In Vitro Double Oxidation of n-Heptane with Direct Cofactor Regeneration

Christina A. Müller,a Beneeta Akkapurathu,a Till Winkler,b Svenja Staudt,c Werner Hummel,b Harald Gröger,c,d and Ulrich Schwaneberg a,*

a Institute of Biotechnology, RWTH Aachen, Worringerweg 1, 52074 Aachen, Germany
Fax: (+49)-241-80-22387; phone: (+49)-241-80-24170; e-mail: u.schwaneberg@biotec.rwth-aachen.de
b Institute of Molecular Enzyme Technology at the Heinrich-Heine-University of Düsseldorf, Research Centre Jülich, Stetternicher Forst, 52426 Jülich, Germany
c Department of Chemistry and Pharmacy, University of Erlangen-Nürnberg, Henkestrasse 42, 91054 Erlangen, Germany
d Faculty of Chemistry, Bielefeld University, Universitätsstrasse 25, 33615 Bielefeld, Germany

Received: February 20, 2013; Published online: May 16, 2013

Abstract: A novel concept for the direct oxidation of cycloalkanes to the corresponding cyclic ketones in a one-pot synthesis in water with molecular oxygen as sole oxidizing agent was reported recently. Based on this concept we have developed a new strategy for the double oxidation of n-heptane to enable a biocatalytic resolution for the direct synthesis of heptanone and (R)-heptanols in a one-pot reaction. The biocatalytic cascade employs an NADH driven P450 BM3 monooxygenase variant (WTNADH, 19A12 NADH or CM1 NADH) and an (S)-enantioselective alcohol dehydrogenase (RE-ADH). In the initial step n-heptane is hydroxylated under consumption of NADH to produce (R/S)-heptanol. In the second oxidation step the (S)-heptanol enantiomers are transformed to the corresponding ketones, reducing and thereby regenerating the cofactor. Characterization of initial hydroxylation step revealed high turnover frequencies (TOF) of up to 600 min$^{-1}$, as well as high coupling efficiencies using NADH as cofactor (up to 44%). In the cascade reaction a nearly 2-fold improved product formation was achieved, compared to the single hydroxylation reaction. The total product concentration reached 1.1 mM, corresponding to a total turnover number (TTN) of 2500. Implementation of an additional cofactor regeneration system (d-glucose/glucose dehydrogenase) enabled a further enhancement in product formation with a total product concentration of 1.8 mM and a TTN of 3500.

Keywords: alkanes; cascade reactions; C–H activation; directed evolution; enzymatic resolution; oxidation; P450 BM3

Introduction

Petrochemical feedstocks are currently used as main raw materials for the production of bulk and fine chemicals. A selective oxyfunctionalization of hydrocarbons remains one of the most challenging tasks in chemistry, especially with regard to simple alkanes and arenes,[1] mainly due to a low reactivity of C–H bonds[2] and poor chemo- and stereoselectivity of most chemocatalysts.[3] The Wacker oxidation[4] represents one of the most efficient chemical methods for oxyfunctionalization of olefins and enables the synthesis of aldehydes and ketones.[5] Prochiral ketones and olefins are further used for synthesis of chiral alcohols, either by asymmetric reduction with chiral boron reagents[6] and chiral phosphorus ligands[7] or by enzymatic ketone reduction.[8]

Chiral alcohols are used as building blocks for agrochemicals and pharmaceuticals.[9] The stoichiometric need of transition metal reagents,[10] or large quantities of organic solvents, and the laborious separation and recycling steps for products, solvents and catalysts renders these chemical synthesis routes unfavourable in regard to environmental impact and sustainability.[11]

Biotechnological processes offer an economical attractive and environmentally preferable access to chiral alcohols.[12] Nowadays most biocatalytic processes rely on reduction of ketones making use of ketoreductases.[8] The direct biocatalytical activation of alkanes is an interesting and more cost-effective alter-
native to the use of ketones in stereochemical synthesis, making the enzymatic oxyfunctionalization a key parameter for a successful application.[13]

The enzyme-catalyzed oxidation of non-activated alkanes is in general achieved with higher selectivities than with chemocatalysts.[14] The main enzyme classes that catalyze alkane oxygenation reactions are oxygenses and peroxidases.[15] Peroxidases catalyze oxygen transfer reactions, such as olefin epoxidations and sulfoxidations. Recently, the peroxidase from Agrocybe aegerita (AaP) was reported as the first peroxidase to hydroxylate aliphatic chains.[16] Oxygenases comprise a more diverse catalytic family due to their intrinsic physiological role in detoxification, synthesis of secondary metabolites or hydrocarbon degradation.[15] Nevertheless, industrial applications of oxygenases are often hindered, mainly due to their low reaction rates, stabilities and cofactor dependency.[17] To overcome the “cofactor challenge” in cell-free biocatalysis two basic strategies have been investigated: the use of enzymatic regeneration systems[18] and regeneration by chemical, electrochemical or photochemical set-ups.[19] The enzymatic regeneration strategy is commonly employed using dehydrogenases with cosubstrates like isopropyl alcohol or formate.[16]

P450 monoxygenases are one class of heme-containing oxygenases, capable of oxidizing non-activated C–C bonds at room temperature, in water with oxygen as sole oxidant.[20] One atom of molecular dioxygen is often incorporated stereo- and regioselectively into the substrate, while the second atom is reduced to water. The electron transfer takes place from the nicotinamide cofactor NAD(P)H to the heme iron via an FAD-FMN electron transfer chain.[20,21]

P450 BM3 from Bacillus megaterium is particularly interesting for in vitro applications and chemical synthesis since it is a soluble and catalytically self-sufficient monooxygenase with the FAD-FMN reductase and hemoprotein domain on one polypeptide chain.[22] P450 BM3 has been engineered extensively to broaden the substrate spectra, to enhance activities, to increase thermal or process stability, and to accept alternative electron transfer systems.[23]

In particular, P450 BM3 mutants with changed substrate specificity and selectivity for medium- and short-chain alkanes were successfully reengineered.[24] P450 BM3 wild-type hydroxylates C_6–C_{10} alkanes with low activities, but does not convert short-chain alkanes (< C_6).[24] The engineered variant 19A12 prefers as substrates short-chain alkanes (C_3–C_5). It hydroxylates propane with a turnover frequency (TOF) of 420 min^{-1} and a total turnover number (TTN) of 10550.[25]

Recently, we reported a novel process concept for the double oxidation of cycloalkanes to produce cycloalkanones in a one-pot synthesis.[26] This bimolecular reaction with a P450 monoxygenase and an alcohol dehydrogenase takes place in water and employs molecular oxygen as sole oxidizing agent. Furthermore, for regeneration of the nicotinamide cofactor no additional co-substrate is supplemented.

