Enzyme Catalysis

DOI: 10.1002/anie.201207916

Asymmetric Enzymatic Hydration of Hydroxystyrene Derivatives**

Christiane Wuensch, Johannes Gross, Georg Steinkellner, Karl Gruber, Silvia M. Glueck, and Kurt Faber**

The stereoselective addition of water across C=C bonds transforms prochiral alkenes to nonracemic alcohols and represents a major challenge in synthetic organic chemistry. In general, alkene hydration is an equilibrium reaction slightly favoring the alcohol side in 1,4-additions and somewhat disfavored on isolated C=C bonds.[1] Acid-catalyzed alkene hydration, which follows the rule of Markovnikov, usually proceeds with low regioselectivity and is often accompanied by rearrangement yielding regioisomeric product mixtures; with a few exceptions,[2] no generally applicable protocol has been developed so far. Likewise, base-catalyzed 1,4-addition of water to α,β-unsaturated (Michael) acceptors is impeded by the poor nucleophilicity of water.[3] Overall, an astonishingly limited number of asymmetric alkene-hydration protocols are reported: 1) The stereoselective hydration of α,β-unsaturated carboxylic acids by using a heterobimetallic chiral biopolymer (wool–Pd^{II}–Co^{II}) catalyst furnished β-hydroxy carboxylic acids in high optical purities,[4] and 2) the asymmetric *syn*-hydration of α,β-unsaturated acyl imidazoles while applying a DNA-based Cu^{II} catalyst yielded β-hydroxy carbonyl compounds with moderate *ee* values.[5] To compensate for the insufficient nucleophilicity of water, indirect methods using strong nucleophiles (alkoxides, *N*-silyloxycarbamates, oximes, silicon and boron reagents) have been employed, which require cumbersome reductive or oxidative follow-up chemistry to yield the desired β-hydroxy carbonyl compounds.[3]

Asymmetric addition of water across isolated or conjugated C=C bonds is an important process in biology,[1] which is catalyzed by lyases (termed "hydro-lyases" or "hydratases"). Mechanistically, these enzymes can be divided into two categories: 1) acting through (Lewis) acid-catalyzed 1,2-addition and 2) acting through (Michael-type) nucleophilic 1,4- or 1,6-addition involving quinone methide enolates.[6]

Among group (1), acetylene hydratase (AH) is a rare tungsten-dependent protein that catalyzes the hydration of acetylene to furnish acetaldehyde.[7] Likewise, several enzymes catalyze the hydration of nonconjugated C=C bonds in fatty acids (for example, oleate hydratase),[8] natural products (kievitone and phaseollidin hydratase,[9] carotenoid 1,2-hydratase),[10] and terpenoids (linalool dehydratase-isomerase).[11] Unfortunately, the high substrate specificity of these enzymes severely limits their practical applicability.

In contrast, enzymes of group (2) appear to be more flexible: Fumarase is industrially applied for the *anti*-hydration of fumarate yielding (*S*)-malate (ca. 2000 t/a).[12] However, fumarase and its relatives malease, citraconase, and mesaconate hydratase exhibit a very narrow substrate spectrum.[13] Enoyl-CoA hydratases are key enzymes in the β-oxidation pathway and require the (ATP-consuming) activation of their monoacid substrates through formation of a thioester bond to the cofactor coenzyme A (CoA).[14] Despite their complexity, a few processes operate on industrial scale.[12] Owing to the dependence on ATP, whole cells are employed. In a related fashion, hydroxycinnamoyl-CoA hydratase-lyase (HCHL) requires ATP-dependent substrate activation with CoA and catalyzes the two-step degradation of feruloyl-CoA through stereospecific hydration of the C=C bond, followed by retroaldol C–C-cleavage to yield vanillin and acetaldehyde.[6,15] Recently, Michael hydratase alcohol dehydrogenase (MhyADH)[1] was shown to be a bifunctional enzyme requiring Mo, Fe, and Zn as cofactors.[16] MhyADH catalyzes the 1,4-hydration of a range of α,β-unsaturated carbonyl compounds followed by oxidation of the β-hydroxy moiety to yield the corresponding β-oxo-aldehydes or -ketones in the presence of an oxidant; in the absence of an electron acceptor, the hydration product could be identified.[17] In view of the general narrow substrate tolerance of hydratases, MhyADH is exceptional owing to its broad substrate spectrum.

