Alkane Oxidation

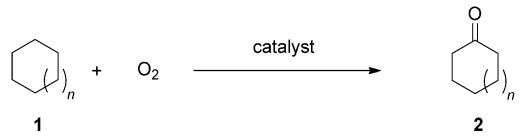
DOI: 10.1002/anie.201204464

Direct Oxidation of Cycloalkanes to Cycloalkanones with Oxygen in Water**

*Svenja Staudt, Edyta Burda, Carolin Giese, Christina A. Müller, Jan Marienhagen, Ulrich Schwaneberg, Werner Hummel, Karlheinz Drauz, and Harald Gröger**

Dedicated to Professor Heribert Offermanns on the occasion of his 75th birthday

The direct functionalization of alkanes to alkanones by means of molecular oxygen represents one of the so-called "dream reactions".[1] A reaction type of particular interest in this area is the oxidation of (higher homologous) cycloalkanes to the corresponding cycloalkanones according to the synthetic concept shown in Scheme 1. The synthesis of cyclohexanone



1 2

Scheme 1. Reaction type of the direct oxidation of cycloalkanes to cycloalkanones with oxygen.

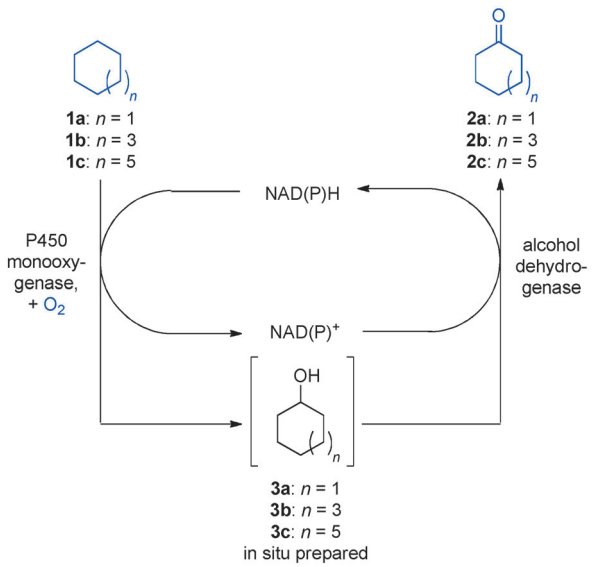
(**2a**), cyclooctanone (**2b**), as well as cyclododecanone by this route is of particular interest due to the economic importance of these compounds (although other synthetic approaches to them also exist).[2] For example, cyclohexanone (**2a**) is a building block in the large industrial scale manufacture of ε-caprolactam, which is required for the production of nylon-6, as well as for the synthesis of adipic acid, which is required for the production of nylon-6.6. Cyclooctanone (**2b**) is a monomeric building block that serves as the starting material for the production of polymers. In addition, cyclododecanone is needed for the production of laurin lactam and polyamides (nylon-12, nylon-6.12), and is also used industrially in the production of fragrances and UV absorbers.

A current route for the production of cycloalkanones, in particular higher homologue cycloalkanones with eight or more carbon atoms, on a large industrial scale is based on the use of molecular oxygen as the oxidation agent according to the Bashkirov process.[3] However, drawbacks of this multistep procedure include the need for a stoichiometric amount of boric acid, the selectivity of the cycloalkane oxidation, which is acceptable only at low conversions ($< 40\,\%$), as well as the extensive distillation-based separation procedure of the product mixture formed because of the low conversion.[3] In recent years, nitrous oxide has been used instead of oxygen as the oxidation agent for the efficient industrial transformation of cycloalkenes to cycloalkanones.[4]