Based on this concept a novel strategy for double oxidation of medium-chain alkanes using n-heptane as substrate was developed (Scheme 1). In this report we have implemented an enzyme cascade (Scheme 2) consisting of two steps for the enantioselective synthesis of chiral alcohols starting from alkanes: the first reaction comprises the P450 BM3-catalyzed hydroxylation of an alkyl chain (1), followed by

Scheme 1. Reaction scheme for the bimolecular direct oxidation of n-heptane to 2-heptanone in aqueous medium, using a monoxygenase–dehydrogenase cascade and oxygen as sole oxidizing agent.

Scheme 2. Double oxidation of n-heptane using P450 BM3 monooxygenases and an alcohol dehydrogenase from Rhodococcus erythropolis (RE-ADH) as cascade catalysts. In the first oxidation step n-heptane (1) is converted by a P450 BM3 monooxygenase (WT NADH, 19A12 NADH, and CM1 NADH) to heptanol [enantiomeric mixture of (S)/(R)-2] under NADH consumption yielding NAD^+. In the second oxidation step enzymatic resolution[28] of the in situ produced (R)-heptanols [(R)-2] and synthesis of the corresponding ketones (3) is achieved under reduction of NAD^+ to NADH (cofactor regeneration). R = CH_3, C_2H_5 or C_3H_7; R’ = C_5H_{11}, C_4H_9 or C_3H_7.
In Vitro Double Oxidation of n-Heptane with Direct Cofactor Regeneration

The one-pot double oxidation of n-heptane by direct coupling of two enzymes, the P450 BM3 monooxygenase and the alcohol dehydrogenase from R. erythropolis, was developed.

As monooxygenase the wild-type (WT) enzyme and two reengineered P450 BM3 variants displaying higher activity for n-heptane (19A12, CM1) were tested. The switch in cofactor specificity from NADPH to NADH was achieved through introduction of the reported substitutions W1046S and R966D. For simplicity reasons the variants with altered cofactor specificity are named WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, and CM1\textsuperscript{NADH}.

These three monooxygenase variants were characterized in detail (activity, coupling efficiency, selectivity and productivity) for the preliminary oxidation of n-heptane to 1-, 2-, 3-, or 4-heptanol. Finally, the entire reaction concept was evaluated by determining product formation and total turnover numbers. The double oxidation reaction was performed \textit{in vitro}, using purified enzymes (0.5 µM P450 BM3/10 U RE-ADH) in a reaction scale of 1 mL.

Engineering of the P450 BM3 Mutant CM1 for Improved n-Heptane Hydroxylation

The P450 BM3 monooxygenase 19A12 (containing substitutions R47C, L52I, V78F, A82S, K94I, P142S, T175I, A184V, L188P, F205C, S226R, H236Q, E252G, R255S, A290V, A328F, L353V, I366V)\textsuperscript{25} was previously reported as a reengineered variant for regioselective hydroxylation of alkanes (propane, butane)\textsuperscript{25,26} and cycloalkanes (cyclohexane, cyclooctane and cyclooctane).\textsuperscript{26} Accumulation of 18 mutations in this P450 BM3 variant resulted in a significantly decreased expression (E. coli BL21 Gold (DE3) lacI\textsuperscript{30}) yielding 6% of the wild-type production (see the Supporting Information, Figure S1). To investigate the effect of different amino acid substitutions on the hydroxylation of n-heptane, the 18 previously reported mutated positions were saturated individually in BM3 WT using degenerated NNK oligonucleotides\textsuperscript{31} (the targeted codon is randomized to all 20 canonical amino acids; N=G/A/T/C; K=G/T). Screening of 180 clones per saturation mutagenesis library with the NADPH depletion assay\textsuperscript{32} showed that position 255 (32-fold improved product formation) and to a lesser extent the positions R47, S226, H236 and L188 (up to 10-fold improvement) contributed individually to an optimized n-heptane hydroxylation. Substitution of arginine by proline at position 255 yielded the best result in terms of NADPH consumption and product formation (see the Supporting Information, Figure S2). A second substitution from proline to histidine at position 329 was introduced in BM3 R255P via site-directed mutagenesis to finally generate the variant P450 BM3 CM1 (substitutions R255P/P329H). Characterization of this double mutant for the hydroxylation of n-heptane using cell-free extracts yielded a turnover frequency (TOF) of 567 min\textsuperscript{1} compared to 13 min\textsuperscript{1} in case of the wild-type, representing a 43-fold improved product formation. The highly active P450 BM3 CM1 mutant was therefore chosen for evaluation of the monooxygenase-dehydrogenase cascade reaction in comparison to the wild-type, and to the mutant 19A12\textsuperscript{25} as reference catalyst.

Characterization of NADH-Dependent P450 BM3 WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, and CM1\textsuperscript{NADH} Monooxygenases in the Initial Step of the Cascade Reaction

P450 BM3 has a high specificity for NADPH over NADH.\textsuperscript{33} Maurer et al. as well as Neeli et al. identified that mutations in the codon of W1046 in the reductase domain of P450 BM3 can cause a switch in cofactor specificity to NADH.\textsuperscript{29,33} In this study, the previously reported substitutions W1046S and R966D\textsuperscript{28} were introduced in the P450 BM3 wild-type, 19A12 and CM1 by site-directed mutagenesis to generate the three NADH-dependent (\textsuperscript{NADH}) BM3 variants (WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, and CM1\textsuperscript{NADH}, see Figure 1; light grey; corresponding NADPH consumption values are shown in Figure 1 in dark grey). WT\textsuperscript{NADH} shows with both cofactors and n-heptane a low activity (132 min\textsuperscript{1} for NADH; Figure 1A) and a moderate coupling efficiency (18% for NADPH; Figure 1C). The variants 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} prefer NADH over NADPH in contrast to WT\textsuperscript{NADH} (Figure 1A and B).
The oxidation rate of NADH with variant 19A12NADH is 9% higher than with the variant CM1NADH (Figure 1A). Nevertheless, CM1 NADH shows a 24% higher turnover frequency (TOF, Figure 1B) than 19A12NADH, which can mainly be attributed to its higher coupling efficiency (44% vs. 32%; Figure 1C).