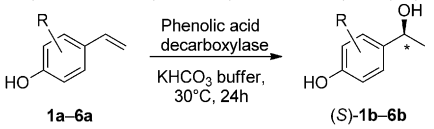
Herein we describe the unprecedented stereoselective asymmetric hydration of hydroxystyrene-type substrates by employing phenolic acid decarboxylases (PADs; Table 1). The promiscuous[18] catalytic "hydratase activity" of these enzymes was discovered during studies on the regioselective β-carboxylation of *p*-vinylphenol,[19] which unexpectedly furnished (*S*)-1-(*p*-hydroxyphenyl)ethanol derived through

[*] C. Wuensch, J. Gross, Dr. S. M. Glueck
ACIB GmbH c/o Department of Chemistry
Organic & Bioorganic Chemistry, University of Graz
Heinrichstrasse 28, 8010 Graz (Austria)
E-mail: si.glueck@uni-graz.at
Dr. G. Steinkellner
ACIB GmbH c/o Institute of Molecular Biosciences
University of Graz, Humboldtstrasse 50, 8010 Graz (Austria)
Prof. K. Gruber
Institute of Molecular Biosciences
University of Graz, Humboldtstrasse 50, 8010 Graz (Austria)
Prof. K. Faber
Department of Chemistry, Organic & Bioorganic Chemistry
University of Graz, Heinrichstrasse 28, 8010 Graz (Austria)
E-mail: Kurt.Faber@Uni-Graz.at

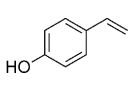
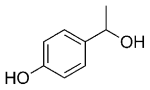
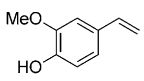
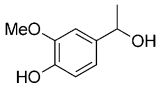
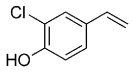
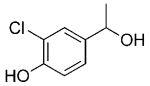
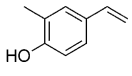
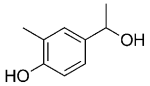
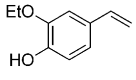
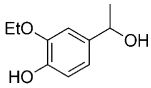
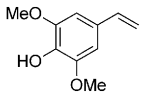
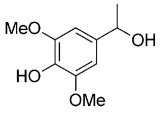
[**] This work has been supported by the Austrian BMWFJ, BMVIT, SFG, Standortagentur Tirol, and ZIT through the Austrian FFG-COMET-Funding Program. Byung-Gee Kim (School of Chemical and Biological Engineering, Seoul National University, Seoul, South Korea) is cordially thanked for the generous donation of phenolic acid decarboxylase plasmids.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201207916.

Table 1: Conversions and stereoselectivities of the asymmetric enzymatic hydration of hydroxystyrenes **1a**–**6a**.[a]



1a–6a (*S*)-**1b–6b**

Entry	Substrate	Product	PAD_Lp conv. [%] (*ee* [%])	PAD_Ba conv. [%] (*ee* [%])	PAD_Mc conv. [%] (*ee* [%])	PAD_Ms conv. [%] (*ee* [%])	PAD_Ps conv. [%] (*ee* [%])	PAD_Ll conv. [%] (*ee* [%])	FDC_Es conv. [%] (*ee* [%])	Control[b] conv. [%] (*ee* [%])
1			82 (43 (*S*))	64 (53 (*S*))	2 (n.d.)	77[c] (40 (*S*))	75[c] (36 (*S*))	73[c] (25 (*S*))	77[c] (41 (*S*))	6 (*rac*)
	1a	**1b**								
2			< 1 (–)	30 (50 (*S*))	< 1 (–)	24[c] (28 (*S*))	39[c] (12 (*S*))	45[c] (28 (*S*))	27[c] (8 (*S*))	14 (*rac*)
	2a	**2b**								
3			73 (28 (*S*))	73 (36 (*S*))	< 1 (–)	63 (27 (*S*))	76 (10 (*S*))	73 (20 (*S*))	74[c] (8 (*S*))	17 (*rac*)
	3a	**3b**								
4			46 (29 (*S*))	46 (44 (*S*))	< 1 (–)	45[c] (37 (*S*))	44[c] (32 (*S*))	47[c] (18 (*S*))	34[c] (71 (*S*))	39 (*rac*)
	4a	**4b**								
5			8 (n.d.)	3 (n.d.)	< 1 (–)	5 (n.d.)	12 (3)[d]	35 (8)[d]	17 (10)[d]	10 (*rac*)
	5a	**5b**								
6			< 1 (–)	< 1 (–)	< 1 (–)	< 1 (–)	< 1 (–)	< 1 (–)	< 1 (–)	< 1 (–)
	6a	**6b**								

