,147] In Table 13 the results of the double oxidation of 0.1mmol **1a** with addition of 2-propanol (2.5‰ (v/v)) are summarized (SOP 15).

Table 13. Results of the double oxidation of **1a** with addition of 2-propanol



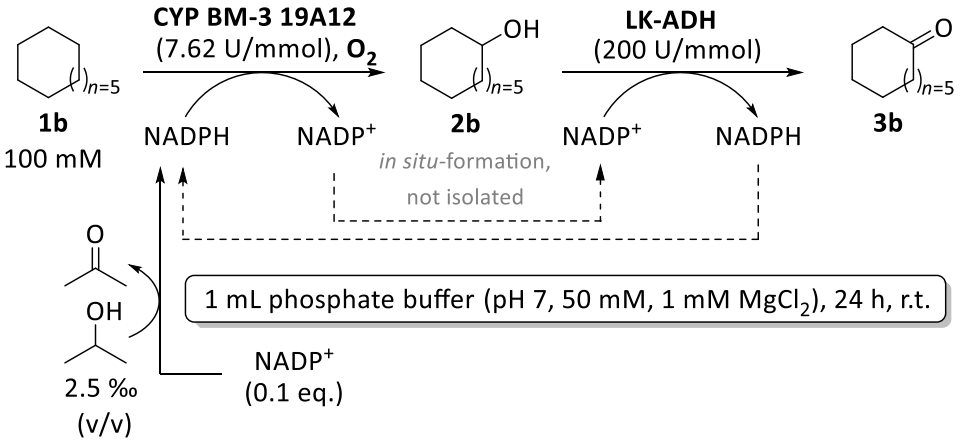
Entry	Activity CYP BM-3 [U]/[mg]	LK-ADH [U/mmol]/[µL]	$NADP^+$ [mol%]	Recovery/formation[c] [g/L] of …**1a**	…**2a**	…**3a**
1	WT 0.762/109.2[a]	200/326	10	0.75	0.25	0.35
2	19A12 0.762/5.2[b]	200/326	10	1.39	0.68	0.19
3	19A12 0.762/5.2[b]	200/326	10	0.91	0.40	0.18

[a]The content of CYP BM-3 WT amounts to 1.260 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [c]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

Higher product amounts (0.35 g/L) were obtained by applying the wildtype enzyme, probably due to the high protein loading and the associated macromolecular crowding. However, this extremely increased need for biomass renders this enzyme unattractive for further applications. The experiments with the mutant CYP BM-3 19A12 showed a product formation of 0.18 resp. 0.19 g/L that lag far behind the product formations without 2-propanol-starter (0.41 g/L)(5.2.4). This is the reason why this type of biotransformation was generally conducted in the absence of 2-propanol.

In Table 14 the results of the double oxidation of 0.1 mmol **1b** with addition of 2-propanol (2.5‰ (v/v)) are summarized (SOP 15).

Table 14. Results of the double oxidation of cyclodecane (**1b**) with CYP BM-3 19A12



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	$NADP^+$ [mol%]	Recovery/formation[c] [g/L] of …**1b**	…**2b**	…**3b**
1	0.762/19.6[a]	200/91	10	7.92	0.00	0.24
2	0.762/14.9[b]	200/166	10	9.66	0.00	0.27
3	0.762/14.9[b]	200/166	10	9.38	0.00	0.27
4	0.762/14.9[b]	200/166	10	10.28	0.00	0.25

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

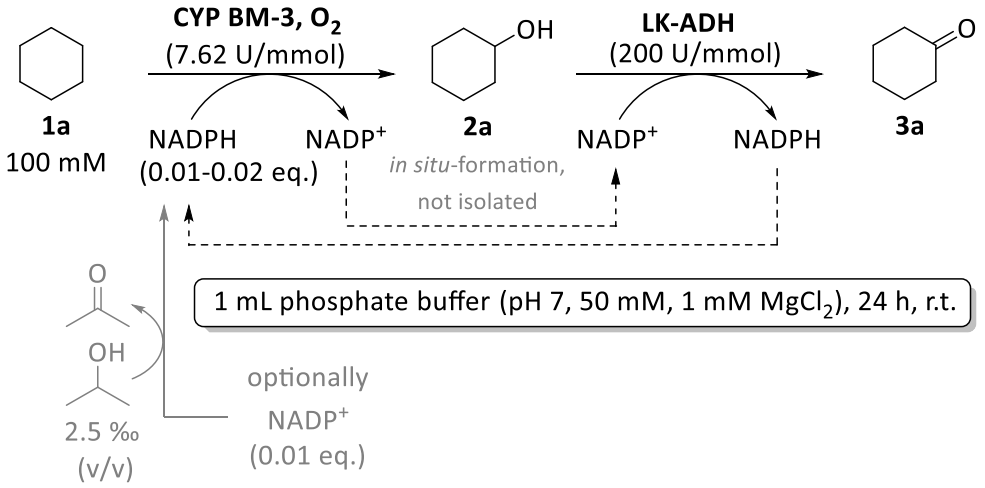
Entry 1 shows one of the early experiments of the double oxidation of cyclodecane (**1b**) with CYP BM-3 19A12 and LK-ADH, in which a product formation of 0.24 g/L cyclodecanone (**1b**) was detected. Since the double oxidation of **1b** without the 2-propanol-starter gave slightly poorer results (0.20 g/L) this type of biotransformation was generally conducted in the presence of 2-propanol. A reason for this phenomenon, namely the slightly higher product formation in the presence of low concentrations of 2-propanol (2.5‰ (v/v)) could be the following: Not only the amount of NADPH, necessary to start the first step of the biotransformation is generated by this system. Also the wastage of NADPH, an undesired result from three possible additional shunt pathways consuming NADPH decoupled from the substrate oxidation, is compensated by the regeneration of NADPH with 2-propanol. The experiments shown in entry 2-4, were conducted one year after the experiment shown in entry 1 and the obtained result of entry 1 could be reproduced precisely accurate for three times.

Like in the experiments without 2-propanol-starter (5.2.4) no intermediate was detected in contrast to the conversion of **1a**. But if the result of the enzyme activity test is regarded (5.2.1), where it is shown that the substrate cyclodecanol (**2b**) is accepted 3 to 6 times better by the LK-ADH than the substrate cyclohexanol (**2a**), it becomes clear that **2b** is quickly converted into **3b** and therefore did not accumulate.

5.2.6 Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH with reduced amount of cofactor

Aiming to find out if an economically profitable reduction of the high-priced cofactor quantity - and therefore a notable decrease of the total turn over number (TTN) - is possible, several experiments are conducted with reduced amount of cofactor. Compared to the experiments described so far in which generally 10 mol% of cofactor were applied, the input of only 1 or 2 mol% cofactor was analyzed (SOP 16). The results of the double oxidation of 0.1 mmol **1a** with addition of only 1 or 2 mol% cofactor are summarized in Table 15.

Table 15. Results of the double oxidation of cyclohexane (**1a**) with different concentrations of cofactor NADPH



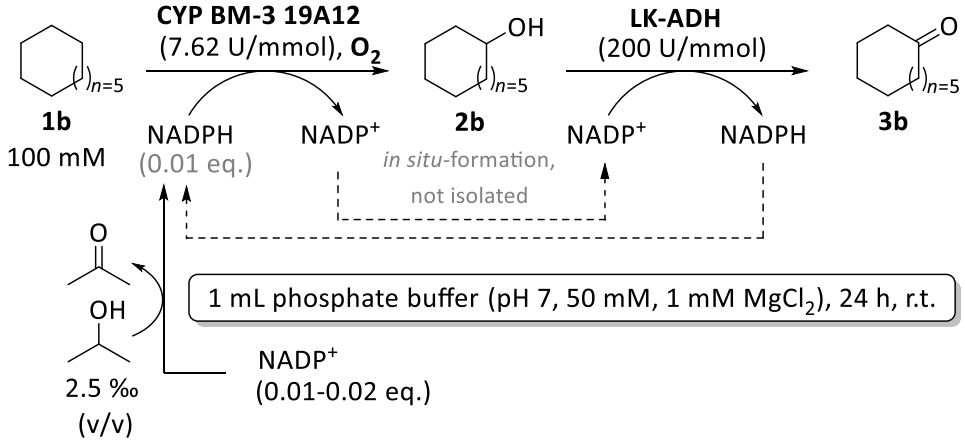
En-try	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	NADP(H)	Recovery/formation[e] [g/L] of		
				…**1a**	…**2a**	…**3a**
1	0.762/5.2[a]	200/825	1 mol%	0.42	0.09	0.08
2	0.762/5.2[a]	200/825	1 mol%	1.04	0.09	0.10
3	0.762/5.2[a]	200/825	2 mol%	1.07	0.11	0.11
4	0.762/5.2[a]	200/825	2 mol%[d]	0.98	0.09	0.10
5	0.762/10.3[b]	200/528[c]	1 mol%	0.31	0.19	0.28
6	0.762/10.3[b]	200/528[c]	10 mol%	1.61	0.16	0.99

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]each 1 mol% cofactor in oxidized + reduced form; [e]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

For the experiments shown in entry 1-4, always the same amount of both enzymes (CYP BM-3 and LK-ADH) was applied to obtain comparable results. The product formation is pretty much the same employing 1 or 2 mol% of NADPH. Also the application of both, each 1 mol % of the reduced as well as of the oxidized form of cofactor gave no notable increase on the product formation. This is not surprising for the initially necessary NADPH in den comparative tests is not formed *via* a 2-propanol-starter. In entry 5, the amount of CYP BM-3 19A12 was twice as high (10.3 mg) as in the previously conducted experiments (entry 1-4; 5.2 mg) and additionally the amount of LK-ADH (528 µL crude extract instead of 825 µL crude extract diluted 1:1 in glycerol) is increased. As already depicted in 5.2.4, macromolecular crowding is, besides an optimized work up, hence the explanation for this threefold higher product formation.

Comparing the experiments in entry 5 and 6, that differ only in the amount of cofactor, it is apparent that scarcely ⅓ of the benchmark product formation can be accomplished with 1/10 amount of cofactor. The results of the double oxidation of 0.1mmol **1b** with addition of only 1 or 2 mol% cofactor are summarized in Table 16.

Table 16. Results of the double oxidation of cyclodecane (**1b**) with different concentrations of cofactor $NADP^+$



En-try	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	$NADP^+$	Recovery/formation[e] [g/L] of …**1b**	…**2b**	…**3b**
1	0.762/19.6[a]	200/241	1 mol%	7.34	n.d.[f]	0.26
2	0.762/19.6[a]	200/241	1 mol%	3.25	n.d.[f]	0.29
3	0.762/19.6[a]	200/241	2 mol%	7.08	n.d.[f]	0.25
4	0.762/19.6[a]	200/241	2 mol%[d]	4.23	n.d.[f]	0.35
5	0.762/14.9[b]	200/83[c]	1 mol%	7.88	n.d.[f]	0.22
6	0.762/14.9[b]	200/166	10 mol%	9.38	n.d.[f]	0.27

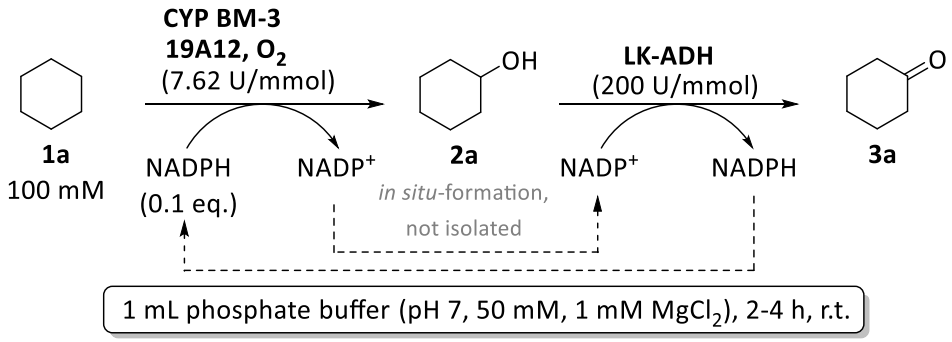
[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]each 1 mol% cofactor in oxidized + reduced form; [e]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography; [f]not detected.

Entry 6 shows the benchmark experiment with a product formation of 0.27 g/L. The biotransformations applying only 1-2 mol% $NADP^+$ gave nearly the same product formation which was 0.26 g/L on an average (entry 1-3, 5). A slightly higher product formation was realized when using each 1 mol% of the reduced as well as of the oxidized form of cofactor (entry 4). This is not surprising since only half of the amount of the cofactor NADPH has to be produced *in situ* and the reaction could start immediately without waiting for the starter-step.

5.2.7 Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH with reduction of the reaction time

Since a shortened reaction time enables the performance of more experiments and decreases the consumption of resources, the biotransformationes with cyclohexane (**1a**) as well as with cyclodecane (**1b**) as substrates were analyzed with regard to this parameter (SOP 17). The results of the biotransformation of cyclohexane (**1a**) are summarized in Table 17 and can be compared easily to the benchmark experiment (entry 3), for the same amounts of both enzymes (CYP BM-3 19A12 and LK-ADH) were applied.

Table 17. Results of the double oxidation of cyclohexane (**1a**) with shortened reaction time

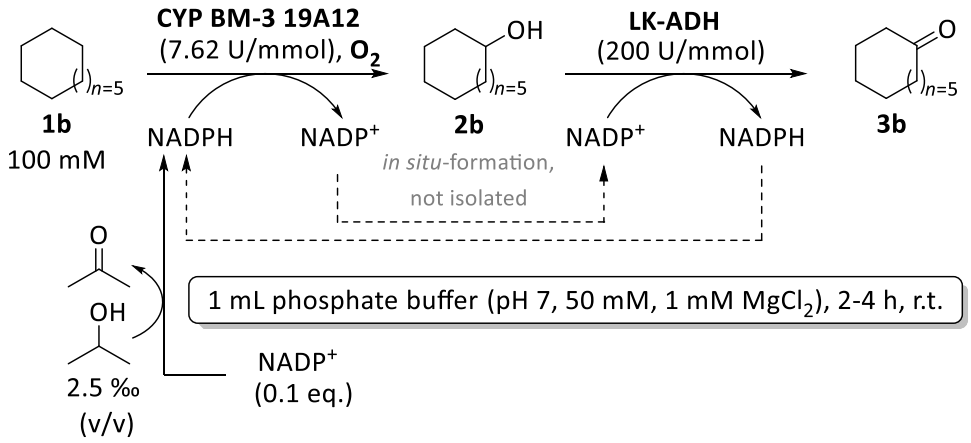


Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Time [h]	Recovery/formation[c] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/10.3[a]	200/528[b]	2	1.09	0.22	0.37
2	0.762/10.3[a]	200/528[b]	4	0.66	0.23	0.51
3	0.762/10.3[a]	200/528[b]	24	1.61	0.16	0.99

[a]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b]Addition of LK-ADH without dilution in glycerol; [c]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

After 4 h, which is one sixth of the generally used reaction time of 24 h, the product formation was already at 0.51 g/L, therefore the half of 0.99 g/L. The results of the biotransformation of cyclodecane (**1b**) are summarized in Table 18.

Table 18. Results of the double oxidation of cyclodecane (**1b**) with shortened reaction time



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Time [h]	Recovery/formation[b] [g/L] of		
				…**1a**	…**2a**	…**3a**
1	0.762/14.9[a]	200/166	2	11.81	0.00	0.21
2	0.762/14.9[a]	200/166	4	10.98	0.00	0.23
3	0.762/14.9[a]	200/166	24	9.38	0.00	0.27

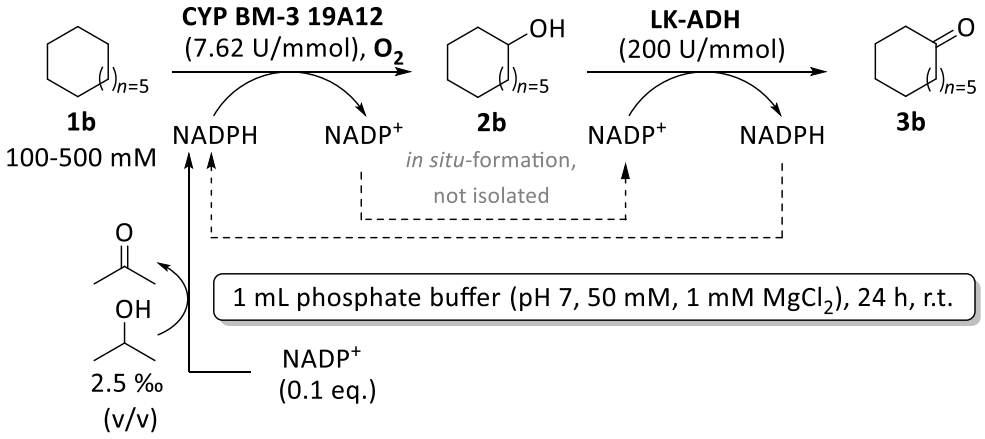
[a]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

The experiments using cyclodecane (**1b**) as substrate are - in contrast - more easy to abbreviate, for the shortening of the reaction time brought along only a marginal decrease of the product formation. The reason for this divergence - namely the different product formation of **3a** and **3b** at shortened reaction times of the double oxidation - can be found in the fact that the biotransformation of **1b** is faster compared to the biotransformation of **1a**. Cyclodecanol (**2b**) is accepted 3 to 6 times better by the LK-ADH than cyclohexanol (**2a**) what makes the reaction faster and provides the reduced form of the cofactor more quickly to the first oxidation step.

5.2.8 Biocatalyzed double oxidation of cyclodecane (1b) with increased initial substrate concentration

To compare the influence of the substrate concentration on the product formation, one experiment with fivefold substrate concentration was conducted (SOP 18). The results are summarized in Table 19.

Table 19. Results of the double oxidation of cyclodecane (**1b**) with increased substrate concentration



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	**1b** [mmol]	Recovery/formation[b)] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/14.9[a)]	200/166	0.1	10.28	0.00	0.25
2	0.762/14.9[a)]	200/166	0.5	64.32	0.00	0.82

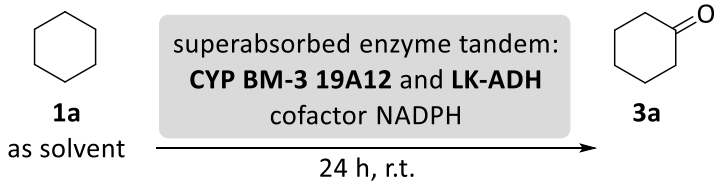
[a)]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b)]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

The increase of the substrate concentration leads to higher product formations, but the correlation is not linear. However, this slightly more than three-times higher product formation can easify the analytic that is quite complex at low product formations.

5.2.9 Double oxidation of cyclohexane (1a) using a superabsorbed enzyme tandem

Aiming to make the presented biotransformation even more attractive by enhancing the conversion in relation to the catalysts by using the catalysts more often than once, a co-immobilisate of enzymes, hereinafter referred to as 'superabsorbed enzyme tandem', was prepared (SOP 19). This superabsorbed enzyme tandem consisted of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) and an alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) together with the cofactor NADPH and was applied for the enzymatic two-step one-pot process. To obtain this hydrogel matrix, the superabsorbent polymer Favor SXM 9155 was employed. The results of the biotransformation are summarized in Table 20 (SOP 20).

Table 20. Double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem

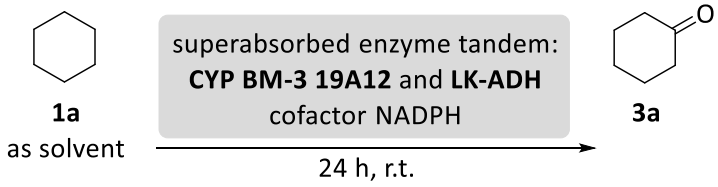


Entry[a)]	Method (SOP 19)	Flask	Cyclohexane (**1a**) [g/L]	Cyclohexanol (**2a**) [g/L]	Cyclohexanone (**3a**) [g/L]
1	A	10 mL	--[d)]	0.00	0.15
2	B	50 mL[c)]	--[d)]	0.00	0.16
3[b)]	B	50 mL[c)]	--[d)]	0.00	0.18

[a)]GC-measurements are conducted in a twofold determination; [b)]Extraction as described in SOP 20, but instead of dichloromethane, **1a** (saturated with water to avoid the unwanted reduction of the aqueous quantity) was used; [c)]Enlargement of the air volume in the flask to rule out the deficiency of molecular oxygen; [d)]Concentration of **1a** is higher than the detection limit of the straight calibration line.

As apparent from Table 20, the product formation was five to six times lower than in the benchmark experiment using non-immobilized free catalysts, where 0.99 g/L of **3a** were formed (5.2.4). Even in the experiments shown in entry 2-3, in which the immobilisate contained twice the amount of all components (SOP 19) no increase was observed. The reason for this phenomenon is most likely to be found in the course of process engineering. The preparation of the heterogenized enzyme tandem was performed according to HEIDLINDEMANN *et al*.[141], keeping the ratio aqueous phase/superabsorbent polymer constant with the result that a quite voluminous hydrogel was formed. It is easy conceivable that the reaction proceeds mainly on the surface of the immobilisate while the inlying enzymes are passive. Additionally, the proteins on the surface might be denaturated due to the contact with organic solvent. The extraction of the hydrogel by gently swirling might be not enough to isolate the product completely. The addition of 5 mL cyclohexane (**1a**) were necessary to surround the voluminous hydrogel with solvent. The decision to use the substrate as solvent arose from the fact that many common solvents are as well accepted as substrates by the cytochrome P450 monooxygenase. This increases the over-all substrate concentration enormously. Despite the increased substrate concentration and the complete missing of a further solvent, the reaction takes place and a proof of concept for the co-immobilization of two enzymes and the cofactor to realize the above presented double oxidation of cycloalkanes **1** was given. Also the recycling of the co-immobilisate was analyzed (SOP 20). However, the system showed no conversion after the second cycle and could be optimized (Table 21).

Table 21. Double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem for 1-3 cycles



Entry[a)]	Cycle	Cyclohexane (**1a**) [g/L]	Cyclohexanol (**2a**) [g/L]	Cyclohexanone (**3a**) [g/L immobilized aqueous phase]
1	1	--[b)]	0.00	0.18
2	2	--[b)]	0.00	0.04
3	3	--[b)]	0.00	< 0.01

[a)]GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a twofold dataset is calculated; [b)]Concentration of **1a** over the detection limit of the straight calibration line.

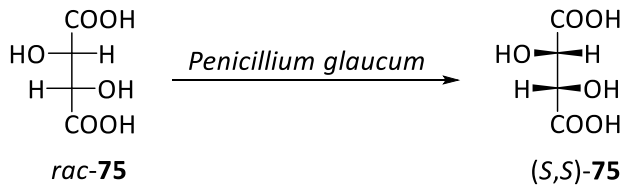
A possible explanation why the system shows no conversion after the second cycle is the denaturation of the biocatalyst on the outer shell of the immobilisate. Furthermore the incomplete extraction after the first (and every following reaction cycle) leads to the accumulation of organic materials in the hydrogel compartiment, causing a destabilization of the biocatalyst.

6 Biocatalyzed double oxidation of cycloalkanes 1 *via* whole cell catalysis

6.1 Introduction, state of the art and aim of this section

Already in ancient times, mankind employed microorganisms - and therefore catalytically active whole cells - within daily life. Completely unaware of their tiny assistants the Sumerians from Mesopotamia started to brew beer since 6000 BC. Today it`s known that for the activation of the fermentation process the dominant operating organism is *Saccharomyces cerevisiae*, already present on the barley. The situation is equal to the wine: Squashed berry juice was fermented yielding alcohol by yeast growing on the berries. As well as the production of these fermented beverages, the manufacturing of bread and cheese is based on the application of living microorganisms.[148,149]

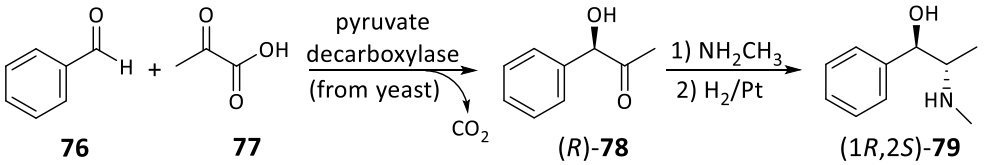
For preparative and scientific purposes, microorganisms were applied 1858 for the first time by L. PASTEUR when he realized the first microbial kinetic resolution with the fungus *Penicillium glaucum*. This fungus is converting exclusively the (*R,R*)-tartaric acid ((*R*,*R*)-**75**) of racemic ammonium tartrate (*rac*-**75**), not touching the (*S*,*S*)-tartaric acid ((*S*,*S*)-**75**). The non-metabolizable enantiomer is accumulating to an excess amount of (*S*,*S*)-tartaric acid ((*S*,*S*)-**75**) (Scheme 41).[132,150,]



rac-**75** (*S*,*S*)-**75**

Scheme 41. Kinetic resolution of racemic tartaric acid (*rac*-**75**) yielding (*S*,*S*)-tartaric acid ((*S*,*S*)-**75**)

But not only on a laboratory scale whole cell biotransformations are highly successful. One of the first biocatalytic reactions using whole cells as catalysts for the industrial synthesis of considerable intermediates is shown in Scheme 42 and further outstanding processes shoud be developed.[151] In 1921, NEUBERG and HIRSCH published a procedure in which benzaldehyde (**76**) and pyruvate (**77**) undergo C-C bond formation in the presence of *Saccharomyces cerevisiae*, yielding the optically active key intermediate (*R*)-phenyl-acetylcarbinol ((*R*)-**78**).[152] Applying two further chemical steps, the nearly enantiomerically pure L-ephedrine ((1*R*,2*S*)-**79**) is easily synthesized from the precursor (*R*)-**78** (Scheme 42).