In the following we describe a biotechnological process, in which the desired cycloalkanone **2** is obtained directly by a one-pot process starting from a cycloalkane **1**. This concept is characterized by the reaction taking place in water, by molecular oxygen being used as the oxidation agent, and by no further cosubstrate being required.[5] Thus, this process fulfills the criteria of the "dream reaction" according to the synthetic concept shown in Scheme 1. In the initial step of this process, the cycloalkane **1** is hydroxylated with molecular oxygen by means of a P450 monooxygenase to afford the corresponding cycloalkanol **3** (Scheme 2). The reduced form of the cofactor NAD(P)H is required for this step, and is then oxidized to $NAD(P)^+$. This is followed by a further alcohol dehydrogenase catalyzed oxidation of the cycloalkanol **3** to afford the desired cycloalkanone **2**. This transformation of **3** proceeds in situ (without isolation of intermediate **3**), and in this step the oxidized form of the cofactor (resulting from the first step) is converted back to the reduced form NAD(P)H by means of the alcohol dehydrogenase. This regenerated (recycled) reduced form of the cofactor NAD(P)H is then available for the next monooxygenase-catalyzed hydroxylation step. Accordingly, only cycloalkane and molecular oxygen are needed as reagents. A P450 monooxygenase, an alcohol dehydrogenase, and the cofactor NAD(P)H are used as the catalytic components. In contrast, the addition of stoichiometric amounts of ("external") cosubstrates for regeneration of the cofactor, which is a typical feature of

[*] S. Staudt, E. Burda, Prof. Dr. H. Gröger
Department of Chemistry and Pharmacy
University of Erlangen-Nürnberg
Henkestrasse 42, 91054 Erlangen (Germany)
C. Giese, Prof. Dr. H. Gröger
Faculty of Chemistry, Bielefeld University
Universitätsstrasse 25, 33615 Bielefeld (Germany)
E-mail: harald.groeger@uni-bielefeld.de
C. A. Müller, Dr. J. Marienhagen, Prof. Dr. U. Schwaneberg
Institute of Biotechnology, RWTH Aachen
Worringerweg 1, 52074 Aachen (Germany)
Prof. Dr. W. Hummel
Institute of Molecular Enzyme Technology at the
Heinrich-Heine-University of Düsseldorf, Research Centre Jülich
Stetternicher Forst, 52426 Jülich (Germany)
Prof. Dr. K. Drauz
Innovation Management Chemicals
International Scientific Relations, Evonik Degussa GmbH
Rodenbacher Chaussee 4, 63457 Hanau-Wolfgang (Germany)

[**] We thank the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) for generous support and promotion of this work within the collaborative research centre SFB583 "Redox-Active Metal Complexes" (Sonderforschungsbereich SFB583 "Redoxaktive Metallkomplexe"). E.B. thanks the Graduate School of Molecular Science at the University of Erlangen-Nürnberg for the support of her research work.

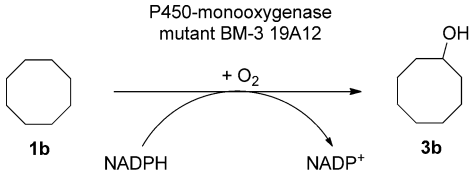


Scheme 2. Concept of an enzymatic direct oxidation of cycloalkanes to cycloalkanones with oxygen.

enzymatic redox reactions with monooxygenases and dehydrogenases, is not required in this double-oxidation concept (with the exception of O_2). The reaction system described above is shown schematically in Scheme 2. Another type of double oxidation, consisting of the combination of an alcohol dehydrogenase with a Baeyer–Villiger monooxygenase, was proven in an earlier study by Willetts et al. to be suitable for the preparation of lactones.[6]

In our initial experiments, an enzyme screening (P450 monooxygenases and alcohol dehydrogenases) was conducted for both oxidation steps individually. The recombinant P450 monooxygenase BM-3 from *Bacillus megaterium* was the starting point for the initial hydroxylation step.[7–9] Recently, there have been multiple reports on the introduction of suitable mutations to improve the catalytic properties of this hydroxylating enzyme, which in its native form only shows poor activity toward alkanes and cycloalkanes. For example, by means of protein engineering, Urlacher and co-workers obtained turnover frequency (TOF) values (oxidation rates) of up to 230 min^{-1} for cycloalkanes,[10] Drone and co-workers achieved total turnover numbers (TTNs) of up to 410 for alkanes,[11] Arnold and co-workers observed TTN values of up to 33 400 for alkanes,[12] and product formation of up to 8.4 mM accompanied by turnover numbers (TONs) up to 3632 was reached by Reetz and co-workers for *n*-butane.[13] All of these transformations, however, required the use of an "external" cosubstrate or the addition of a stoichiometric amount of the cofactor.