[a] All conversion data in Table 1 are corrected for the control in the absence of biocatalyst; reaction conditions: whole lyophilized cells containing the overexpressed enzyme (30 mg), substrate (10 mM), $KHCO_3$ (3 M), phosphate buffer (pH 8.5, 100 mM), 30°C, 120 rpm (orbital shaker), 24 h; n.d. = not determined owing to low conversion; phenolic acid decarboxylases from *Lactobacillus plantarum* (PAD_Lp), *Bacillus amyloliquefaciens* (PAD_Ba), *Mycobacterium colombiense* (PAD_Mc), *Methylobacterium* sp. (PAD_Ms), *Pantoea* sp. (PAD_Ps), *Lactoccocus lactis* (PAD_Ll), and ferulic acid decarboxylase from *Enterobacter* sp. (FDC_Es) were used; [b] spontaneous (nonenzymatic) hydration in the absence of biocatalyst; [c] the corresponding (substituted) benzaldehyde was detected as minor side product; [d] absolute configuration not determined owing to low *ee*.

enzymatic hydration of the C=C bond, next to the expected carboxylation product *p*-coumaric acid.

Detailed analysis revealed that recombinant phenolic acid decarboxylases from *Lactobacillus plantarum* (PAD_Lp) and from *Bacillus amyloliquefaciens* (PAD_Ba) were able to hydrate the C=C bond of *p*-vinylphenol (**1a**), thereby forming (*S*)-4-(1-hydroxyethyl)phenol (**1b**) with good conversions and remarkable stereoselectivities (PAD_Lp: conv. 82%, *ee* 43%; PAD_Ba: conv. 64%, *ee* 53%, Table 1, entry 1) in the presence of carbonate buffer (3 M, pH 8.5) used as CO_2 source for carboxylation. Interestingly, the expected carboxylation product (*p*-coumaric acid) was observed only as minor side product ($\leq$5%).[*] To exploit the potential of this method, the following enzymes were chosen based on a similarity search (40–80% sequence identity): phenolic acid decarboxylases from *Mycobacterium colombiense* (PAD_Mc), *Methylobacterium* sp. (PAD_Ms), *Pantoea* sp. (PAD_Ps), *Lactoccocus lactis* (PAD_Ll), ferulic acid decarboxylase from *Enterobacter* sp. (FDC_Es), and (for reason of comparison) *p*-hydroxycinnamoyl CoA hydratase-lyase from *Pseudomonas fluorescens* (HCHL). All genes were synthesized by Geneart AG (Regensburg) and subcloned in a pET vector (pET 21a or pET 28a). A standard *E. coli* BL21(DE3) host was transformed with the obtained plasmids and IPTG was used to induce overexpression. According to SDS-PAGE analysis, successful overexpression was obtained for all enzymes, which were employed as lyophilized whole-cell biocatalyst to a range of substrates. The absence of competing hydratase activity of empty *E. coli* host cells was verified by separate control experiments. To our delight, almost all decarboxylases were able to catalyze the hydration of hydroxystyrene-type substrates **1a**–**5a** except for PAD_Mc from *Mycobacterium colombiense* (Table 1). The broad range of hydratase activities was even more remarkable, since the

[*] For enzymes, which showed very low hydration activity, such as PAD_Mc, the carboxylation reaction was dominant.