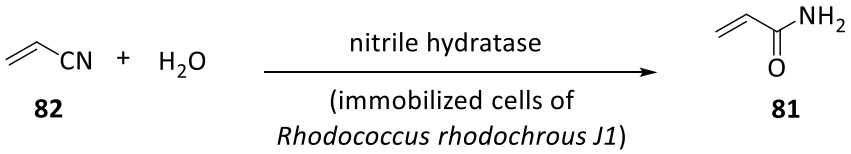


76 **77** (*R*)-**78** (1*R*,2*S*)-**79**

Scheme 42. L-Ephedrine production in the presence of yeast and two further chemical steps, since 1930 by Knoll-AG, Ludwigshafen (Germany)[8,151-154]

For the purpose of cost- and atom efficiency, acetaldehyde (**80**) can be employed in preference to pyruvate (**77**).[8] This is one of the oldest biocatalytic processes still applied, for ephedrine **79** is synthesized until today following this procedure.[154]

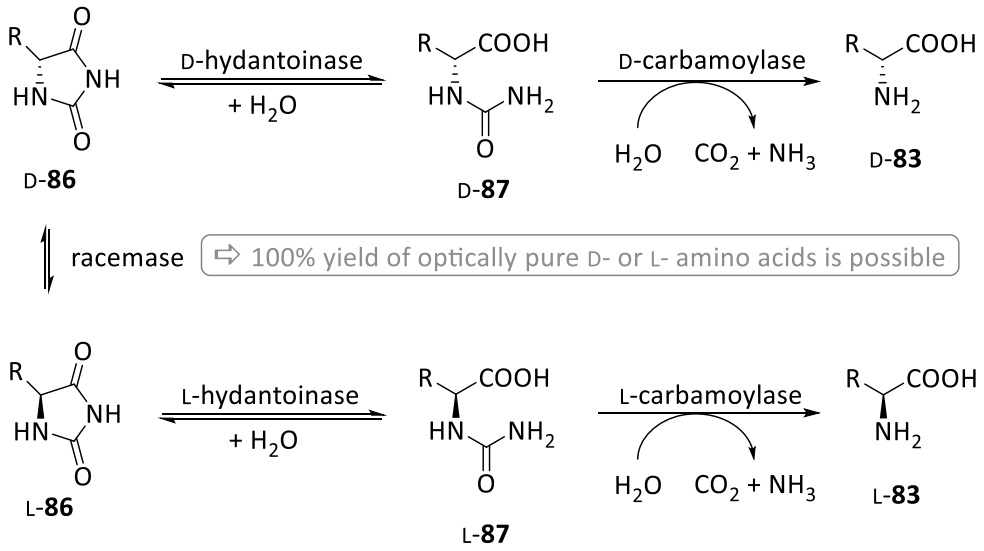
Another important process based on whole cell catalysis, the synthesis of acrylamide (**81**) from acrylonitrile (**82**), is often serving as prime example to demonstrate the commercial relevance of biocatalysis in industry. Acrylamide (**81**) ranks among the most significant chemical commodities for it is applied as versatile starting material for polymers. This importance is mirrored by the global consumption of about 200 000 tons per year worldwide.[132] Quite recently, in the year 2013, it was published that the reaction shown in Scheme 43 is now performed on a scale of > 40 000 tons per year, which represents ⅕ of the global demand.[155]



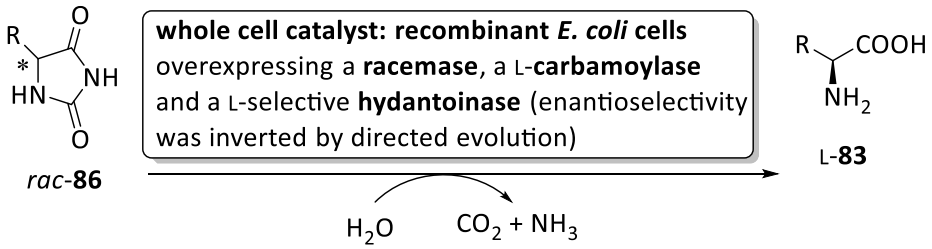
82 **81**

Scheme 43. Acrylamide-Process based on immobilized whole cells, since 1985 by Nitto Chemical Ltd (Japan)

The last example that should emphasize the enormous significance of whole cells in biocatalysis is the hydantoinase process. The D-hydantoinase process (Scheme 44, above) is applied since the 1970s, providing a direct access to D-amino acids (D-**83**) and in this particular case to D-phenylglycine (D-**83a**) and *p*-hydroxyphenyl-glycine (D-**83b**). Nowadays, more than 1000 tons per year of D-**83a** and D-**83b** are produced as these compounds serve as side chain building blocks for the β-lactam antibiotics ampicillin **84** and amoxicilin **85**.[156] A process of highest importance is without doubt the L-hydantoinase process (Scheme 44, below), producing L-amino acids (L-**83**) with the aid of a tailor-made recombinant whole-cell biocatalyst (Scheme 45).[156]



Scheme 44. Reaction scheme of the D-hydantoinase process (above) and the L-hydantoinase process (below) for the production of D- resp. L-amino acids (D-**83** resp. L-**83**), starting from D- resp. L-hydantoin (D-**86** resp. L-**86**) *via* the corresponding D- resp. L-carbamoyl amino acid (D-**87** resp. L-**87**) [156]



Scheme 45. Reaction scheme of the L-hydantoinase process yielding L-amino acids (L-**83**) [156]

As pointed out above, there are several processes that are based on whole cell biocatalysts and their application is known to have clear advantages. There is no universal answer to the question if a certain reaction should be conducted in the presence of whole cells or isolated enzymes. Some criterions helpful to balance the pros and cons are - without raising a claim to completeness - summarized in Table 22.[18]

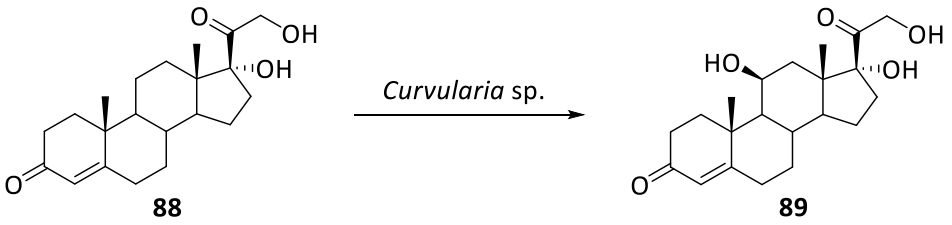
Table 22. Pros and cons of non-recombinant whole cells and isolated enzymes as catalysts according to JEROMIN[18]

Non-recombinant whole cells as catalyst	Isolated enzymes as catalyst
Cofactor recycling occurs in the cell	Demand for expensive cofactors in stoichiometric amounts or need for an external cofactor regeneration system
Side reactions are characteristic for a multi-enzyme system, a previous statement concerning the selectivities is difficult	Side reactions are rare for this highly selective and definable system what makes the reaction good manageable and a process more controllable
Biotransformations with extracellularly not stable or applicable enzymes are possible. However, the substrate has to overcome the cell membrane to enter the cytosol	Absence of a protective cell membrane requires suitable reaction conditions to avoid the denaturation or the complete loss of activity of the enzyme
Working up might cause difficulties as a result of the biomass	Working up is traditionally rather easy

Due to these advantages, especially with regard to the process management, it is worthy to analyze if results that are obtained with isolated enzymes as catalysts can be reproduced in a comparative whole cell biotransfomation. In particular problems like side reactions, catalyzed by other enzymes in the wildtype cells, can be avoided by using recombinant whole cell catalysts.

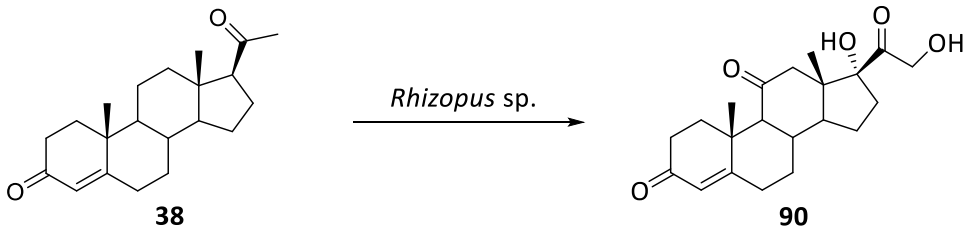
In the case of cytochrome P450 monooxygenases (CYPs), this approach is promising for there are already several whole cell biotransformations with this class of enzymes. Indeed, the technical application of these enzymes is still constrained to a limited number of whole cell processes[67], recently reviewed by BERNHARDT and URLACHER.[157] The reason behind this fact is that isolated enzymes are considered to be inappropriate basically because of their low stability, their stoichiometric demand for the high priced cofactors NAD(P)H and the low solubility of many substrates in aqueous reaction media.[67] Indeed it is not necessary to add stoichiometric amounts of cofactor if a regeneration system is applied. However, additional cofactor has to be regenerated to compensate the losses within the shunt pathways.

Microbial oxidations of steroids are good examples of commercial applications of cytochrome P450 monooxygenases. 11-Deoxycortisol (**88**) for example can be transformed into hydrocortisone (**89**) *via* hydroxylation catalyzed by a CYP from *Curvularia* sp. This process is applied by Schering AG (in 2006 acquired by Merck, Germany) and performed on an industrial large scale of about 100 tons per year (Scheme 46).[94,157,158]



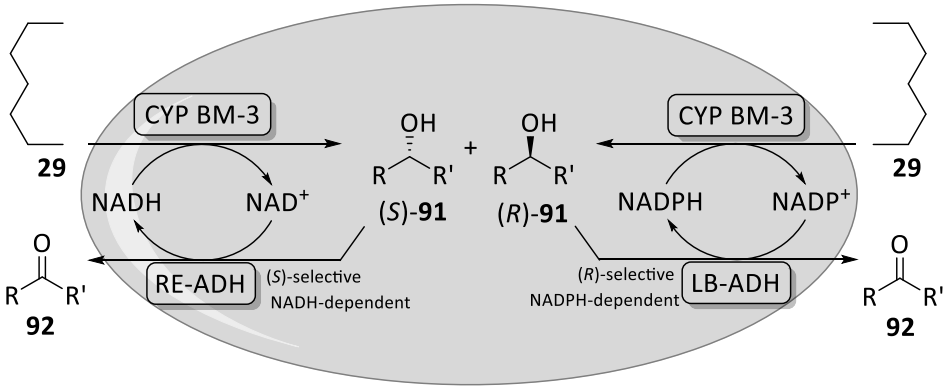
Scheme 46. Reaction scheme of the hydroxylation of 11-deoxycortisol (**88**)

A further example already established in the 1950s is the application of *Rhizopus* sp. for the regioselective hydroxylation of progesterone (**38**) yielding cortisone (**90**) (Scheme 47) conducted by Pharmacia & Upjohn (meanwhile acquired by Pfizer Inc., USA).[157,159]



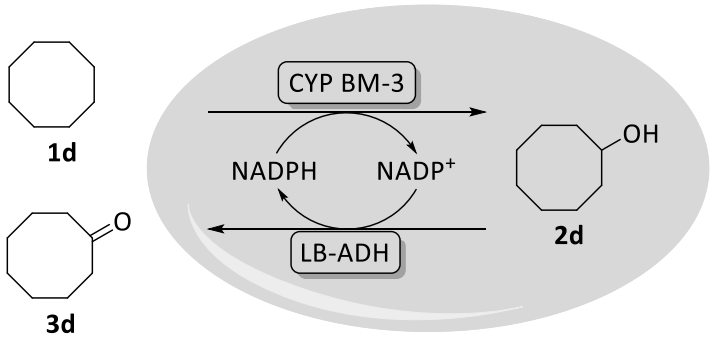
Scheme 47. Reaction scheme of the regioselective hydroxylation of progesterone (**38**) yielding cortisone (**90**)

But also in the particular case of CYP-based whole cell biotransformations with (cyclic) alkanes as substrates researchers already started their investigations.[31,32] Taking their results into consideration, there is a reasonably confidence for the quite similar substrates cyclohexane (**1a**) and cyclodecane (**1b**) applied in this work. The first whole cell double oxidation of *n*-heptane (**29**) was recently reported by MÜLLER *et al.*[31,32] and is shown in Scheme 48. While the two-enzyme system (consisting of CYP BM-3 and one ADH) enables the chiral resolution of the (*R*)- resp. (*S*)-heptanols ((*R*)- resp. (*S*)-**91**) (RE-ADH: (*S*)-selective; LB-ADH: (*R*)-selective), the three-enzyme system converts the alcohols **91** completely into the corresponding ketones **92**.[31,32]



Scheme 48. Reaction scheme of the whole cell double oxidation of *n*-heptane (**29**) with a three-enzyme catalyst, converting the intermediates (*R*)- resp. (*S*)-heptanols ((*R*)- resp. (*S*)-**91**) completely into the corresponding ketones **92**[31,32]

This concept was furthermore successfully adapted to cyclooctane (**1d**) and the application of the three-enzyme system CYP BM-3 19A12^{NADH}-RE-ADH-LB-ADH yielded 94% cyclooctanone (**3d**) (652 mg/l). With the two-enzyme system containing CYP BM-3 19A12-LB-ADH the third highest TTN of 23700 for CYP BM-3 was achieved (Scheme 49).[32]



Scheme 49. Reaction scheme of the whole cell double oxidation of cyclooctane (**1d**) yielding the corresponding ketone **3d**[32]

Cyclohexane (**1a**) and cyclodecane (**1b**) turned out to be the most promising substrates in both, the monohydroxylation experiments and the double oxidation approaches. In this section it is analyzed if the cyclic alkanes cyclohexane (**1a**) and cyclodecane (**1b**) can be applied as substrates in a whole cell biotransformation as good as the encouraging substrate cyclooctane (**1d**).[32]

6.2 Results and discussion

6.2.1 Cultivation of the recombinant cells

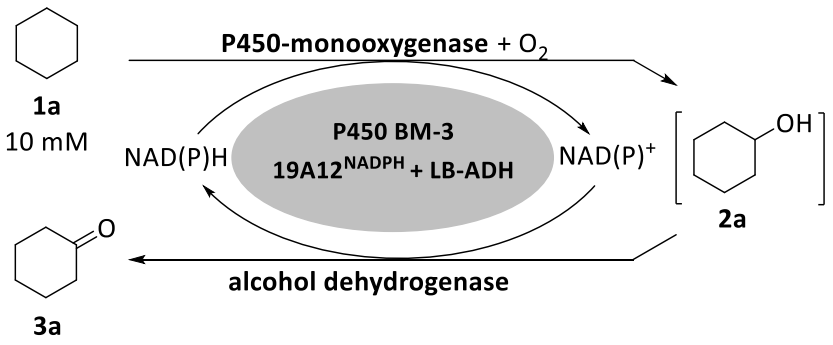
To investigate cyclohexane (**1a**) and cyclodecane (**1b**) in a whole cell biotransformation two different constructs were applied. These two constructs (*E. coli* BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}) and *E. coli* BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R})) were previously used in the

above-mentioned biotransformation of cyclooctane (**1d**).[31,32] In both cases recombinant cells from *E. coli* are used in which the respective enzymes were coexpressed. At one point, the monooxygenase and the LB-ADH were coexpressed on two different plasmids while the other time, the RE-ADH was coexpressed on the same plasmid with the monooxygenase while the LB-ADH was coexpressed on a second plasmid. The strains were cultivated according to SOP 21 and for the whole cell experiments the cells were defrosted and suspended in buffer.

6.2.2 Oxidation of cyclohexane (1a) using a two- and a three-enzyme system

In this section the whole cell biotransformation of cyclohexane (**1a**) is described which was carried out according to SOP 22. The reaction scheme is shown in Table 23 - Table 24 and the model reaction was studied at different reaction times. The concentration of the substrate **1a**, the intermediate **2a** and the product **3a** was analyzed *via* gas chromatography and the results are summarized in Figure 23 - Figure 24.

Table 23. Oxidation of cyclohexane (**1a**) using construct A, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}); OD_{600} = 40.2, CDW = 8.7 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1a** [g/L]	**2a** [g/L]	**3a** [g/L]	Σ**2a**+**3a** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	0.5	0.06	0.10	0.02	0.12	14
2	1	0.01	0.16	0.08	0.24	28
3	2	0.02	0.21	0.14	0.35	40
4	4	0.03	0.26	0.15	0.41	94
5	18	0.02	0.36	0.04	0.40	47
6	24	0.02	0.38	0.04	0.42	48

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

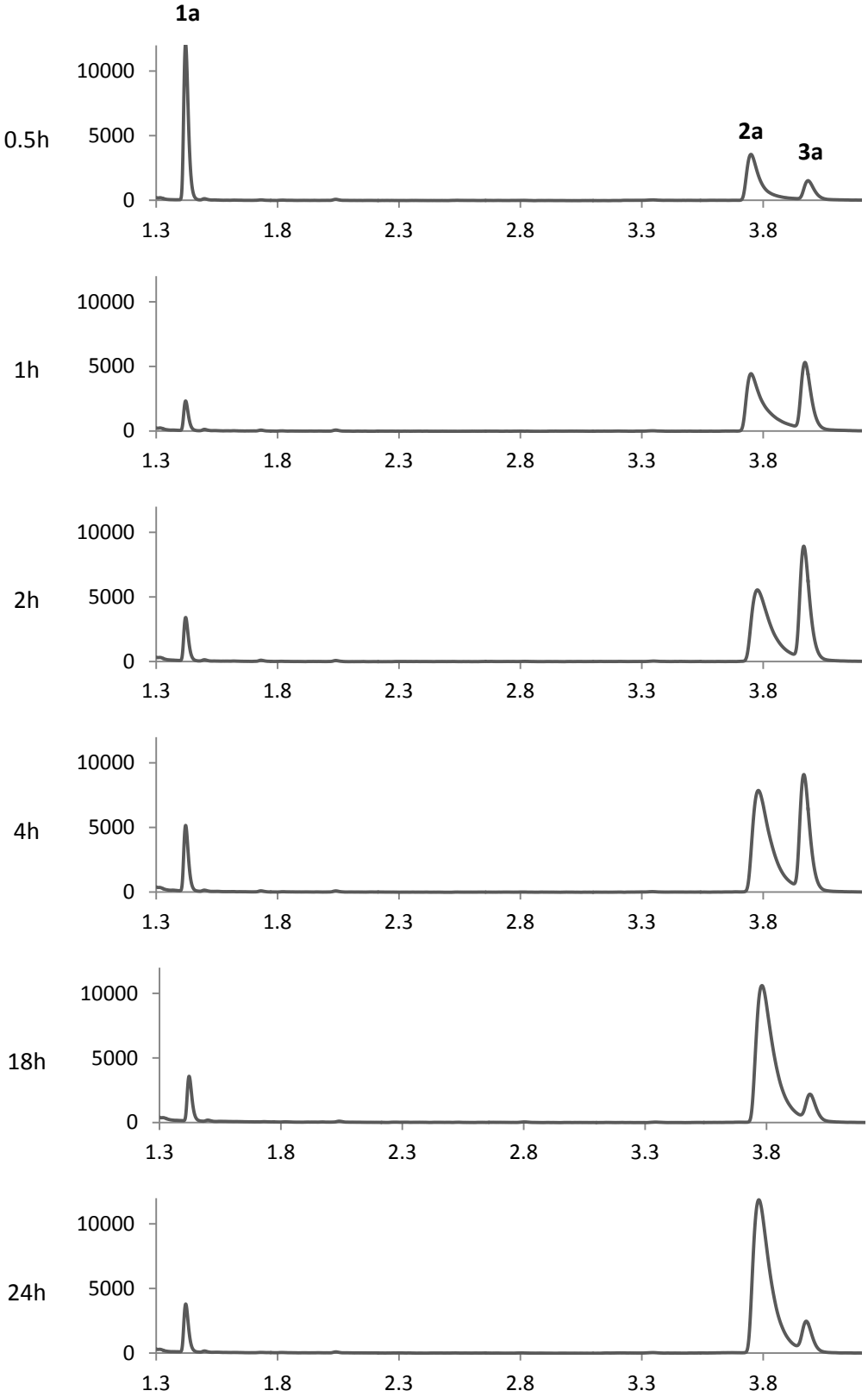
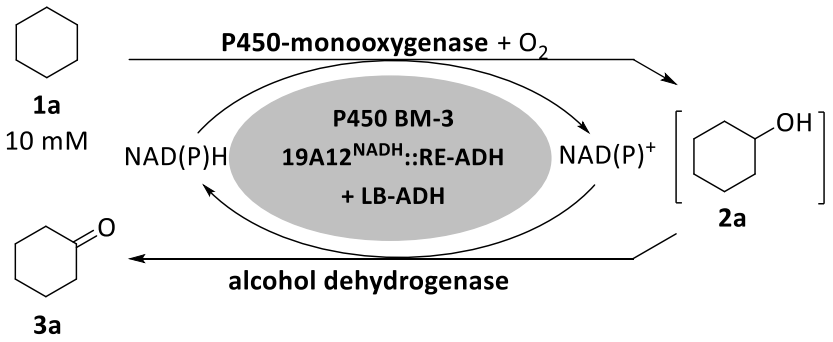


Figure 23. Oxidation of cyclohexane (**1a**) using construct A, BL21 (DE3) Gold $LacI^{Q1}$ pALXtreme-1a P450 BM-3 $19A12^{NADPH}(Kan^{R})$ + pKA1 LB-ADH (Cm^{R}) over the course of time

Table 24. Oxidation of cyclohexane (**1a**) using construct B, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R}); OD_{600} = 39.0; CDW = 5.0 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1a** [g/L]	**2a** [g/L]	**3a** [g/L]	Σ**2a**+**3a** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	0.5	0.05	0.08	0.03	0.11	22
2	1	0.03	0.14	0.09	0.23	46
3	2	0.02	0.18	0.15	0.33	66
4	4	0.01	0.25	0.21	0.46	92
5	18	0.01	0.41	0.06	0.47	94
6	24	0.01	0.43	0.08	0.51	102

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

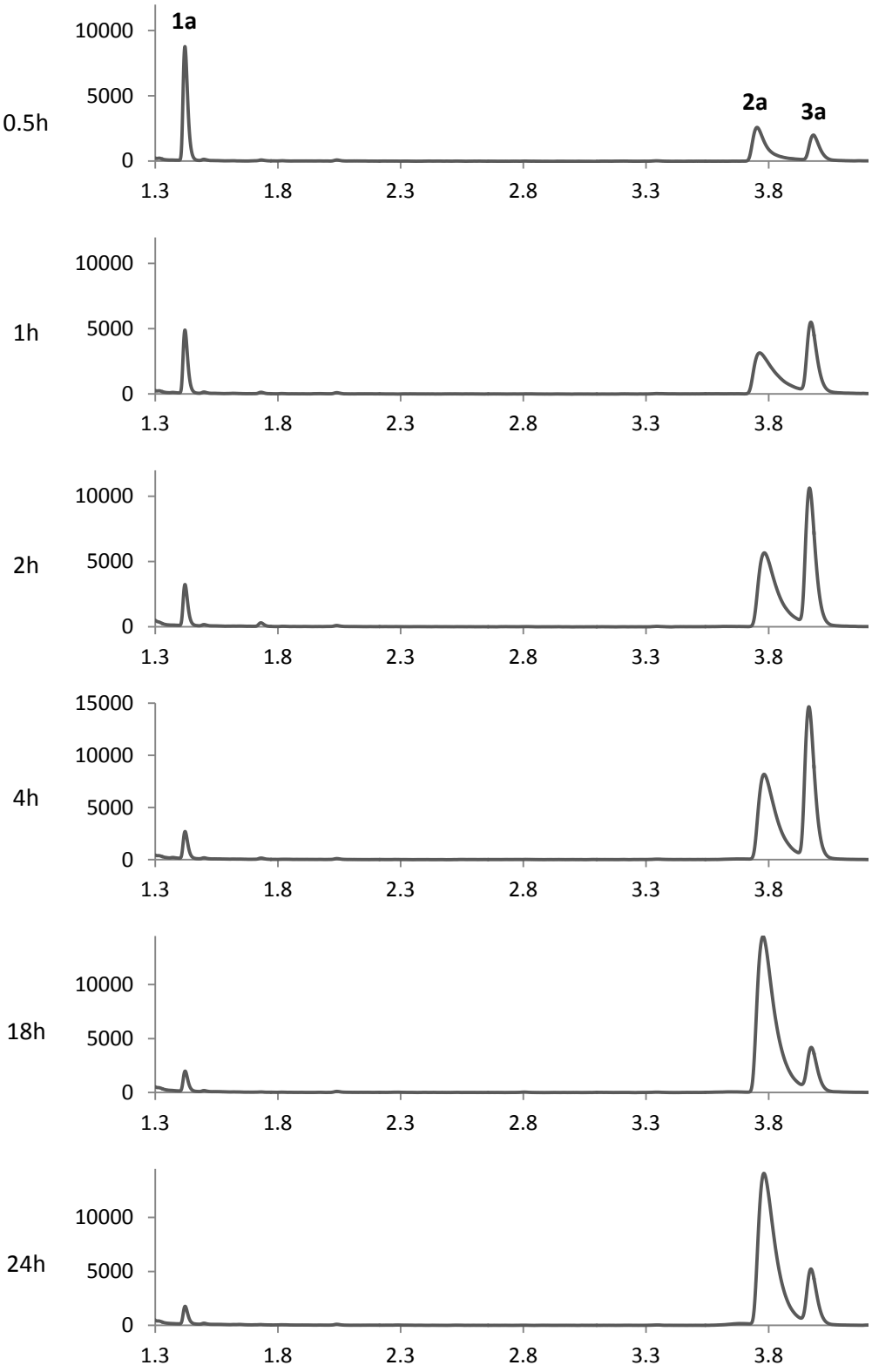


Figure 24. Oxidation of cyclohexane (**1a**) using construct B, BL21 (DE3) Gold $LacI^{Q1}$ pALXtreme-1a P450 BM-3 $19A12^{NADH}$::RE-ADH (Kan^R) + pKA1 LB-ADH (Cm^R) over the course of time

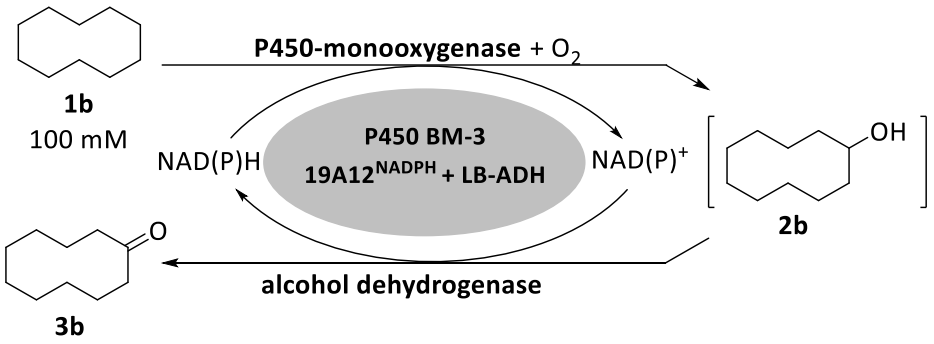
Both, the series of experiments with the two-enzyme system (consisting of CYP BM-3 19A12^{NADPH} and LB-ADH) and the three-enzyme system (consisting of CYP BM-3 19A12^{NADH}, RE-ADH and LB-ADH) (Scheme 48 - Scheme 49 and Figure 23 - Figure 24) showed a very similar progress: in the beginning of the whole cell catalyzed reaction, the concentration of the substrate cyclohexane (**1a**) is continually decreasing. At about the same time, the concentration of the intermediate cyclohexanol (**2a**) and the product cyclohexanone (**3a**) is continually increasing. After the 4 initial hours of the reaction, the equilibrium is strongly shifted from the product cyclohexanone (**3a**) towards the intermediate cyclohexanol (**2a**). A conceivable explanation for this phenomenon is the low stability of the cytochrome P450 monooxygenase and the associated, chronological following disruption of the first oxidation step. As a consequence, the reduced form of the cofactor NAD(P)H accumulates within the cells. This excess causes the increased activity of the alcohol dehydrogenase in the reductive direction.

The experiments with the three-enzyme system (CYP BM-3 19A12^{NADH}+ RE-ADH + LB-ADH) showed a tendentially higher product formation (up to 0.51 g/l **2a** and **3a**) than the experiments with the two-enzyme system (CYP BM-3 19A12^{NADPH}+ LB-ADH) where up to 0.42 g/l **2a** and **3a** are produced. An explanation for these findings is the varying intracellular concentration of CYP BM-3 that can be ascribed to a varying expression level of the recombinant protein in the cells. This value, specified as mg_{P450}/g_{CDW}, is generally determined *via* CO-difference spectroscopy. For the experiments described in this chapter, the intracellular concentration of CYP BM-3 was not determined. Nevertheless, it is published by MÜLLER *et al.* that the cells containing three enzymes (CYP BM-3 19A12^{NADH}+ RE-ADH + LB-ADH) include a fourfold higher concentration of the monooxygenase (12 mg_{P450}/g_{CDW}) than the cells containing only two enzymes (3 mg_{P450}/g_{CDW} with CYP BM-3 19A12^{NADPH}+ LB-ADH). Within the whole cell double oxidation of *n*-heptane, this considerably increased content of CYP BM-3 causes an improvement in product formation.[31,32] Assuming that this a general tendency, this makes the increased formation of the oxidation products **2a** and **3a** with the three-enzyme system (CYP BM-3 19A12^{NADH}+ RE-ADH + LB-ADH) more comprehensible.

