Enzyme Catalysis

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Introducing an In Situ Capping Strategy in Systems Biocatalysis To Access 6-Aminohexanoic acid**

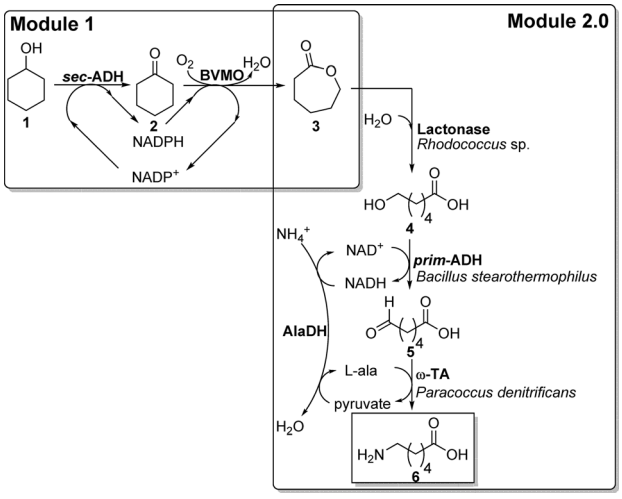
*Johann H. Sattler, Michael Fuchs, Francesco G. Mutti, Barbara Grischek, Philip Engel, Jan Pfeffer, John M. Woodley, and Wolfgang Kroutil**

Abstract: *The combination of two cofactor self-sufficient biocatalytic cascade modules allowed the successful transformation of cyclohexanol into the nylon-6 monomer 6-aminohexanoic acid at the expense of only oxygen and ammonia. A hitherto unprecedented carboxylic acid capping strategy was introduced to minimize the formation of the dead-end intermediate 6-hydroxyhexanoic acid. For this purpose, the precursor ε-caprolactone was converted in aqueous medium in the presence of methanol into the corresponding methyl ester instead of the acid. Hence, it was shown for the first time that esterases—specifically horse liver esterase—can perform the selective ring-opening of ε-caprolactone with a clear preference for methanol over water as the nucleophile.*

In recent years, metabolic engineering and synthetic biology have been successfully employed to modify organisms for the preparation of fuels and drugs in vivo.[1] The metabolic intermediates of the host organism represent the key building blocks to synthesize the desired target molecules. In contrast, "systems biocatalysis"[2] is the concept of organizing enzymes from various organisms in vitro[3] to generate an artificial metabolism[4] for the synthetic transformation of non-natural precursors. Herein, we exemplify the approach of systems biocatalysis for the preparation of 6-aminohexanoic acid, the open-chain analogue of the polymer building block ε-caprolactam.

Industrially, cyclohexanone is the precursor used to prepare ε-caprolactam, which is required for the production of nylon-6.[5] The most common synthetic route involves 1) oxime formation (with NH_2OH, prepared from NH_3 and H_2O_2) and 2) a Beckmann rearrangement in the presence of strong acids as the key step, which leads to significant amounts of salts as by-products.[5,6] With a global demand of 4.2 million tons year^{-1}, the production of ε-caprolactam represents one of the largest and most prominent industrial processes.[7] The aim of this study is to show a proof of concept for a new and environmentally benign biocatalyst system to obtain the hydrolyzed ε-caprolactam derivative, 6-aminohexanoic acid, in water and requiring only molecular oxygen and ammonia as stoichiometric reagents. 6-Aminohexanoic acid can be transformed into ε-caprolactam in the presence of, for example, superheated steam.[8] For redox-balance reasons, cyclohexanol turned out to be the starting material of choice.

In an initial approach, a system was designed that consisted of two modules to transform cyclohexanol (**1**) into 6-aminohexanoic acid (**6**; Scheme 1). Each module involves one oxidation step requiring $NAD(P)^+$ and one step consuming NAD(P)H in a linear sequence, thereby allowing the design of self-sufficient redox cascades[3c,9] with respect to the



Scheme 1. Initial design of a biocatalytic system to transform cyclohexanol into 6-aminohexanoic acid based on two modules, each requiring a different nicotinamide cofactor.