TOF values were calculated based on GC quantification of the overall amount of products (see the Supporting Information, Figure S3). The TOF values reached with NADH as cofactor are 485 min·mol P450⁻¹/C₀ and 503 U·g P450⁻¹/C₀ for 19A12NADH and CM1NADH, respectively.

Figure 2 shows the regioselectivity of P450 BM3 WT NADH, 19A12NADH and CM1NADH. WT NADH is a subterminal hydroxylase with a similar regioselectivity (2-, 3- or 4-heptanol) to the hydroxylation pattern of fatty acids. WT NADH produces all 3 subterminal hydroxylated heptanol regioisomers in amounts higher than 24%.

In contrast, P450 BM3 monooxygenase 19A12NADH and CM1NADH show a changed regiospecificity and a significant hydroxylation preference for two subterminal carbon atoms. 19A12NADH hydroxylates mainly the C-2 atom (64% 2-heptanol) followed by C-3 (33% 3-heptanol). CM1NADH produces preferentially 4-heptanol (48%) followed by 3-heptanol (42%) and 2-heptanol (9%) (for the original GC spectra see the Supporting Information, Figure S4).

Interestingly, the stereopreferences for chiral heptanols differ between WT NADH, 19A12NADH, and CM1NADH (Table 1). WT NADH produces preferentially (R)-heptanols [ee of 49% for (R)-3-heptanol and 74% for (R)-2-heptanol], whereas 19A12NADH produces (S)-3-heptanol with a high preference (ee 86%) and a nearly racemic mixture of 2-heptanol [ee 3.9% for (S)-2-heptanol]. CM1NADH shows also a slight excess of (S)-3-heptanol (ee 12%) and a clear preference for (R)-2-heptanol (ee 46%). All details for the determination of enantioselectivity and the original GC spectra can be found in the Supporting Information, Figure S5 and Figure S6.

Double Oxidation Reaction of n-Heptane: Conversion and Total Turnover Numbers

Figure 3 shows the performance of WT NADH, 19A12NADH, and CM1NADH in the initial hydroxylation step (upper part) and in the double oxidation reaction (lower part).

The (S)-specific alcohol dehydrogenase from Rhodococcus erythropolis was selected for the oxidation of 2-heptanol due to its high activity and selectivity. The volumetric activity of the semi-purified RE-ADH
towards the heptanol isomers was determined under the experimental conditions for the double oxidation cascade. (S)-2- and (S)-3-heptanol are the preferred heptanol isomers of the RE-ADH, which had a volumetric activity of 46.7 and 6.8 U mg\(^{-1}\) for each enantiomer, respectively. Activities for 1- and 4-heptanol as substrates reached only 1.3 and 0.8 U mg\(^{-1}\).

During the initial oxidation reaction \(n\)-heptane is hydroxylated by BM3 WT\(^{NADH}\) or variants (19A12\(^{NADH}\), CM1\(^{NADH}\)) to the heptanol isomers [see Scheme 2, product (S)/(R)-2]. In the subsequent step the (S)-specific RE-ADH catalyzes the further oxidation of the preferred alcohols (S)-2 to the corresponding ketones (see Scheme 2; product 3), thus enabling an \textit{in situ} resolution of the (R)-enantiomers [see Scheme 2; product (R)-2]. The second oxidation is accompanied by the reduction of NAD\(^{+}\) to NADH. The regenerated cofactor NADH can be reused by the monoxygenase for hydroxylation of \(n\)-heptane.

The \(n\)-heptane conversion in a single hydroxylation reaction with WT\(^{NADH}\), 19A12\(^{NADH}\), or CM1\(^{NADH}\) stops nearly after 1 hour yielding product concentrations of 0.23, 0.69, and 0.62 mM heptanols, respectively. In contrast, during the double oxidation reaction the product formation increases continuously over more than 4 h. This can be attributed to the NADH cofactor recycling during the double oxidation. A detailed comparison in terms of total product concentrations shows an increase from 0.23 to 0.56 mM for WT\(^{NADH}\) (Figure 3A and D), from 0.69 to 1.3 mM for 19A12\(^{NADH}\) (Figure 3B and E), and from 0.62 to 1.1 mM CM1\(^{NADH}\) (Figure 3C and F).

RE-ADH determines the product ratio between heptanones and heptanols in the coupled reaction due to its specificity for the (S)-isomers. This ratio differs significantly between 19A12\(^{NADH}\) and CM1\(^{NADH}\). 19A12\(^{NADH}/\text{RE-ADH}\) produces twice the amount of heptanones (0.85 mM vs. 0.43 mM heptanol), whereas CM1\(^{NADH}/\text{RE-ADH}\) produces twice the amount of heptanols (0.78 mM vs. 0.35 mM heptanone). BM3 WT\(^{NADH}\) achieves a product concentration of approximately 0.56 mM when coupled to the alcohol dehydrogenase (Figure 3D). Nevertheless, the concentration of heptanones achieved with BM3 WT\(^{NADH}\) is

**Table 1.** Enantioselectivity of the \(n\)-heptane hydroxylation catalyzed by P450 BM3 WT\(^{NADH}\), 19A12\(^{NADH}\) and CM1\(^{NADH}\).

<table>
<thead>
<tr>
<th>P450 BM3 variant</th>
<th>2-Heptanol(^{[a]}) (ee) [%]</th>
<th>3-Heptanol(^{[b]}) (ee) [%]</th>
<th>2-Heptanol ((S:\mathit{R}))</th>
<th>3-Heptanol ((S:\mathit{R}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(^{NADH})</td>
<td>74 ± 0.1 (R)</td>
<td>50 ± 3 (R)</td>
<td>13:87</td>
<td>50:50</td>
</tr>
<tr>
<td>19A12(^{NADH})</td>
<td>4 ± 3 (S)(^{[c]})</td>
<td>86 ± 2 (S)</td>
<td>52:48</td>
<td>25:75</td>
</tr>
<tr>
<td>CM1(^{NADH})</td>
<td>46 ± 0.2 (R)</td>
<td>12 ± 1 (S)</td>
<td>27:73</td>
<td>93:7</td>
</tr>
</tbody>
</table>

\(^{[a]}\) Enantiomeric excess (\(ee\)) and enantiomeric ratio (\(er\)) were determined by chiral GC, for 2-heptanol using a subsequent lipase-catalyzed kinetic resolution [(\(R\))-2-heptanol acylation].