'real' hydratase, hydroxycinnamoyl-CoA hydratase-lyase did not show any hydratase activity at all.

All active enzymes displayed similar levels of conversion with respect to a given substrate (conv. 64–82% for **1a**, conv. 24–45% for **2a**, conv. 63–76% for **3a**, conv. 34–47% for **4a**, conv. 3–35% for **5a**, conv. <1% for **6a**). The hydroxy group in the *para*-position appears to be mandatory for enzyme activity, *meta*-analogues were unreactive, and *ortho*-substituted substrates turned out to be unstable. Overall, the data indicate that steric effects play a major role, because *p*-vinylphenol (**1a**) and the chloro analogue **3a** gave the highest conversions (conv. 63–82%, Table 1, entries 1,3), while the conversion continuously dropped upon increasing size and/or number of substituents. An additional methyl or methoxy group (**2a**, **4a**) led to moderate conversions (conv. 24–47%, Table 1, entries 2,4), and more bulky substituents like on **5a** resulted in a significant decrease of conversion (Table 1, entry 5). Compound **6a**, carrying three substituents on the phenyl ring, was not accepted (conv. <1, Table 1, entry 6). Comparison of results obtained with substrates bearing alkyl, alkoxy, and chloro groups shows that electronic effects play a minor role.

To elucidate the effect of bicarbonate, a set of experiments with various buffer systems were performed using *p*-vinylphenol (**1a**) and whole cells of *E. coli* harboring PAD_Lp (Figure 1). The data clearly show that the hydration indeed

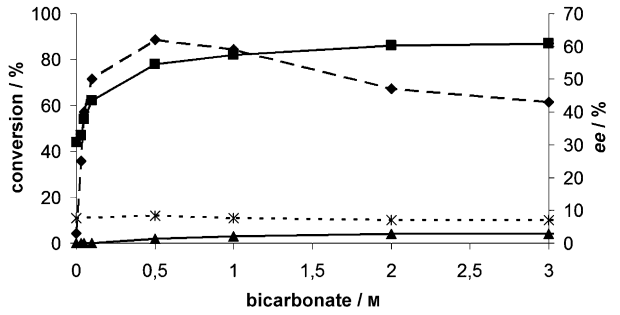
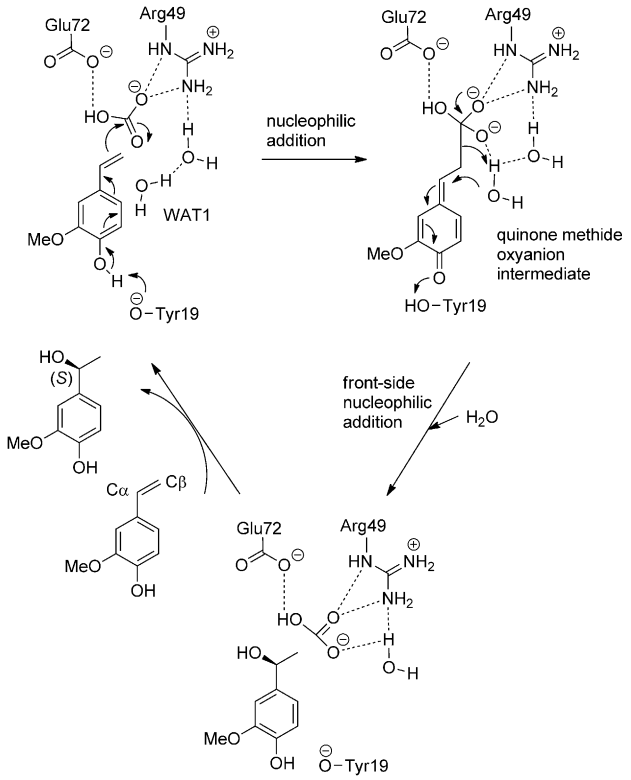