To find a monooxygenase mutant optimized with respect to the hydroxylation of cycloalkanes we screened a BM-3 mutant library, with cyclooctane (**1b**) chosen as the cycloalkane substrate. The molar amount of the corresponding P450 monooxygenase in the lyophilisate was first determined (in μmol g^{-1} of lyophilized crude extract) and subsequently the specific activity in the form of the corresponding oxidation rate (expressed as TOF in min^{-1}) of the converted cofactor NADPH was measured spectrophotometrically. By using the already known mutant 19A12,[14] for which we recently reported an expression system for its preparation,[15] in our determination of the photometric activity for cyclooctane, we were pleased to find a high specific activity of 174.9 U g^{-1} for the lyophilisate at a P450 monooxygenase content of 0.123 μmol g^{-1} in the lyophilized crude extract, which corresponds to a TOF of 1422 min^{-1} (Scheme 3).

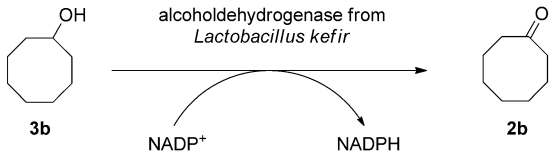


P450 monooxygenase mutant BM-3 19A12: activity: 174.9 U g^{-1} lyophilisate
(content: 0.123 μmol g^{-1} lyophilisate) TOF: 1422 min^{-1}

Scheme 3. P450 monooxygenase-catalyzed hydroxylation of cyclooctane.

Accordingly, we selected this BM-3 mutant 19A12 as well as the BM-3 F87V variant—which had already been identified to be suitable in earlier studies[10,16] and for which we determined spectrophotometrically a TOF of 14 min^{-1}—for the subsequent synthetic studies towards the cosubstrate-free double oxidation.

We identified (among others) an ADH from *Lactobacillus kefir* as an alcohol dehydrogenase (ADH) suitable for the conversion of cycloalkanols **3** into cycloalkanones **2** under transformation of the cofactor $NADP^+$ into NADPH (Scheme 4).[17,18] We observed an enzyme activity of 84 %



alcohol dehydrogenase from *Lactobacillus kefir*: 19.7 U mg^{-1} specific enzyme activity
84% relative enzyme activity
(in comparison to phenylethan-1-ol, 100%)

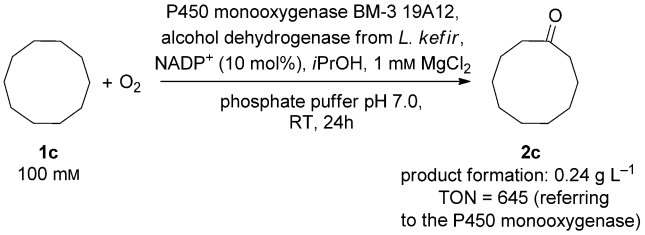
Scheme 4. Alcohol dehydrogenase-catalyzed oxidation of cyclooctanol.

(corresponding to a specific activity of 19.7 U mg^{-1}) for the conversion of cyclooctanol (**3b**) into cyclooctanone (**2b**); this value is in a similar range to that for the oxidation of the reference substrate 1-phenylethanol (100 %, corresponding to 23.4 U mg^{-1} [17]).