6.2.3 Oxidation of cyclodecane (1b) using a two- and a three-enzyme system

In this section the whole cell biotransformation of cyclodecane (**1b**) is described which was carried out according to SOP 23. The reaction scheme is shown in Table 25 - Table 26 and the model reaction was studied at different reaction times. The concentration of the substrate **1b**, the intermediate **2b** and the product **3b** was analyzed *via* gas chromatography and the results are summarized in Figure 25 - Figure 26.

Table 25. Oxidation of cyclodecane (**1b**) using construct A, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}); OD_{600} = 39.4; CDW = 14.9 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1b** [g/L]	**2b** [g/L]	**3b** [g/L]	Σ**2b**+**3b** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	1	8.00	--	0.06	0.06	4
2	4.5	6.42	--	0.10	0.10	7
3	20	1.99	--	0.06	0.06	4

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

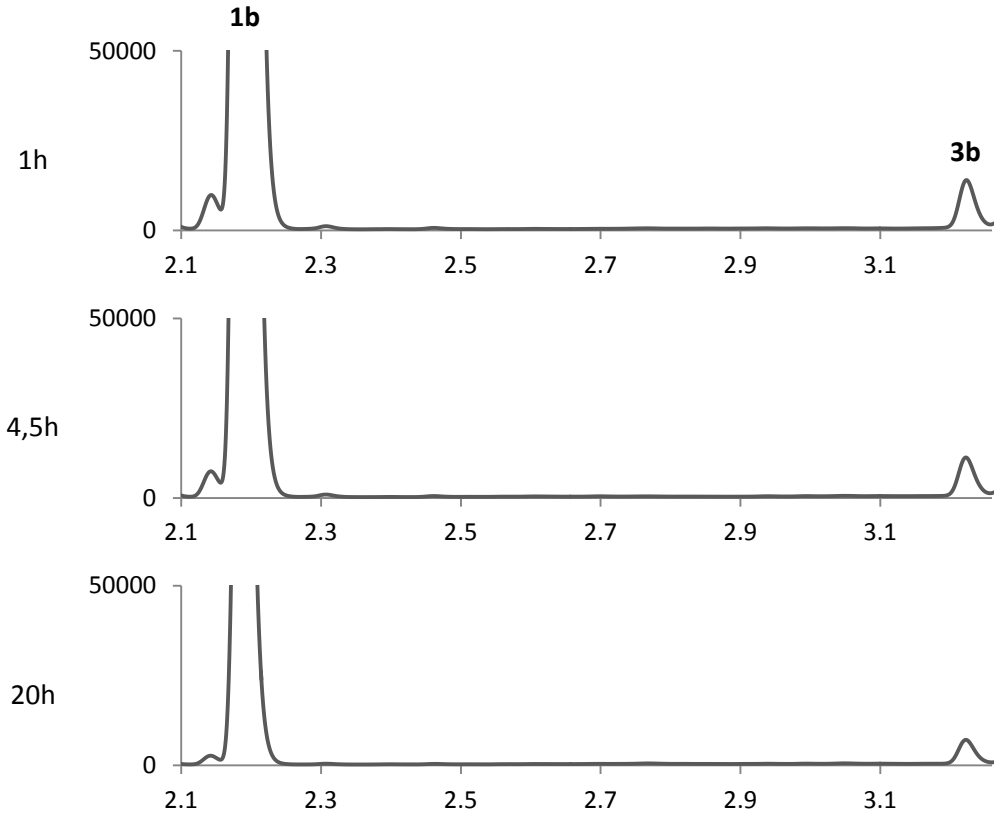
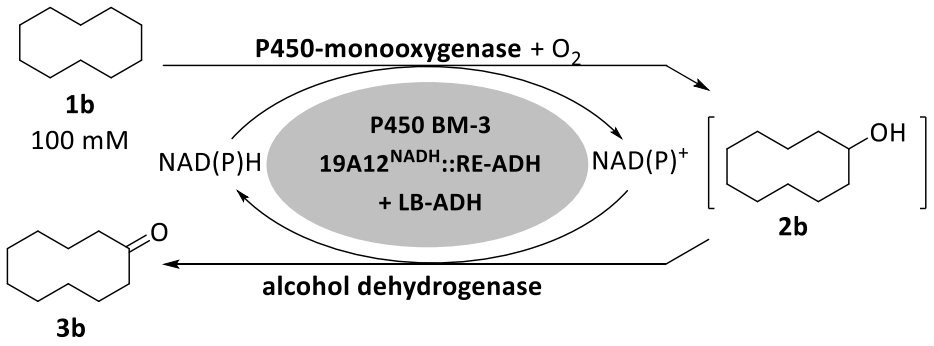


Figure 25. Oxidation of cyclodecane (**1b**) using construct A, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}) over the course of time

Table 26. Oxidation of cyclodecane (**1b**) using construct B, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R}); OD_{600} = 33.6; CDW = 10.4 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	time [h]	**1b** [g/L]	**2b** [g/L]	**3b** [g/L]	Σ**2b**+**3b** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	1	7.97	--	0.12	0.12	12
2	4.5	7.17	--	0.12	0.12	12
3	20	4.95	--	0.12	0.12	12

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

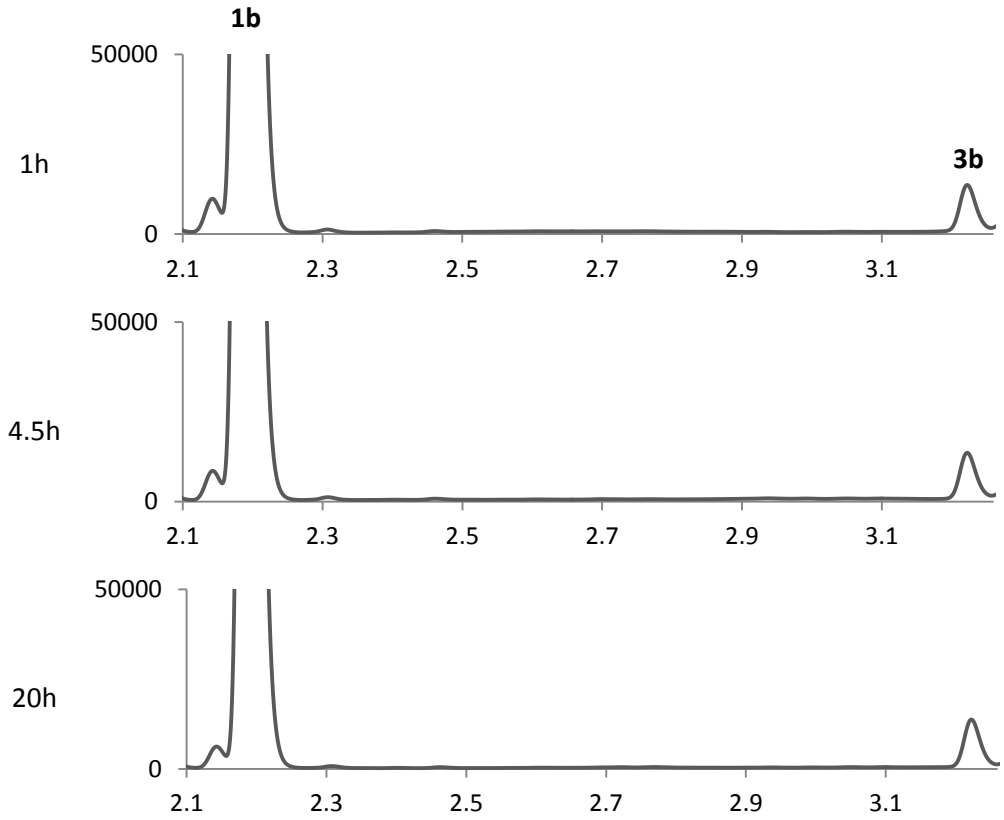


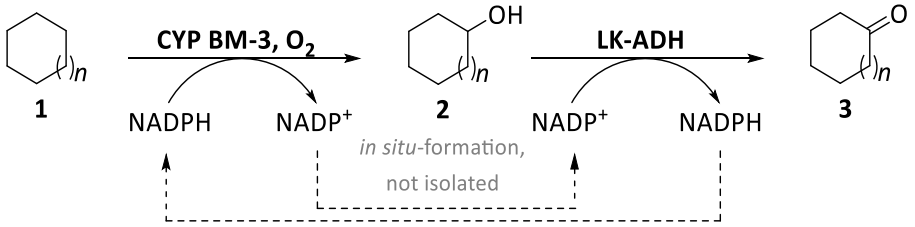
Figure 26. Oxidation of cyclodecane (**1b**) using construct B, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R}) over the course of time

Both, the series of experiments with the two-enzyme system (consisting of CYP BM-3 19A12^{NADPH} and LB-ADH) and the three-enzyme system (consisting of CYP BM-3 19A12^{NADH}, RE-ADH and LB-ADH) (Table 25 - Table 26 and Figure 25 - Figure 26) show a very similar progress: over the course of time the concentration of the substrate cyclodecane (**1b**) is continually decreasing. On the other hand, the concentration of the product cyclodecanone (**3b**) remains constant (disregarding one deviation of measurement, Table 25, entry 2). For the intermediate cyclodecanol (**2b**) is not detected at any time, a reasonable explanation for the decreasing concentration of **1b** might be a further- or multiple oxidation. Gas chromatography was used as analytical method for the absolute and simultaneous quantification of all cycloalkanes **1**, cycloalkanols **2** and cycloalkanones **3** by a straight calibration line. Multiple oxidation products would give signals at higher retention times than the analyzed cycloalkanes **1**, cycloalkanols **2** and cycloalkanones **3**. Therefore it is conceivable that multiple oxidation products of cyclodecane (**1b**) are not detected by the time-optimized method of measurement which was designed for quantification of the oxidation products cyclodecanol (**2b**) and cyclodecanone (**3b**) only. This approach can serve as starting point for further investigation on this topic.

As already described for the substrate cyclohexane (**1a**), the formation of the oxidation products **2b** and **3b** with the three-enzyme system (CYP BM-3 19A12^{NADH}+ RE-ADH + LB-ADH) is increased compared to the whole cell catalysis with the two-enzyme system (CYP BM-3 19A12^{NADPH}+ LB-ADH). This is the result of the tendential higher intracellular concentration of CYP BM-3 within the three-enzyme system.[31,32]

7 Summary

The aim of this thesis was predominantly to develop a more contemporary and green procedure for the direct functionalization of cycloalkanes **1** to cycloalkanones **3**, theoretically able to substitute traditionally applied oxidation procedures. The concept was a biotechnological approach, in which the desired cycloalkanone **3** is obtained directly by a one-pot process starting from the corresponding cycloalkane **1** and using atmospheric oxygen as oxidizing agent (Scheme 50).



Scheme 50. Double oxidation of cycloalkanes **1** (*n* = 1, 3)

Besides the cycloalkanes **1** mentioned above, a range of substrates, for various reasons attractive to be hydroxylated by cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3), was analyzed in this context. Cyclooctyne (**17**) was accepted by none of the tested mutants while two mutants showed activity with cyclododecene (**18**). However, the formation of an oxidation product was not detected and the previously measured activity towards **18** was attributed to an undefined background reaction consuming the cofactor NADPH resp. uncoupling in general. The substrate pentanal (**19**) was expected to be suitable for an enzymatic oxidation since a specific activity of 29 U/g towards this compound was found with the mutant CYP BM-3 19A12. Nevertheless, the precisely identified new compound was not the product of an oxidation reaction but the corresponding alcohol **53**, a product of the reduction of pentanal (**19**). Probably this was the result of an undesired background activity caused by an alcohol dehydrogenase from the host organism *E. coli*, operating in reductive direction.

Prior to preparative oxidation experiments with cycloalkanes **1**, preliminary studies like the simulation of the reaction conditions and the working up were conducted to develop an overall process that is only marginal error-prone under realistic conditions. Taking sources of error like the presence of biomass into consideration, an efficiently optimized reaction progress and working up led to a likewise high isolation rate of 94% cyclododecanone (**3c**) after 24 h, when 100 mM of **3c** were applied in a simulated reaction. Even concentrations down to 10 mM of the substrate cyclododecane (**1c**) and the intermediate cyclododecanol (**2c**) showed a constant deviation of < 10% and mostly far below, based on an ideal recovery of 100%.

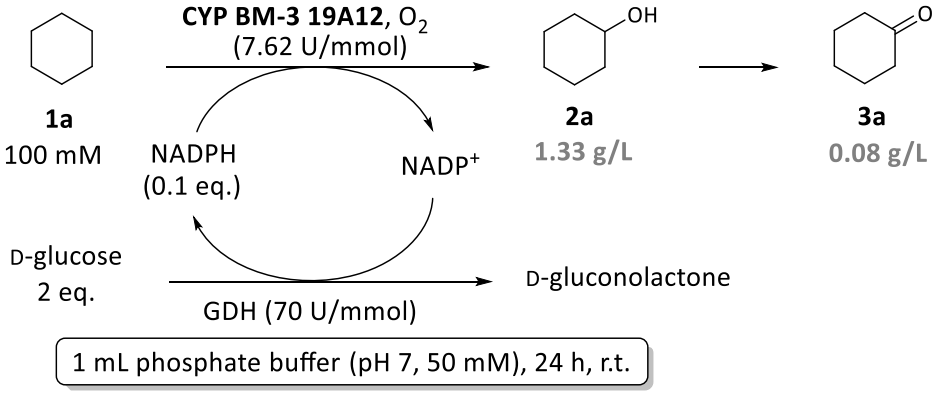
To facilitate the simultaneous quantification of the particular cycloalkanes **1**, cycloalkanols **2** and cycloalkanones **3** from the extraction mixture *via* gas chromatography, multipoint calibration lines were acquired. Additionally, the methods detection limit (MDL) was investigated to ensure quantitatively correct results and guarantee the reproducibility of the measurements. The C12-compounds **1-3c** can be detected in concentrations down to 0.005 g/L, covering the relevant concentration range of interest. Also the quantification of the C10-compounds **1-3b** is realized and valid results are obtained in microgram quantities. Even at a concentration of 0.025 g/L, the deviation was not exceeding more than 10% of the ideal recovery (100%) at any time. For the C6-compounds **1-3a** the simultaneous and reliable quantification can be realized in a concentration range down to 0.1 g/L, a result that is entirely sufficient for the planned syntheses.

In preliminary stages prior to the preparative biotransformations, the enzyme activities of selected mutants were determined *via* UV/VIS-spectrophotometric assays. The most promising substrates that came out of these enzyme activity studies with the cycloalkanes **1** were then applied in preparative biotransformations. For the not readily soluble substrate cyclododecane (**1c**), the kinetics according to MICHAELIS-MENTEN were investigated to ensure that the biotransformation can be conducted at maximum reaction rate. Since the enzyme is already saturated with substrate at a substrate concentration of 50 µM, addition of more substrate would not affect the reaction rate and under the standard reaction conditions the maximum reaction rate is reached.

Since only the mutants CYP BM-3 F87A A328V and CYP BM-3 F87P seemed to be promising for the conversion of the substrate cyclododecane (**1c**) (15-34 U/mg; TOFs of 31 resp. 145 min^{-1}) while all other mutants showed no or only poor activity, these mutants were used for the preparative biotransformations of **1c**. In summary it can be stated that all tested mutants showed a low acceptance towards **1c** in the preparative experiments although activity was shown in preliminary enzyme activity tests.

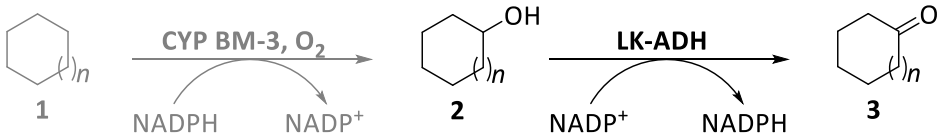
The highest specific activity for the oxidation of cyclodecane (**1b**) by far (39-56 U/g; TOFs of 317-850 min^{-1}) was shown by the mutant CYP BM-3 19A12 and therefore it was preferred for preparative experiments with this substrate. In order to consider the hydroxylation of the cycloalkane **1** in isolation, a cofactor regeneration system using glucose dehydrogenase (GDH) was applied. In an experimentally performed hydroxylation of cyclodecane (**1b**) (100 mM) with CYP BM-3 19A12, 0.15 g/L of the desired product cyclodecanol (**2b**) were formed. Therefore **1b** turned out to be a suitable substrate for the planned double oxidation. Just as with the substrate cyclodecane (**1b**), the mutant CYP BM-3 19A12 turned out to be as well by far the best recombinant enzyme to catalyze the oxidation of cyclohexane (**1a**) and without exception, all other tested enzymes lag far behind this remarkable activity. In summary, specific activities from 131 U/g through to 161 U/g and TOFs from 1165 to 2183 min^{-1} were found for the different formulations.

Therefore, CYP BM-3 19A12 was used extensively for preparative experiments. In the following hydroxylation experiment, remarkable 1.33 g/L of the product **2a** were formed while as a product of further oxidation 0.08 g/L **3a** were detected (Scheme 51). Keeping the synthetic concept - namely the intention to synthesize cycloalkanones **3** from cycloalkanes **1** - in mind, the further oxidation is supporting the LK-ADH-catalyzed reaction step (Scheme 50) by enhancing the desired product quantity.



Scheme 51. Oxidation of cyclohexane (**1a**) with CYP BM-3 19A12

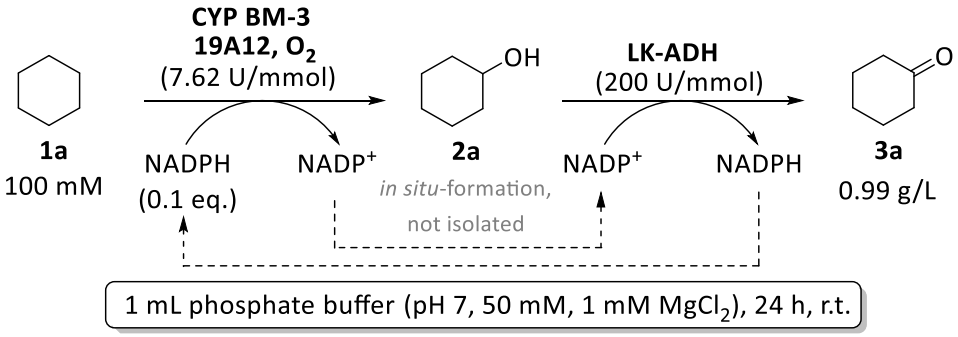
This successful P450 monooxygenase-catalyzed hydroxylation using and consuming molecular oxygen (O_2) represents the initial step of the planned two step one-pot process starting from a cycloalkane **1** and aiming to directly synthesize the desired cycloalkanone **3**. For the realization, a further alcohol dehydrogenase-catalyzed oxidation of the cycloalkanol **2** into the desired cycloalkanone **3** was planned, which proceeds *in situ* and transforms the oxidized form of the cofactor resulting from the initial hydroxylation back to the reduced form NAD(P)H (Scheme 52).



Scheme 52. Double oxidation of cycloalkanes **1** (*n* = 1, 3) focussing the second LK-ADH-catalyzed oxidation step

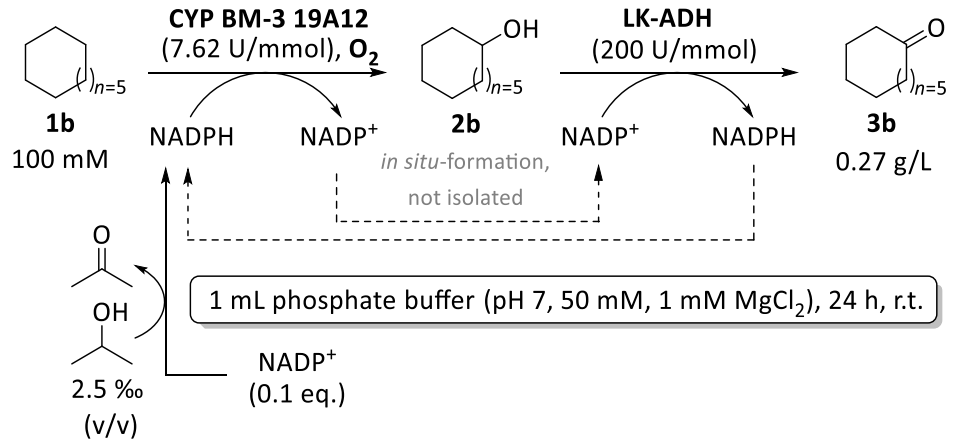
Spectrophotometric assays were performed to determine the enzyme activity of the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) towards the substrates of most interest, cyclohexanol (**2a**) and cyclodecanol (**2b**). Average relative activities up to 42% referred to 1-phenylethanol (**71**) were determined for the transformation of **2a**. The enzyme activity studies with **2b** in contrast revealed 1.5 times higher relative activities of Ø 155% referred to the reference compound **71**. The double oxidations of cyclohexane (**1a**) and cyclodecane (**1b**) were realized in the presence of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) in phosphate buffer.

To oxidize **1a**, the cofactor NADPH was added directly to start the reaction. The approach of adding 2-propanol to generate the reduced cofactor form NADPH - that is required for the initial hydroxylation step - *in situ* from $NADP^+$ was discarded since in experiments with the mutant CYP BM-3 19A12 the product formation of 0.19 g/L was lower by half of the product formations without 2-propanol (0.41 g/L). Using the subsequent system the best results were obtained whereby it was notable that early experiments yielded 0.41 g/L while later conducted experiments gave 0.98 g/L on an average (Scheme 53). Since quantitatively more biomass, nearly the double amount, was applied in the later experiments, 'Macromolecular Crowding' - caused by an increased overall protein loading - is hence, besides the optimized work up, an explanation for the more than twofold higher productivity.



Scheme 53. Double oxidation of cyclohexane (**1a**) with CYP BM-3 19A12

The formation of cyclohexanone (**3a**) on a gram scale constitutes an excellent productivity for this type of reaction. In comparison to cyclohexane (**1a**), slightly better results were achieved for the double oxidation of cyclodecane (**1b**) by adding the 2-propanol-starter. An average product formation of 0.26 g/L was obtained by admixing $NADP^+$ and 2-propanol (Scheme 54). The intermediate **2b** was not detected at any time in contrast to intermediate **2a**. This is owed to a fact clarified by the enzyme activity assays: cyclodecanol (**2b**) revealed 1.5 times higher relative activities of Ø 155% referred to the reference compound **71**, compared to cyclohexanol (**2a**) with only 42%. Therefore **2b** is quickly converted into **3b** and was not accumulated.



Scheme 54. Double oxidation of cyclodecane (**1b**) with CYP BM-3 19A12

Furthermore the shift in product formation caused by a shortened reaction time was investigated. The double oxidation of cyclohexane (**1a**) was performed under constant conditions with constant amounts of both enzymes (CYP BM-3 19A12 and LK-ADH) and various reaction times. After 4 h the product formation was already 0.51 g/L and therefore half the benchmark product formation after 24 h, which was 0.99 g/L. Even more easy to abbreviate were the experiments using cyclodecane (**1b**) as substrate, since a shorter reaction time brought along only a marginal decrease of product formation: After 2 h it was 0.21 g/L and after 4 h it was 0.23 g/L while 24 h gave 0.27 g/L of cyclodecanone (**3b**). The reason for this divergence - namely the different product formation of **3a** and **3b** at shortened reaction times of the double oxidation - can be found in the fact that the biotransformation of **1b** is faster compared to the biotransformation of **1a**. Cyclodecanol (**2b**) is accepted 3 to 6 times better by the LK-ADH than cyclohexanol (**2a**) what makes the reaction faster and provides the reduced form of the cofactor more quickly to the first oxidation step.

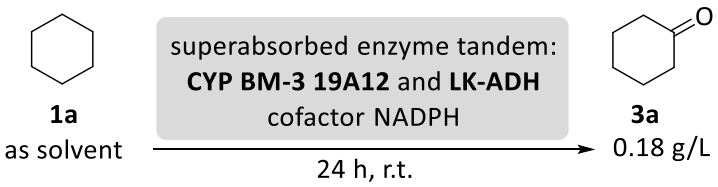
Moreover the effect of decreasing the usually applied concentration of cofactor $NADP^+$/NADPH (10 mol%) was investigated. Under constant conditions, the double oxidation of cyclohexane (**1a**) yielded a product formation that remained unchanged employing 1 or 2 mol% of NADPH (Ø 0.10 g/L) and also the application of both, each 1 mol % of the reduced as well as of the oxidized form of cofactor resulted in the same productivity. This was not surprising because the initially necessary NADPH in den comparative tests had as well not to be formed *via* a 2-propanol-starter and the reaction could start immediately in any case. By directly comparing experiments that differ only in the amount of cofactor it was found that scarcely ⅓ of the benchmark product formation can be accomplished with $1/_{10}$ amount of cofactor: The application of 10 mol% cofactor led to a product formation of 0.99 g/L while using only 1 mol% cofactor effected a product formation of 0.28 g/L.

With cyclodecane (**1b**) as substrate, the biotransformations applying only 1-2 mol% $NADP^+$ resulted in a nearly unchanged product formation, which was 0.26 g/L on an average, compared to a benchmark product formation of 0.27 g/L. However, a slight but noteworthy increased product formation of 0.35 g/L was obtained when each 1 mol% of the reduced as well as of the oxidized form of cofactor were added. This can be attributed to the fact that 1 mol% of the reduced cofactor was provided directly to start the reaction and only half of the amount of the cofactor NADPH had to be produced *in situ* from $NADP^+$. The direct comparison of the experiments that differ only in the amount of cofactor showed that the benchmark product formation (0.27 g/L at 10 mol% cofactor) can be achieved with $1/_{10}$ amount of cofactor (Ø 0.26 g/L at 1 mol% cofactor).

A shortened reaction time - explained in the section above - illustrated that the enzyme catalyzed formation of cyclodecanone (**3b**) proceeds faster than the formation of cyclohexanone (**3a**), probably due to a higher acceptance of cyclodecanol (**2b**) by the LK-ADH.

Therefore the considerably reduced cofactor quantity is more quickly provided to the first hydroxylation step in the oxidation of cyclodecane (**1b**) whereas the double oxidation of cyclohexane (**1a**) is not fast enough to compensate the reduced amount of cofactor. To investigate the impact of substrate concentration on product formation, an experiment with fivefold substrate concentration was performed. The increase of the substrate concentration led to higher product formations, but the correlation was not linear: Applying 100 mM **1b** in a biotransformation, the product formation was 0.25 g/L of **3b**. A fivefold higher substrate concentration, namely 500 mM resulted in a product formation of 0.82 g/L **3b**.