Figure 1. Hydration (resulting in **1b**; ——■——) versus carboxylation (resulting in *p*-coumaric acid; ——▲——) of *p*-vinylphenol (**1a**) employing PAD_Lp at various concentrations of bicarbonate. For **1b** the *ee* values are plotted (– – –◆– – –). The control (·····✳·····) corresponds to spontaneous (nonenzymatic) hydration in the absence of biocatalyst.

depends on the bicarbonate concentration, and the conversion reached its maximum at approximately 3 M. Moreover, the stereoselectivity of hydration (expressed as *ee* of **1b**) also strongly increased from near racemic (in the absence of bicarbonate) to reach a maximum (ca. 62% *ee*) at a bicarbonate concentration of approximately 0.5 M, followed by a continuous decrease to a plateau of approximately 43% *ee*. In contrast, the carboxylation product (*p*-coumaric acid) was observed only as minor side product (≤5%) independent on the bicarbonate concentration. These results strongly suggest that bicarbonate is mechanistically involved in the enzymatic hydration.

To provide a rationale for the promiscuous catalytic hydratase activity of phenolic acid decarboxylases, detailed literature studies revealed puzzling similarities between the (proposed) mechanism of *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*,[20] ferulic acid decarboxylase from *Enterobacter* sp.,[21] and hydroxycinnamoyl-CoA hydratase-lyase from *Pseudomonas fluorescens*:[1,6,21] All enzymes act via a central quinone methide intermediate,[*] which represents a Michael-acceptor for the nucleophilic 1,6-addition of water. Based on literature[1,6,15a,20,21] and experimental results, in silico docking and energy minimization studies (based on the crystal structure of ferulic acid decarboxylase from *E.* sp.) were performed,[21] which result in the mechanistic proposal depicted in Scheme 1.



Scheme 1. Proposed catalytic mechanism for hydration of substrate **2a** supported by docking experiments based on the crystal structure of ferulic acid decarboxylase from *Enterobacter* sp.[21] (see Figure 2, PDB-Code: 3NX2). WAT1 = water molecule 1, see Figure 2.

In contrast to the mechanism suggested by Gu et al.[21] we recognized that the active-site pocket provides productive substrate binding through formation of hydrogen bonds to Glu72 and Arg49, supported by a hydrophobic environment for the aromatic system (Ile29, Ile41, Trp70, Val78, Leu80, and Ile93). Tyr19 lies within an appropriate distance for the deprotonation of the *p*-hydroxy group of the substrate (supported by Tyr21, Figure 2), thereby initiating an electron flow along the *p*-hydroxy styrene structure. The shift of electrons enhances the nucleophilicity of the Cβ atom, which

[*] Also reported for vanillyl-alcohol oxidase[22] and 4-ethylphenol methylenehydroxylase.[23]

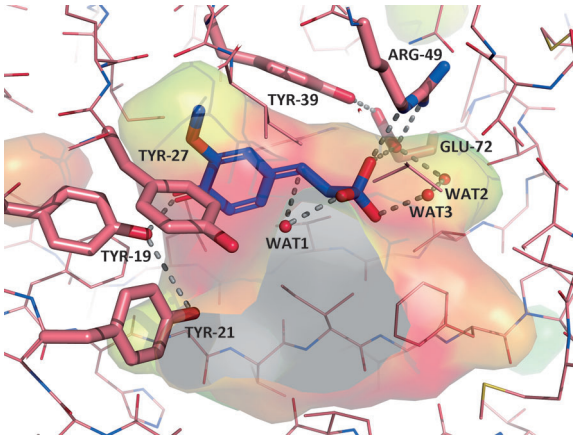


Figure 2. Energy-minimized oxyanion quinone methide intermediate of substrate **2a** (blue) in the active site of ferulic acid decarboxylase from *Enterobacter.* sp. (PDB-Code: 3NX2).[21] The active-site surface is colored by hydrophobicity from red (hydrophobic) to blue (hydrophilic). Coordinated water molecules (WAT) are shown as balls; WAT1 is ideally positioned to launch a nucleophilic front-side attack resulting in the formation of (*S*)-alcohol **2b** (see Scheme 1 and Table 1). Figure was prepared by using PyMOL.[24]

performs a nucleophilic attack onto bicarbonate, which is coordinated by Glu72 and Arg49 through hydrogen bonds and salt bridges, respectively. The latter results in an oxyanion-like *p*-quinone methide intermediate, which is stabilized by water molecules WAT1–WAT3 (Figure 2),[*] which were found in the original crystal structure.