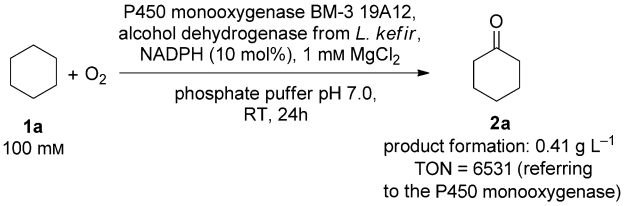
Subsequently, the identified P450 monooxygenases and alcohol dehydrogenase were used in preparative transformations according to the synthetic concept of the double oxidation mentioned above (Scheme 2). The direct oxidation of cyclooctane (**1b**) to cyclooctanone (**2b**) was initially chosen as the target reaction, and we were pleased that we could successfully demonstrate the proof of concept for this novel synthetic conception of the biocatalytic direct oxidation of

cycloalkanes **1** to cycloalkanones **2** with molecular oxygen in water according to Scheme 2. For the analytical determination of cyclooctanone we used the method employed by Schmid, Urlacher, and co-workers,[7e] who made conclusions about productivity by means of measuring the product concentration/amount of product (in mM and $g L^{-1}$, respectively). Since a significant amount of the substrate cyclooctane can in general evaporate during the reaction (as well as work-up), this analytical method for determining the amount of product appeared to be a more accurate indicator for the progress of the reaction than determining a conversion. The experiments were carried out preferentially using $NADP^+$ as a cofactor (instead of the direct use of NADPH) as well as 2-propanol to initiate the reaction through in situ formation of the reduced cofactor form NADPH, which is required for the initial hydroxylation step.[19] A first proof of concept of this new synthetic approach of double oxidation could already be demonstrated in preliminary experiments at the elevated substrate concentration of 100 mM of cyclooctane (**1b**) and even in the presence of the less active mutant BM-3 F87V, with the desired product cyclooctanone (**2b**) being obtained at a concentration of 3.9 mM (Table 1, entry 1). This corresponds to a product amount of $0.49 g L^{-1}$ and a turnover number (TON) of 72. An even more pleasing result was obtained when using the P450 mutant BM-3 19A12 in combination with the alcohol dehydrogenase from *L. kefir* for the double oxidation of cyclooctane: in this case, cyclooctanone (**2b**) was formed with a high product concentration of 6.3 mM, which corresponds to a product amount of $0.80 g L^{-1}$ and a TON of 11641, by using a significantly lower amount of P450 monooxygenase (entry 2). This product concentration as well as the TON value are among the highest values reported so far for synthetic biotransformations with P450 monooxygenases under in situ regeneration of the cofactor.[7–15]

In addition, the enzymatic double oxidation process turned out to be suitable for the synthesis of cyclodecanone (**2c**). The oxidative direct transformation of cyclodecane (**1c**) to cyclodecanone (**2c**) proceeds under formation of the desired product **2c** in a product amount of $0.24 g L^{-1}$ (Scheme 5). Since the experiments thus far were performed using a (relatively high) "standard amount" of 10 mol% of cofactor, the use of a lower amount of the cofactor was investigated. In this study we found that the oxidation of **1c** in

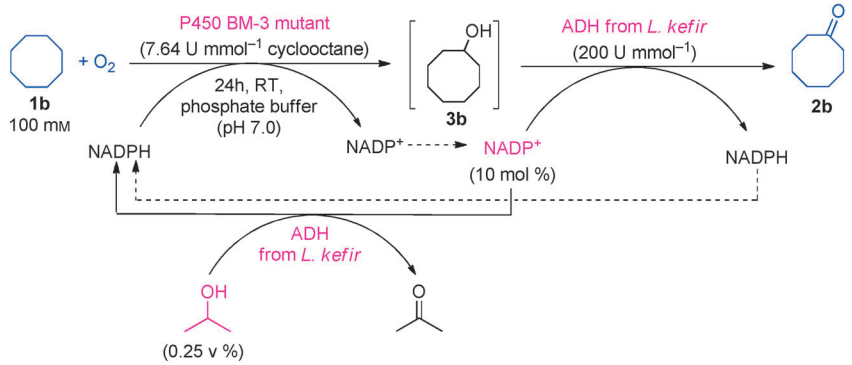


Scheme 5. Enzymatic double oxidation of cyclodecane.



Scheme 6. Enzymatic double oxidation of cyclohexane.

Table 1: Double oxidation of cyclooctane depending on the mutant used.