[*] Dr. J. H. Sattler, Dr. M. Fuchs, Dr. F. G. Mutti, B. Grischek, Prof. Dr. W. Kroutil
Institut für Chemie, Organische und Bioorganische Chemie
University of Graz
Heinrichstrasse 28, 8010 Graz (Austria)
E-mail: wolfgang.kroutil@uni-graz.at
Dr. J. H. Sattler
Austrian Centre of Industrial Biotechnology (ACIB)
Petersgasse 14, 8010 Graz (Austria)
Dr. P. Engel, Dr. J. Pfeffer
Evonik Industries AG, CREAVIS Technologies & Innovation
Paul-Baumann-Strasse 1, 45772 Marl (Germany)
Prof. Dr. J. M. Woodley
Department of Chemical and Biochemical Engineering
Technical University of Denmark
Søltofts Plads, 2800, Kgs. Lyngby (Denmark)

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nicotinamide cofactor. Hence, the $NAD(P)^+$ required in the first oxidative step is regenerated in the subsequent step. More specifically, cyclohexanol (**1**) is oxidized by an alcohol dehydrogenase (ADH) to cyclohexanone (**2**) at the expense of $NADP^+$ to give NADPH; the latter is consumed during the following NADPH-dependent Baeyer–Villiger monooxygenase (BVMO) step to give ε-caprolactone (**3**) and thereby regenerating the $NADP^+$ for the first step. It is important to note that since the first module is NADP-dependent, the second module has to be NAD-dependent to allow both modules to run simultaneously and independently without interference. Hence, the second module starts with the hydrolysis of **3** to liberate the alcohol moiety to give 6-hydroxyhexanoic acid (**4**). For the amination of the primary alcohol,[3b,10] alcohol **4** is oxidized to 6-oxohexanoic acid (**5**) and finally aminated by an ω-transaminase (ω-TA) in the presence of an alanine dehydrogenase (AlaDH) to give 6-aminohexanoic acid (**6**). The latter reaction requires L-alanine as an amine donor to give pyruvate as a co-product, which is recycled by the AlaDH to L-alanine, thereby consuming the ammonia and NADH. Since NADH is generated in the oxidation step, module 2 again represents a self-sufficient redox sequence.

In each module, an alcohol moiety is oxidized: this is a secondary alcohol in module 1 and a primary alcohol in module 2.0. To avoid interference of the two modules, the alcohol dehydrogenases for each step need to be carefully selected to preferentially transform the intended alcohol exclusively. Similarly, the ω-transaminase must aminate exclusively compound **5** but not cyclohexanone (**2**). Therefore, it has to exhibit perfect chemoselectivity to distinguish between a ketone and an aldehyde.

For the first module, the BVMO originating from *Acinetobacter calcoaceticus*[11] turned out to be most suitable for our purpose, with the C376 L M400I double mutant applied because of its higher oxidative stability compared to the wild-type enzyme.[12] Since the BVMO was NADPH-dependent, an $NADP^+$-dependent ADH was selected (ADH from *Lactobacillus brevis*).[13] Although the ADH/BVMO cascade has been investigated by others,[14] it turned out that the selection of the BVMO variant was crucial to achieve excellent conversion (up to 98%) into ε-caprolactone (**3**), even at a relatively high substrate loading (96% at 20 $g\,L^{-1}$, 200 mM; Table 1). This result could be confirmed on a preparative scale (100 mg substrate **1**), which resulted in 99% conversion, with 96% formation of ε-caprolactone (**3**) and a 75% yield of the isolated product.