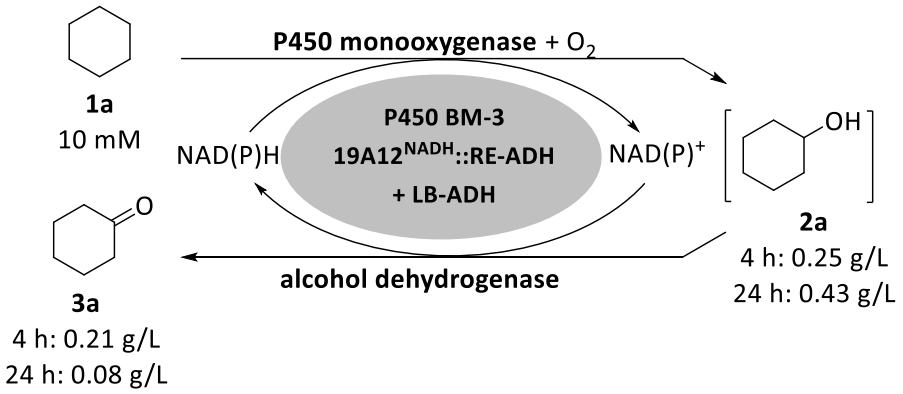
An extended aim was to test a co-immobilisate of enzymes, hereinafter referred to as 'superabsorbed enzyme tandem', that would render the presented biotransformation even more attractive by reusing the catalysts. This tandem consisted of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) and an alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) together with the cofactor NADPH and was applied for the enzymatic two-step one-pot process (Scheme 55).



Scheme 55. Double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem

To obtain this hydrogel matrix, the superabsorbent polymer Favor SXM 9155 was employed. On an average, the product formation was about six times lower using this not optimized system than in the benchmark experiment using non-immobilized free catalysts, where 0.99 g/L of **3a** were formed. Moreover, the system showed no conversion after the second cycle.

Finally the biocatalyzed double oxidation *via* whole cell catalysis was investigated for cyclohexane (**1a**) and cyclodecane (**1b**) and two different constructs were used for this project. At one point, monooxygenase and LB-ADH were coexpressed on two different plasmids while the other time, an RE-ADH was coexpressed on the same plasmid with the monooxygenase while the LB-ADH was coexpressed on a second plasmid. The oxidation of **1a** was performed using the two- and the three-enzyme system and the model reaction was studied at different reaction times. The series of experiments with the two-enzyme system (consisting of CYP BM-3 19A12^{NADPH} and LB-ADH) and the three-enzyme system (consisting of CYP BM-3 19A12^{NADH}, RE-ADH and LB-ADH) showed a very similar progress and one selected reaction scheme is shown exemplarily in Scheme 56.



Scheme 56. Oxidation of cyclohexane (**1a**) using the three-enzyme system (consisting of CYP BM-3 19A12^{NADH}, RE-ADH and LB-ADH)

At the beginning of the whole cell biotransformation the concentration of **1a** was continually decreasing while the amount of the intermediate **2a** and the product **3a** increased significantly. Following this trend for 4 hours, the equilibrium was then strongly shifted from the product **3a** towards the intermediate **2a**. A disruption of the first step might cause the accumulation of the reduced form of the cofactor NAD(P)H within the cells and this excess effects the increased activity of the alcohol dehydrogenase in reductive direction. Conceivable due to the varying expression level of the recombinant protein in the cells, the experiments with the three-enzyme system showed a slightly higher product formation (up to 0.51 g/L **2a** and **3a** after 24 h) than the experiments with the two-enzyme system where up to 0.42 g/L **2a** and **3a** were produced after 24 h.

The oxidation of **1b** was also analyzed using the two- and the three-enzyme system over the time and with the two-enzyme system, 0.10 g/L **3b** were obtained after 4.5 h. As well as with **1a** as substrate, the experiments with the two- and the three-enzyme system showed an almost identical progression. The concentration of the substrate **1b** was decreasing while the concentration of **3b** remained most widely constant. The intermediate cyclodecanol (**2b**) was not detected at any time. A reasonable explanation for the decreasing concentration of **1b** - without visible product formation - might be the formation of multiple oxidation products which are not detected by the time-optimized method of measurement which was designed for the quantification of the oxidation products **2b** and **3b** only.

In summary a biotechnological process was developed in which the desired cycloalkanones **3**, namely cyclohexanone (**3a**) and cyclodecanone (**3b**), were obtained in a one-pot process starting from the corresponding cycloalkane **1**. This system, in which the reaction takes place in water, atmospheric oxygen is used as oxidation agent and no further co-substrate is required, renders the necessity of interim stages with isolation of intermediates obsolete. Furthermore this technology was successful performed in a superabsorbed enzyme tandem consisting of both enzymes as well as the cofactor NADPH. Equally this type of reaction can be realized using whole cell catalysis.

8 Experimental Section

8.1 Materials, instruments and methods

Chemicals:
The commercially available chemicals used for this project were purchased from Acros Organics®, Sigma-Aldrich®, ABCR®, Thermo Fisher Scientific®, TCI Europe® or Fluka® and except as noted otherwise used without further purification. The superabsorbent polymer „Favor SXM9155“ is commercially available from Evonik Industries, Stockhausen.

Enzymes and cofactors:
The cytochrome P450 monooxygenases (CYP BM-3) used for this project (CYP BM-3 WT[81], CYP BM-3 19A12[160,161], CYP BM-3 F87V[89,161], CYP BM-3 F87A A328V[92,162], CYP BM-3 R255P-P329H[32,163], CYP BM-3 F87P[164]) were made available through the courtesy of the research group Prof. Dr. U. Schwaneberg (RWTH Aachen). The alcohol dehydrogenases from *Lactobacillus kefir* (LK-ADH) and *Rhodococcus* sp. (Rsp.-ADH)[165-167] were made available through the courtesy of the research group Prof. Dr. W. Hummel (Research Centre Jülich). The ADH-200 is commercially available from evocatal[168] (catalogue entry evo-1.1.200, CAS: 9031-72-5). The glucose dehydrogenase from *Bacillus* sp. (Amano 2) was purchased from Amano Enzyme Inc. (Nagoya, Japan). The cofactors NADPH and $NADP^+$ were purchased from Oriental Yeast Co. Ltd., Japan.

Buffer and media:
Buffer solutions were prepared from sodium dihydrogenphosphate and equilibrated by adding sodium hydroxide until the desired pH was reached. 1 l LB-medium is prepared by dissolving 10 g peptone, 5 g yeast extract and 10 g NaCl in deionized water and filling up to a final volume of 1 l. The medium is autoclaved. 1 l TB-medium is mixed on demand from 800 mL solution A and 200 mL solution B. For the preparation of solution A, 12 g peptone, 24 g yeast extract und 4 g glycerol are dissolved in deionized water, filled up to 800 mL and autoclaved. For the preparation of solution B, KH_2PO_4 (2.31 g) und K_2HPO_4 (12.54 g) are dissolved in deionized water, filled up to 200 mL and autoclaved.

The following instruments have been used for analytic and preparative works:

NMR-spectroscopy:
^1H and ^{13}C NMR spectra were recorded on an Avance DRX 500 spectrometer (Bruker) in deuterochloroform ($CDCl_3$), deuteriumoxide (D_2O), dimethylsulfoxide-d6 (DMSO-d6) or deuterodichloromethane (CD_2Cl_2) on a 500 MHz instrument. Chemical shifts were reported in parts per million (ppm, δ) downfield from residual solvent signals: $CHCl_3$: δ(^1H), 7.26 ppm; δ(^{13}C), 77.16 ppm. D_2O: δ(^1H), 4.79 ppm. CD_2Cl_2: δ(^1H), 5.32 ppm; δ(^{13}C), 53.84 ppm. DMSO-d6: δ(^1H), 2.50 ppm; δ(^{13}C), 39.52 ppm. Proton coupling patterns are described as singlet (s), doublet (d), triplet (t), doublet of triplet (dt), quartet (q) and multiplet (m).

Spectrophotometer:
The data for activity determination of the enzymes was recorded with a UV/VIS-spectrophotometer V-630 from Jasco.

Thermomixer:
Extractions under constant shaking and isothermic conditions were conducted using a thermomixer comfort 5355 from Eppendorf.

Centrifuge:
Phase separations during work-up were achieved using a microliter centrifuge (model CT15RE, VWR). Samples were centrifuged for 10 - 30 minutes at 13000 rpm and room temperature.

Gas chromatography:
All qualitative and quantitative measurements *via* gas chromatography were performed with a Shimadzu GC 2010 (Autoinjector AOC-20i (Shimadzu)) using a Rt-βDEXm (Restek®) column (Length: 30 m; Inner Diameter: 0.25 mm; Film Thickness: 0.25 µm) (used for method A) or a Shimadzu GC 2010 Plus (Autoinjector AOC-20i (Shimadzu), Autosampler AOC-20s (Shimadzu)) using a Rxi-5ms (Restek®) column (Length: 25 m; Inner Diameter: 0.2 mm; Film Thickness: 0.33 µm) (used for method B). Molecular nitrogen (N_2) is used as carrier gas at a pressure of 100 kPa and as gas chromatographic detector a flame ionization detector (FID) is applied. Since technical deviation of the retention times occur to a certain degree, the samples are spiked at regular intervals. The measurements are conducted according to the following temperature programs:

1-3c:
Method A (0.1 - 10 g/L): Shimadzu GC 2010: starting at 150°C, heating 10°C/min up to 220°C. Old column: t_R (**1c**): 2.5 min; t_R (**2c**): 4.9 min; t_R (**3c**): 4.5 min; new column: t_R (**1c**): 2.7 min; t_R (**2c**): 5.2 min; t_R (**3c**): 4.7 min.
Method B (0.025 - 0.2 g/L): Shimadzu GC 2010 Plus: isotherm at 170°C for 5.5 min. t_R (**1c**): 2.7 min; t_R (**2c**): 5.2 min; t_R (**3c**): 4.7 min.

1-3b:
Method A (0.1 - 10 g/L): Shimadzu GC 2010: starting at 150°C, heating 10°C/min up to 220°C. Old column: t_R (**1b**): 1.8 min; t_R (**2b**): 3.4 min; t_R (**3b**): 3.0 min; new column: t_R (**1b**): 1.9 min; t_R (**2b**): 3.7 min; t_R (**3b**): 3.2 min.
Method B (0.025 - 0.80 g/L): Shimadzu GC 2010 Plus: starting at 125°C, heating 15°C/min up to 185°C. t_R (**1b**): 2.2 min; t_R (**2b**): 3.6 min; t_R (**3b**): 3.3 min.

1-3a:
Method A (0.15 - 1.5 g/L): Shimadzu GC 2010: starting at 70°C, heating 3.5°C/min up to 95°C. Old column: t_R (**1a**): 1.5 min; t_R (**2a**): 5.6 min; t_R (**3a**): 5.1 min; new column: t_R (**1a**): 1.6 min; t_R (**2a**): 6.3 min; t_R (**3a**): 5.7 min.
Method B (0.025 - 0.80 g/L): Shimadzu GC 2010 Plus: isotherm at 70°C for 4.5 min. t_R (**1a**): 1.5 min; t_R (**2a**): 3.8 min; t_R (**3a**): 4.0 min

General methods for working up:

Standard Operating Procedure A (SOP A):
After the individual reaction time the aqueous phase is poured into an Eppendorf tube and 1 mL dichloromethane is added. The biphasic system is extracted for 30 min by shaking in a Thermomixer Comfort (type 5355) with 550 rpm at 25°C. In order to get a well-defined phase boundary the mixture is centrifuged at 13000 rpm for 10 min. The supernatant aqueous phase is transferred into another Eppendorf tube and the extraction as described above is repeated twice, whereby the final phase separation is obtained by centrifugation at 13000 rpm for 30 min. The combined organic phases are transferred completely into a 5 mL volumetric flask which is filled with dichloromethane. By means of a straight calibration line the concentration is analyzed *via* gas chromatography.

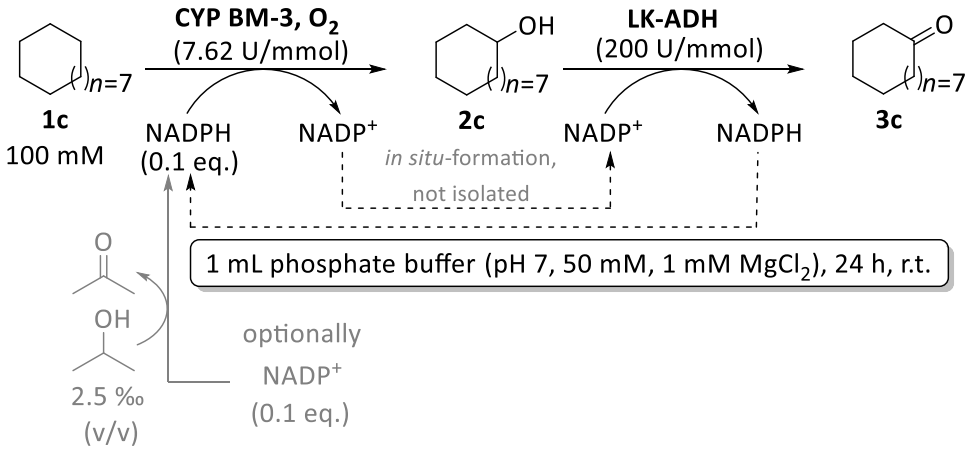
Standard Operating Procedure B (SOP B):
After the individual reaction time 1 mL dichloromethane is added and the biphasic system is poured into an Eppendorf tube. The biphasic system is extracted for 30 min by shaking in a Thermomixer Comfort (type 5355) with 550 rpm at 25°C. In order to get a well-defined phase boundary the mixture is centrifuged at 13000 rpm for 10 min. After removing the organic phase the extraction as described above is repeated twice, whereby the final phase separation is obtained by centrifugation at 13000 rpm for 30 min. The combined organic phases are transferred completely into a 3 mL volumetric flask which is filled with dichloromethane. By means of a straight calibration line the concentration is analyzed *via* gas chromatography.

8.2 Syntheses and analytical data

8.2.1 Standard operation procedure 1 (SOP 1): Biocatalyzed double oxidation of cyclododecane (1c) with CYP BM-3 and LK-ADH

In a 25 mL round bottom flask cyclododecane (**1c**) (16.8 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) resp. 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to cyclodecane (**1b**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) resp. $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and worked up according to SOP A.

Table 27. Results of the double oxidation of cyclododecane (**1c**)



Entry	CYP BM-3 (biomass)	IPA	Cofactor	Cyclododecanone (**3c**) [g/l]]	Recovery of substrate **1c** [%]
1	CYP BM-3 19A12 (0) (19.6 mg)[a)]	✓	$NADP^+$	0	92
2	CYP BM-3 F87V (127.0 mg)[b)]	-	NADPH	0	83

[a)]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b)]The content of CYP BM-3 F87V amounts to 0.525 µmol/g lyophilized crude extract.

8.2.2 Standard operation procedure 2 (SOP 2): Simulation of reaction conditions and working up for cyclododecane (1c), cyclododecanol (2c) and cyclododecanone (3c)

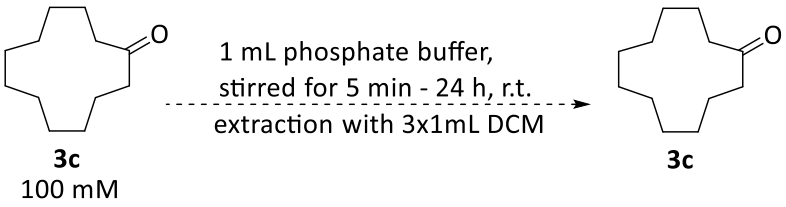
In a 25 mL round bottom flask cyclododecanone (**3c**) (18.13 - 18.54 mg, 0.10 mmol) resp. cyclododecanol (**2c**) (0.184 -18.72 mg, 0.001 - 0.10 mmol) resp. cyclododecane (**1c**) (0.168 - 16.97 mg, 0.001 - 0.10 mmol) is mixed with 1 mL

phosphate buffer (pH 7.0, 50 mM) (**3c**) or 900 µL phosphate buffer (pH 7.0, 50 mM) and 100 µL DMSO (**1c**, **2c**). Then 0 - 30 mg of the denaturated (60°C, 20 h) cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, lyophilisate) is added and the mixture is stirred for 5 min up to 24 h at room temperature, each time covered with a plug. Then 1 mL dichloromethane is added and the biphasic system is poured into an Eppendorf tube. The biphasic system is extracted for 30 min by shaking in a Thermomixer Comfort (type 5355) with 550 rpm at 25°C. After removing the organic phase the extraction as described above is repeated twice (whereby with **1c** and **2c**, the third extraction is conducted only with 500 µL dichloromethane) and the final phase separation is obtained by centrifugation at 13000 rpm for 30 min. The combined organic phases are transferred completely into a 2 mL (**1c**, **2c**) resp. 5 mL (**3c**) volumetric flask which is filled with dichloromethane. By means of a straight calibration line the concentration is analyzed *via* gas chromatography.

8.2.2.1 Cyclododecanone (3c)

In a 25 mL round bottom flask cyclododecanone (**3c**) (18.13 - 18.54 mg, 0.10 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM) and 0 - 20 mg of the cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, lyophilisate) and stirred for 5 min - 24 h at room temperature covered with a plug and worked up according to SOP B, but a 5 mL volumetric flask was used instead of a 3 mL volumetric flask.

Table 28. Results of the simulation of reaction conditions and working up for cyclododecanone (**3c**)



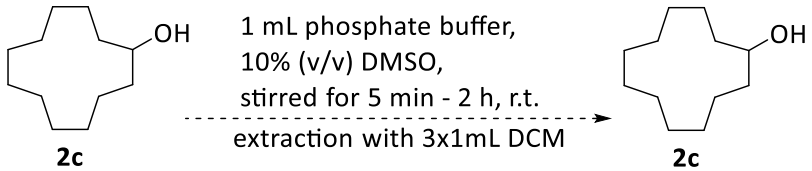
Entry	Time	Initial weight [mg]	Output weight [mg][b)]	Recovery [%]
1	5 min	18.40	17.29	94
2	24 h	18.13	17.29	95
3 [a)]	24 h	18.40	--[c)]	--
4	5 min	18.54	17.04	92
5	24 h	18.15	17.28	95
6 [a)]	24 h	18.44	17.32	94

[a)]20 mg denaturated CYP BM-3 19A12 are added; [b)] calculated *via* GC; [c)]lost during working up.

8.2.2.2 Cyclododecanol (2c)

In a 25 mL round bottom flask cyclododecanol (**2c**) (0.184 - 18.72 mg, 0.001 - 0.10 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) and 100 µL DMSO. Then 0 - 30 mg of the denaturated (60°C, 20 h) cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, lyophilisate) are added. The mixture is stirred for 5 min - 2 h at room temperature covered with a plug and worked up according to SOP B, but a 2 mL volumetric flask was used instead of a 3 mL volumetric flask and the third extraction is conducted only with 500 µL dichloromethane (Table 29).

Table 29. Results of the simulation of reaction conditions and working up for cyclododecanol (**2c**)



2c 1/10/100 mM — **2c**

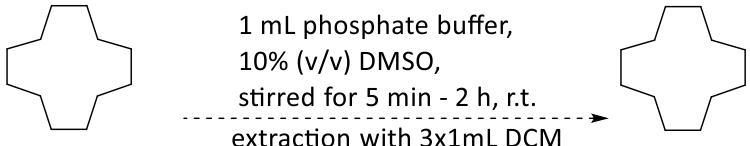
Entry[a]	**2c** [mmol]	Time	Initial weight [mg]	Output weight [mg][d]	Deviation[e] [%]
1	0.1	5 min	18.35	20.04	+9
2	0.1	2 h	18.72	20.16	+8
3[b]	0.1	2 h	18.35	18.45	+1
4	0.01	5 min	2.21	2.27	+3
5	0.01	2 h	2.18	2.19	0
6[b]	0.01	2 h	2.20	2.13	-3
7	0.001	5 min	0.184[c]	0.151	-18
8	0.001	2 h	0.184[c]	0.150	-19
9[b]	0.001	2 h	0.184[c]	0.136	-26

[a]All measurements are conducted in a twofold determination; [b]30 mg denaturated CYP BM-3 19A12 are added; [c]weigh in *via* stock solution; [d]calculated *via* GC; [e]average deviation from a recovery of 100%.

8.2.2.3 Cyclododecane (1c)

In a 25 mL round bottom flask cyclododecane (**1c**) (0.168 - 16.97 mg, 0.001 - 0.10 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) and 100 µL DMSO. Then 0 - 30 mg of the denaturated (60°C, 20 h) cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, lyophilisate) are added. The mixture is stirred for 5 min - 2 h at room temperature covered with a plug and worked up according to SOP B, but a 2 mL volumetric flask was used instead of a 3 mL volumetric flask and the third extraction is conducted only with 500 µL dichloromethane (Table 30).

Table 30. Results of the simulation of reaction conditions and working up for cyclododecane (**1c**)



1c
1/10/100 mM

1c

Entry[a)]	**1c** [mmol]	Time	Initial weight [mg]	Output weight [mg][d)]	Deviation[f)] [%]
1	0.1	5 min	16.89	18.26	+8
2	0.1	2 h	16.95	18.35	+8
3[b)]	0.1	2 h	16.97	18.03	+6
4	0.01	5 min	1.80	1.87	+4
5	0.01	2 h	1.75	1.79	+2
6[b)]	0.01	2 h	1.92	1.86[e)]	-3
7	0.001	5 min	0.168[c)]	0.162	-4
8	0.001	2 h	0.168[c)]	0.106	-37
9[b)]	0.001	2 h	0.168[c)]	0.039	-77

[a)]All measurements are conducted in a twofold determination; [b)]30 mg denaturated CYP BM-3 19A12 are added; [c)]weigh in *via* stock solution; [d)]calculated *via* GC; [e)]loss of one drop during working up; [f)]average deviation from a recovery of 100%.

8.2.3 Standard operation procedure 3 (SOP 3): Simultaneous quantification of cycloalkanes 1, cycloalkanols 2 and cycloalkanones 3 *via* gas chromatography

In a 100 mL volumetric flask a stock solution in acetone is prepared containing the substrate **1**, the intermediate **2** and the product **3**, each in a concentration of 10 g/L (A) resp. 0.2 g/L (B) for the compounds **1-3c**; 10 g/L (A) resp. 0.80 g/L (B) for the compounds **1-3b**; 15 g/L (A) resp. 0.80 g/L (B) for the compounds **1-3a**. A serial dilution is prepared to measure the following concentrations *via* gas chromatography:
1-3c: Method A (0.1 - 10 g/L): 10 g/L, 5 g/L, 1 g/L, 0.5 g/L, 0.3 g/L, 0.1 g/L; Method B (0.025 - 0.2 g/L): 0.2 g/L, 0.1 g/L, 0.05 g/L, 0.025 g/L.
1-3b: Method A (0.1 - 10 g/L): 10 g/L, 1 g/L, 0.5 g/L, 0.3 g/L, 0.1 g/L; Method B (0.025 - 0.80 g/L): 0.80 g/L, 0.40 g/L, 0.20 g/L, 0.10 g/L, 0.05 g/L, 0.025 g/L.
1-3a: Method A (0.15 - 1.5 g/L): 1.5 g/L, 1.0 g/L, 0.75 g/L, 0.5 g/L, 0.3 g/L, 0.15 g/L; Method B (0.025 - 0.80 g/L): 0.80 g/L, 0.40 g/L, 0.20 g/L, 0.10 g/L, 0.05 g/L, 0.025 g/L.

All measurements are conducted in a sixfold determination and different concentrations of **1**, **2** and **3** are analyzed to verify the respective method.

8.2.3.1 Quantification of cyclododecane (1c), cyclododecanol (2c), cyclododecanone (3c)

According to SOP 3 a stock solution in acetone is prepared containing the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product cyclododecanone (**3c**). A serial dilution is prepared to measure different concentrations *via* gas chromatography. Straight calibration lines are prepared from the obtained data and different concentrations are analyzed to verify these methods.

8.2.3.2 Definition of the methods detection limit (MDL)

The detection limit of cyclododecane (**1c**), cyclododecanol (**2c**) and cyclododecanone (**3c**) is defined *via* gas chromatography. A stock solution in acetone is therefore prepared containing the substrate cyclododecane (**1c**), the intermediate cyclododecanol (**2c**) and the product cyclodecanone (**3c**), each in a concentration of 0.04 g/L. A serial dilution is prepared to measure the following concentrations *via* gas chromatography: 0.04 g/L, 0.02 g/L, 0.01 g/L, 0.005 g/L, 0.002 g/L. All measurements are conducted in a twofold determination (Table 31).

Table 31. GC-determined percental deviation from the prepared concentrations of cyclododecane (**1c**), cyclododecanol (**2c**) and cyclododecanone (**3c**) (B)

Entry[a)]	Compound	Deviation[b)] at a concentration of…				
		…0.04 g/L	…0.02 g/L	…0.01 g/L	…0.005 g/L	…0.002 g/L
1	**1c**	+3%	-7%	-9%	-13%	-31%
2	**2c**	-1%	-6%	-6%	-2%	+6%
3	**3c**	-6%	-8%	-9%	-13%	-41%

[a)]All measurements are conducted in a twofold determination; [b)]average deviation from a recovery of 100%.

8.2.3.3 Quantification of cyclodecane (1b), cyclodecanol (2b), cyclodecanone (3b)

According to SOP 3 a stock solution in acetone is prepared containing the substrate cyclodecane (**1b**), the intermediate cyclodecanol (**2b**) and the product cyclodecanone (**3b**). A serial dilution is prepared to measure different concentrations *via* gas chromatography. Straight calibration lines are prepared from the obtained data and different concentrations are analyzed to verify these methods (Table 32).

Table 32. Results of the verification of the GC-method for **1b**, **2b** and **3b**

Entry[a)] (method A)	Compound	Deviation[c)] at a concentration of…		
		…7.0 g/L	…1.4 g/L	…0.2 g/L
1	**1b**	-1.7%	-3.6%	0.0%
2	**2b**	+2.9%	n.d.	-5.0%
3	**3b**	-1.4%	-2.7%	-8.6%
Entry[b)] (method B)	Compound	Deviation[c)] at a concentration of…		
		…0.8 g/L	…0.1 g/L	…0.025 g/L
1	**1b**	-1.4%	-2.7%	-10.0%
2	**2b**	-0.5%	+0.1%	+0.8%
3	**3b**	-1.5%	-2.1%	-5.0%

[a)]All measurements are conducted in a fourfold determination; [b)]All measurements are conducted in a twofold determination; [c)]average deviation from a recovery of 100%.

8.2.3.4 Quantification of cyclohexane (1a), cyclohexanol (2a), cyclohexanone (3a)

According to SOP 3 a stock solution in acetone is prepared containing the substrate cyclohexane (**1a**), the intermediate cyclohexanol (**2a**) and the product cyclohexanone (**3a**). A serial dilution is prepared to measure different concentrations *via* gas chromatography. Straight calibration lines are prepared from the obtained data and different concentrations are analyzed to verify these methods (Table 33).

Table 33. Results of the verification of the GC-method for **1a**, **2a** and **3a**

Entry[a)] (method A)	Compound	Deviation[c)] at a concentration of…		
		…1.3 g/L	…0.9 g/L	…0.2 g/L
1	**1a**	+7.8%	+12.2%	+13.9%
2	**2a**	-1.3%	-10.6%.	-4.5%
3	**3a**	-5.5%	-6.9%	-6.7%
Entry[b)] (method B)	Compound	Deviation[c)] at a concentration of…		
		…0.8 g/L	…0.1 g/L	…0.025 g/L
1	**1a**	+5.0%	+9.5%	+21.4%
2	**2a**	+4.5%	+1.9%	+2.1%
3	**3a**	-1.8%	-5.3%	-10.8%

[a)]All measurements are conducted in a fourfold determination; [b)]All measurements are conducted in a twofold determination; [c)]average deviation from a recovery of 100%.

