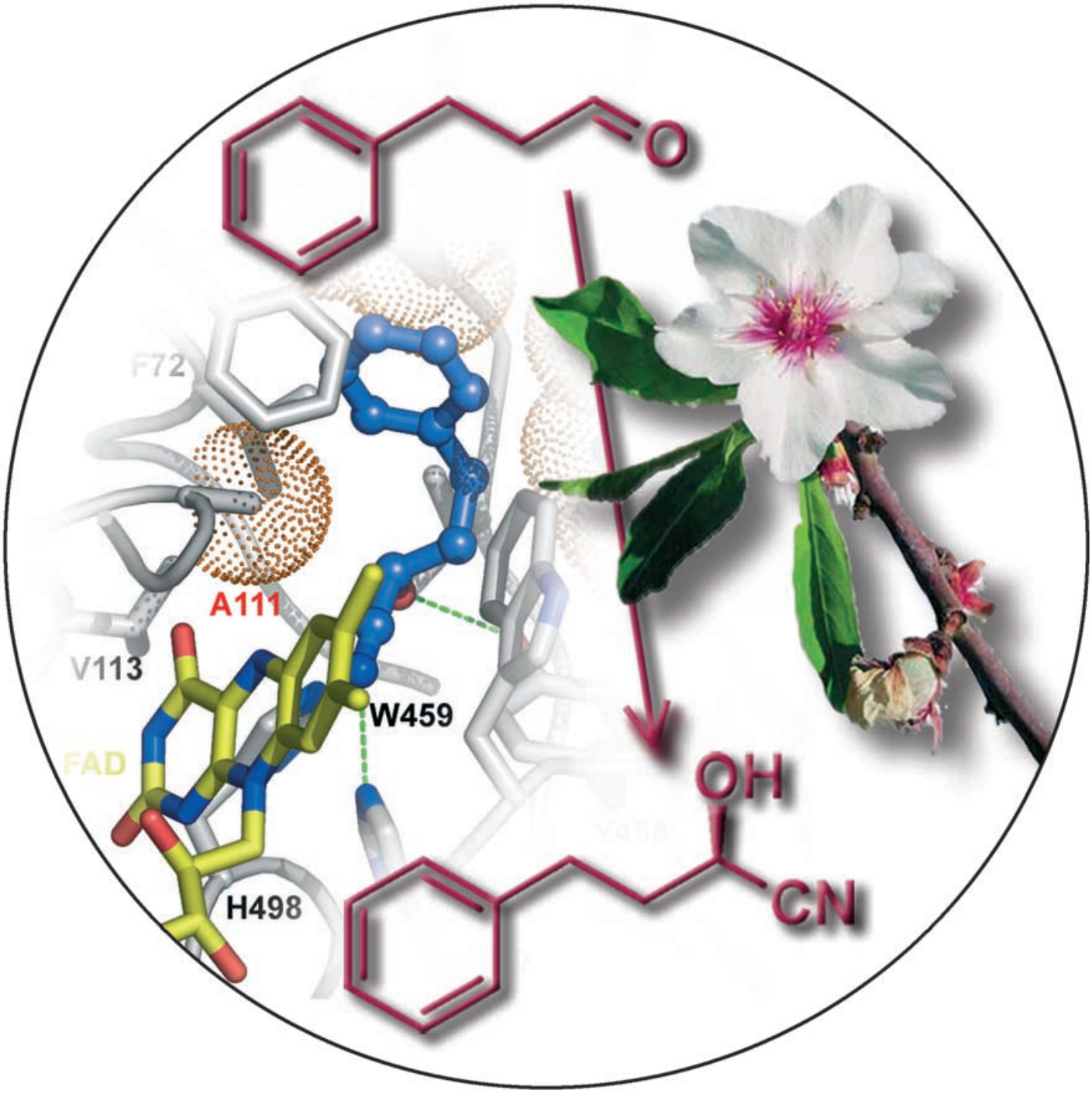
Communications



The active site of almond hydroxynitrile lyase was redesigned to give highly enantioselective enzyme variants for the synthesis of (*R*)-2-hydroxy-4-phenylbutyronitrile. K. Gruber, A. Glieder et al. describe their rationalization and realization of these biocatalysts in their communication on the following pages.