\(^{[b]}\) For 3-heptanol, the absolute configuration was determined by polarimetry (see Experimental Section).

\(^{[c]}\) High deviation is caused by low differences in values of enantiomer “peaks”.

---

**Figure 3.** Conversion of \(n\)-heptane to heptanols (initial hydroxylation step: \textit{upper part}) and conversion of \(n\)-heptane to heptanones (double oxidation; \textit{lower part}). Initial hydroxylations are shown for (A) WT\(^{NADH}\), (B) 19A12\(^{NADH}\) or (C) CM1\(^{NADH}\) and the double oxidations in presence of the RE-ADH: (D) WT\(^{NADH}\), (E) 19A12\(^{NADH}\), and (F) CM1\(^{NADH}\). Total heptanol (○) and total heptanone (○) concentrations are shown as sum of the corresponding regioisomers (1-, 2-, 3-, and 4-heptanols) which were quantified via GC after a reaction time of 0.17, 0.5, 1, 2, 4 and 24 h (see details in Experimental Section).
very low (69 μM vs. 0.5 mM heptanol) and differs significantly from 19A12NADH and CM1NADH.

Notably, when starting from the alkane n-heptane the double oxidation concept also allows the production of (R)-alcohols due to the selectivity of the RE-ADH. Table 2 summarizes the yields obtained on (R)-2- and (R)-3-heptanol in the double oxidation cascade. The yields from the enzymatic resolution experiment are calculated based on the overall amount of the chiral [(R)/(S)-2- and (R)/(S)-3-heptanols] and non-chiral products (2- and 3-heptanones), which were set to 100% disregarding the conversion of the non-chiral 1- and 4-heptanols. The highest yields on (R)-heptanols were obtained with BM3 WT NADH [89% (R)-2-heptanol], followed by CM1NADH [72% (R)-2- and 58% (R)-3-heptanol]. Comparably low yields were achieved with 19A12NADH [51% (R)-2- and 20% (R)-3-heptanol].

The calculated yields for the resolution of (R)-heptanols are higher than the expected values from the single, non-coupled hydroxylation reaction (Table 2 vs. Table 1). The differences are caused by a possible shift in the product distribution during the double oxidation due to the regeneration and therefore higher availability of the cofactor.

In order to explore whether the cofactor regeneration is a limiting factor in the double oxidation cascade an additional NADH regeneration system based on glucose/glucose dehydrogenase (GDH) was implemented (see the Supporting Information, Scheme S1). Addition of cofactor regeneration over glucose/GDH can improve the regeneration capacity and compensate for cofactor losses as a result of the low coupling efficiencies of the P450 monooxygenases, where NADH is utilized for undesired (uncoupling) reactions.

Figure 4 shows the results of the double oxidation with WTNADH, 19A12NADH, and CM1NADH in the presence of the d-glucose/GDH regeneration system. Produced NADH during the initial oxidation step is utilized simultaneously by the RE-ADH and the GDH for the second coupled oxidation/regeneration step. As both NADH-consuming reactions run in parallel it was interesting to characterize more in detail the cofactor demand of each regenerating enzyme. Therefore, the Km and Vmax values for NADH of both enzymes were determined. The Km value of the RE-ADH for NADH using 2-heptanol as substrate is 84 ± 9 μM and Vmax reaches 0.005 sec⁻¹. The GDH from Pseudomonas sp. displays a lower affinity for NADH with a Km of 113 ± 15 μM when d-glucose is used as substrate, and achieves a nearly 3-fold higher Vmax of 0.017 sec⁻¹ compared to RE-ADH (see the Supporting Information, Figure S7).

Based on the Km and Vmax values it was deduced that a key performance parameter was to adjust the glucose concentration to ensure sufficient NADH supply for the RE-ADH-catalyzed oxidation in order to maximize product formation (see the Supporting Information, Figure S8). The best result in terms of product concentration and ratio between heptanone and heptanol was obtained with a d-glucose concentration between 2 and 5 mM. A concentration of 2 mM was finally employed for the enzyme cascade as shown in Figure 4.

Total product concentrations in the double oxidation cascades with supporting cofactor regeneration (d-glucose/GDH) are significantly higher. In the case of BM3 WTNADH a maximal product concentration of

---

**Table 2.** Obtainable yields of the RE-ADH-catalyzed resolution of (R)/(S)-heptanols in the 2nd step of the double oxidation cascade.

<table>
<thead>
<tr>
<th>Yield [%]a</th>
<th>(R)-2-Heptanol</th>
<th>(R)-3-Heptanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>WTNADH</td>
<td>89 ± 1</td>
<td>82 ± 2</td>
</tr>
<tr>
<td>19A12NADH</td>
<td>51 ± 3</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>CM1NADH</td>
<td>72 ± 3</td>
<td>58 ± 3</td>
</tr>
</tbody>
</table>

[a] See Table 1 for details on the theoretical composition (enantiomeric ratio er) of the heptanol mixture which serves as substrate. Conversion of (S)-2-heptanol and (S)-3-heptanol by RE-ADH was determined to be > 99%.

---

**Figure 4.** Double oxidation of n-heptane with a supporting regeneration system (d-glucose/GDH) for the cofactor NADH. Panels A–C show: (A) BM3 WTNADH, (B) 19A12NADH and (C) CM1NADH. Total heptanol (●) and total heptanone (○) concentrations are displayed as sum of the corresponding regioisomers which were quantified via GC after a reaction time of 0.17, 0.5, 1, 2, 4 and 24 h (see details in the Experimental Section).
In Vitro Double Oxidation of n-Heptane with Direct Cofactor Regeneration

Table 3. Total turnover numbers of n-heptane oxidation with BM3 WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} in the absence of RE-ADH (entry 1), in presence of RE-ADH (entry 2) and in presence of RE-ADH with additional NADH regeneration (d-glucose/GDH; entry 3).