8.2.4 Standard operation procedure 4 (SOP 4): Photometric assay to determine the enzyme activity of different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3)

The enzyme activities can be determined with an UV/VIS-spectrophotometer for NAD(P)H is used as cofactor. The consumption of NAD(P)H through oxidation to $NAD(P)^+$ is measured spectrophotometrically at a wavelength of 340 nm in the presence of the tested enzyme and one of the following compounds: Cyclohexane (**1a**), cyclodecane (**1b**), cyclododecane (**1c**), pentanal (**19**), cyclooctyne (**17**) or cyclododecene (**18**). A solution of the respective substrate (12 - 25 mM resp. 0.5 - 16 mM **1c** for kinetics, assuming that the substrate will dissolve completely) is prepared in DMSO. For this purpose the substrates are weigh in a 5 mL volumetric flask which is filled up with DMSO.

A cuvette (1 mL) is filled with 690 µl phosphate buffer (pH 7.0, 50 mM) and 10 µL of the substrate solution in DMSO (12 - 25 mM). Then 100 µL of the enzyme solution (10 mg/mL) are pipetted in the cuvette and mixed by inversion. After 5 min, 200 µl of a buffered solution of the cofactor NAD(P)H (NAD(P)H: 0.6 - 0.8 mM; phosphate buffer: pH 7.0, 50 mM) are pipetted in the cuvette and mixed by inversion. Then the measurement is started immediately.

At first a measurement of the blank value (DMSO without substrate) is taken in which 690 µl of the phosphate buffer (pH 7.0, 50 mM), 10 µl DMSO and 100 µl of the enzyme solution (10 mg/mL) are pipetted in the cuvette and mixed by inversion. After 5 min, 200 µl of a buffered solution of the cofactor NAD(P)H (NAD(P)H: 0.6-0.8 mM; phosphate buffer: pH 7.0, 50 mM) are pipetted in the cuvette and mixed by inversion. Then the measurement is started immediately. This blank value is subtracted from the measured values resulting from the measurements including the respective substrate solutions. By means of the initial slope of the absorption curve and the following formula the volumetric enzyme activity resp. the kinetics resp. the stability can be determined.

$$\frac{U}{mL} = \frac{\Delta E_{340nm}\, V_t\, f}{\varepsilon\, V_s\, t\, d}$$

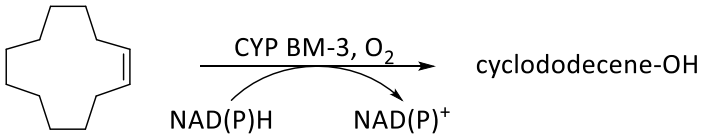
8.2.4.1 Enzyme activity towards monounsaturated cyclic hydrocarbons

8.2.4.1.1 Enzyme activity towards cyclododecene (18)

The measurements are performed as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclododecene (**18**). A solution of **18** (12 mM, assuming that **18** will dissolve completely) is prepared in DMSO. For this purpose 9.98 mg (0.06 mmol) of **18** are weigh in a 5 mL volumetric flask which is filled up with DMSO.

A 0.6 mM solution of NAD(P)H is used for the measurements. For all preparative experiments 0.762 U relative to **18** are used (Table 34).

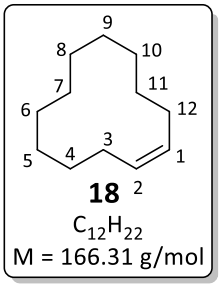
Table 34. Results of the enzyme activity test with cyclododecene (**18**) as substrate



18

Entry	CYP BM-3[a)]	Specific activity [U/mg]	0.762 U	Content of CYP [nmol/g lyophilisate][d)]	TOF
1	19A12 (1)	--	--	60	--
2	F87P (2)	0.040	19.05 mg	223	179
3	F87V	--	--	525	--
4	F87A A328V (2)	0.040	19.05 mg	989	40
5	WT	--	--	1260	--
6	R255P-P329H[b)]	0.003	254.0 mg	220	14

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm; [a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]determined *via* CO-difference spectroscopy.



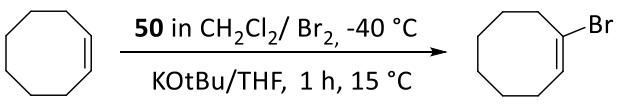
18
$C_{12}H_{22}$
M = 166.31 g/mol

^{1}H-NMR (500 MHz, CD_2Cl_2):
δ (ppm) = 5.28-5.39 (2H, m, H-C**1**,**2**), 2.04-2.13 (4H, m, H-C**3**, **12**), 1.25-1.47 (16H, m, H-C**4-11**).

8.2.4.1.2 Synthesis and enzyme activity towards cyclooctyne (17)

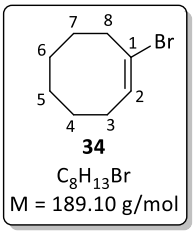
Two-step synthesis:

1) Synthesis of 1-bromocyclooctene (**51**))[125]:



50 **51**

Scheme 57. Synthesis of 1-bromocyclooctene (**51**) according to BRANDSMA *et al.*[125]



34
$C_8H_{13}Br$
M = 189.10 g/mol

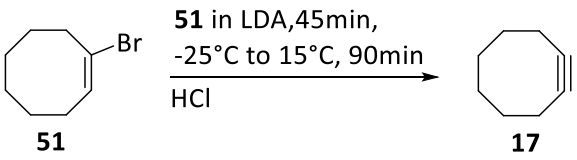
The synthesis of 1-bromocyclooctene (**51**) is carried out according to BRANDSMA *et al.* starting from cyclooctene (**50**).[125] To a solution of cyclooctene (**50**; 11.02 g, 0.1 mol) in dichloromethane (35 mL) bromine (5.15 mL, 0.1 mol) is added with cooling at about -40°C until the brown colour persists. After removal of the solvent *in vacuo* the residue is dissolved in dry THF (40 mL) and a suspension of potassium *t*-butoxide (16.85 g, 0.15 mol) in 50 mL THF is added in 20 min to the vigorously stirred mixture, keeping its temperature at 0 °C. After the addition, stirring is continued for 1 h at 15 °C. The mixture is then poured into ice/water (50 mL) and the organic products are extracted with MTBE. The organic phase is dried over magnesium sulfate. After evaporation of the solvent *in vacuo* the crude product **51** is obtained as pale beige oil and purified *via* fractional distillation.

Yield: 12.90 g (68%)

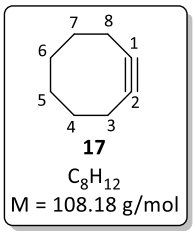
^{1}H-NMR (500 MHz, $CDCl_3$):
δ (ppm) = 6.05 (1H, t, *J* = 12.5 Hz, H-C**2**), 2.60-2.62 (2H, m, H-C**8**), 2.08-2.13 (2H, m, H-C**3**), 1.46-1.66 (8H, m, H-C**4**-**7**).

The compound **51** is further confirmed through conversion into the product **17** and its analytical characterization.

2) Synthesis of cyclooctyne (**17**))[125]:



Scheme 58. Synthesis of cyclooctyne (**17**) according to BRANDSMA *et al.*[125]



17
C_8H_{12}
M = 108.18 g/mol

The above product (**51**; 12.90 g, 68.22 mmol) is added at once to a solution of lithium diisopropylamide (1.5 M) in THF, cooled at -25 °C. The temperature of the reaction mixture is allowed to rise gradually over a period of 45 min to 15 °C and is kept at this level for another 90 min. It is then poured into a cold solution of 3 normal hydrochloric acid. Five extractions with *n*-heptane (5 x 15 mL) are carried out. The combined extracts are washed several times with water in order to remove the THF and are then dried over magnesium sulphate. The solvents are removed *in vacuo* keeping the bath temperature at 0 °C. Distillation of the crude product by using a vigreux column gives cyclooctyne (**17**).

Yield: 0.87 g (12%)

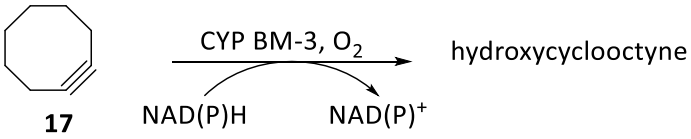
^{1}H-NMR (500 MHz, $CDCl_3$):
δ (ppm) = 2.15-2.18 (4H, m, H-C**4**-**7**), 1.86-1.87 (4H, m, H-C**4**-**7**), 1.60-1.64 (4H, m, H-C**3**,**8**).

The spectral data are in accordance with literature.[126]

Enzyme activity:

The measurements are taken as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclooctyne (**17**). A solution of **17** (12 mM, assuming that **17** will dissolve completely) is prepared in DMSO. For this purpose 6.49 mg (0.06 mmol) of **17** are weigh in a 5 mL volumetric flask which is filled up with DMSO. A 0.6 mM solution of NAD(P)H is used for the measurements (Table 35).

Table 35. Results of the enzyme activity test with CYP BM-3 and cyclooctyne (**17**)



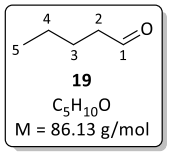
17

Entry	CYP BM-3[a)]	Substrate	Specific activity [U/mg]	0.762 U
2	F87A A328V (2)	cyclooctyne (**17**)	--	--
4	F87P (2)	cyclooctyne (**17**)	--	--

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm [a)]In parentheses the different enzyme formulations are given.

8.2.4.2 Enzyme activity towards pentanal (19)

Stability of pentanal (**19**) in water over the time:



19
$C_5H_{10}O$
M = 86.13 g/mol

The stability of pentanal (**19**) in water is analyzed over a period of 24 h in the presence of equimolar amounts sodium acetate as external standard. For this purpose pentanal (**19**) (8.6 mg, 0.1 mmol) is weigh in a 25 mL round bottom flask and 1 mL D_2O is added. The mixture is stirred for up to 24 h and then transferred into a NMR-tube filled with sodium acetate (8.2 mg, 0.1 mmol). The formation of the corresponding carboxylic acid was found only to very small extent of 5.4% on an average, identified on the basis of the characteristic triplet caused by the two C_α-protons at 2.2 ppm. Pentanal (**19**) is high vacuum distilled prior use and purity is examined *via* ^{1}H-NMR-spectroscopy.

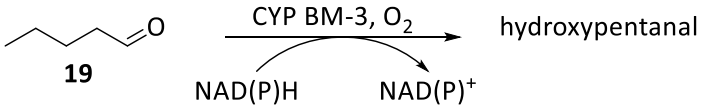
^{1}H-NMR (500 MHz, CD_2Cl_2):

δ (ppm) = 9.73 (1H, t, *J* = 1.8 Hz, H-C**1**), 2.40 (2H, td, *J* = 7.4 Hz, 1.8 Hz, H-C**2**), 1.55-1.62 (2H, m, H-C**3**), 1.31-1.38 (2H, m, H-C**4**), 0.92 (3H, t, *J* = 7.4 Hz, H-C**5**)

Enzyme activity

The measurements are performed as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for pentanal (**19**). A solution of **19** (12 mM, assuming that **19** will dissolve completely) is prepared in DMSO. For this purpose 5.17 mg (0.06 mmol) of **19** are weigh in a 5 mL volumetric flask which is filled up with DMSO. A 0.6 mM solution of NAD(P)H is used for the measurements. For all preparative experiments 0.762 U relative to **19** are used (Table 36).

Table 36. Results of the enzyme activity test with CYP BM-3 for pentanal (**19**)



Entry	CYP BM-3[a)]	Specific activity [U/mg]	0.762 U	Content of CYP[d)] [nmol/g lyophilisate]	TOF [min^{-1}]
1	F87A A328V (2)	0.010	76.2 mg	989	10
2	F87P (2)	0.021	36.3 mg	223	94
3	19A12 (2)	0.029	26.3 mg	120	242
4	F87V	0.009	84.7 mg	525	17
5	R255P P329H[b)]	--	--	220	--
6	WT[c)]	0.009	84.7 mg	1260	7

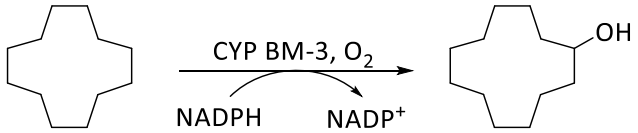
V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm; [a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]5 mg/mL enzyme; [d)]determined *via* CO-difference spectroscopy.

8.2.4.3 Enzyme activity towards higher homologous cycloalkanes

8.2.4.3.1 Enzyme activity towards cyclododecane (1c)

The measurements are performed as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclododecane (**1c**). A solution of **1c** (25 mM: entry 1-10; 12.5 mM: entry 11-13; assuming that **1c** will dissolve completely) is prepared in DMSO. For this purpose 10.5 mg resp. 21 mg (0.06 mmol resp. 0.12 mmol) are weigh in a 5 mL volumetric flask which is filled up with DMSO. A 0.8 mM (entry 1-2) resp. 0.6 mM (entry 3-13) solution of NAD(P)H was used for the measurements. For all preparative experiments 0.762 U relative to **1c** are used (Table 37).

Table 37. Results of the enzyme activity test with cyclododecane (**1c**) as substrate



1c **2c**

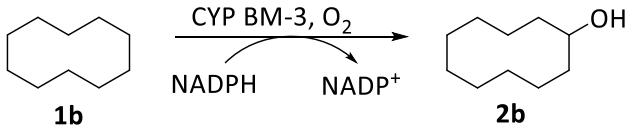
Entry	CYP BM-3[a)]	Specific activity [U/mg]	0.762 U	Content of CYP[d)] [nmol/g lyophilisate]	TOF [min^{-1}]
1	19A12 (0)	0.003	254.0 mg	123	24
2	F87P (1)	0.052	14.7 mg	235	221
3	19A12 (1)	--	--	60	--
4	19A12 (2)	--	--	120	--
5	19A12 (3)	0.001	762 mg	115	9
6	F87P (1)	0.034	22.4 mg	235	145
7	F87V	0.001	762 mg	525	2
8	F87A A328V (1)	0.015	50.8 mg	480	31
9	WT	--	--	1260	--
10	R255P-P329H[b)]	--	--	220	--
11	F87A A328V (2)	0.027	28.2 mg	989	27
12	F87A A328V (2)[c)]	0.005	152.4 mg	989	5
13	F87A A328V (2)	0.023	33.1 mg	989	23

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm; [a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]10% (v/v) DMSO by adding 600 µL phosphate buffer (pH 7.0, 50 mM)/ 90 µL DMSO and 10 µL of **1c** in DMSO (12.5 mM) instead of 690 µl phosphate buffer (pH 7.0, 50 mM) and 10 µL of **1c** in DMSO (12.5 mM); [d)]determined *via* CO-difference spectroscopy.

8.2.4.3.2 Enzyme activity towards cyclodecane (1b)

The measurements are performed as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenases from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclodecane (**1b**). A solution of **1b** (25 mM: entry 1-4, 6-13; 12 mM: entry 5, assuming that **1b** will dissolve completely) is prepared in DMSO. For this purpose 8.4 mg resp. 17.5 mg (0.06 mmol resp. 0.12 mmol) are weigh in a 5 mL volumetric flask which is filled up with DMSO. A 0.8 mM (entry 1-4) resp. 0.6 mM (entry 5-13) solution of NAD(P)H was used for the measurements. For all preparative experiments 0.762 U relative to **1b** are used (Table 38).

Table 38. Results of the enzyme activity test with cyclodecane (**1b**) as substrate



1b **2b**

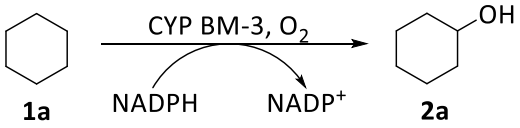
Entry	CYP BM-3[a)]	Specific activity [U/mg]	0.762 U	Content of CYP[c)] [nmol/g lyophilisate]	TOF [min^{-1}]
1	F87V	0.006	127.0 mg	525	11
2	19A12 (0)	0.039	19.6 mg	123	317
3	WT	0.008	95.3 mg	1260	6
4	Empty vector *E.coli* BL21	0.001	762.0 mg	--	--
5	19A12 (2)	0.051	14.9 mg	120	425
6	19A12 (1)	0.051	14.9 mg	60	850
7	19A12 (2)	0.052	14.7 mg	120	433
8	19A12 (3)	0.056	13.6 mg	115	487
9	F87P (1)	0.076	10.0 mg	235	323
10	F87V	0.005	152.4 mg	525	10
11	F87A A328V (1)	0.020	38.1 mg	480	42
12	WT	0.004	190.5 mg	1260	3
13	R255P-P329H[b)]	0.014	54.4 mg	220	64

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm; [a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]determined *via* CO-difference spectroscopy.

8.2.4.3.3 Enzyme activity towards cyclohexane (1a)

The measurements are performed as described above (SOP 4) to determine the enzyme activity of different cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (solution of the lyophilisate: 10 mg/mL, NAD(P)H-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclohexane (**1a**). A solution of **1a** (25 mM: entry 1-12; 12 mM: entry 13-14, assuming that **1a** will dissolve completely) is prepared in DMSO. For this purpose 5.1 mg resp. 10.5 mg (0.06 mmol resp. 0.12 mmol) are weigh in a 5 mL volumetric flask which is filled up with DMSO. A 0.8 mM (entry 1-4) resp. 0.6 mM (entry 5-14) solution of NAD(P)H was used for the measurements. For all preparative experiments 0.762 U relative to **1a** are used (Table 39).

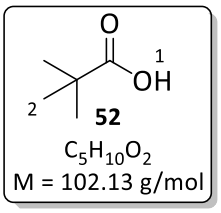
Table 39. Results of the enzyme activity test with cyclohexane (**1a**) as substrate



Entry	CYP BM-3[a)]	Specific activity [U/mg]	0.762 U	Content of CYP[c)] [nmol/g lyophilisate]	TOF [min^{-1}]
1	F87V	0.002	343.24 mg	525	4
2	19A12 (0)	0.146	5.22 mg	123	1187
3	WT	0.007	109.17 mg	1260	6
4	Empty vector *E.coli* BL21	0.004	1.772 g	--	--
5	19A12 (1)	0.131	5.8 mg	60	2183
6	19A12 (2)	0.161	4.7 mg	120	1342
7	19A12 (3)	0.134	5.7 mg	115	1165
8	F87P (1)	0.025	30.5 mg	235	106
9	F87V	0	--	525	--
10	F87A A328V (1)	0.010	76.2 mg	480	21
11	WT	0.001	762.0 mg	1260	1
12	R255P-P329H[b)]	0.009	84.7 mg	220	41
13	19A12 (2)	0.074	10.3 mg	120	617
14	19A12 (2)	0.047	16.2 mg	120	392

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm; [a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]determined *via* CO-difference spectroscopy.

8.2.5 Standard operation procedure 5 (SOP 5): Analysis of the kinetics according to MICHAELIS-MENTEN of the cyclododecanol (2b)-formation

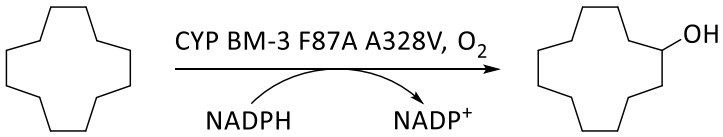


The concentration of the saturated solution (~16 mM) of **1c** in DMSO is identified *via* ^{1}H-NMR-spectroscopy with pivalic acid (**52**) as standard. **^{1}H-NMR** (**1c**) (500 MHz, DMSO): δ (ppm) = 1.30 (24H, s); **^{1}H-NMR** (**52**) (500 MHz, DMSO): δ (ppm) = 12.03 (1H, s, HO-C**1**), 1.11 (9H, s, H-C**2**)

The measurements are performed as described above (SOP 4) to determine the enzyme activity of the cytochrome P450 monooxygenase mutant from *Bacillus megaterium* CYP BM-3 F87A A328V (1) (solution of the lyophilisate: 10 mg/mL, NADPH-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclododecane (**1c**). Solutions of **1c** (0.5-16 mM) are prepared in DMSO.

For this purpose a stock solution of the substrate cyclododecane (**1c**) is prepared in DMSO in a 25 mL volumetric flask at a concentration of 16 mM. A set of serial dilution (16 mM, 12 mM, 8 mM, 4 mM, 2 mM, 1 mM, 0.5 mM) is prepared to measure the following absolute concentrations in a photometric assay: 160 µM, 120 µM, 80 µM, 40 µM, 20 µM, 10 µM and 5 µM. A 0.6 mM solution of NADPH was used for the measurements (Table 40). In this way the kinetics according to MICHAELIS-MENTEN of the cyclododecanol (**2c**)-formation can be determined *via* an enzyme activity test with the CYP BM-3 and cyclododecane (**1c**) as substrate.

Table 40. Results of the enzyme activity test with cyclododecane (**1c**) as substrate



1c
5-160 µM

2c

Entry	Substrate concentration [mM]	Initial slope v_o [1/min]
1	0.16	0.133
2	0.12	0.131
3	0.08	0.127
4	0.04	0.121
5	0.02	0.105
6	0.01	0.081
7	0.005	0.057

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm

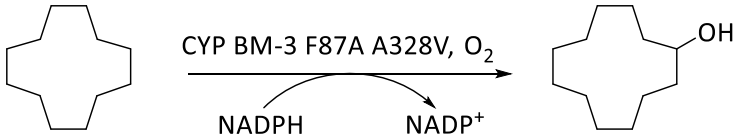
8.2.6 Standard operation procedure 6 (SOP 6): Analysis of the stability of CYP BM-3 F87A A328V

The measurements are performed in the manner of SOP 4 to determine the enzyme activities of the cytochrome P450 monooxygenase mutant from *Bacillus megaterium* CYP BM-3 F87A A328V (1) (solution of the lyophilisate: 10 mg/mL, NADPH-dependent, phosphate buffer: pH 7.0, 50 mM) for cyclododecane (**1c**) as a function of time. In a 25 mL round bottom flask 50.8 mg CYP BM-3 F87A A328V (0.762 U) and D-glucose monohydrate (**22**) (3.96 mg, 0.02 mmol) are mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) and 100 µL DMSO (10% v/v). The mixture is stirred and samples are taken at (regular) intervals over a period of 28h.

A cuvette (1 mL) is filled with 680 µl phosphate buffer (pH 7.0, 50 mM) and 100 µL of **1c** in DMSO (1.2 mM) is added. Then 20 µL of the enzyme containing mixture (50 mg/mL) are pipetted in the cuvette and mixed by inversion.

After 5 min 200 µl of a buffered solution of the cofactor NADPH (NADPH: 0.6 mM; phosphate buffer: pH 7.0, 50 mM) are pipetted in the cuvette and mixed by inversion. Hereafter the measurement is started immediately. Before every sampling at a certain time (Table 41), at first a measurement of the blank value (DMSO without substrate) is taken according to SOP 4 (680 µl phosphate buffer (pH 7.0, 50 mM), 100 µl DMSO, 20 µl of the enzyme containing mixture (50 mg/mL), 200 µl of a buffered solution of the cofactor NADPH (NADPH: 0.6 mM; phosphate buffer: pH 7.0, 50 mM)).

Table 41. Enzyme activity of CYP BM-3 F87A A328V as a function of time



1c
stirred up to 28h

2c

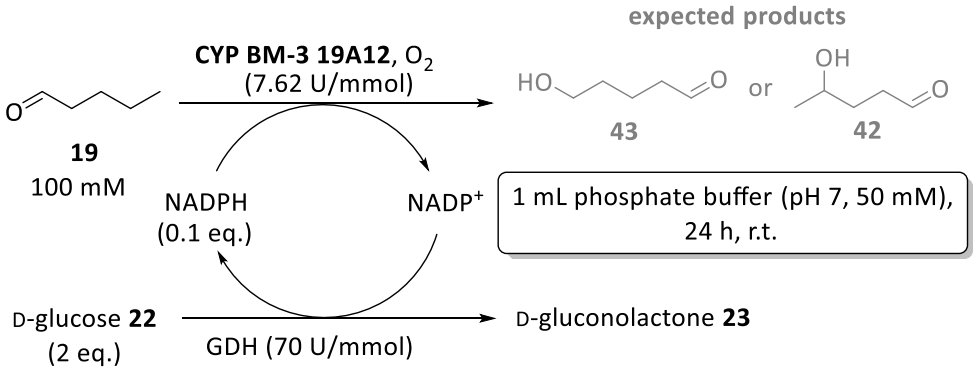
Entry	Time [h]	Initial slope v_0 [1/min]
1	0.167	0.0725
2	1	0.0741
3	2	0.0874
4	4	0.0689
5	6	0.0904
6	28	0.0585

V_t = 1 mL, f = 1, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.1 mL, d = 1 cm

8.2.7 Standard operation procedure 7 (SOP 7): Biocatalyzed oxidation of cyclododecene (18) with different CYP BM-3 and glucose dehydrogenase (GDH)

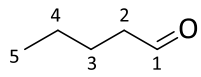
In a 25 mL round bottom flask cyclododecene (**18**) (16.63 mg, 0.1 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 F87P resp. CYP BM-3 F87A A328V) (0.762 U, 19.05 mg) and 100 µl GDH (7 U) dissolved in phosphate buffer (pH 7.0, 50 mM) and glycerol (1:1) are added. Then D-glucose monohydrate (**22**) (39.6 mg, 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 mg, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and is worked up according to SOP B using deuterated dichloromethane. This solution is analyzed *via* ^{1}H-NMR-spectroscopy and compared to the spectra of the substrate **18**. The formation of an oxidation product was not detected, neither with CYP BM-3 F87P nor with CYP BM-3 F87A A328V.