Entry[a]	Mutant	Productivity in terms of cyclooctanone (**2b**) formation		
		[$g L^{-1}$]	[mmol L^{-1}]	TON[b]
1	F87V	0.49	3.9	72
2	19A12	0.80	6.3	11641

[a] For the experimental procedure and determination of the formed amount of product, see the Experimental Section. Blue: reagents used and the resulting product, purple: enzymes used as well as the cofactor and 2-propanol (for initial formation of the cofactor form NADPH required for the first step). [b] The TON value refers to the P450 monooxygenase and was calculated on the basis of the amount of product **2b** formed as well as the amount of P450 monooxygenase used.

the presence of a lower (and not yet optimized) amount of the cofactor (1 mol%) leads to a similar result.[20]

Furthermore, the oxidative direct transformation of cyclohexane (**1a**) to cyclohexanone (**2a**) proceeded successfully. Once again, the P450 monooxygenase mutant BM-3 19A12 turned out to be most efficient for this synthesis, and led to the formation of the desired product **2a** in $0.41 g L^{-1}$ (Scheme 6).[21]

In summary, we have reported an enzymatic one-pot two-step process, in which a cycloalkanone **2** is obtained directly from the corresponding cycloalkane **1**. This oxidation process proceeds in water and requires only molecular oxygen as the oxidation agent, with the P450 monooxygenase and an alcohol dehydrogenase, two

enzymes that are complementary with respect to the required cofactor form (NADPH, $NADP^+$), used as catalysts. The products **2** were obtained with high product concentrations, amounts of product, and TON values which are in a good range for P450 monooxygenases. One focus of our current studies in this field is process optimization by using improved recombinant biocatalysts, with the goal of increased TON values (in particular refering to the monooxygenase and cofactor) and amount of product formed. Furthermore, the molecular biological optimization of the P450 monooxygenase with respect to activity, stability, and improvement of the "coupling efficiency" represents a current challenge. Extension of this concept of biocatalytic double oxidation towards the synthesis of other ketones is also in progress.

Experimental Section

The corresponding cycloalkane (**1**, 0.1 mmol) was treated with a mixture of phosphate buffer (997.5 μL; pH 7.0, 50 mM, 1 mM $MgCl_2$) and 2-propanol (2.5 μL) in a 25 mL round-bottom flask. A P450 monooxygenase mutant of type BM-3 19A12 or BM-3 F87V (in each case as a lyophilized crude extract, 0.764 U refering to the cycloalkane **1**) and an alcohol dehydrogenase from *Lactobacillus kefir* (LK-ADH, crude extract, diluted with glycerol (1:1), 200 U $mmol^{-1}$ cycloalkanol, **3**) were then added successively. The reaction was then started through addition of the cofactor $NADP^+$ (0.01 mmol), and the reaction mixture stirred for 24 h at room temperature in the closed round-bottom flask. Subsequently, the reaction mixture was extracted with methylene chloride (3 × 1 mL). For determination of the conversion, the work-up of the combined organic phases depended on the cycloalkane component and was carried out either by 1) transfer into a 5 mL volumetric flask and filling with methylene chloride, followed by subsequent determination of the absolute amount of cycloalkane by means of a calibration curve obtained by gas chromatography (GC instrument: Shimadzu GC-2010; capillary column: Rt-BDEXm; carrier gas: molecular nitrogen (N_2), 100 kPa; detector: flame ionization detector) or 2) careful removal of the solvent in vacuo (at 900 mbar) and subsequent determination of the absolute amount of the cycloalkanone by ^1H NMR spectroscopic analysis of the crude product with the aid of an external standard (pyridine) and the weigh-out quantity, respectively. Method (1) was used for **2a**,**c**, and method (2) for **2b**. The absolute amount of the cycloalkanone product **2** determined was set relative to the volume of the solvent used (1 mL) and stated in mmol L^{-1} and g L^{-1}, respectively.