The more demanding module 2.0 was tested first by employing the following enzymes: a lactonase from *Rhodococcus* sp.[15] for the hydrolytic ring-opening of ε-caprolactone (**3**), a primary ADH from *E. coli*[16] for the oxidation of alcohol **4**, an alanine dehydrogenase (AlaDH) from *Bacillus subtilis*, and various ω-transaminases for the amination. In this system, substrate **3** was transformed only into alcohol **4** with a high conversion (99%), but formation of amine **6** was insignificant (1%), irrespective of the transaminase employed (see Table S4, entries 1–4 in the Supporting Information). Testing module 2.0 with alternative ADHs (horse liver ADH E-isoenzyme, ADH from *Bacillus stearothermophilus*, two ADHs from *Candida tropicalis*) led to the same result. An investigation of the amination of aldehyde **5** exclusively showed that all the transaminases converted this substrate with high conversion (see Table S5 in the Supporting Information) and also that the AlaDH was not inhibited. Subsequently, it turned out that all the tested ADHs were unable to oxidize the primary alcohol moiety of the 6-hydroxycarboxylic acid (**4**). Although an ADH from *Acinetobacter* NCIB 9871[17] as well as an isoenzyme from *Brevibacterium epidermitis* strain HCU has been reported[18] to perform the oxidation of 6-hydroxycarboxylic acid (**4**), these enzymes turned out to be unsuitable for our reaction at elevated substrate concentrations because of their poor stability and problems with expression. Interestingly, related substrates such as 1-hexanol and the corresponding ethyl ester of **4** (ethyl 6-hydroxyhexanoate) were readily oxidized by all the primary ADHs employed and were efficiently converted into the corresponding amines with an ADH/ω-TA/AlaDH system (see Table S4, entries 5–12 in the Supporting Information). Thus, it was concluded that the carboxylic acid moiety of 6-hydroxyhexanoic acid (**4**) inhibits the oxidation by the ADHs tested. For example, the K_i value of **4** for the *prim*-ADH from *Bacillus stearothermophilus* (ADH-ht)[19] was determined to be 98 μM. Since the ethyl and methyl esters of **4** were substrates for oxidation as well as for the overall amination, an alternative reaction sequence was envisioned in which an ester of **4** was generated as an intermediate. To achieve this, lactone **3** has to be opened in aqueous buffer with an alcohol (ethanol/methanol) rather than water as the nucleophile. This should afford the corresponding ester and thereby introduce capping of the carboxylic acid moiety. Methanol was the nucleophile of choice over ethanol, because the primary ADHs employed oxidize ethanol, thus leading to an unwanted side reaction, while the oxidation of methanol was negligible. Enzymes catalyzing the ring opening of ε-caprolactone (**3**) have in general been described for polymerization in organic solvents,[20] since hydrolysis to the corresponding carboxylic acid is expected in aqueous solution. In fact, to the best of our knowledge, the ring opening of a lactone in water with an alcoholic nucleophile has not been described before.

On testing various hydrolytic enzymes for the unprecedented transformation of ε-caprolactone (**3**) into the corresponding methyl ester in aqueous buffer, it turned out that neither the lactonase from *Rhodococcus* sp. nor any lipase (12 tested) led to useful ester formation (see Table S6, entries 1–13 in the Supporting Information). However, out of the 18 esterases tested, four showed activity, namely an esterase from *Bacillus subtilis* (esterase 008-SD), two esterases from

Table 1: Synthesis of ε-caprolactone from cyclohexanol through a redox self-sufficient cascade reaction (module 1, Scheme 1).[a]

Entry	Conc. **1** [mM]	Conv. [%]	Ketone **2** [%]	Lactone **3** [%]
1	50	>99	2	98
2[b]	200	99	3	96

[a] Reaction conditions: Na_2HPO_4/KH_2PO_4 buffer pH 8.0, 2 mM $MgCl_2$, 21 °C, 170 rpm orbital shaker, 20 h, 1 bar O_2; 0.2 U ADH and 0.2 U BVMO, 0.3 mg $NADP^+$. [b] 0.8 U ADH and 0.8 U BVMO, 1.2 mg $NADP^+$.

pig liver (PLE03 and PLE06), and a crude horse liver esterase preparation. For the first three enzymes the amino acid sequences have been described and all three can be expressed heterologously.[21] Interestingly, out of the six isoenzymes of pig liver esterase[21a,22] tested, only two variants showed the desired activity (PLE03 and PLE06; see Table S6, entries 28 and 30 in the Supporting Information). Since the active recombinant esterase from pig liver can be expected to be related to the active enzyme from horse liver,[23] it is very likely that an esterase from horse liver is indeed responsible for the activity and not any other enzyme in the crude commercial preparation. Although various esterases from pig liver can be produced in recombinant form, the esterases from horse liver have not yet been expressed heterologously.