Interestingly, the polar residues that interact with the substrate and those forming the hydrophobic environment for the aromatic substrate moiety are strictly conserved among the enzymes used in this study (Figure S3 in the Supporting Information), although the overall sequence identities are only between 40 and 80%.

In the carboxylation reaction that is catalyzed by the enzyme in nature, deprotonation at Cβ (accompanied by tautomerization) yields cinnamic acid derivatives as products. In contrast, hydration takes place through nucleophilic 1,6-addition of a water molecule (WAT1, Figure 2) across the quinone methide intermediate, followed by collapse of the oxyanion, thereby causing a heterolytic cleavage of the Cβ–$C_{carboxylate}$ bond through protonation of Cβ by WAT1. The latter yields the hydration product and reforms bicarbonate. The preference for (*S*)-alcohols is explained by a nucleophilic front-side attack, which is favored by the highly coordinated water network (WAT1–3).

In summary, we have presented for the first time the enzyme-catalyzed addition of water to the C=C double bond of hydroxystyrene-type substrates in an asymmetric fashion.

[*] The docking calculations were initially performed without water molecules present. During energy minimization water molecules were added again automatically.

Received: October 1, 2012
Revised: December 10, 2012
Published online: January 17, 2013

Keywords: biotransformations · catalytic promiscuity · decarboxylases · enzyme catalysis · hydratases