  DOI: 10.1002/anie.200500435

Protein Engineering

Carving the Active Site of Almond *R*-HNL for Increased Enantioselectivity**

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Hydroxynitrile lyases (HNLs) catalyze quantitative, stereoselective carbon–carbon bond formation in the addition of HCN to aldehydes or ketones yielding enantiopure cyanohydrins, which are key intermediates for numerous synthetic routes.[1] Both *R*- and *S*-selective HNLs are widely present in nature, and several genes have been cloned and expressed.[1] Recombinant almond (*Prunus amygdalus*) (*R*)-HNL isoenzyme 5 (*Pa*HNL5) is secreted to the culture supernatant of *Pichia pastoris*, which can be directly used for biocatalytic conversions in water or biphasic systems without prior enzyme purification or immobilization. Further prerequisites for an industrial application of this enzyme are its stability at acidic pH (suppression of the unselective non-enzymatic background reaction) and its enantioselectivity. Although the high stability of recombinant *Pa*HNL5 already enabled stereoselective enzymatic syntheses in water or biphasic systems even with slow-reacting substrates,[1,2] the enantioselectivity of *Pa*HNL5 is in some cases still too low for biocatalysis on a large scale.

Among the broad substrate range accepted by *Pa*HNL5[3] those with the aldehyde functionality separated from an aromatic moiety by an aliphatic linker represent an especially interesting group. The aromatic ring, which plays an important role in the correct recognition and binding of the natural substrate mandelonitrile (benzaldehyde cyanohydrin),[4] still has to fit into the closer region around the active site. On the other hand its distance from the functional group and the conformational flexibility of the alkyl linker reduce its influence on the stereoselectivity of the reaction. Among such aromatic substrates, 3-phenylpropionaldehyde (**1a**) and 3-phenylpropenal (**2a**, *trans*-cinnamaldehyde), and their corresponding cyanohydrins (*R*)-2-hydroxy-4-phenylbutyronitrile (**1b**) and (*R*)-2-hydroxy-4-phenyl-3-butene nitrile (**2b**) emerged as the most interesting representatives in terms of commercial use. Enantiopure α-hydroxycarboxylic acids, which are important intermediates for the synthesis of a class of angiotensin-converting enzyme inhibitors (ACEi) known as "prils",[5] can be derived from these cyanohydrins by acid hydrolysis. Compound **2a** was described as a notoriously recalcitrant substrate, but palladium-catalyzed hydrogenation of **2b** also yields (*R*)-2-hydroxy-4-phenylbutyric acid.[6] In addition **2b** can be used as a versatile intermediate for asymmetric epoxidation, dihydroxylation, and halogen addition.[7] Only *R* enantiomer **2b** is a building block of pharmacologically active "prils", and commercial production calls for high enantiomeric excesses (> 95 % *ee*), high yields (> 95 %), an environmentally benign process, and economic reaction times with a low enzyme/substrate ratio. Enantiomer separation is feasible by preferential crystallization of diastereomers, although with limited yields (68 %).[8] Other synthetic routes, for example, enantioselective reduction of keto or diketo esters, suffer from complicated procedures or require expensive starting compounds.[5,9]

Considering implementation of these chiral building blocks on a large scale, we investigated a biocatalytic route using very low amounts (i.e. 17 μg enzyme per mmol **1a**) of recombinant *Pa*HNL5 (Scheme 1). This route starts with cheap substrates, minimizes the number of unit operations, and offers optional crystallization after cyanohydrin hydrolysis to recover pure product. Conversion of **1a** to **1b** using recombinant *Pa*HNL5 was almost complete (93 %) after 4 h with 89.4 % *ee* (Table 1, entry 1). However, to avoid enantioselective crystallization as an additional step in the synthetic route, we needed a more selective mutein.

Directed evolution of enzymes has proved to be an efficient tool to influence the enantioselectivity of many (mostly bacterial) enzymes.[10] However, although expression of *Pa*HNL isoenzymes by *E. coli* is feasible, highly active *Pa*HNL5 could only be expressed in *Pichia pastoris*,[1,11] which is not a reliable host system for laboratory evolution. Furthermore a codon dilemma[12] impedes access to all possible amino acid exchanges by point mutations, especially when only a few thousand mutants can be screened. Because of our recent success in designing a more active mutein for sterically demanding mandelonitrile derivatives,[1a] we started a structure-guided approach to increase the enantioselectivity of this lyase. To our knowledge this is the first report on a successful structure-guided design of lyases to improve enantioselectivity, although rational approaches to *Manihot esculenta* HNL variants with reduced substrate transport limitations resulted in improved enantioselectivity for a variety of substrates as well.[13]

We modeled the complexes of *Pa*HNL5 with (*R*)-**1b** and (*S*)-**1b.** For both substrates equivalent binding modes were observed with respect to the position of the phenyl group as well as the mechanistically important polar interactions of the hydroxy and cyano groups with His 498 and His 460 (Figure 1).[4] Differences were observed only for the interactions of *Pa*HNL5 with the alkyl linker chain of **1b**, which was oriented towards Ala 111 in the *S* enantiomer and towards Val 360 in the *R* enantiomer (Figure 1, see also

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[**] This research was supported by DSM, the FFG, the province of Styria, the SFG, and the city of Graz. We thank H. Mandl from the Research Centre Applied Biocatalysis, I. Wirth and O. Maurer from DSM for excellent technical support, and K. Faber, C. Kratky, and W. Kroutil for valuable comments on the manuscript, and P. Naggl for the color artwork.