When following the ring opening of ε-caprolactone (**3**) in buffer over time in the presence of methanol (10% v/v) it became evident that the esterase from *Bacillus subtilis* catalyzed the formation of the methyl ester as well as hydrolysis, with ester formation being faster and leading to about double the amount of ester compared to acid after 120 min (see Figure S24 in the Supporting Information). Surprisingly, the horse liver esterase preparation catalyzed first the formation methyl ester **7** exclusively until all the ε-caprolactone (**3**) had been consumed (within 30 min), before slow hydrolysis of the methyl ester **7** to the corresponding acid **4** was detectable (Figure 1). The esterase from horse liver was also superior to PLE03 and PLE06 in terms of the chemoselectivity (data not shown).

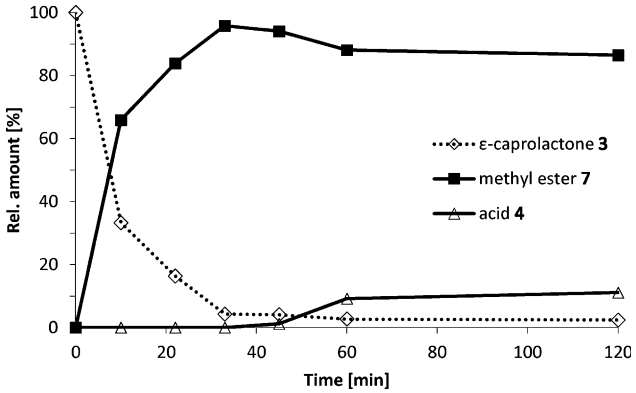
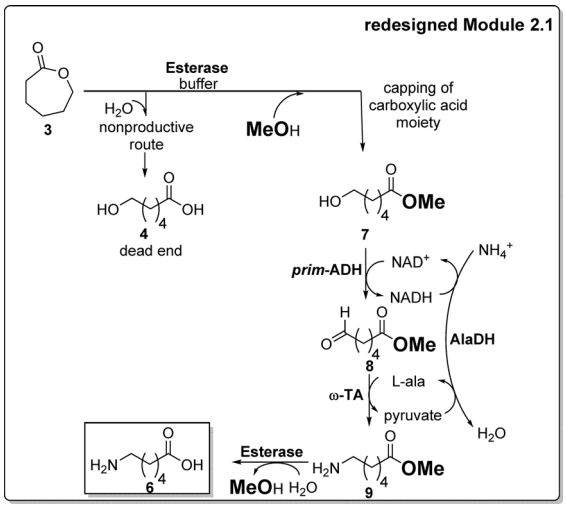


Figure 1. Ring opening of ε-caprolactone **3** (50 mM) in the presence of buffer (pH 7.5) and MeOH (10% v/v), which in the presence of a horse liver esterase preparation leads to either to hydrolysis (formation of acid **4**) or formation of methyl ester **7**. Reaction conditions (1 mL total volume): HL-Esterase (4 mg, 2.04 U), MeOH (100 µL, 10% v/v), Na_2KPO_4-buffer (50 mM, pH 7.5), ε-caprolactone (5.5 mg, 50 mM).

Having now a tool in hand to introduce a capping group for the carboxylic acid moiety of **4**, module 2.0 was redesigned by introducing the esterase-catalyzed ring opening of ε-caprolactone (**3**) to give the 6-hydroxyhexanoic acid methyl ester (**7**; Scheme 2).

The subsequent steps were identical to module 2.0, with oxidation of the alcohol **7** to the 6-oxo ester **8** and amination to give the 6-amino ester **9** followed by hydrolysis of the ester



Scheme 2. Redesigned module 2.1 to transform ε-caprolactone (**3**) into 6-aminohexanoic acid (**6**) by capping of the carboxylic acid moiety as methyl ester (**7**).

to finally yield 6-aminohexanoic acid (**6**). Although the amination was in general rather fast, there is also the possibility that the 6-oxo ester **8** is partly hydrolyzed to the oxo-acid **5** before it gets aminated.

Testing module 1 and 2.1 simultaneously, thus starting the transformation of cyclohexanol (**1**; 50 mM) with all six enzymes and all components at the same time, revealed that the MeOH concentration is problematic for the BVMO. Indeed, the low stability of the wild-type enzyme towards MeOH has been reported previously.[24] Nevertheless, running modules 1 and 2.1 at low concentrations of methanol (2% v/v) led to the successful formation of 24% of 6-aminohexanoic acid (**6**; 35% remaining cyclohexanol (**1**), 10% cyclohexanone (**2**), 1% ε-caprolactone (**3**), 30% 6-hydroxyhexanoic acid (**4**); all other intermediates were below the detection limit).