<table>
<thead>
<tr>
<th>Entry</th>
<th>WT\textsuperscript{NADH}</th>
<th>TTN\textsuperscript{[a]}</th>
<th>CM1\textsuperscript{NADH}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1\textsuperscript{[b]}</td>
<td>only P450</td>
<td>462 ± 56</td>
<td>1374 ± 2</td>
</tr>
<tr>
<td>2\textsuperscript{[c]}</td>
<td>P450 + RE-ADH</td>
<td>1128 ± 27</td>
<td>2514 ± 92</td>
</tr>
<tr>
<td>3\textsuperscript{[d]}</td>
<td>P450 + RE-ADH + GDH</td>
<td>1457 ± 24</td>
<td>3146 ± 213</td>
</tr>
</tbody>
</table>

\textsuperscript{[a]} TTN: total turnover number (\mu mol total product/\mu mol catalyst\textsuperscript{[1]}); total product is the sum of all quantified heptanol and heptane isomers (24 h).

\textsuperscript{[b]} The reaction mixture contained n-heptane (50 mM) in KPi buffer (1 mL, 100 mM; pH 8.0), ethanol (1.3% v/v), NADH (2 mM), catalase (600 U), P450 BM3 (0.5 \mu M).

\textsuperscript{[c]} Additionally to entry 1: RE-ADH (10 U).

\textsuperscript{[d]} Additionally to entry 1: RE-ADH (10 U), d-glucose (2 mM) and GDH (1 U).

0.73 mM is obtained (215% higher than without GDH; Figure 4A). The variants 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} produced 1.57 and 1.8 mM total product, which correspond to a 129% and 188% increased product formation (Figure 4B and C). Product distribution patterns in the double oxidation cascades as well as conversion and yields in the second oxidation step remained within standard deviations in the presence and absence of the d-glucose/GDH regeneration system.

Based on the quantified product concentrations after a reaction time of 24 h, the total turnover numbers (TTN) were determined for the single and the double oxidation reactions as well as for the double oxidation reactions with supporting cofactor regeneration (d-glucose/GDH) (see Table 3). The employed monoxygenases (WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, CM1\textsuperscript{NADH}) displayed a >1.8-fold improved TTN in the double oxidation cascades compared to the single hydroxylation reaction in absence of RE-ADH (Table 3, entry 1 vs. 2).

Supplementation of an NADH regeneration system with d-glucose as a co-substrate and GDH yielded higher product concentrations for all three BM3 variants and consequently TTNs increased up to 3-fold (Table 3, entry 1 vs. 3). 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} achieved comparably high TTN values (up to 3591) which are under all tested reaction conditions more than 2-fold higher than the corresponding TTN-values of WT\textsuperscript{NADH} (Table 3).

Discussion

Monoxygenases are rarely employed on an industrial scale, despite their synthetic potential for oxyfunctionalization of alkanes. C–H bond activation with O\textsubscript{2} as clean oxidant has been described as a dream reaction in organic chemistry.\textsuperscript{[11]}

Enzymatic cascade reactions are interesting as an alternative to chemical synthesis routes in that challenges in cofactor supply, insufficient coupling rates, and stability issues can be addressed successfully. In this regard, significant progress has been achieved to improve stabilities and coupling rates of monoxygenases by rational design and/or directed evolution.\textsuperscript{[23c,25,36]} The concept of the double oxidation cascade using molecular oxygen as sole oxidant and direct regeneration of the cofactor NADH is synthetically attractive since use of additional cosubstrates can be omitted or at least be reduced.

To validate the one-pot double oxidation n-heptane was selected as substrate for the coupled direct oxidation with P450 BM3 monoxygenases (WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, CM1\textsuperscript{NADH}) and the (S)-enantioselective alcohol dehydrogenase from R. erythropolis (RE-ADH). In all three P450s the cofactor specificity was switched from NADPH to NADH. With the variants 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} not only the cofactor preference changes but also higher product yields using NADH were achieved (43% more for 19A12\textsuperscript{NADH} and 80% more for CM1\textsuperscript{NADH}).

P450 BM3 WT\textsuperscript{NADH} 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} were characterized in detail with regard to activity, coupling efficiency, selectivity and product formation in the oxidation of n-heptane to 1-, 2-, 3-, or 4-heptanol and in the double oxidation cascades without and with a supporting cofactor regeneration system.

Remarkably, the regio- and stereoselectivity of WT\textsuperscript{NADH}, 19A12\textsuperscript{NADH}, and CM1\textsuperscript{NADH} differs significantly (Figure 2, Table 1). For instance, WT\textsuperscript{NADH} is (R)-selective for the production of 2- and 3-heptanols, whereas 19A12\textsuperscript{NADH} and CM1\textsuperscript{NADH} are (S)-selective with respect to 3-heptanol. In this case only two substitutions (R255P, P329H) are required in CM1\textsuperscript{NADH} to partially invert the stereoselectivity of the enzymes. Additionally, 19A12\textsuperscript{NADH} is poorly stereoselective for the 2-heptanol production in contrast to the (R)-enantioselective WT\textsuperscript{NADH} and CM1\textsuperscript{NADH}.
19A12NADH was selected as reference based on its catalytic performance for the conversion of n-heptane (18 substitutions) showing a TTN of 5360 ± 1020, which is to the best of our knowledge the highest reported value for biocatalytic hydroxylation of this compound. Our total turnover numbers for 19A12NADH reach up to 3150. Differences to the previously reported TTN might be attributed to the altered cofactor specificity (NADH instead of NADPH) and the employed regeneration system (isocitrate dehydrogenase vs. GDH) as well as the reaction conditions.

The catalytic characterization of BM3 CM1NADH showed a comparably high activity with n-heptane (TOF value of 604 min⁻¹ or 10 s⁻¹ with NADH; Figure 1B) as found for other engineered P450 BM3 variants for the oxidation of non-natural substrates like octane (variant 53-5H, TOF of 660 min⁻¹ using NADPH). A fine compilation of $k_{cat}$ values for P450 BM3 is reported in the review by Whitehouse et al. (38).