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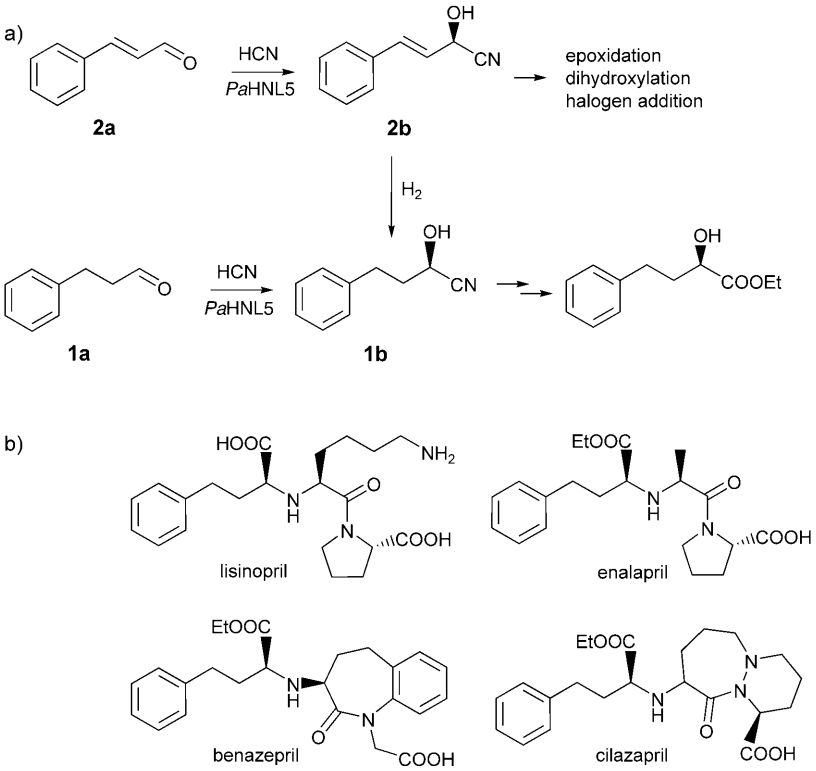

Scheme 1. a) Enantioselective synthesis of (*R*)-2-hydroxy-4-phenylbutyronitrile (**1b**) and (*R*)-2-hydroxy-4-phenylbutene nitrile (**2b**) from 3-phenylpropionaldehyde (**1a**) and 3-phenylpropenal (**2a**), respectively, for the production of (*R*)-2-hydroxy-4-phenylbutyric acid, an intermediate for "prils". b) Examples of "prils".

Table 1: Comparison of the constructed muteins to the unmodified *Pa*HNL5 for the enantioselective hydrocyanation of **1a**.[a]

Entry	Enzyme	Conv. [%]	*ee* [%]
1	*Pa*HNL5	93	89.4
2	A111VL331A	30	≈0
3	A111G	40	15.2
4	A111VL343A	40	27.6
5	A111V	16	60.2
6	L331F	80	63.1
7	A111GV360M	75	64.7
8	L343A	83	68.8
9	L343F	95	74.4
10	V360M	93	86.7
11	A111GV360I	92	87.6
12	L331A	94	88
13	V360I	98	95.3

[a] Reaction conditions: 30 mmol of **1a** and 0.5 mg of enzyme (17 μg mmol^{-1} substrate), 4 h, 10 °C, pH 3.4.