Testing module 2.1 on its own, ε-caprolactone (**3**) was successfully transformed into 6-aminohexanoic acid (**6**) at a 50 mM substrate concentration. Using the *Bacillus subtilis* esterase, an overall conversion of 47% was achieved at 10% v/v methanol (Table 2, entry 1). Higher as well as lower concentrations of methanol led to lower conversion into the final product **6** within 20 h. The scalability of the reaction could also be shown, with one gram ε-caprolactone (**3**) leading to 55% conversion of product **6**. Employing PLE03 as the esterase led to the formation of 29% of the final product **6** (entry 4, 10% v/v MeOH). The best results were obtained with horse liver esterase, which gave 6-aminohexanoic acid (**6**) with 75% conversion within 20 h (entry 5).

Bio-approaches to synthesize polymer building blocks involving enzymes are under intense investigation.[25] The raw materials for nylon-6 production are phenol or cyclohexane, which are converted into cyclohexanol and cyclohexanone as starting points for further processing. The organization of the here-presented self-sufficient redox modules into two orthogonal oxidation/reduction modules with respect to cofactor usage allows entry to the cascade at two different starting

Table 2: Transformation of ε-caprolactone (**3**) into 6-aminohexanoic acid (**6**) according to module 2.1 (Scheme 2).[a] The concentrations of all intermediates not mentioned were below detection limit.

Entry	Esterase	MeOH [% *v*/*v*]	6-Hydroxyhexanoic acid **4** [%]	6-Aminohexanoic acid **6** [%]
1	*B. subtilis*	10	53	47
2	*B. subtilis*	15	60	40
3	PLE03	5	80	20
4	PLE03	10	71	29
5	Horse liver	10	25	75

[a] Reaction conditions (1 mL total volume): HL-esterase (4 mg, 2.04 U) or esterase from *Bacillus subtilis* (5 mg, 3.5 U) or esterase PLE03 (5 mg, 0.8 U), ADH-hT (100 μL, 0.18 $U_{6\text{-hydroxyhexanoic acid}}$), AlaDH (10 μL, 0.22 $U_{alanine}$), transaminase ParDen (lyophilized cells, 20 mg), MeOH (100 μL, 10% v/v), Na_2KPO_4 buffer (120 mM, pH 8.5), L-alanine (22.3 mg, 250 mM), NH_4Cl (13.4 mg, 250 mM), NAD^+ (0.3 mg, 0.4 μmol), PLP (0.05 mg, 0.2 μmol), ε-caprolactone (**3**, 5.5 mg, 50 mM), 20 h, 30 °C. PLP = pyridoxal 5′-phosphate.

points: for module 1, cyclohexanol is required, which is in general available from fossil sources, while ε-caprolactone, which can be produced from fructose,[25c] a renewable resource, is the substrate for module 2.1. Thus, the choice of modules provides particular flexibility with respect to substrate availability and price. Nevertheless, the research presented herein merely represents a proof of concept. Further improvement is required; for example, increasing the stability of the BVMO towards MeOH and fine-tuning the esterase. Most importantly, the heterologous expression and structural as well as functional characterization of the different isoenzymes of horse liver esterase would be of outstanding interest.

In summary, a highly atom-efficient one-pot combination of two cofactor self-sufficient cascade modules was developed to transform either cyclohexanol (**1**) or ε-caprolactone (**3**) into 6-aminohexanoic acid (**6**) at the expense of oxygen and ammonia as the only stoichiometric reagents. The key to success was to circumvent/minimize the formation of the dead-end intermediate 6-hydroxyhexanoic acid (**4**) in the cascade. For this purpose ε-caprolactone (**3**) had to be transformed into the methyl ester; thus the methyl ester served as a cap for the acid moiety. For the first time, it was shown that ε-caprolactone (**3**) can be transformed selectively into the corresponding methyl ester in aqueous medium in the presence of methanol, most efficiently by a horse liver esterase preparation. This study offers a novel alternative bio-route to prepare one of the most demanded polymer building blocks and introduces the concept of the designed capping of inhibiting moieties in systems biocatalysis; the latter was achieved here by the unprecedented opening of a lactone to the corresponding ester instead of the acid in buffer.