8.2.8 Standard operation procedure (SOP 8): Biocatalyzed oxidation of pentanal (19) with different CYP BM-3 and glucose dehydrogenase (GDH)



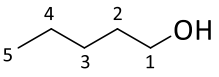
Scheme 59. Anticipated biocatalyzed oxidation of pentanal (**19**)

In a 25 mL round bottom flask pentanal (**19**) (8.6 mg, 0.1 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM). Then 0.762 U of a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12 (26.3 mg) resp. CYP BM-3 F87P (36.3 mg)) and 100 µl GDH (7 U) dissolved in phosphate buffer (pH 7.0, 50 mM) and glycerol (1:1) are added. Then D-glucose monohydrate (**22**) (39.6 mg, 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 mg, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and is worked up according to SOP B using 0.5 mL deuterated dichloromethane. The combined organic phases are transferred completely into a NMR-tube. This solution is analyzed *via* ^{1}H-NMR-spectroscopy and compared to the spectrum of the substrate **19**. Instead of the desired oxidation of pentanal (**19**), its reduction yielding 1-pentanol (**53**) took place, clearly identified *via* ^{1}H-NMR-spectroscopy:



19
$C_5H_{10}O$
M = 86.13 g/mol

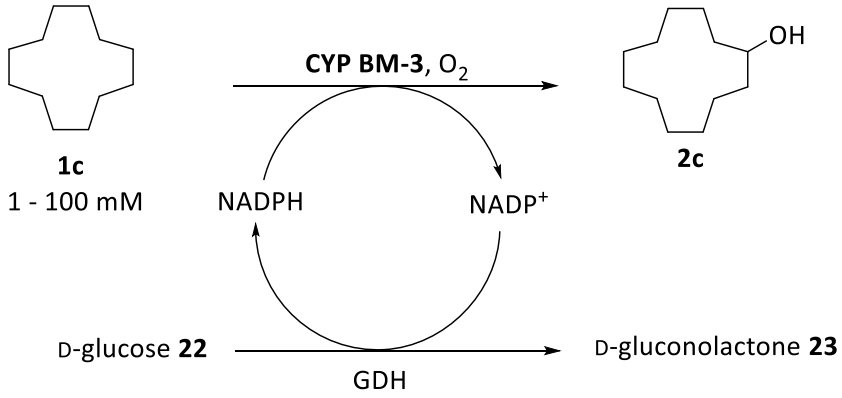
Pentanal (**19**): **^{1}H-NMR** (500 MHz, CD_2Cl_2):
δ (ppm) = 9.73 (1H, t, J = 1.8 Hz, H-C**1**), 2.40 (2H, td, J = 7.4, 1.8 Hz, H-C**2**), 1.55-1.62 (2H, m, H-C**3**), 1.31-1.38 (2H, m, H-C**4**), 0.92 (3H, t, J = 7.4 Hz, H-C**5**)



53
$C_5H_{12}O$
M = 88.15 g/mol

1-Pentanol (**53**): **^{1}H-NMR** (500 MHz, CD_2Cl_2):
δ (ppm) = 3.58 (2H, t, J = 5.0 Hz, H-C**1**), 1.51-1.61 (2H, m, H-C**2**), 1.26-1.37 (4H, m, H-C**3**, **4**), 0.89-0.93 (3H, m, H-C**5**).

8.2.9 Standard operation procedure (SOP 9): Biocatalyzed hydroxylation of the higher homologous cycloalkane 1c with different CYP BM-3 and glucose dehydrogenase (GDH)



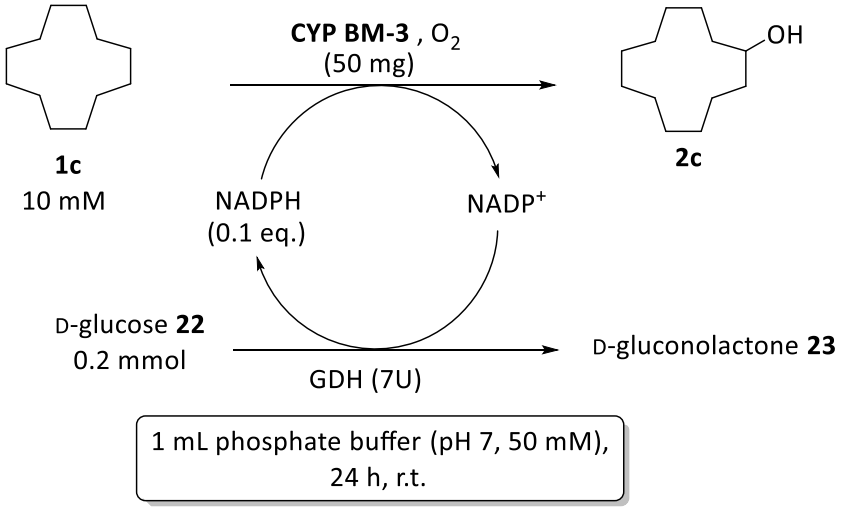
Scheme 60. Biocatalyzed hydroxylation of cyclododecane (**1c**)

In a 25 mL round bottom flask cyclododecane (**1c**) (0.168 - 16.8 mg, 0.001 - 0.1 mmol) is mixed with 800 - 900 µL phosphate buffer (pH 7.0, 50 mM) depending on the amount of DMSO. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (0.15 - 1.52 U), 0 - 200 µl GDH (0 - 14 U) diluted in phosphate buffer (pH 7.0, 50 mM) and 0 - 100 µL DMSO (containing the 0.0001 mmol **1c**) are added. Then D-glucose monohydrate (**22**) (0 - 39.6 mg, 0 - 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 - 83.4 mg, 0.01 - 0.1 mmol) resp. NADH (0.67 mg, 0.001 mmol) is added. The reaction mixture is stirred for 2-24 h at room temperature covered with a plug and is worked up according to SOP B.

8.2.9.1 Hydroxylation of cyclododecane (1c) with spectrophotometrically inactive mutants of CYP BM-3

According to SOP 9 cyclododecane (**1c**) (1.68 mg, 0.01 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (50 mg) and 100 µl GDH (7 U) diluted in phosphate buffer (pH 7.0, 50 mM) are added. Then D-glucose monohydrate (**22**) (3.96 mg, 0.02 mmol) is weigh in. To start the reaction cofactor NADPH (0.83 mg, 0.001 mmol) resp. NADH (0.67 mg, 0.001 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 9 (Table 42).

Table 42. Results of the hydroxylation of cyclododecane (**1c**) with spectrophotometrically inactive mutants of CYP BM-3



Entry	CYP BM-3[a)]	Cyclododecanol **2c** [mg/L][c)]	Conversion [%]
1	19A12 (1)	<5	--
2	19A12 (2)	<5	--
3	19A12 (3)	<5	--
4	F87V	~3	0.75
5	WT	<5	--
6	R255P P329H[b)]	<5	--

[a)]In parentheses the different enzyme formulations are given; [b)]NADH-dependent; [c)]Analysis *via* gas chromatography (method B).

8.2.9.2 Hydroxylation of cyclododecane (1c) with different CYP BM-3

According to SOP 9 cyclododecane (**1c**) (1.68 - 16.8 mg, 0.01 - 0.1 mmol) is mixed with 800 - 900 µL phosphate buffer (pH 7.0, 50 mM) in a 25 mL round bottom flask, depending on the amount of DMSO. Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*CYP BM-3, 0.15 - 1.52 U), 0 - 200 µl GDH (0 - 14 U) diluted in phosphate buffer (pH 7.0, 50 mM) and 0 - 100 µL DMSO are added. Then D-glucose monohydrate (**22**) (0 - 39.6 mg, 0 - 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (0.83 - 83.4 mg, 0.001 - 0.1 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 9 (Table 43).

Table 43. Results of the hydroxylation of cyclododecane (**1c**) with different CYP BM-3 according to Scheme 60

Entry[a,b)]	CYP BM-3	Activity CYP BM-3 [U]	GDH [U]	Substrate [mM]	Glucose [mmol]	NADPH [mol%]	DMSO [% (v/v)]
1	F87P	0.762	7	100	0.2	10	0
2	F87P	0.762	--	100	--	30	0
3	F87P	1.524	7	100	0.2	10	0
4	F87P	0.762	14	100	0.2	10	0
5	F87P	0.762	7	100	0.2	10	2
6	F87P	0.762	7	100	0.2	10	0
7	F87P	0.762	7	100	0.2	10	5
8	F87P	0.762	7	100	0.2	10	10
9	F87P	0.762	7	10	0.2	100	10
10	19A12	0.15	7	10	0.2	100	10

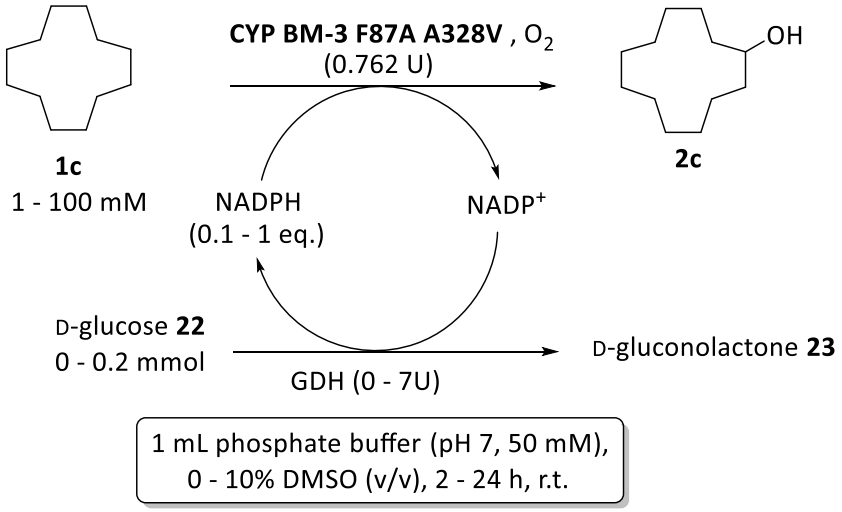
[a)]Analysis *via* gas chromatography (method B); [b)]The combined organic phases are transferred completely into a 2 mL volumetric flask for GC-analysis.

In all experiments shown in Table 43 the formation of cyclododecanol (**2c**) was below 5 mg/L and conversion could not be determined.

8.2.9.3 Hydroxylation of cyclododecane (1c) with CYP BM-3 F87A A328V

According to SOP 9 cyclododecane (**1c**) (0.168 - 16.8 mg, 0.001 - 0.1 mmol) is mixed with 800 - 900 µL phosphate buffer (pH 7.0, 50 mM) (depending on the amount of DMSO) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*CYP BM-3 F87A A328V, 0.762U), 0 - 100 µl GDH (0 - 7 U) diluted in phosphate buffer (pH 7.0, 50 mM) and 0 - 100 µL DMSO are added. Then D-glucose monohydrate (**22**) (3.96 - 39.6 mg, 0.02 - 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (0.83 - 83.4 mg, 0.001 - 0.1 mmol) is added. The reaction mixture is stirred for 2 - 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 9 (Table 44).

Table 44. Results of the oxidation of cyclododecane (**1c**) with CYP BM-3 F87A A328V



Entry	GDH [U]	Substrate [mM]	Glucose [mmol]	NADPH [mol%]	DMSO [% (v/v)]	Time [h]	**2c** [mg/L][a)]	Conversion [%]
1	7	100	0.2	10	0	24	<5	--
2	--	100	--	30	0	24	<5	--
3	7	100	0.2	10	5	24	33	0.17
4	7	100	0.2	10	10	24	40	0.19
5	7	10	0.2	100	10	24	<5	--
6	7	100	0.2	10	10	24	35	0.17
7	--	100	--	100	10	24	n.d.	n.d.
8	7	10	0.02	10	10	24	27	1.76
9	--	10	--	100	10	24	n.d.	n.d.
10	7	10	0.02	10	10	24	24	1.87
11	7	1	0.02	100	10	24	n.d.	n.d.
12	7	10	0.02	10	10	2	16	0.72
13	7	1	0.02	100	10	2	30	44.6

[a)]Analysis *via* gas chromatography (new calibration line); [b)]The combined organic phases are transferred completely into a 3 mL volumetric flask for GC-analysis.

8.2.10 Standard operation procedure 10 (SOP 10): Biocatalyzed hydroxylation of the higher homologous cycloalkanes 1a and 1b with CYP BM-3 19A12 and glucose dehydrogenase (GDH)

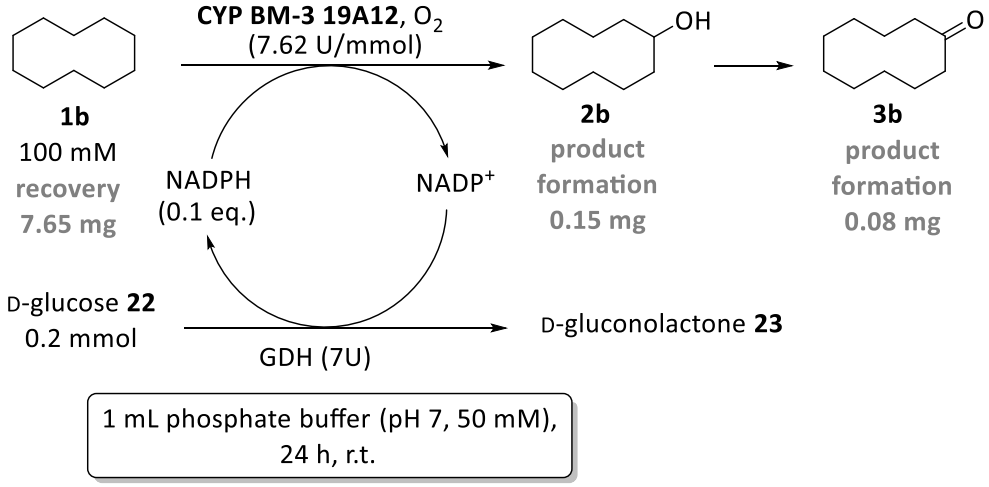
In a 25 mL round bottom flask a cycloalkane **1** (8.4 - 14.0 mg, 0.1 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) (0.762 U,

10.3 - 14.9 mg) and 100 µl GDH (7 U) dissolved in phosphate buffer (pH 7.0, 50 mM) and glycerol (1:1) are added. Then D-glucose monohydrate (**22**) (39.6 mg, 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and worked up according to SOP B.

8.2.10.1 Hydroxylation of cyclodecane (1b) with CYP BM-3 19A12

According to SOP 10 cyclodecane (**1b**) (14.0 mg, 0.1 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*CYP BM-3 19A12, 0.762U, 14.9 mg, the content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract) and 100 µl GDH (7U) dissolved in phosphate buffer (pH 7.0, 50 mM) and glycerol (1:1) are added. Then D-glucose monohydrate (**22**) (39.6 mg, 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP B (Table 45).

Table 45. Results of the oxidation of cyclodecane (**1b**) with CYP BM-3 19A12



Entry	Activity 19A12 [U]/[mg]	GDH [U]	Substrate [mmol]	Glucose [mmol]	NADPH [mol%]	Time [h]	Product formation [g/L]
1	0.762/14.9[a)]	7	0.1	0.2	10	24	0.15

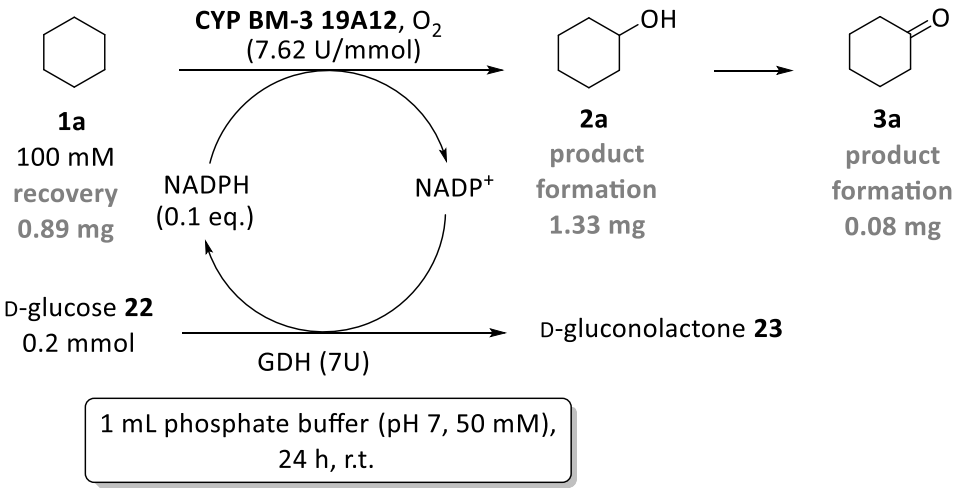
[a)]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract.

8.2.10.2 Hydroxylation of cyclohexane (1a) with CYP BM-3 19A12

According to SOP 10 cyclohexane (**1a**) (8.4 mg, 0.1 mmol) is mixed with 900 µL phosphate buffer (pH 7.0, 50 mM) in a 25 mL round bottom flask.

Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*CYP BM-3 19A12, 0.762U, 10.3 mg, the content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract) and 100 µl GDH (7U) dissolved in phosphate buffer (pH 7.0, 50 mM) and glycerol (1:1) are added. Then D-glucose monohydrate (**22**) (39.6 mg, 0.2 mmol) is weigh in. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP B (Table 46).

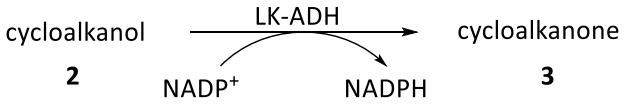
Table 46. Results of the oxidation of cyclohexane (**1a**) with CYP BM-3 19A12



Entry	activity 19A12 [U]/[mg]	GDH [U]	substrate [mmol]	glucose [mmol]	NADPH [mol%]	time [h]	product formation [g/L]
1	0.762/10.3[a)]	7	0.1	0.2	10	24	1.33

[a)]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract.

8.2.11 Standard operation procedure 11 (SOP 11): Photometric assay to determine the enzyme activity of the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH)



Scheme 61. General reaction equation of the photometric assay with LK-ADH

The enzyme activities can be determined with an UV/VIS-spectrophotometer for $NADP^+$ is used as cofactor. The formation of NADPH through reduction of $NADP^+$ is measured spectrophotometrically at a wavelength of 340 nm in the presence of the LK-ADH and each one of the following compounds: 1-phenylethanol (**71**), cyclohexanol (**2a**) and cyclodecanol (**2b**).

A 10 mM solution of the reference compound **71** and the cycloalkanols **2a** and **2b** (10 mM, assuming that **71** and **2** will dissolve completely) is prepared in phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$). For this purpose 5.0 - 7.8 mg (mmol) of **2a**, **2b** and **71** are weigh in a 5 mL volumetric flask which is filled up with phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$). A cuvette (1 mL) is filled with each 960 µl of a buffered solution of 1-phenylethanol (**71**), cyclohexanol (**2a**) or cyclodecanol (**2b**) (**71** and **2a** - **b**, phosphate buffer: pH 7.0, 50 mM, 1 mM $MgCl_2$). Then 20 µl of a buffered solution of the cofactor $NADP^+$ ($NADP^+$: 12.5 mM; phosphate buffer: pH 7.0, 50 mM, 1 mM $MgCl_2$) are pipetted in the cuvette and mixed by inversion. After that 20 µL enzyme solution (diluted 1:160 resp. 1:300 with phosphate buffer: pH 7.0, 50 mM, 1 mM $MgCl_2$) are pipetted in the cuvette and mixed by inversion. Then the measurement is started immediately. At first a measurement of the blank value is taken in which 960 µl of the phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$), 20 µl buffered solution of the cofactor $NADP^+$ ($NADP^+$: 12.5 mM; phosphate buffer: pH 7.0, 50 mM, 1 mM $MgCl_2$) and 20 µL enzyme solution (diluted 1:160 resp. 1:300 with phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$)) are pipetted in the cuvette, mixed by inversion and measured immediately. This blank value is subtracted from the measured values resulting from the measurements including the substrate solutions of **71** and **2a-b**. By means of the initial slope of the absorption curve and the following formula, the volumetric enzyme activity can be determined.

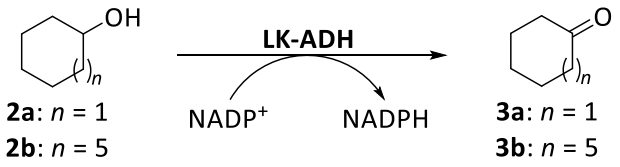
$$\frac{U}{mL} = \frac{\Delta E_{340nm}\, V_t\, f}{\varepsilon\, V_s\, t\, d}$$

With U/mL = volumetric enzyme activity; $\Delta E_{340nm}/t$ = initial slope of the absorption curve, V_t = total volume [mL]; f = dilution factor; ε = molar extinction coefficient for NAD(P)H [6.3 mL*µmol^{-1}*cm^{-1}]; V_S = sample volume [mL]; d = path length of the cuvette [cm]

8.2.11.1 Photometric assay with cyclohexanol (2a) resp. cyclodecanol (2b) as substrate

The measurements are taken as described above (SOP 11) to determine the enzyme activity of the LK-ADH (dilution of the crude extract: 1:160 or 1:300 with phosphate buffer: pH 7.0, 50 mM, 1 mM $MgCl_2$, NADPH-dependent). For all preparative experiments 200 U/mmol relative to cyclohexanol (**2a**) resp. cyclodecanol (**2b**) are used (Table 47 - Table 50).

Table 47 - Table 50. Summarized results of all enzyme activity tests with LK-ADH and **2a** - **b** as substrates



2a: *n* = 1
2b: *n* = 5

3a: *n* = 1
3b: *n* = 5

Table 47. Results of the enzyme activity test with LK-ADH and **2a** - **b** as substrates

Entry	Substrate	Vol. activity [U/mL]	200 U/mmol	Relative activity [%]
1	1-phenylethanol (**71**)	291.43	--	100
2	cyclohexanol (**2a**)	122.67	326 µL[a)]	42
3	1-phenylethanol (**71**)	286.54	--	100
4	cyclodecanol (**2b**)	441.59	91 µL[a)]	154

V_t = 1 mL, f = 160, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.02 mL, d = 1 cm; [a)]1:1-dilution in glycerol.

Table 48. Results of the enzyme activity test with LK-ADH and **2a** - **b** as substrates

Entry	Substrate	Vol. activity [U/mL]	200 U/mmol	Relative activity [%]
1	cyclohexanol (**2a**)	24.25	825 µL[a)]	n.d.[b)]
2	cyclodecanol (**2b**)	82.92	241 µL[a)]	n.d.[b)]

V_t = 1 mL, f = 160, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.02 mL, d = 1 cm; [a)]1:1-dilution in glycerol [b)]not determined.

Table 49. Results of the enzyme activity test with LK-ADH and **2a** - **b** as substrates

Entry	Substrate	Vol. activity [U/mL]	200 U/mmol	Relative activity [%]
1	1-phenylethanol (**71**)	154.52	--	100
2	cyclohexanol (**2a**)	37.86	528 µL[a)]	25
3	cyclodecanol (**2b**)	241.43	83 µL[a)]	156

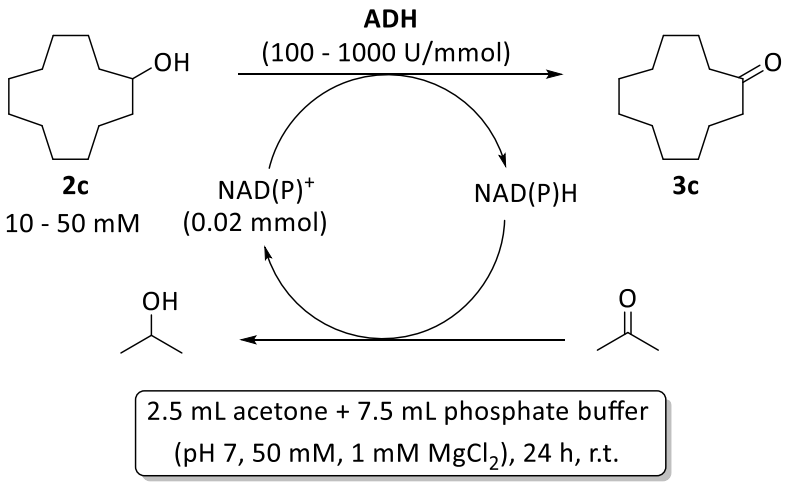
V_t = 1 mL, f = 300, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.02 mL, d = 1 cm; [a)]crude extract without glycerol.

Table 50. Results of the enzyme activity test with LK-ADH and **2a** as substrate

Entry	Substrate	Vol. activity [U/mL]	200 U/mmol	Relative activity [%]
1	1-phenylethanol (**71**)	171.4	--	100
2	cyclohexanol (**2a**)	45.2	443 µL[a)]	26

V_t = 1 mL, f = 300, ε = 6.3 mL*µmol^{-1}*cm^{-1}, V_s = 0.02 mL, d = 1 cm; [a)]crude extract without glycerol.

8.2.12 Standard operation procedure 12 (SOP 12): Biocatalyzed oxidation of cyclododecanol (2c) with different alcohol dehydrogenases



Scheme 62. ADH-catalyzed oxidation of cyclododecanol (**2c**)

In a 25 mL round bottom flask cyclododecanol (**2c**) (18.4 - 92.2 mg, 0.1 -0.5 mmol) is mixed with 2.5 mL acetone and 7.5 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$). Then 0.02 mmol cofactor $NAD(P)^+$ (13.3 - 15.8 mg) is added. To start the reaction an alcohol dehydrogenase (100 U/mmol - 1000 U/mmol, referred to the respective standard substrate (LK-ADH: acetophenone (**72**) (1544 U/mL[a)]); Rsp.-ADH: *p*-chloroacetophenone (**73**) (49 U/mL[a)]); ADH evo-1.1.200: ethyl acetoacetate (**63**) (4.5 U/mg) ([a)]diluted 1:1 in glycerol))) is added. The reaction mixture is stirred for 24 h at room temperature each time covered with a plug. Then the aqueous phase is extracted with dichloromethane (3 x 50 mL) very gently to get a well-defined phase boundary. The unified organic phase is dried over magnesium sulfate. After evaporation of the solvent the crude product is dissolved in 1.5 mL acetone again and the conversion is analyzed *via* gas chromatography. For this purpose the GC-areas of the substrate **2c** and the product **3c** are compared. In order to verify this method a mixture of each 0.25 mmol **2c** and **3c** is weigh in and measured, the resulting area-related conversion is compared to the expected weight-in conversion (Table 51).

Table 51. Comparison of the weigh-in conversion to the area-related conversion

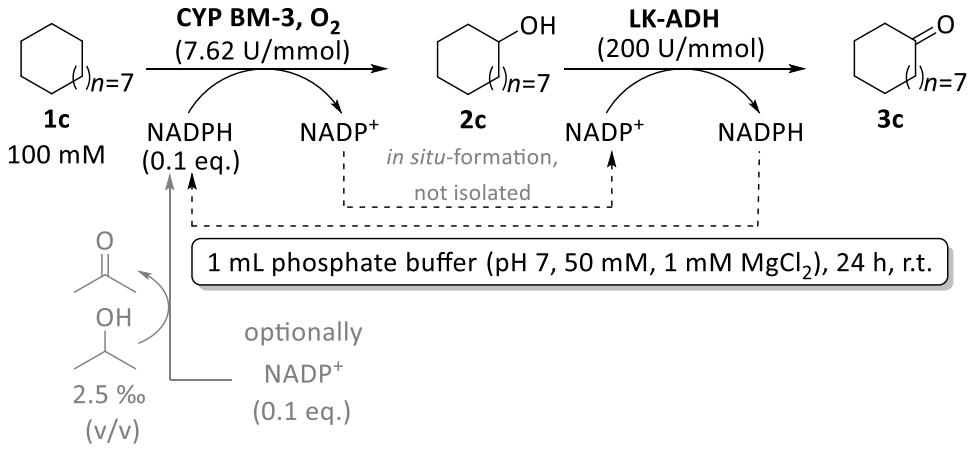
2c 0.25 mmol (46.08 mg)	**3c** 0.25 mmol (45.58 mg)	Conversion [%]
weigh in: 46.15 mg	weigh in: 46.56 mg	Weigh-in: 50.22% GC-Area-related: 49.99%

Table 52. Results of the ADH-catalyzed oxidation of cyclododecanol (**2c**) according to Scheme 62

Entry	ADH/µL [a)]	Activity [b)] [U/mmol]	Cofactor	Substrate [mmol]	Conversion [%]
1	LK-ADH/32.4 µL[a)]	100	$NADP^+$	0.5	2
2	Rsp.-ADH/2.5 mL[a)]	100	NAD^+	0.5	1
3	ADH evo-1.1.200/11.1 mg	100	NAD^+	0.5	2
4	LK-ADH/64.8 µL[a)]	1000	$NADP^+$	0.1	10
5	ADH evo-1.1.200/22.2 mg	1000	NAD^+	0.1	5

[a)]diluted 1:1 in glycerol; [b)]referred to the respective standard substrate (LK-ADH: acetophenone (**72**) (1544 U/mL[a)]); Rsp.-ADH: *p*-chloroacetophenone (**73**) (49 U/mL[a)]); ADH evo-1.1.200: ethyl acetoacetate (**63**) (4.5 U/mg)).