video material in the Supporting Information). We took advantage of this pseudo mirror symmetry and redesigned the substrate binding site of *Pa*HNL5 to tailor the accessible volume for one or the other enantiomer, similarly to other successful examples of laboratory-evolved enzymes.[14] Amino acids in the binding site were exchanged with residues of different sizes while preserving hydrophobicity. Ala111 and Val360 interact with the alkyl chain of the substrate from opposite sides and therefore are promising sites for mutations. The same is true for Leu331 and Leu343, which mainly interact with the phenyl group and form part of the access tunnel leading to the active site. In total, we studied 24 mutants in silico, and 12 of those—with the largest predicted changes in enantioselectivity—were prepared and analyzed experimentally. In a first test of the hydrocyanation of **1a** with only low amounts of each mutein, V360I yielded the most selective enzyme (Table 1, entry 13). As expected, A111G was less stereoselective. In a more detailed examination, 1 mg each of the best four muteins (entries 10–13) were analyzed for conversion of 2 g (15 mmol) of **1a** (i.e. 67 μg enzyme per mmol substrate). L331A, V360M, and V360I (Table 2, entries 3–5) resulted in almost complete substrate conversion within 4 h with significantly improved enantioselectivity. At the end of the conversion **1b** produced with mutein A111GV360I also showed slightly higher enantiopurity (91.8% *ee*), although the conversion was a little slower.

To explore turnover rates, the amount of the enzyme was adjusted such that with each individual mutein the conversion of 2 g (15 mM) of **1a** was in a roughly linear range during the first 20 min. Specific activities under these conditions (Table 2) served for comparison of the individual muteins for cyanohydrin synthesis, although due to the nonlinear kinetics of *Pa*HNL5-catalyzed reactions in biphasic systems, such numbers cannot be translated to k_{cat} values for higher enzyme concentrations. The introduction of isoleucine at position 360 raised the *ee* to more than 96% and accelerated the transformation of **1a** almost sixfold. Thus the conversion

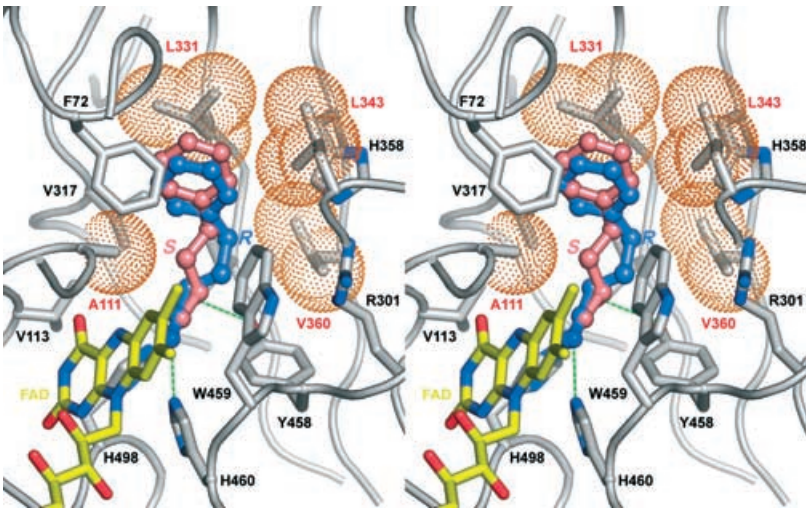

Figure 1. Stereoimage of the superposition of modeled complexes of (*R*)-**1b** (blue) and (*S*)-**1b** (pink) bound to the active site of *Pa*HNL5. Amino acid side chains are shown in light gray, the FAD cofactor in yellow. N and O atoms are shown in blue and red, respectively. Residues selected for mutagenesis are labeled in red, and their van der Waals volumes are represented by small spheres. The image was prepared with the software PyMol (http://www.pymol.org).

was complete within a couple of hours despite a low enzyme/substrate ratio. In contrast, in a recent report at least 30 times more enzyme[6] was employed. Mutations L331A and, surprisingly, A111GV360I also resulted in a moderately increased catalytic rate. Compared to other muteins, however, the turnover rate of A111GV360I decreased during the course of the reaction.

All muteins with increased enantioselectivity for the synthesis of **1b** were also tested in the conversion of 15 mmol of the more rigid **2a** to **2b** (Table 3). Two muteins were also more selective with **2a**, reaching ≈98% *ee* with mutation V360I. In addition all mutations except V360M caused a faster conversion of **2a** than with the original enzyme *Pa*HNL5. The higher rigidity of **2a** compared to **1a** and the larger volume of the methionine residue are likely reasons for the weaker activity of the V360M variant. With only 27 μg of *Pa*HNL5-V360I per mmol **2a**, conversion to **2b** was almost complete after 3 h with an excellent enantiomeric excess of ≈98%. In contrast, Gerrits and co-workers reported a reaction time of 168 h for 97% yield and 98% *ee*.[15] Recently also the Sheldon lab reported highly enantioselective and fast reactions by employing cross-linked enzyme aggregates of native almond HNL.[16]