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[1] a) M. B. Quin, C. Schmidt-Dannert, *Curr. Opin. Biotechnol.* **2014**, *29*, 55–61; b) B. M. Woolston, S. Edgar, G. Stephanopoulos, *Annu. Rev. Chem. Biomol. Eng.* **2013**, *4*, 259–288; c) C. J. Paddon, P. J. Westfall, D. J. Pitera, K. Benjamin, K. Fisher, D. McPhee, M. D. Leavell, A. Tai, A. Main, D. Eng, et al., *Nature* **2013**, *496*, 528–532; d) Y. J. Choi, S. Y. Lee, *Nature* **2013**, *502*, 571–574; e) B. B. Bond-Watts, R. J. Bellerose, M. C. Y. Chang, *Nat. Chem. Biol.* **2011**, *7*, 222–227; f) H. Yim, R. Haselbeck, W. Niu, C. Pujol-Baxley, A. Burgard, J. Boldt, J. Khandurina, J. D. Trawick, R. E. Osterhout, R. Stephen, J. Estadilla, S. Teisan, H. B. Schreyer, S. Andrae, T. H. Yang, S. Y. Lee, M. J. Burk, S. Van Dien, *Nat. Chem. Biol.* **2011**, *7*, 445–452.

[2] S. Servi, R. Wohlgemuth, W.-D. Fessner, CMST COST Action CM1303: Systems biocatalysis, **2014**, http://www.cost.eu/domains_actions/cmst/Actions/CM1303.

[3] a) P. Welch, R. K. Scopes, *J. Biotechnol.* **1985**, *2*, 257–273; b) C. V. Voss, C. C. Gruber, K. Faber, T. Knaus, P. Macheroux, W. Kroutil, *J. Am. Chem. Soc.* **2008**, *130*, 13969–13972; c) J. H. Sattler, M. Fuchs, K. Tauber, F. G. Mutti, K. Faber, J. Pfeffer, T. Haas, W. Kroutil, *Angew. Chem. Int. Ed.* **2012**, *51*, 9156–9159; *Angew. Chem.* **2012**, *124*, 9290–9293; d) M. Bujara, M. Schümperli, S. Billerbeck, M. Heinemann, S. Panke, *Biotechnol. Bioeng.* **2010**, *106*, 376–389; e) J.-K. Guterl, D. Garbe, J. Carsten, F. Steffler, B. Sommer, S. Reiße, A. Philipp, M. Haack, B. Rühmann, A. Koltermann, U. Kettling, T. Brück, V. Sieber, *ChemSusChem* **2012**, *5*, 2165–2172; f) J. S. Martín del Campo, J. Rollin, S. Myung, Y. Chun, S. Chandrayan, R. Patiño, M. W. W. Adams, Y.-H. P. Zhang, *Angew. Chem. Int. Ed.* **2013**, *52*, 4587–4590; *Angew. Chem.* **2013**, *125*, 4685–4688.

[4] W.-D. Fessner, O. Eyrisch, *Angew. Chem. Int. Ed. Engl.* **1992**, *31*, 56–58; *Angew. Chem.* **1992**, *104*, 76–78.

[5] H. A. Wittcoff, B. G. Reuben, J. S. Plotkin in *Ind. Org. Chem.*, Wiley, Hoboken, NJ, USA, **2012**, pp. 349–354.

[6] G. Bellussi, C. Perego, *CATTECH* **2000**, *4*, 4–16.

[7] J. Ritz, H. Fuchs, H. Kieczka, W. C. Moran in *Ullmann's Encycl. Ind. Chem.*, Wiley-VCH, Weinheim, **2011**, DOI: 10.1002/14356007.a05_031.pub2.

[8] R. P. M. Guit, D. D. T. Van, L. M. Raamsdonk, (DSM IP Assets B. V.), US 2013/0030146 A1, **2013**.

[9] J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti, W. Kroutil, *Curr. Opin. Chem. Biol.* **2011**, *15*, 249–256.