Received: June 8, 2012
Revised: August 31, 2012
Published online: January 21, 2013

Keywords: alkanes · enzyme catalysis · ketones · oxidation

[1] The term "dream reactions" is used to characterize sustainable synthetic methods with high atom economy and efficiency; for overviews about the topic of sustainable synthetic methods, see a) P. Anastas, J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, Oxford, **1998**; b) R. A. Sheldon, I. Arends, U. Hanefeld, *Green Chemistry and Catalysis*, Wiley-VCH, Weinheim, **2007**.

[2] Overview about the industrial production of bulk chemicals: K. Weissermel, H.-J. Arpe, *Industrial Organic Chemistry*, 4th ed., Wiley-VCH, Weinheim, **2003**.

[3] T. Schiffer, G. Oenbrink, *Ullmann's Encyclopedia of Industrial Chemistry*, 6th ed., electronic release, Wiley-VCH, Weinheim, **2000**.

[4] a) J. Teles, B. Rößler, R. Pinkos, T. Genger, T. Preiss (BASF SE), Eur. Patent EP 1663932, **2008**; b) J. Teles, B. Rössler, R. Pinkos, G. Tebben, C. Müller (BASF SE), Eur. Patent EP 2041060, **2009**.

[5] Prior to this publication, a patent application covering this double oxidation concept (with some very early, preliminary experiments) was also filed by some of us, see H. Gröger, E. Burda, U. Schwaneberg, J. Marienhagen, K. Drauz (Evonik Degussa GmbH), PCT Patent Appl. WO2010/070086A2, **2010**.

[6] A. J. Willetts, C. J. Knowles, M. S. Levitt, S. M. Roberts, H. Sandeya, N. F. Shipston, *J. Chem. Soc. Perkin Trans. 1* **1991**, 1608–1610.

[7] The suitability of a P450 monooxygenase from *Bacillus megaterium* and related mutants, respectively, for the hydroxylation of alkanes and cycloalkanes was demonstrated in pioneering work by the research groups of Adam, Schmid, Schwaneberg, Urlacher, and Arnold; for selected contributions, see a) W. Adam, Z. Lukacs, C. R. Saha-Möller, B. Weckerle, P. Schreier, *Eur. J. Org. Chem.* **2000**, 2923–2926; b) D. Appel, S. Lutz-Wahl, P. Fischer, U. Schwaneberg, R. D. Schmid, *J. Biotechnol.* **2001**, *88*, 167–171; c) E. T. Farinas, U. Schwaneberg, A. Glieder, F. H. Arnold, *Adv. Synth. Catal.* **2001**, *343*, 601–606; d) S. C. Maurer, H. Schulze, R. D. Schmid, V. Urlacher, *Adv. Synth. Catal.* **2003**, *345*, 802–810; e) S. C. Maurer, K. Kühnel, L. A. Kaysser, S. Eiben, R. D. Schmid, V. B. Urlacher, *Adv. Synth. Catal.* **2005**, *347*, 1090–1098; f) J. Kuper, T. S. Wong, D. Roccatano, M. Wilmanns, U. Schwaneberg, *J. Am. Chem. Soc.* **2007**, *129*, 5786–5788.

[8] Reviews: a) V. B. Urlacher, R. D. Schmid, *Curr. Opin. Chem. Biol.* **2006**, *10*, 156–161; b) V. B. Urlacher, S. Eiben, *Trends Biotechnol.* **2006**, *24*, 324–330; c) V. B. Urlacher, M. Girhard in *Enzyme Catalysis in Organic Synthesis*, *Vol. 3*, 3rd ed. (Eds.: K. Drauz, H. Gröger, O. May), Wiley-VCH, Weinheim, **2012**, chap. 30, pp. 1227–1267; d) R. Fasan, *ACS Catal.* **2012**, *2*, 647–666.

[9] For overviews with industrial examples of enzymatic oxidation reactions, see a) A. Liese, K. Seelbach, C. Wandrey, *Industrial Biotransformations*, 2nd ed., Wiley-VCH, Weinheim, **2006**; b) U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185–194.

[10] E. Weber, A. Seifert, M. Antonovici, C. Geinitz, J. Pleiss, V. B. Urlacher, *Chem. Commun.* **2011**, *47*, 944–946.