[1] J. Jin, U. Hanefeld, *Chem. Commun.* **2011**, *47*, 2502–2510.
[2] H.-D. Hahn, G. Dämbkes, N. Rupprich, H. Bahl, *Butanols* in *Ullmann's Encyclopedia of Industrial Chemistry*, Electronic version, Wiley-VCH, Weinheim, **2010**.
[3] E. Hartmann, D. J. Vyas, M. Oesterreich, *Chem. Commun.* **2011**, *47*, 7917–7932.
[4] S.-Q. Wang, Z.-W. Wang, L.-C. Yang, J.-I. Dong, C.-Q. Chi, D.-N. Sui, Y.-Z. Wang, J.-G. Ren, M.-Y. Hung, Y.-Y. Jiang, *J. Mol. Catal. A* **2007**, *264*, 60–65.
[5] A. J. Boersma, D. Coquiere, D. Geerdink, F. Rosati, B. L. Feringa, G. Roelfes, *Nat. Chem.* **2010**, *2*, 991–995.
[6] J. P. Bennett, L. Bertin, B. Moulton, I. J. S. Fairlamb, A. M. Brzozowski, N. J. Walton, G. Grogan, *Biochem. J.* **2008**, *414*, 281–289.
[7] a) F. tenBrink, B. Schink, P. M. H. Kroneck, *J. Bacteriol.* **2011**, *193*, 1229–1236; b) R.-Z. Liao, F. Himo, *ACS Catal.* **2011**, *1*, 937–944; c) R.-Z. Liao, J.-G. Yu, F. Himo, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 22523–22527; d) G. B. Seiffert, G. M. Ullmann, A. Messerschmidt, B. Schink, P. M. H. Kroneck, O. Einsle, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 3073–3077.
[8] a) L. E. Bevers, M. W. H. Pinkse, P. D. E. M. Verhaert, W. R. Hagen, *J. Bacteriol.* **2009**, *191*, 5010–5012; b) A. Volkov, A. Liavonchanka, O. Kamneva, T. Fiedler, C. Goebel, B. Kreikemeyer, I. Feussner, *J. Biol. Chem.* **2010**, *285*, 10353–10361.
[9] C. S. Turbek, D. A. Simth, C. L. Schardl, *FEMS Microbiol. Lett.* **1992**, *94*, 187–190.
[10] J. A. Maresca, J. E. Graham, A. D. Pryant, *Photosynth. Res.* **2008**, *97*, 121–140.
[11] D. Brodkorb, M. Gottschall, R. Marmulla, F. Lüddeke, J. Harder, *J. Biol. Chem.* **2010**, *285*, 30436–30442.
[12] A. Liese, K. Seelbach, A. Buchholz, J. Haberland in *Industrial Biotransformations*, 2nd ed. (Eds.: A. Liese, K. Seelbach, C. Wandrey), Wiley-VCH, Weinheim, **2006**, p. 465 and p. 488.
[13] M. Wubbolts in *Enzyme Catalysis in Organic Synthesis*, 2nd ed (Eds.: K. Drauz, H. Waldmann), Wiley-VCH, Weinheim, **2002**, pp. 686–697.
[14] a) G. Agnihotri, H.-w. Liu, *Bioorg. Med. Chem.* **2003**, *11*, 9–20; b) B. J. Bahnson, V. E. Anderson, G. A. Petsko, *Biochemistry* **2002**, *41*, 2621–2629; c) A. F. Bell, Y. Feng, H. A. Hofstein, S. Parikh, J. Wu, M. J. Rudolph, C. Kisker, A. Whitty, P. J. Tonge, *Chem. Biol.* **2002**, *9*, 1247–1255.
[15] a) P. M. Leonard, A. M. Brzozowski, A. Lebedev, C. M. Marshall, D. J. Smith, C. S. Verma, N. J. Walton, G. Grogan, *Acta Crystallogr. Sect. D* **2006**, *62*, 1494–1501; b) P. M. Leonard, C. M. Marshall, E. J. Dodson, N. J. Walton, G. Grogan, *Acta Crystallogr. Sect. D* **2004**, *60*, 2343–2345; c) A. Mitra, Y. Kitamura, M. J. Gasson, A. Narbad, A. J. Parr, J. Payne, M. J. C. Rhodes, C. Sewter, N. J. Walton, *Arch. Biochem. Biophys.* **1999**, *365*, 10–16.
[16] J. Jin, A. J. J. Straathof, M. W. H. Pinske, U. Hanefeld, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1831–1840.
[17] J. Jin, P. C. Oskam, S. K. Karmee, A. J. J. Straathof, U. Hanefeld, *Chem. Commun.* **2010**, *46*, 8588–8590.
[18] a) U. T. Bornscheuer, R. J. Kazlauskas, *Angew. Chem.* **2004**, *116*, 6156–6165; *Angew. Chem. Int. Ed.* **2004**, *43*, 6032–6040; b) M. Svedendahl Humble, P. Berglund, *Eur. J. Org. Chem.* **2011**, 3391–3401.
[19] C. Wuensch, S. M. Glueck, J. Gross, D. Koszelewski, M. Schober, K. Faber, *Org. Lett.* **2012**, *14*, 1974–1977.

[20] H. Rodriguez, I. Angulo, B. de Las Rivas, N. Campillo, J. A. Paez, R. Munoz, J. M. Mancheno, *Proteins Struct. Funct. Bioinf.* **2010**, *78*, 1662 – 1676.
[21] W. Gu, J. Yang, Z. Lou, L. Liang, Y. Sun, J. Huang, X. Li, Y. Cao, Z. Meng, K.-Q. Zhang, *PLoS ONE* **2011**, *6*, e16262.
[22] a) R. H. H. van den Heuvel, M. W. Fraaije, W. J. H. van Berkel, *FEBS Lett.* **2000**, *481*, 109 – 112; b) A. Mattevi, M. W. Fraaije, A. Mozzarelli, L. Olivi, A. Coda, W. J. H. Van Berkel, *Structure* **1997**, *5*, 907 – 920.
[23] a) D. J. Hopper, L. Cottrell, *Appl. Environ. Microbiol.* **2003**, *69*, 3650 – 3652; b) C. D. Reeve, M. A. Carver, D. J. Hopper, *Biochem. J.* **1989**, *263*, 431 – 437.
[24] E. Krieger, G. Koraimann, G. Vriend, *Proteins Struct. Funct. Bioinf.* **2002**, *47*, 393 – 402.