[10] a) M. Schrewe, N. Ladkau, B. Bühler, A. Schmid, *Adv. Synth. Catal.* **2013**, *355*, 1693–1697; b) J.-W. Song, J.-H. Lee, U. T. Bornscheuer, J.-B. Park, *Adv. Synth. Catal.* **2014**, *356*, 1782–1788.

[11] D. E. T. Pazmiño, A. Riebel, J. de Lange, F. Rudroff, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* **2009**, *10*, 2595–2598.

[12] D. J. Opperman, M. T. Reetz, *ChemBioChem* **2010**, *11*, 2589–2596.

[13] K. Niefind, J. Müller, B. Riebel, W. Hummel, D. Schomburg, *J. Mol. Biol.* **2003**, *327*, 317–328.

[14] a) H. Mallin, H. Wulf, U. T. Bornscheuer, *Enzyme Microb. Technol.* **2013**, *53*, 283–287; b) S. Staudt, U. T. Bornscheuer, U. Menyes, W. Hummel, H. Gröger, *Enzyme Microb. Technol.* **2013**, *53*, 288–292.

[15] C. J. van der Vlugt-Bergmans, M. J. van der Werf, *Appl. Environ. Microbiol.* **2001**, *67*, 733–741.

[16] A. Karlsson, M. El-Ahmad, K. Johansson, J. Shafqat, H. Jörnvall, H. Eklund, S. Ramaswamy, *Chem.-Biol. Interact.* **2003**, *143–144*, 239–245.

[17] N. A. Donoghue, P. W. Trudgill, *Eur. J. Biochem.* **1975**, *60*, 1–7.

[18] P. C. Brzostowicz, M. S. Blasko, P. E. Rouvière, *Appl. Microbiol. Biotechnol.* **2002**, *58*, 781 – 789.
[19] X. Zhang, T. C. Bruice, *Biochemistry* **2007**, *46*, 837 – 843.
[20] a) J. Zhang, H. Shi, D. Wu, Z. Xing, A. Zhang, Y. Yang, Q. Li, *Process Biochem.* **2014**, *49*, 797 – 806; b) S. Kundu, A. S. Bhangale, W. E. Wallace, K. M. Flynn, C. M. Guttman, R. A. Gross, K. L. Beers, *J. Am. Chem. Soc.* **2011**, *133*, 6006 – 6011.
[21] a) H. J. Rozeboom, L. F. Godinho, M. Nardini, W. J. Quax, B. W. Dijkstra, *Biochim. Biophys. Acta Proteins Proteomics* **2014**, *1844*, 567 – 575; b) A. Hummel, E. Brüsehaber, D. Böttcher, H. Trauthwein, K. Doderer, U. T. Bornscheuer, *Angew. Chem. Int. Ed.* **2007**, *46*, 8492 – 8494; *Angew. Chem.* **2007**, *119*, 8644 – 8646.
[22] M. Hermann, M. U. Kietzmann, M. Ivancić, C. Zenzmaier, R. G. M. Luiten, W. Skranc, M. Wubbolts, M. Winkler, R. Birner-Gruenberger, H. Pichler, H. Schwab, *J. Biotechnol.* **2008**, *133*, 301 – 310.
[23] R. S. Holmes, L. A. Cox, J. L. Vandeberg, *Comp. Biochem. Physiol. Part D* **2009**, *4*, 54 – 65.
[24] F. Secundo, S. Fialà, M. W. Fraaije, G. de Gonzalo, M. Meli, F. Zambianchi, G. Ottolina, *Biotechnol. Bioeng.* **2011**, *108*, 491 – 499.
[25] a) S. Schaffer, T. Haas, *Org. Process Res. Dev.* **2014**, *18*, 752 – 766; b) M. C. R. Franssen, P. Steunenberg, E. L. Scott, H. Zuilhof, J. P. M. Sanders, *Chem. Soc. Rev.* **2013**, *42*, 6491 – 6533; c) T. Buntara, S. Noel, P. H. Phua, I. Melián-Cabrera, J. G. de Vries, H. J. Heeres, *Angew. Chem. Int. Ed.* **2011**, *50*, 7083 – 7087; *Angew. Chem.* **2011**, *123*, 7221 – 7225; d) W. Lu, J. E. Ness, W. Xie, X. Zhang, J. Minshull, R. A. Gross, *J. Am. Chem. Soc.* **2010**, *132*, 15451 – 15455.