[11] M. Bordeaux, A. Galarneau, F. Fajula, J. Drone, *Angew. Chem.* **2011**, *123*, 2123–2127; *Angew. Chem. Int. Ed.* **2011**, *50*, 2075–2079.

[12] R. Fasan, Y. T. Meharenna, C. D. Snow, T. L. Poulos, F. H. Arnold, *J. Mol. Biol.* **2008**, *383*, 1069–1080.

[13] F. E. Zilly, J. P. Acevedo, W. Augustyniak, A. Deege, U. W. Häusig, M. T. Reetz, *Angew. Chem.* **2011**, *123*, 2772–2776; *Angew. Chem. Int. Ed.* **2011**, *50*, 2720–2724.

[14] R. Fasan, M. M. Chen, N. C. Crook, F. H. Arnold, *Angew. Chem.* **2007**, *119*, 8566–8570; *Angew. Chem. Int. Ed.* **2007**, *46*, 8414–8418.

[15] S. Staudt, C. A. Müller, J. Marienhagen, C. Böing, S. Buchholz, U. Schwaneberg, H. Gröger, *Beilstein J. Org. Chem.* **2012**, *8*, 186–191.

[16] Furthermore, this mutant, in which L-phenylalanine was replaced by L-valine in position 87, proved advantageous in earlier studies of the following applications: a) transformation of a P450 monoxygenase BM-3 into a regio- and stereoselective arachidonic acid epoxygenase: S. Graham-Lorence, G. Truan, J. A. Peterson, J. R. Falck, S. Wei, C. Helvig, J. H. Capdevila, *J. Biol. Chem.* **1997**, *272*, 1127–1135; b) hydroxylation of aromatic substrates with increased enzyme activity: W. T. Sulistyaning-

dyah, J. Ogawa, Q.-S. Li, C. Maeda, Y. Yano, R. D. Schmid, S. Shimizu, *Appl. Microbiol. Biotechnol.* **2005**, *67*, 556 – 562.

[17] For the preparation of the *R*-alcohol dehydrogenase from *Lactobacillus kefir*, see A. Weckbecker, W. Hummel, *Biocatal. Biotransform.* **2006**, *24*, 380 – 389.

[18] For examples of synthetic applications of the *R*-alcohol dehydrogenase from *Lactobacillus kefir* in the asymmetric reduction of ketones, see a) C. W. Bradshaw, W. Hummel, C.-H. Wong, *J. Org. Chem.* **1992**, *57*, 1532 – 1536; b) H. Gröger, F. Chamouleau, N. Orologas, C. Rollmann, K. Drauz, W. Hummel, A. Weckbecker, O. May, *Angew. Chem.* **2006**, *118*, 5806 – 5809; *Angew. Chem. Int. Ed.* **2006**, *45*, 5677 – 5681.

[19] Alternatively, NADPH can also be used directly, although slightly lower levels of product were formed: S. Staudt, E. Burda, C. Giese, H. Gröger, unpublished results. An explanation for the higher product formation in the presence of a (small) amount of 2-propanol of 0.25 v% could be the compensation of losses of NADPH, which result from the known undesired side reaction of P450 monooxygenases (consumption of NADPH under generation of H_2O_2 and $NADP^+$; these losses of NADPH can be expressed and quantified by the so-called "coupling efficiency") by regeneration of NADPH with 2-propanol.

[20] Only low turnover numbers in terms of the cofactor (cofactor-related TON) of <1 are obtained when using 10 mol% of cofactor as a (high) "standard amount" in the proof of concept reactions, whereas when 1 mol% of the cofactor is used, the turnover number is already >1 (cofactor-related TON with cyclodecane as a substrate: 2). Further optimization of the amount of cofactor and the increase of the cofactor-related TON is the subject of current studies on process development.

[21] In this experiment the cofactor NADPH was used directly, whereas in the other experiments, which are shown in Table 1 and Scheme 5, the NADPH for the initial hydroxylation was generated in situ from $NADP^+$ and 2-propanol.