8.2.13 Standard operation procedure 13 (SOP 13): Biocatalyzed double oxidation of cyclododecane (1c) with CYP BM-3 and LK-ADH



Scheme 63. Double oxidation of cyclododecane (**1c**)

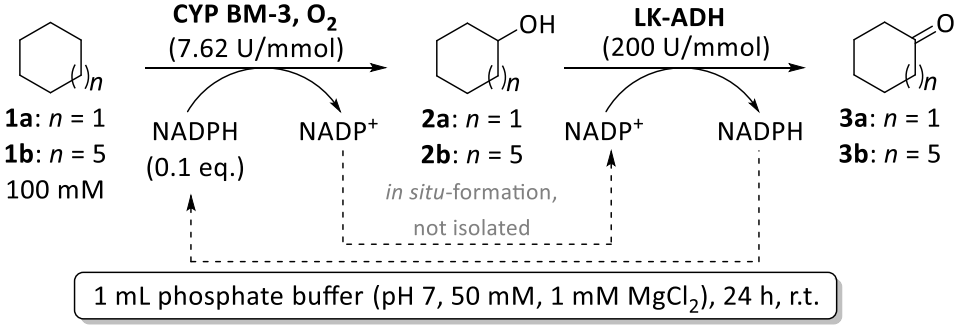
In a 25 mL round bottom flask cyclododecane (**1c**) (16.8 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) resp. 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to cyclodecane (**1b**) (entry 1-2) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol, in total 91 µL) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) resp. $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and worked up according to SOP A. The results are summarized in Table 53.

Table 53. Results of the double oxidation of cyclododecane (**1c**) according to Scheme 63

Entry	CYP BM-3 (biomass)	IPA	Cofactor	Cyclododecanone (**3c**) [g/l]]	Recovery of substrate **1c** [%]
1	19A12 (19.6 mg)[a)]	✓	$NADP^+$	0	92
2	F87V (127.0 mg)[b)]	-	NADPH	0	83

[a)]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b)]The content of CYP BM-3 F87V amounts to 0.525 µmol/g lyophilized crude extract.

8.2.14 Standard operation procedure 14 (SOP 14): Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH



Scheme 64. Double oxidation of cycloalkanes **1**

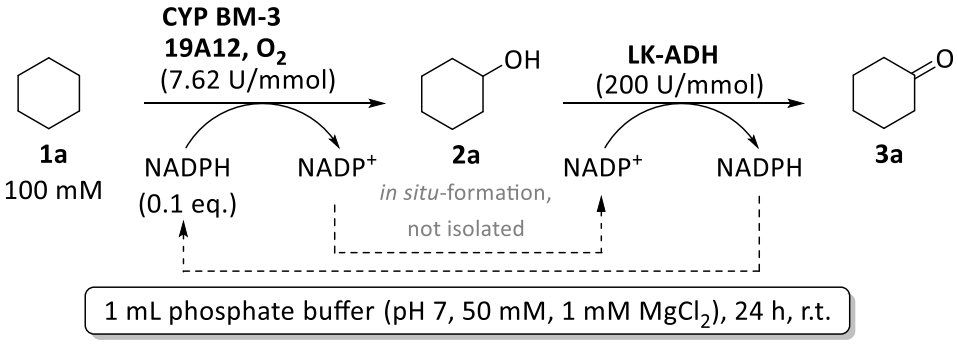
In a 25 mL round bottom flask a cycloalkane **1** (8.4 - 14.0 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to the corresponding cycloalkane **1**) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to the corresponding cycloalkanol **2**, diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature and worked up according to SOP A resp. SOP B.

8.2.14.1 Double oxidation of cyclohexane (1a)

According to SOP 14 cyclohexane (**1a**) (8.4 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (5.2 - 10.3 mg CYP BM-3 19A12, lyophilisate, 0.762 U referred to cyclohexane (**1a**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclohexanol (**2a**), diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug.

After that time the mixture is worked up according to SOP 14 (SOP A: entry 1-2; SOP B: entry 3-4) (Table 54).

Table 54. Results of the double oxidation of cyclohexane (**1a**) with CYP BM-3 19A12



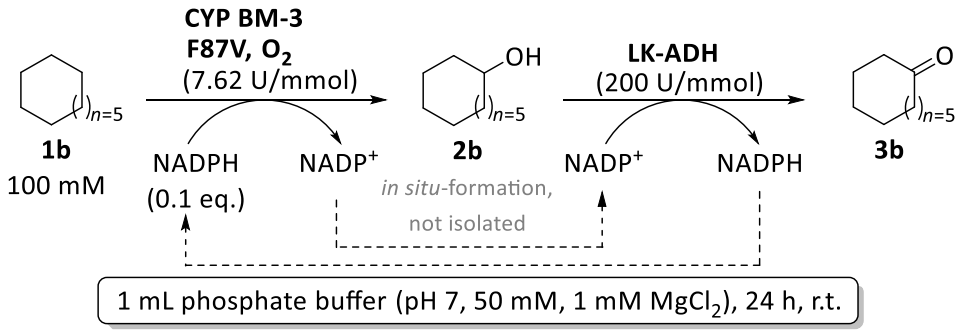
Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	NADPH [mol%]	Recovery/formation[d] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/5.2[a]	200/326	10	1.01	0.43	0.41
2	0.762/5.2[a]	200/326	10	1.05	n.d.[e]	0.40
3	0.762/10.3[b]	200/1057	10	0.61	0.53	0.96
4	0.762/10.3[b]	200/528[c]	10	1.61	0.16	0.99

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography; [e]n.d.: not determined, no clear assignment possible.

8.2.14.2 Double oxidation of cyclodecane (1b)

According to SOP 14 cyclodecane (**1b**) (14.0 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to cyclodecane (**1b**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 14 (Table 55).

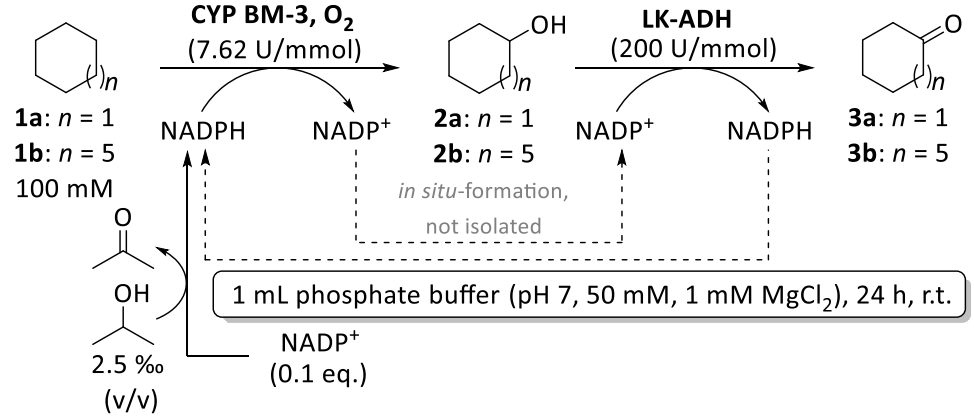
Table 55. Results of the double oxidation of cyclodecane (**1b**)



Entry	Activity F87V [U]/[mg]	LK-ADH [U/mmol]/[µL]	NADPH [mol%]	Recovery/formation[b] [g/L] of …**1b**	…**2b**	…**3b**
1	0.762/127[a]	200/91	10	3.21	n.d.	0.20

[a]The content of CYP BM-3 F87V amounts to 0.525 µmol/g lyophilized crude extract; [b]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.15 Standard operation procedure 15 (SOP 15): Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH and addition of 2-propanol



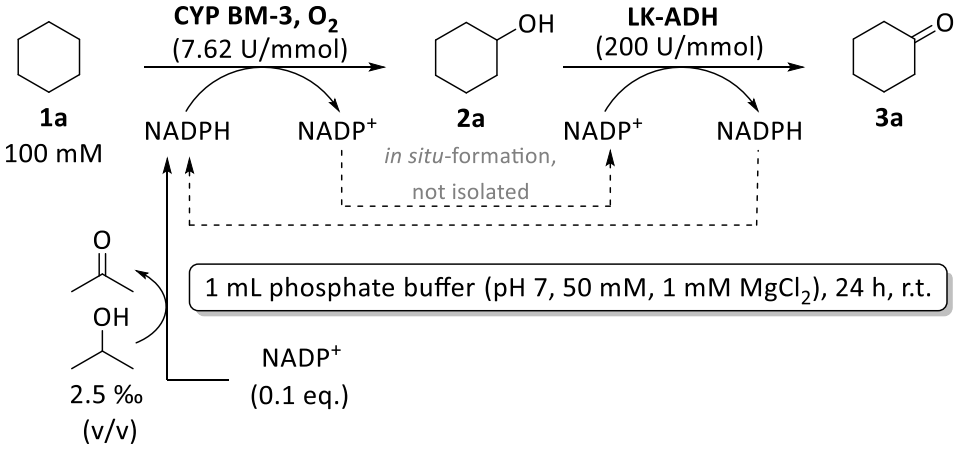
Scheme 65. Double oxidation of cycloalkanes **1** with addition of 2-propanol

In a 25 mL round bottom flask a cycloalkane **1** (8.4 - 14.0 mg, 0.1 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to the corresponding cycloalkane **1**) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to the corresponding cycloalkanol **2**, diluted 1:1 with glycerol) are added. To start the reaction cofactor $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and worked up according to SOP A resp. SOP B.

8.2.15.1 Double oxidation of cyclohexane (1a) with addition of 2-propanol

According to SOP 15 cyclohexane (**1a**) (8.4 mg, 0.1 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to cyclohexane (**1a**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclohexanol (**2a**), diluted 1:1 with glycerol) are added. To start the reaction cofactor $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP A.

Table 56. Results of the double oxidation of **1a** with addition of 2-propanol



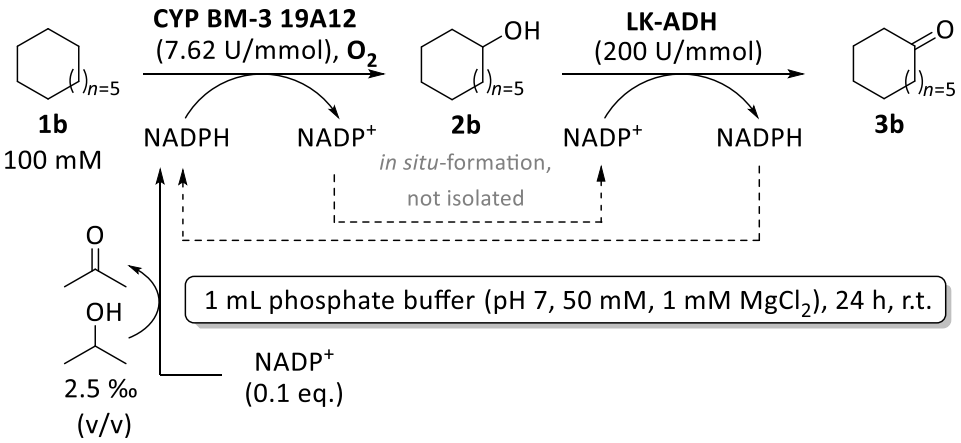
Entry	Activity CYP BM-3 [U]/[mg]	LK-ADH [U/mmol]/[µL]	$NADP^+$ [mol%]	Recovery/formation[c] [g/L] of …**1a**	…**2a**	…**3a**
1	WT 0.762/109.2[a]	200/326	10	0.75	0.25	0.35
2	19A12 0.762/5.2[b]	200/326	10	1.39	0.68	0.19
3	19A12 0.762/5.2[b]	200/326	10	0.91	0.40	0.18

[a] The content of CYP BM-3 WT amounts to 1.260 µmol/g lyophilized crude extract; [b] The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [c] The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.15.2 Double oxidation of cyclodecane (1b) with addition of 2-propanol

According to SOP 15 cyclodecane (**1b**) (14.0 mg, 0.1 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*14.9 - 19.6 mg lyophilisate CYP BM-3 19A12, 0.762U referred to cyclodecane (**1b**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction cofactor $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 15 (SOP A: entry 1; SOP B: entry 2-4) (Table 57).

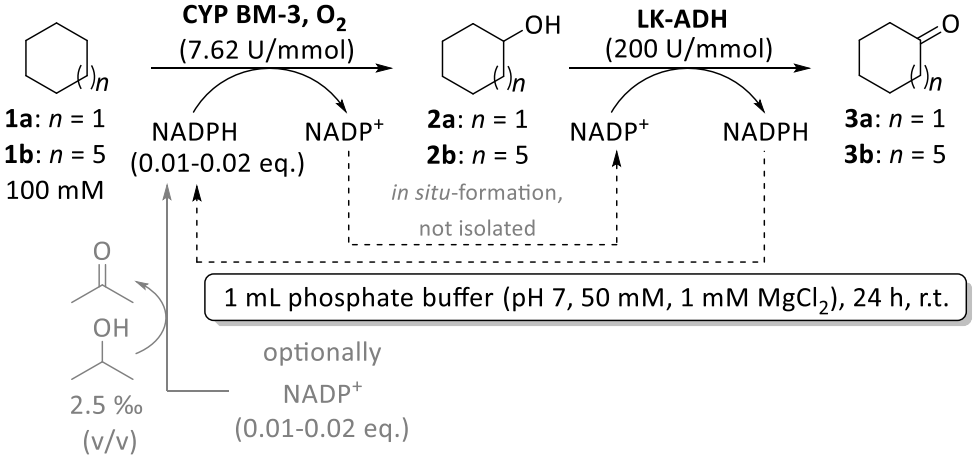
Table 57. Results of the double oxidation of cyclodecane (**1b**) with CYP BM-3 19A12



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	$NADP^+$ [mol%]	Recovery/formation[c] [g/L] of …**1b**	…**2b**	…**3b**
1	0.762/19.6[a]	200/91	10	7.92	0.00	0.24
2	0.762/14.9[b]	200/166	10	9.66	0.00	0.27
3	0.762/14.9[b]	200/166	10	9.38	0.00	0.27
4	0.762/14.9[b]	200/166	10	10.28	0.00	0.25

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.16 Standard operation procedure 16 (SOP 16): Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH with reduced amount of cofactor



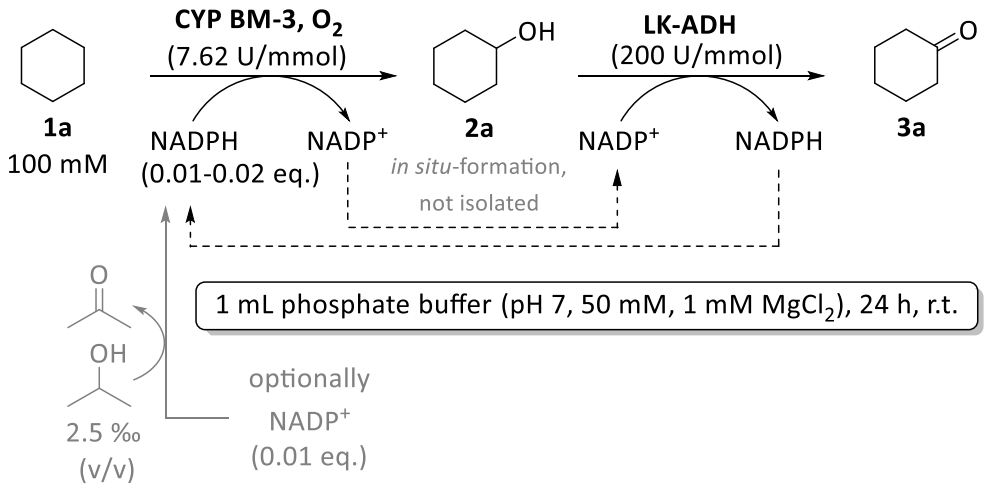
Scheme 66. Double oxidation of cycloalkanes **1** with reduced amount of cofactor

In a 25 mL round bottom flask a cycloalkane **1** (8.4 - 14.0 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) resp. 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to the corresponding cycloalkane **1**) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to the corresponding cycloalkanol **2**, diluted 1:1 with glycerol) are added. To start the reaction 1 resp. 2 mol% of cofactor (NADPH: 0.83 - 1.67 mg, 0.001 - 0.002; $NADP^+$: 0.79 mg - 1.57 mg, 0.001 - 0.002 mmol) resp. 1 mol% NADPH (0.83 mg, 0.001 mmol) as well as 1 mol% $NADP^+$ (0.79 mg, 0.001 mmol) is added. The reaction mixture is stirred for 24 h at room temperature and worked up according to SOP A resp. SOP B.

8.2.16.1 Double oxidation of cyclohexane (1a) with reduced amount of cofactor

According to SOP 16 cyclohexane (**1a**) (8.4 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) (lyophilisate, 0.762 U referred to cyclohexane (**1a**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclohexanol (**2a**), diluted 1:1 with glycerol) are added. To start the reaction 1 or 2 mol% of cofactor NADPH (0.83 - 1.67 mg, 0.001 - 0.002 mmol) resp. 1 mol% NADPH (0.83 mg, 0.001 mmol) as well as 1 mol% $NADP^+$ (0.79 mg, 0.001 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 16 (SOP A: entry 1-4; SOP B: entry 5) (Table 58).

Table 58. Results of the double oxidation of cyclohexane (**1a**) with different concentrations of cofactor NADPH



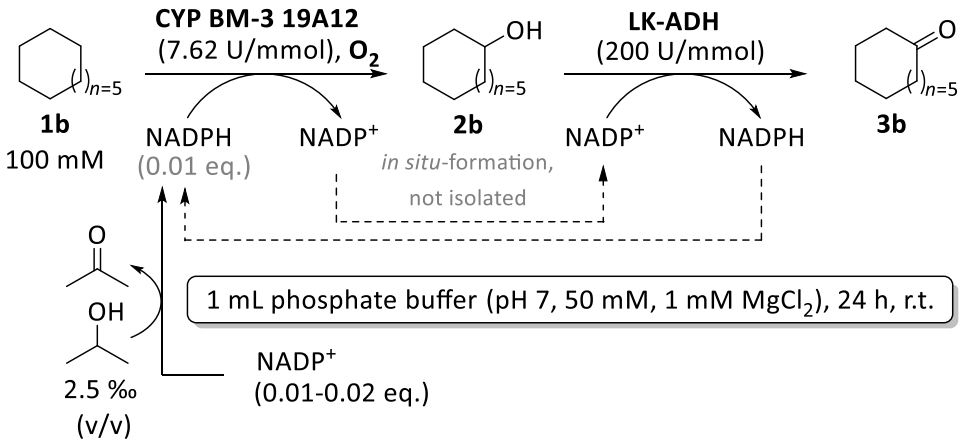
En-try	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Cofactor	Recovery/formation[e] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/5.2[a]	200/825	1 mol%	0.42	0.09	0.08
2	0.762/5.2[a]	200/825	1 mol%	1.04	0.09	0.10
3	0.762/5.2[a]	200/825	2 mol%	1.07	0.11	0.11
4	0.762/5.2[a]	200/825	2 mol%[d]	0.98	0.09	0.10
5	0.762/10.3[b]	200/528[c]	1 mol%	0.31	0.19	0.28

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]each 1 mol% cofactor in oxidized + reduced form; [e]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.16.2 Double oxidation of cyclodecane (1b) with reduced amount of cofactor

According to SOP 16 cyclodecane (**1b**) (14.0 mg, 0.1 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12) (lyophilisate, 0.762 U referred to cyclodecane (**1b**)) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction 1 resp. 2 mol% of cofactor $NADP^+$ (0.79 - 1.57 mg, 0.001 - 0.002 mmol) resp. 1 mol% NADPH (0.83 mg, 0.001 mmol) as well as 1 mol% $NADP^+$ (0.79 mg, 0.001 mmol) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP 16 (SOP A: entry 1-4; SOP B: entry 5) (Table 59).

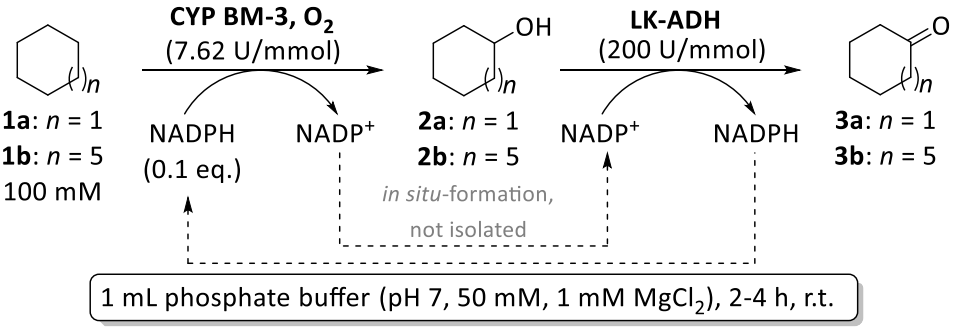
Table 59. Results of the double oxidation of cyclodecane (**1b**) with different concentrations of cofactor $NADP^+$



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Cofactor	Recovery/formation[e] [g/L] of …**1b**	…**2b**	…**3b**
1	0.762/19.a[a]	200/241	1 mol%	7.34	n.d.[f]	0.26
2	0.762/19.6[a]	200/241	1 mol%	3.25	n.d.[f]	0.29
3	0.762/19.6[a]	200/241	2 mol%	7.08	n.d.[f]	0.25
4	0.762/19.6[a]	200/241	2 mol%[d]	4.23	n.d.[f]	0.35
5	0.762/14.9[b]	200/83[c]	1 mol%	7.88	n.d.[f]	0.22

[a]The content of CYP BM-3 19A12 (0) amounts to 0.123 µmol/g lyophilized crude extract; [b]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [c]Addition of LK-ADH without dilution in glycerol; [d]each 1 mol% cofactor in oxidized + reduced form; [e]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography; [f]not detected.

8.2.17 Standard operation procedure 17 (SOP 17): Biocatalyzed double oxidation of cycloalkanes 1 with different CYP BM-3 and LK-ADH and reduction of the reaction time



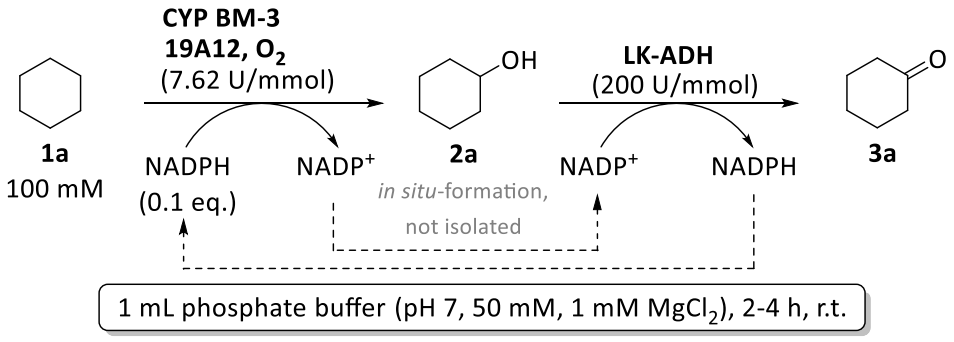
Scheme 67. Double oxidation of cycloalkanes **1**

In a 25 mL round bottom flask a cycloalkane **1** (8.4 - 14.0 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) resp. 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3) (lyophilisate, 0.762 U referred to the corresponding cycloalkane **1**) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to the corresponding cycloalkanol **2**, diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) resp. $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 2 - 4 h at room temperature covered with a plug and worked up according to SOP B.

8.2.17.1 Double oxidation of cyclohexane (1a) with shortened reaction time

According to SOP 17 cyclohexane (**1a**) (8.4 mg, 0.1 mmol) is mixed with 1 mL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, 0.762 U, 10.3 mg) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclohexanol (**2a**), diluted 1:1 with glycerol) are added. To start the reaction cofactor NADPH (8.3 mg, 0.01 mmol) is added. The reaction mixture is stirred for 2 - 4 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP B (Table 60).

Table 60. Results of the double oxidation of cyclohexane (**1a**) with shortened reaction time



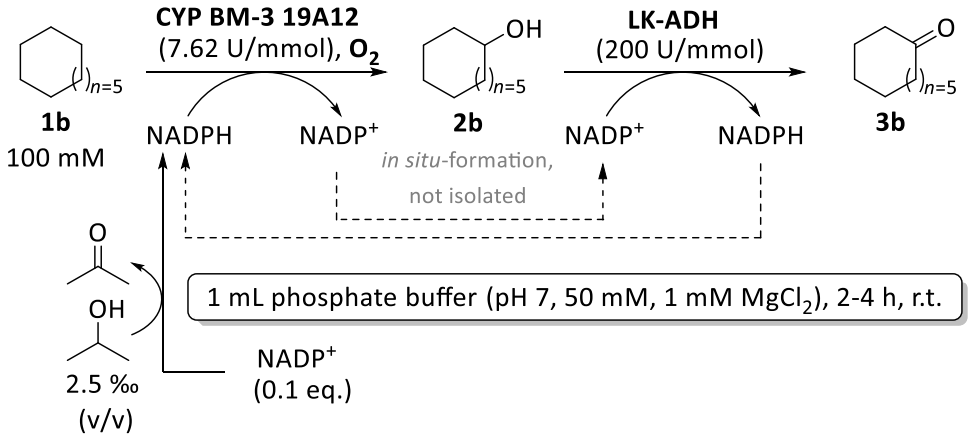
Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Time [h]	Recovery/formation[c] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/10.3[a]	200/528[b]	2	1.09	0.22	0.37
2	0.762/10.3[a]	200/528[b]	4	0.66	0.23	0.51

[a] The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b] Addition of LK-ADH without dilution in glycerol; [c] The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.17.2 Double oxidation of cyclodecane (1b) with shortened reaction time

According to SOP 17 cyclodecane (**1b**) (14.0 mg, 0.1 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)) in a 25 mL round bottom flask. Then a cytochrome P450 monooxygenase from *Bacillus megaterium (*CYP BM-3 19A12, 0.762 U, 14.9 mg) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction cofactor $NADP^+$ (7.9 mg, 0.01 mmol) is added. The reaction mixture is stirred for 2 - 4 h at room temperature covered with a plug. After that time the mixture is worked up according to SOP B (Table 61).