The oxyfunctionalization of alkanes under mild conditions was summarized in a recent review. Oxidation of n-heptane to the corresponding alcohols and ketones was reported for a Mn(TDCPP)Cl complex together with H₂O₂ as oxidant with a TTN of 202 (38,39). Total turnover numbers for 19A12NADH and CM1NADH are 15-fold higher (see Table 2) and the catalytic performance for the conversion of (18 substitutions) showing a TTN of 5360 ± 1020, which is to the best of our knowledge the highest reported value for biocatalytic hydroxylation of this compound. The regioselectivity of P450 BM3 could also be improved significantly for non-native substrates like limonene, steroids, and 2-arylacetic acid esters (39). In this regard, tuning of P450 enzymes for non-native transformations was recently reviewed in a comprehensive way by Fasan (44).

The stereopreferences of the employed ADH opens up the possibility of a non-racemic resolution of enantiomers starting from an n-alkane substrate and yielding (R)- or (S)-alcohols as value added products in double oxidation cascade reactions. The specificity of the RE-ADH [(S)-selective], which accepts only (S)-alcohols for NADH production reduces the amount of regenerated cofactor. In an ideal case two enzymes with matching $K_M$ values and specificities, and a highly coupled cofactor oxidation would be sufficient to overcome constraints in the cofactor availability.

The developed double oxidation concept is a tunable and modular system in which the employed enzymes (monooxygenase and alcohol dehydrogenase) can be exchanged depending on the targeted specificity and selectivity. General options are to focus on the resolution of enantiomeric mixtures for the production of chiral alcohols or to maximize the ketone production.

Conclusions

A double oxidation cascade concept using the transformation of n-heptane into chiral heptanols and heptanone as a model reaction has been developed by coupling a monooxygenase- and an alcohol dehydrogenase-catalyzed reaction with and without a supporting cofactor regeneration system. The cascade oxidation reactions were performed in water at room temperature using molecular oxygen as an oxidant and the obtained TTNs (>3000) exceed the ones from chemical catalysts by 15-fold.

Interestingly, the stereopreferences for n-heptane hydroxylation were partially inverted by substitutions R255P and P329H. The reported bicatalytic cascade is especially attractive for cell-free chemical syntheses, can be handled in chemical synthesis labs (without biosafety specifications) and represents the first concept in which a chiral resolution of alcohols is implemented after functionalization of an alkane.

The double oxidation concept employs alcohol dehydrogenases and monooxygenases in a modular manner. As a toolbox it will enable, for instance, the synthesis of enantiomerically pure alcohols or the corresponding ketones by employing alternative and highly selective enzymes like the ADH from Lactoba-
In Vitro Double Oxidation of n-Heptane with Direct Cofactor Regeneration

cillus brevis (Lb-ADH).

The concept of double oxidation can further be expanded to other stereoselective oxidoreductases or further enzyme classes such as aminotransferases or lyases.

**Experimental Section**

**Chemicals and Reagents**

All chemicals used were of analytical grade or higher and were purchased, if not stated otherwise, from Sigma Aldrich (Steinheim, Germany), AppliChem (Darmstadt, Germany), TCI Europe (Eschborn, Germany) and Carl Roth (Karlsruhe, Germany). Enzymes and dNTPs were obtained from Fermentas (St. Leon-Rot, Germany), New England Biolabs (Frankfurt, Germany) and Sigma Aldrich (Steinheim, Germany). The HPLC-purified oligonucleotides employed for mutagenesis were procured from Eurofins MWG Operon (Ebersberg, Germany).

**Strains and Expression Vectors**

Cloning of the three P450 BM3 monooxygenase variants used in this work (WT, 19A12 and CM1) was performed using the pET28a(+) derived pALXtreme-1a vector and transformation into chemically competent E. coli BL21 Gold (DE3) lacI cells. The construction of the P450 BM3 monooxygenase variant harbouring the 19A12 gene was already described elsewhere. The expression vector pKAl harbouring the (S)-alcohol dehydrogenase from Rhodococcus erythropolis (RE-ADH) was transformed into chemically competent E. coli BL21 Gold (DE3) cells. Chemically competent cells were prepared using a standard protocol yielding a transformation efficiency of 1 x 10^7 and 5 x 10^6 cfu μg⁻¹ pUC19, respectively.

**Generation of the Mutant P450 BM3 CM1**

Altogether 18 previously reported positions of the P450 BM3 monooxygenase variant 19A12 were saturated in the BM3 WT using NNK-degenerated oligonucleotides. A two-stage PCR was performed according to the modified QuikChange Site-Directed mutagenesis protocol. Expression of the mutant libraries and screening for activity towards n-heptane was achieved following a published procedure. The reaction mixture for measuring NADPH depletion contained n-heptane (1 mM), DMSO as cosolvent (4% v/v), crude cell lysate (50 μL) and NADPH (0.2 mM) in a final volume of 250 μL. KPi buffer (100 mM; pH 8.0). These studies led to identification of the key position P255 for enhanced hydroxylation activity towards n-heptane. Based on the NADPH depletion (relative activity) and the sequencing results (MWG Eurofins DNA, Ebersberg, Germany), the improved variant P450 BM3 containing the mutation R255P was identified. A second, previously identified beneficial substitution P329H was combined following oligonucleotide primers were used: R255-forward: 5'-GCTTAGCCACCTGCTGTTCTCCCTATGCG-3', R255-reverse: 5'-GAATCGTAATAATTTGATAMN NAATGTCCATGCTACAGGCA-3', P329H-forward: 5'-GCTATGCCAACCTGCTGTTCTCCCTATGCG-3', P329H-reverse: 5'-GCATTAGGGAAACGCTGAATGGC CATAAGCC-3'.

**Construction of NADH-Dependent Mutants**

To switch cofactor specificity of the P450 BM3 variants the mutations W1046S and R966D were introduced in the P450 BM3 genes (wild type, 19A12 and CM1) by site-directed mutagenesis, using following primers: W1046S-forward: 5'-GATACGCAAAAGACGTGAGCGCTGGGTAATAAGAATTCG-3', W1046S-reverse: 5'-GGCTGATTTGGCATGCTCAGAAGGCTAATGAAAGCG-3', R966D-forward: 5'-GTCCTTAACCGTTTTTTCTGACATGCAAAATGCAC-3', R966D-reverse: 5'-GGCTGATTTGGCATGCTCAGAAGGCTAATGAAAGCG-3'.

The mutagenic PCR using a two-stage modified QuikChange protocol and the transformation procedure were performed as described above. Correct insertion of both mutations was corroborated by sequencing.

**Expression and Purification of P450 BM3 WT NADH, 19A12 NADH and CM1 NADH Mutants**

Expression of the NADH-dependent monooxygenase mutants and preparation of crude cell extracts for further purification were performed as previously described. The fresh cell lysates were subsequently purified by means of anion exchange chromatography using a Toyopearl DEAE 650S matrix (Tosoh Bioscience, Stuttgart, Germany) and an established protocol. An Äktaprime Plus system with UV detector (GE Healthcare, München, Germany) was used for purification and collection of the eluate. The eluted protein fractions were pooled together and concentrated using an Amicon Ultra-4 centrifugation tube (30 kDa cut-off; Millipore, Schwalbach, Germany), followed by desalting and buffer exchange in KPi buffer (100 mM; pH 8.0) using a PD-10 gel-filtration column (GE Healthcare, München, Germany). A purity of 90% was estimated after electrophoresis in a 10% sodium dodecyl polyacrylamide (SDS-PAGE) gel. The purified monooxygenase mutants were finally shock-frozen in liquid nitrogen, lyophilized for 48 h at −54°C in an Alpha 1–2 LD plus Freeze dryer (Christ, Osterode am Harz, Germany) and stored at −20°C until further use.

**Expression and Purification of RE-ADH**

The RE-ADH was expressed and partially purified according to a published protocol with minor modifications. LB medium containing chloramphenicol (50 mM, 1.7 mg) was used and after reaching an OD600 of 5.0 the expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG; 25 μM) and ZnCl2 (1 mM) for 20 h at 30°C. Cells were harvested by centrifugation (10 min, 2900 g at 4°C) and the cell pellet was frozen. For preparation of the cell extract the frozen pellet (1.3 g wet cells) was then resuspended in KPi buffer (20 mM, 100 mM; pH 6.0) prior to disruption with an Avestin Emulsiflex-C3 high pressure homogenizer (Ottawa, ON, Canada) by applying three cycles of 1500 bar. The cell debris was removed by centrifugation (15 min, 16000 g at 4°C) in a Sorvall RC-6 Plus centrifuge (Thermo Scientific, Rockford, IL, USA) and the supernatant was par-
tially purified using heat-treatment incubation for 15 min at 65°C. A second centrifugation for removal of precipitated proteins was performed. The supernatant was further clarified by filtration through a 0.45 μm filter (Roth, Karlsruhe, Germany) and stored at 4°C. A purity of nearly 90% was estimated according to SDS-PAGE visualization.

**Kinetic Characterization of the Purified P450 BM3 Monooxygenases in Terms of Product Formation and Coupling Efficiency**

The concentration of active, purified and lyophilized P450 BM3 monooxygenase was determined from the CO-difference spectra of the reduced heme iron using a standard procedure. CO-difference spectra and NADH oxidation were recorded with a Varian Cary 50 spectrophotometer (Agilent Technologies, Darmstadt, Germany). To assay activity of the P450 BM3 mutants NADH or NADPH depletions was monitored in a cuvette at 340 nm in triplicate. Quantification of the exact amount of consumed NAD(P)H was accomplished using the extinction coefficient $e_{\text{NAD(P)H}} = 6.22 \text{mM}^{-1}\text{cm}^{-1}$. The reaction mixture consisted of $n$-heptane (10 mM), ethanol as cosolvent (2% v/v), P450 BM3 (0.2 μM) in KPi buffer (950 μL, 100 mM; pH 8.0). After 5 min of pre-incubation the reaction was started by addition of NADH or NADPH from a stock solution (50 μL, 8 mM). When the cofactor was almost consumed the reaction was quenched by addition of concentrated HCl (100 μL, 37% v/v) and directly extracted with methyl tert-butyl ether (MTBE; 400 μL) containing decane (1 mM) as internal standard. The organic layer was dried over NaSO$_4$ and analyzed by gas chromatography (GC) for product quantification. Maximal initial product formation rates were calculated for the first 20 sec of the reaction. The molecular weight value of P450 BM3 and variants used in the calculations was 119 kDa.

**Quantification of Heptanol and Heptanone Production**

Quantification of the reaction products was performed using a Shimadzu GC-2010 gas chromatograph coupled to a flame ionization detector (FID) (Shimadzu, Japan). For quantification the GC was fitted with a column FS-Supreme-5 ms (30 m x 0.25 mm x 0.25 μm film thickness; CS-Chromatography, Germany) and following column oven temperature program was used for separation: 40°C for 19.5 min, 20°C min$^{-1}$ to 100°C, 3 min. For each corresponding alcohol and ketone a 6-point calibration curve was generated using authentic standards and a defined amount of decane (1 mM) as internal standard. Retention times for 4-, 3-, 2-, 1-heptanones, 4-, 3-, 2-, 1-heptanols and decane, respectively: 13.6, 15.2, 15.8, 16.4, 17.1, 17.9, 22.7 and 23.3 min (see original GC spectra in the Supporting Information, Figure S3).

**Regioselectivity**

Product distribution (normalized to 100%) was calculated based on GC quantification of each regioisomer (1-, 2-, 3-, 4-heptanol) as mentioned above.

**Enantioselectivity**

Pure enantiomeric standards of 3-heptanol were not commercially available, but the racemic 3-heptanol could be separated on a chiral GC column (see below). Therefore, the absolute configuration of each GC peak was assigned based on polarimetry measurements and correlation to the optical rotation (sign of the rotation) according to literature values [−4.9° for (R)-(−)-3-heptanol] and +4.5°8 for (S)-(+) -3-heptanol]. Samples of each enantiomer in excess were obtained after conversion of (rac)-3-heptanol, either with the (R)-specific alcohol dehydrogenase from Lactobacillus brevis (LbADH) or the (S)-specific ADH from Rhodococcus erythropolis (RE-ADH). The LbADH reaction was performed using recombinant E. coli whole cells (optical density of 22) in KPi-buffer (25 mL, 0.1 M, pH 6.0), containing acetone (2 mL, 8% v/v), MgSO$_4$ (4.25 mg), NADP$^+$ (2 mM) and 3-heptanol (0.4 M), for 24 h at room temperature. The products were extracted twice by addition of chloroform (10 mL). After centrifugation the organic layers were combined, dried over NaSO$_4$ and concentrated to 1 mL using an evaporator IKA RV 10 basic (IKA-Werke, Staufen, Germany). Subsequently, the extracted reaction mixture was used for polarimetric measurement with a Perkin–Elmer 241 MC Polarimeter (Waltham, USA) and for chiral GC analysis.