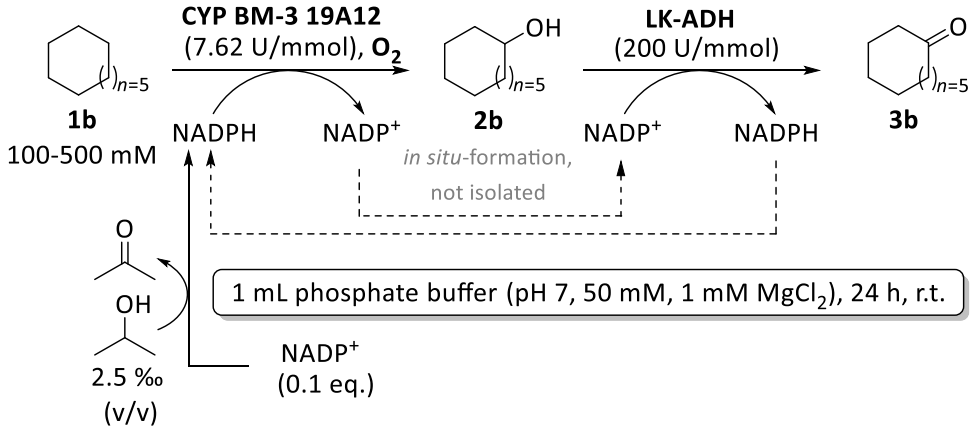
Table 61. Results of the double oxidation of cyclodecane (**1b**) with shortened reaction time



Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	Time [h]	Recovery/formation[b] [g/L] of …**1a**	…**2a**	…**3a**
1	0.762/14.9[a]	200/166	2	11.81	0.00	0.21
2	0.762/14.9[a]	200/166	4	10.98	0.00	0.23

[a]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

8.2.18 Standard operation procedure 18 (SOP 18): Biocatalyzed double oxidation of cyclodecane (1b) with increased initial substrate concentration



Scheme 68. Double oxidation of cycloalkanes **1** with increased substrate concentration

In a 25 mL round bottom flask cyclodecane (**1b**) (14.0 - 70.2 mg, 0.1 - 0.5 mmol) is mixed with 997.5 µL phosphate buffer (pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2.5 µL 2-propanol (2.5 ‰ (v/v)). Then a cytochrome P450 monooxygenase from *Bacillus megaterium* (CYP BM-3 19A12, 0.762 U, 14.9 mg) and the alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH) (crude extract, 200 U/mmol referred to cyclodecanol (**2b**), diluted 1:1 with glycerol) are added. To start the reaction cofactor $NADP^+$ (7.9 - 39.4 mg, 0.01 - 0.05 mmol, 10 mol%) is added. The reaction mixture is stirred for 24 h at room temperature covered with a plug and worked up according to SOP B. The results are summarized in Table 62.

Table 62. Results of the double oxidation of cyclodecane (**1b**) with increased substrate concentration according to Scheme 68

Entry	Activity 19A12 [U]/[mg]	LK-ADH [U/mmol]/[µL]	**1b** [mmol]	Recovery/formation[b] [g/L] of		
				…**1a**	…**2a**	…**3a**
1	0.762/14.9[a]	200/166	0.1	10.28	0.00	0.25
2	0.762/14.9[a]	200/166	0.5	64.32	0.00	0.82

[a]The content of CYP BM-3 19A12 (2) amounts to 0.120 µmol/g lyophilized crude extract; [b]The term "recovery" describes the mass concentration in g/L of a compound initially added to the reaction, that is recovered after workup, here cycloalkane **1**. The term "formation" describes the mass concentration in g/L of a compound which is formed during the reaction and isolated while workup (product formation of cycloalkanol **2** and cycloalkanone **3**). All mass concentrations in g/L were determined *via* gas chromatography.

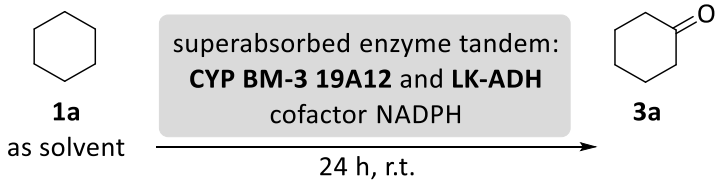
8.2.19 Standard operation procedure 19 (SOP 19): Preparation of the superabsorbed enzyme tandem

To a homogenous mixture consisting of (A) 8.34 - (B) 16.68 mg NADPH, (A) 1 - (B) 2 mL phosphate buffer (pH 7, 50 mM, containing 1 mM $MgCl_2$), (A) 16.2 - (B) 32.4 mg CYP BM-3 19A12 (0.762 U referred to cyclohexane (**1a**)) and (A) 443 - (B) 886 µL LK-ADH (200 U/mmol relative to the ordinarily used 0.1 mmol substrate **1a**) in a (A) 10 - (B) 50 mL round bottom or pear shaped flask were added (A) 248 - (B) 496 mg of the superabsorbent polymer Favor SXM 9155® (Evonik Industries AG). The mixture solidifies within 1 min of stirring.

8.2.20 Standard operation procedure 20 (SOP 20): Double oxidation of cyclohexane (1a) using a superabsorbed enzyme tandem

The superabsorbed enzyme tandem is prepared according to SOP 19 in a 10 mL round bottom or 50 mL pear shaped flask. The immobilizate is then mixed with 5 mL cyclohexane (**1a**) (saturated with water to avoid the unwanted reduction of the aqueous quantity) and stirred at 70 rpm for 24 h at room temperature. The supernatant organic phase is pipetted into a 10 mL volumetric flask while the remaining hydrogel is extracted twice by adding each 2 mL dichloromethane or cyclohexane (**1a**) (saturated with water to avoid the unwanted reduction of the aqueous quantity) and swirling the suspension. The combined organic phases are transferred completely into the 10 mL volumetric flask which is filled with dichloromethane or **1a**. By means of a straight calibration line the concentration is analyzed *via* gas chromatography. Then 5 mL cyclohexane (**1a**) (saturated with water to avoid the unwanted reduction of the aqueous quantity) are added to the immobilisate and the next cycle is started by stirring at 70 rpm for 24 h at room temperature. The results of the double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem are summarized in Table 63, the recycling of the superabsorbed enzyme tandem is shown in Table 64.

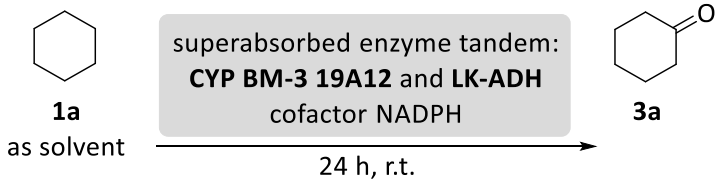
Table 63. Double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem



Entry[a)]	Method	Flask	Cyclohexane (**1a**) [g/L]	Cyclohexanol (**2a**) [g/L]	Cyclohexanone (**3a**) [g/L]
1	A	10 mL, round bottom	-- [d)]	0.00	0.15
2	B	50 mL[c)] pear shaped	-- [d)]	0.00	0.16
3[b)]	B	50 mL[c)] pear shaped	-- [d)]	0.00	0.18

[a)]GC-measurements are conducted in a twofold determination; [b)]Extraction as described above, but instead of dichloromethane, cyclohexane (**1a**) (saturated with water to avoid the unwanted reduction of the aqueous quantity) was used; [c)]Enlargement of the air volume in the flask to rule out the deficiency of molecular oxygen; [d)]Concentration of **1a** is over the detection limit of the straight calibration line.

Table 64. Double oxidation of cyclohexane (**1a**) using the superabsorbed enzyme tandem for 1-3 cycles



Entry[a)]	Cycle	Cyclohexane (**1a**) [g/L]	Cyclohexanol (**2a**) [g/L]	Cyclohexanone (**3a**) [g/L immobilized aqueous phase]
1	1	-- [b)]	0.00	0.18
2	2	-- [b)]	0.00	0.04
3	3	-- [b)]	0.00	< 0.01

[a)]GC-measurements are conducted in a twofold determination; [b)]Concentration of **1a** is over the detection limit of the straight calibration line.

8.2.21 Standard operation procedure 21 (SOP 21): Cultivation of recombinant cells for the biocatalyzed double oxidation of cycloalkanes 1[31,32]

Overnight preculture:

Under sterile conditions a preculture is prepared by inoculating 5 mL LB medium, 5 µL kanamycin (50 g/l, sterile filtered) and 5 µL chloramphenicol (34 g/l, sterile filtered) with 5 µL of the construct A (*E. coli* BL21 (DE3) Gold $LacI^{Q1}$ pALXtreme-1a P450 BM-3 $19A12^{NADPH}(Kan^R)$ + pKA1 LB-ADH (Cm^R)) or construct B (*E. coli* BL21 (DE3) Gold $LacI^{Q1}$ pALXtreme-1a P450 BM-3 $19A12^{NADH}$::RE-ADH (Kan^R) + pKA1 LB-ADH (Cm^R) (1 µL/mL)). The cultures are grown overnight (14 h) by shaking at 37°C.

Expression culture:

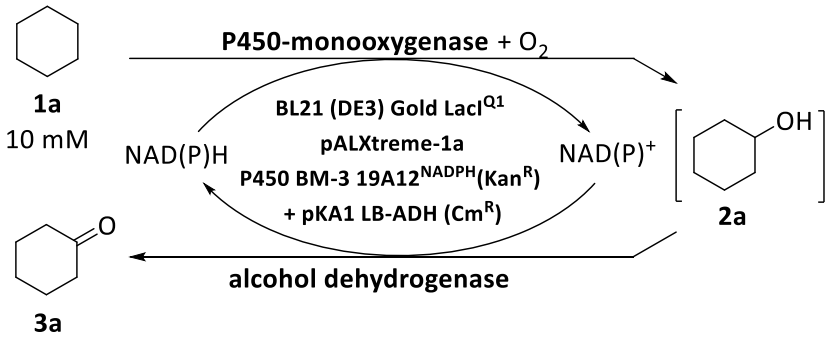
Under sterile conditions an expression culture is prepared. In a 2 L shaking flask 400 mL TB medium is supplemented with 400 µL trace elements (3.40 mM $CaCl_2$, 0.63 mM $ZnSO_4$, 0.59 mM $MnSO_4$, 59.82 mM Na_2-EDTA, 61.79 mM $FeCl_3$, 0.64 mM $CuSO_4$, 0.76 mM $CoCl_2$, autoclaved and sterile filtered) and each 400 µL of the antibiotics kanamycin (50 g/l, sterile filtered) and chloramphenicol (34 g/l, sterile filtered). Then 4 mL of the preculture are added. The cultures are grown by shaking at 37°C. To monitor the bacterial growth, the optical density at 600 nm (OD_{600}) of the bacterial culture is measured spectrophotometrically with an UV/VIS-spectrophotometer (BioPhotometer plus, Eppendorf). The spectrophotometer is blanked by measuring the media without the preculture. After the initial cultivation (construct A: OD_{600} = 0.72 after 3h; construct B: OD_{600} = 0.80 after 4h) the culture is supplemented with each 400 µL aminolevulinic acid (ALA) (0.5 M, sterile filtered), $ZnCl_2$ (1 M, sterile filtered) and thiamine (100 g/l). Then expression is induced by adding 400 µL isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 M, sterile filtered). Expression takes place while incubating the cultures at 25°C and 250 rpm (construct A: 20 h; construct B: 19 h). The *E. coli* cells are transferred into tubes and harvested by centrifugation (10 min, 4000 rpm, 4 °C, Thermo Scientific™ Sorvall™ RC 6 Plus Centrifuge). The supernatant media is discarded and the cells are washed with 80 mL KPi-buffer (pH 8.0, 100 mM). After centrifugation the supernatant is discarded again and the cell pellets are stored at -20°C.

8.2.22 Standard operation procedure (SOP 22): Oxidation of cyclohexane (1a) using whole cells

The cell pellet is resuspended in KPi-buffer (pH 8.0, 100 mM) to an optical density of approximately 40 (construct A: OD_{600} = 40.2; construct B: OD_{600} = 39.0). 50 µL glucose solution (200 g/L, sterile filtered using 0.2 µm filters) are poured into a 10 mL-glass vial with screw plug and 1 mL of the resuspended cells (OD_{600} = ca. 40) are added. This mixture is incubated for 5 min at room temperature before 20 µL of a 0.5 M stock solution of cyclohexane (**1a**) in ethanol are pipetted in the glass vial (10 mM). Then the vial is sealed and the reaction mixture is stirred at room temperature for 0.5 - 24 h.

After different time intervals the experiments are terminated by transferring the reaction mixture into an Eppendorf tube and admix with hydrochloric acid (100 µL, 37%) and MTBE (1 mL). The biphasic system is vortexed for 2 min and the phase separation is obtained by centrifugation at maximal speed for 2 min (Eppendorf centrifuge 5425). The organic phase is pipetted into another Eppendorf tube where it is vortexed and centrifuged as described above in the presence of magnesium sulphate. By means of a straight calibration line the concentration is analyzed *via* gas chromatography (Table 65 and Table 66).

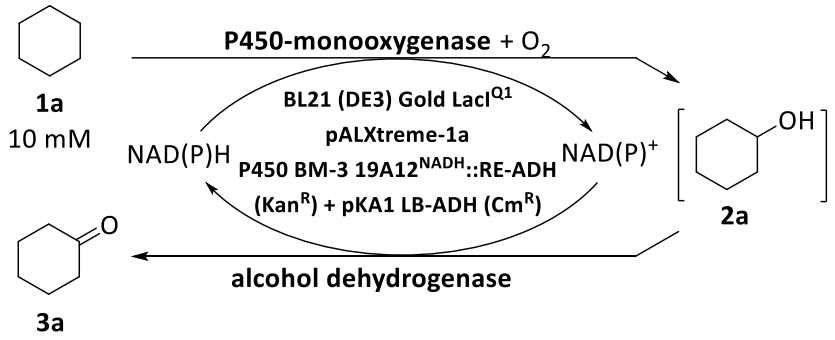
Table 65. Oxidation of cyclohexane (**1a**) using construct A, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}) (SOP 22) OD_{600} = 40.2; CDW = 8.7 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1a** [g/L]	**2a** [g/L]	**3a** [g/L]	Σ**2a**+**3a** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	0.5	0.06	0.10	0.02	0.12	14
2	1	0.01	0.16	0.08	0.24	28
3	2	0.02	0.21	0.14	0.35	40
4	4	0.03	0.26	0.15	0.41	94
5	18	0.02	0.36	0.04	0.40	47
6	24	0.02	0.38	0.04	0.42	48

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

Table 66. Oxidation of cyclohexane (**1a**) using construct B, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R}) (SOP 22) OD_{600} = 39.0; CDW = 5.0 (cell dry weight; in g_{CDW} L^{-1})



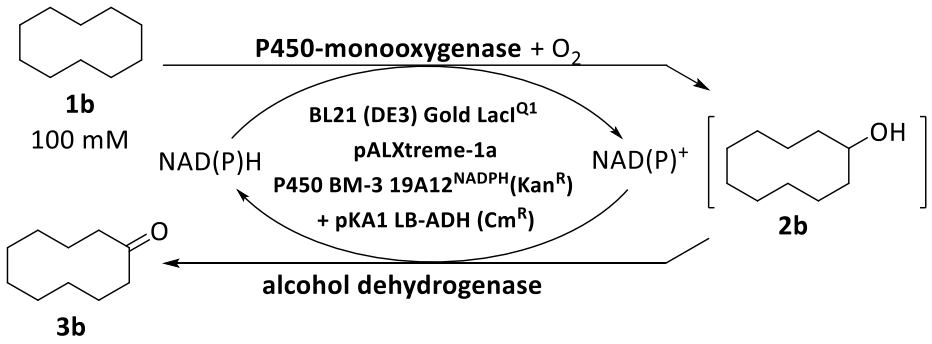
Entry[a)]	Time [h]	**1a** [g/L]	**2a** [g/L]	**3a** [g/L]	Σ**2a**+**3a** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	0.5	0.05	0.08	0.03	0.11	22
2	1	0.03	0.14	0.09	0.23	46
3	2	0.02	0.18	0.15	0.33	66
4	4	0.01	0.25	0.21	0.46	92
5	18	0.01	0.41	0.06	0.47	94
6	24	0.01	0.43	0.08	0.51	102

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

8.2.23 Standard operation procedure (SOP 23): Oxidation of cyclodecane (1b) using whole cells

The cell pellet is resuspended in KPi buffer (pH 8.0, 100 mM) to an optical density of approximately 40 (construct A: OD_{600} = 39.4; construct B: OD_{600} = 33.6). 50 µL glucose solution (200 g/L, sterile filtered using 0.2 µm filters) are poured into a glass vial and 1 mL of the resuspended cells (OD_{600} = ca. 40) are added. This mixture is incubated for 5 min at room temperature before 20 µL of a 5 M stock solution of cyclodecane (**1b**) in ethanol are pipetted in the glass vial (100 mM). Then the vial is sealed and the reaction mixture is stirred at room temperature for 1 - 20 h. After different time intervals the experiments are terminated by transferring the reaction mixture into an Eppendorf tube and admix with hydrochloric acid (100 µL, 37%) and MTBE (1 mL). The biphasic system is vortexed for 2 min and the phase separation is obtained by centrifugation at maximal speed for 2 min (Eppendorf centrifuge 5425). The organic phase is pipetted into another Eppendorf tube where it is vortexed and centrifuged as described above in the presence of magnesium sulphate. By means of a straight calibration line the concentration is analyzed *via* gas chromatography (Table 67 and Table 68).

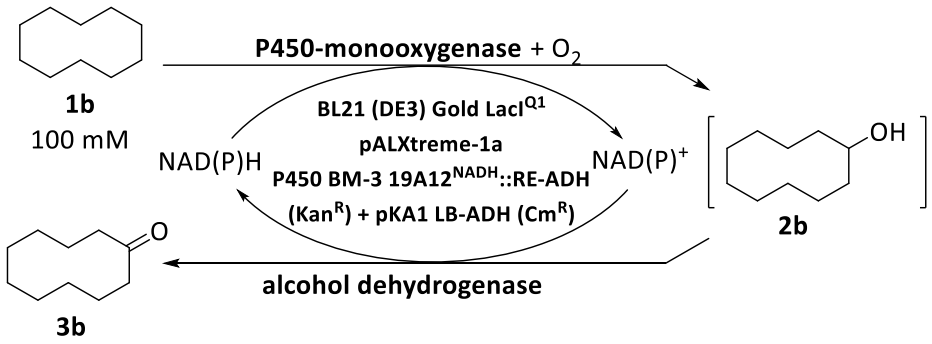
Table 67. Oxidation of cyclodecane (**1b**) using construct A, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^{R}) + pKA1 LB-ADH (Cm^{R}) (SOP 23) OD_{600} = 39.4; CDW = 14.9 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1b** [g/L]	**2b** [g/L]	**3b** [g/L]	Σ**2b**+**3b** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	1	8.00	--	0.06	0.06	4
2	4.5	6.42	--	0.10	0.10	7
3	20	1.99	--	0.06	0.06	4

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

Table 68. Oxidation of cyclodecane (**1b**) using construct B, BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^{R}) + pKA1 LB-ADH (Cm^{R}) (SOP 23) OD_{600} = 33.6; CDW = 10.4 (cell dry weight; in g_{CDW} L^{-1})



Entry[a)]	Time [h]	**1b** [g/L]	**2b** [g/L]	**3b** [g/L]	Σ**2b**+**3b** [g/L]	Product yield on catalyst[b)] [$mg_{product}/g_{CDW}$]
1	1	7.97	--	0.12	0.12	12
2	4.5	7.17	--	0.12	0.12	12
3	20	4.95	--	0.12	0.12	12

[a)]Both, experiments and GC-measurements are conducted in a twofold determination with the result that for each reaction the average of a fourfold dataset is calculated; [b)]Product yield on catalyst describes the ratio between the sum of products mass concentrations (Σ**2**+**3**) in mg/L and the amount of catalyst as cell dry weight in g_{CDW} L^{-1}.

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10 List of Abbreviations

%	percent
(v/v)	volume/volume
[S]	substrate concentration
°C	degree Celsius
µL	mikroliter
µm	micromole
‰	per mille
^{1}H-NMR-spectroscopy	**N**uclear **M**agnetic **R**esonance spectroscopy; studied nucleus: ^{1}H
$^{1}O_2$	singlet oxygen
2p	2p atomic orbital
2s	2s atomic orbital
$^{3}O_2$	triplet oxygen
A	absorption
Å	Ångström (equals 0.1 nm)
*Aae*UPO	*Agrocybe aegerita*
ADH	alcohol dehydrogenase
ADH evo-1.1.200	alcohol dehydrogenase available from evocatal GmbH
ADH-A	alcohol dehydrogenase from *Rhodococcus ruber* DSM 44541
ALA	aminolevulinic acid
API	active pharmaceutical ingredient
Arg47	arginine residue at position 47
AS	auxiliary substrate
BC	before christ
BSA	bovine serum albumin
c	concentration
CD_2Cl_2	deutero dichloromethane; dichloromethane-d2
$CDCl_3$	deutero chloroform
CDW	cell dry weight (in g_{CDW} L^{-1})
Cm^R	chloramphenicol resistance
Construct A	BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADPH}(Kan^R) + pKA1 LB-ADH (Cm^R)
Construct B	BL21 (DE3) Gold LacI^{Q1} pALXtreme-1a P450 BM-3 19A12^{NADH}::RE-ADH (Kan^R) + pKA1 LB-ADH (Cm^R)
content of CYP [µmol/g or nmol/g]	content of the cytochrome P450 monooxygenase from *Bacillus megaterium* in the lyophilized crude extract (in micromole per gram or nanomole per gram)
CPR	NADPH-cytochrome P450 reductase
CYPs	cytochrome P450 monooxygenases
CYP BM-3	cytochrome P450 monooxygenase from *Bacillus megaterium*

CYP BM-3 WT CYP BM-3 19A12 CYP BM-3 F87P CYP BM-3 F87V CYP BM-3 F87A A328V CYP BM-3 R255P- P329H CYP BM-3 139-3 CYP BM-3 R47L Y51F CYP BM-3 A328V	wild type (WT) enzyme and genetically engineered mutants of the cytochrome P450 monooxygenase from *Bacillus megaterium*, mutated at the specified positions of the amino acid sequence
CYP106A2	cytochrome P450 monooxygenase from *B. megaterium* ATCC 13368
CYP153A13a	cytochrome P450 monooxygenase from *Alcanivorax borkumensis* SK2
CYP1A2 CYP2C9 CYP2D6 CYP2E1 CYP3A4	human cytochrome P450 enzymes
CYP CAM	camphor hydroxylase from *Pseudomonas putida*
d	path length of the cuvette
d	doublet
D_2O	deuteriumoxide
d^5	d^5-configuration
DCM	dichloromethane
dd	doublet of doublet
DMSO	dimethyl sulfoxide
DMSO-d6	dimethylsulfoxide-d6
dt	doublet of triplet
E	energy
E. coli	*Escherichia coli*
E.coli BL21	*E. coli* cells containing an empty vector
E. coli BL21 (DE3)	chemically competent *E. coli* cells
Gold LacI^{Q1}	
ee	enantiomeric excess
eq.	equivalents
f	dilution factor
FAD	flavin adenine dinucleotide
Favor SXM 9155®	superabsorbent polymer commercially available from Evonik Industries
FDH	formate dehydrogenase
FdR	ferredoxin reductase
Fdx	ferredoxin
FID	flame ionization detector

FMN	flavin mononucleotide
GC	gas chromatography
GDH	glucose dehydrogenase from *Bacillus* sp.
Glu47	glutamic acid residue at position 47
h	hour
H	hydrogen
HLADH	alcohol dehydrogenase from horse liver
hPa	hectopascal
HPLC	high performance liquid chromatography
hs	high spin
Hz	Hertz
IPA	2-propanol
IPTG	isopropyl-β-D-thiogalactopyranoside
J	scalar coupling constant
K	NERNST distribution coefficient
Kan^R	kanamycin resistance
kDa	kilo Dalton
kJ mol^{-1}	kilo Joule per mole
KOtBu	potassium *t*-butoxide
KPi-buffer	potassium phosphate buffer
LB medium	lysogeny broth medium
LB-ADH	alcohol dehydrogenase from *Lactobacillus brevis*
LDA	lithium diisopropylamide
LK-ADH	alcohol dehydrogenase from *Lactobacillus kefir*
log P value	decadic logarithm of the partition-coefficient
ls	low spin
LY 300164	an orally administered benzodiazepine (Eli Lilly)
m	multiplet
M [g/mol]	molecular weight
MDL	method detection limit
mg	milligram
mg_{P450}/g_{CDW}	expression level of the recombinant P450 monooxygenase in the cells (generally determined *via* CO-difference spectroscopy)
$mg_{product}/g_{CDW}$	product yield on catalyst
MHz	megahertz
min	minute
mL	millilitre
mM	millimolar
mmol	millimol
mol%	mole fraction multiplied by 100
Mt/a	metric tons per year
MTBE	methyl *tert*-butyl ether
mV	milli Volt
n	number of subunits
n.d.	not determined

n.d.	not detected
NADH, NAD^+	nicotinamide-adenine-dinucleotide
NADPH, $NADP^+$	nicotinamide-adenine-dinucleotide-phosphate
NAPQI	*N*-acetyl-*p*-benzoquinone imine
nm	nanometre
NMR	nuclear magnetic resonance
OD_{600}	optical densitiy, measured at a wavelength of 600 nm
OTC drug	over-the-counter drug
P450	"P" stands for pigment and "450" is derived from the UV absorption peak of the carbon monoxide complex
P450 or CYP BM-3; CYP102A1; EC 1.14.14.1	Analogue abbreviations for the cytochrome P450 monooxygenase from the soil bacterium *Bacillus megaterium*
P450balk	P450 monooxygenase from *Alcanivorax borkumensis* SK2
P450RhF	self-sufficient P450 monooxygenase
pAlXtreme-1a	plasmid with P450 BM-3 $19A12^{NADPH}$(Kan^R) resp. P450 BM-3 $19A12^{NADH}$::RE-ADH (Kan^R); kanamycin-resistant
Phe87	phenylalanine at position 87
pKA1	plasmid with LB-ADH; chloramphenicol-resistant
ppm	parts per million
pRED	expression vector
q	quartet
r.t.	room temperature
RE-ADH	alcohol dehydrogenase from *Rhodococcus erythropolis*
resp.	respectively
rpm	rounds per minute
Rsp.-ADH	alcohol dehydrogenase from *Rhodococcus species*
s	singlet
S	bond order
SOP	**S**tandard **O**peration **P**rocedure
SPAAC	strain-promoted alkyne azide cycloaddition
SPP	stoichiometric side product
t	triplet
t/a	tons per year
TB medium	terrific broth medium
td	triplet of doublet
THF	tetrahydrofuran
TOF	turn over frequency
t_R	retention time
TTN	Total Turnover Number
U	enzyme unit (the amount of enzyme that catalyzes the conversion of 1 micromole of substrate per minute)
U/mg	specific enzyme activity; gravimetric enzyme activity
U/mL [$\mu mol \cdot min^{-1} \cdot mL^{-1}$]	volumetric enzyme activity

U/mmol	units per millimole substrate
UV/VIS	ultraviolet/visible
v.s.	*vide supra*
v_0	initial rate
v_{max}	the maximum rate achieved by the (enzymatic) system at maximum saturating substrate concentrations
vol.	volumetric
V_S	sample volume
V_t	total volume
δ	chemical shift
δ [ppm]	chemical shift in parts per million
$\Delta E_{340nm}/t$	initial slope of the absorption curve
ε [6.3 $mL \cdot \mu mol^{-1} \cdot cm^{-1}$]	molar extinction coefficient for NAD(P)H
π	bonding pi-molecular orbital
π^*	anti-bonding pi-molecular orbital
σ_P	bonding sigma(P)-molecular orbital
σ_P^*	anti-bonding sigma(P)-molecular orbital
σ_S	bonding sigma(S)-molecular orbital
σ_S^*	anti-bonding sigma(S)-molecular orbital
ω	ω-positions

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