The 2-heptanol enantiomers could not be separated on a chiral GC column, therefore the calculation of the enantiomeric ratio (er) and enantiomeric excess (ee) was performed based on the lipase-catalyzed resolution of the (S)-2-heptanol enantiomer as described by Patel et al. with some modifications. The products of the P450 BM3-catalyzed hydroxylation of $n$-heptane were extracted in 1 volume of $n$-heptane (1 mL) and split into two equal volumes of 0.4 mL. One volume was treated with lipase B for acylation of the (R)-2-heptanol (20 U, 4 mg of lipase from Candida Antarctica/acyllic resin, Sigma Aldrich, Steinheim, Germany) and succinic anhydride (0.2 mg), one volume without the addition of lipase as reference reaction. Both samples were incubated in parallel (35°C, 24 h, 600 rpm). For chiral separation and quantification of the (R)- and (S)-3-heptanols, as well as analysis of the remaining (S)-2-heptanol after acylation of the (R)-enantiomer, a GC-2010 Plus with an FID detector (Shimadzu, Japan) was fitted with a column FS-Hydrodex β-TBDAC (Macherey-Nagel GmbH, Dueren, Germany). The following oven temperature program was used: 50°C 5°C min$^{-1}$ to 95°C, 9.5 min. Retention times for (R)- and (S)-3-heptanol, respectively: 9.88, 10.01 min; for (S/R)-2-heptanol: 10.65 min (for the original GC spectra see the Supporting Information, Figure S5 and Figure S6).

**Determination of the Selectivity of the P450 BM3 Variants**

The distribution of the regioisomers and the enantiomeric excess (ee values) for the hydroxylation of $n$-heptane were analyzed via gas chromatography. The same set up of the hydroxylation reaction for the determination of the total turnover number, containing solely P450 BM3, was used.

**RE-ADH and GDH Activity Determination**

The activity of the heat-treated RE-ADH and of the commercially available glucose dehydrogenase (GDH) from Pseudomonas sp. (Sigma Aldrich, Steinheim, Germany) was assayed spectrophotometrically with a Varian Cary 50 (Agilent Technologies, Darmstadt, Germany) by measuring the increase in absorption of NADH at 340 nm.
In Vitro Double Oxidation of n-Heptane with Direct Cofactor Regeneration

For RE-ADH: The reaction mixture (999 μL; cuvette) contained the corresponding alcohol (1-, 2-, 3- or 4-heptanol; 10 mM) and NAD⁺ (1 mM) in KPi buffer (100 mM; pH 8.0). The reaction was started by addition of RE-ADH (1 μL; partially purified). One unit was defined as the amount of enzyme that reduces 1 μmol of NAD⁺ per minute. Total protein concentration was determined by the method of Bradford using bovine serum albumin as standard.[31] A control reaction with ethanol (4% v/v), which is used in the cascade reaction as cosolvent, was also recorded and revealed no activity of RE-ADH towards ethanol. The Km value of the RE-ADH for the cofactor NAD⁺ using 2-heptanol as substrate was determined in an analogous manner, varying the NAD⁺ concentration (1 to 4000 μM).

For GDH: the reaction mixture contained KPi buffer (1 mM, 100 mM; pH 8.0), d-glucose (10 mM) and NAD⁺ (1 mM). The reaction was started by addition of GDH (1 μL). The Km value of the GDH for the cofactor NAD⁺ using d-glucose as substrate was determined in an analogous manner, varying the NAD⁺ concentration (3 to 2000 μM).

One-Pot Double Oxidation of n-Heptane by P450 BM3 and RE-ADH, with and without Supplementation of GDH

Conversion experiments and determination of total turnover number (TTN): To determine product formation the in vitro double oxidation of n-heptane was monitored over the first 24 h (1 mL, stirred flask, room temperature). The reaction mixture contained n-heptane (7 μL, 50 mM) and ethanol (13 μL, 1.3% v/v) as cosolvent for enhanced solubility of the organic substrate, P450 BM3 (0.5 μM), RE-ADH (10 U, referring to the activity determined with 2-heptanol) and catalase (600 U, to remove traces of hydrogen peroxide) in KPi buffer (1 mL, 100 mM; pH 8.0). After an incubation period of 5 min the reaction was started by addition of NADH (2 mM). After reaction times of 10, 30, 60, 120, 240 min and 24 h, the reaction mixture (1 mL) was transferred to a reaction vessel containing HCl (100 μL) for stopping the reaction and the aqueous phase was directly extracted with MTBE (400 μL, 1 mM decane as internal standard) for GC quantification as described above. In parallel, a second oxidation reaction containing an additional NADH-regeneration system was performed. In this case glucose dehydrogenase (1 U, referring to the activity determined with d-glucose) and d-glucose (2 mM) were added to the reaction mixture. As a reference reaction the sole hydroxylation of n-heptane, using only P450 BM3 as catalyst was conducted in the same way.

Yields for resolution of (R)-heptanols: Yields were calculated for each (R)-heptanol as product of the second RE-ADH-catalyzed oxidation reaction, starting from the enantiomeric mixture [(R)/(S)-2-heptanol or (R)/(S)-3-heptanol] and not heptane as substrate. For the calculation of yields conversion of the non-chiral 1- and 4-heptanol was not considered. The products were quantified using the area of each enantiomeric peak by chiral GC analysis as mentioned above.

Acknowledgements

This work was supported by Deutsche Bundesstiftung Umwelt (ChemBioTec project AZ 13234; Nachhaltige Biokatalytische Oxidationprozesse). We also would like to thank Dr. Jan Marienhangen (FZ Jülich), Dr. Anna Joelle Ruff and Alexander Dennig (RWTH Aachen) for fruitful discussions.

References

In this concept the term /C28enzymatic resolution/C29 refers to the separation of enantiomers in a non-racemic mixture of (S)- and (R)-heptanols (the composition will depend on the enantioselectivity of the employed P450 BM3 monooxygenase variant).