Structure-guided design was employed to generate recombinant *Pa*HNL5 variants suitable for large-scale stereoselective synthesis of aromatic cyanohydrins in water-based systems. From 24 desigend enzyme variants, 12 of which were prepared, four well-expressed, sit-specific mutants of the *Pa*hnl5 gene with significantly increased stereoselectivity were obtained. All muteins were stable at low pH. As a highlight, the variant *Pa*HNL5-V360I exhibited extraordinary catalytic properties. The enantiomeric excess for the conversion of **1a** was improved to >96% despite a very low enzyme/substrate ratio. This particular mutein reduced the required amount of biocatalyst 10- to 30-fold to produce enantiopure (*R*)-2-hydroxy-4-phenylbutyric acid and derivatives thereof. From *trans*-cinnamaldehyde (**2a**) very low amounts of catalyst with mutation V360I were needed to produce cyanohydrin **2b** with an optical purity of ≈98% *ee* within 3 h. The conversion time for this recalcitrant substrate was reduced from 168 h to 3 h. Interestingly our designed and more selective muteins were also more active. This is in contrast to reports on the design of more selective hydrolases, where just the reactivity with the wrong enantiomer was reduced.[17] *Pa*HNL5-V360I is currently the most efficient catalyst for syntheses of enantiomerically pure building blocks for pharmaceutically active "prils", therefore suggesting itself for industrial application on a large scale.

Received: February 4, 2005
Published online: July 6, 2005

Keywords: ACE inhibitors · asymmetric synthesis · biocatalysis · hydroxynitrile lyase · protein engineering

[1] a) A. Glieder, R. Weis, W. Skranc, P. Poechlauer, I. Dreveny, S. Majer, M. Wubbolts, H. Schwab, K. Gruber, *Angew. Chem.* **2003**, *115*, 4963–4966; *Angew. Chem. Int. Ed.* **2003**, *42*, 4815–4818; b) M. H. Fechter, H. Griengl, *Food Technol. Biotechnol.* **2004**, *42*, 287–294.

[2] a) M. Bauer, R. Geyer, M. Boy, H. Griengl, W. Steiner, *J. Mol. Catal. B* **1998**, *5*, 343–347; b) U. Kragl, U. Niedermeyer, M. R. Kula, C. Wandrey, *Ann. N. Y. Acad. Sci.* **1990**, *613*, 167–175.

[3] a) F. Effenberger, B. Horsch, F. Weingart, T. Ziegler, S. Kuhner, *Tetrahedron Lett.* **1991**, *32*, 2605–2608; b) F. Effenberger, *Angew. Chem.* **1994**, *106*, 1609–1619; *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 1555–1564.

Table 2: Conversion and enantioselectivity of selected muteins and the original *Pa*HNL5 for the hydrocyanation of **1a**.[a]

		Reaction time						
		1 h		2 h		4 h		
Entry	Enzyme	Conv.[b] [%]	*ee* [%]	Conv.[b] [%]	*ee* [%]	Conv.[b] [%]	*ee* [%]	TOF[c] [s^{-1}]
1	*Pa*HNL5	72	89.0	87	90.1	96	90.2	2497 ± 208
2	A111GV360I	70	90	83	90.6	94	91.8	3379 ± 208
3	L331A	78	90.7	90	93.3	95	92.9	2865 ± 312
4	V360M	78	93.6	93	94.0	97	94.6	6812 ± 837
5	V360I	86	96.0	96	96.6	98	96.7	14397 ± 416

[a] Reaction conditions: 15 mmol of **1a** and 1 mg of enzyme (67 μg enzyme mmol^{-1} substrate), 10°C, pH 3.4. [b] Conversions. [c] TOF: turnover frequency.

Table 3: Conversion and enantioselectivity of selected muteins and the parent *Pa*HNL5 for the hydrocyanation of **2a**.[a]

		Reaction time						
		1 h		2 h		3 h		
Entry	Enzyme	Conv.[b]	*ee*	Conv.[b]	*ee*	Conv.[b]	*ee*	TOF[c] [s^{-1}]
1	*Pa*HNL5	13	95.2	22	96.2	30	96.4	110 ± 19
2	V360M	9	90.3	14	92.7	18	92.6	n.d.[d]
3	L331A	19	94.5	29	95.4	39	95.7	n.d.[d]
4	A111GV360I	21	95.8	36	96.8	47	96.8	149 ± 16
5	V360I	70	98.0	90	97.9	97	97.6	458 ± 32

[a] Reaction conditions: 15 mmol of **2a** and 0.4 mg of enzyme (27 μg enzyme mmol^{-1} substrate), 10°C, pH 3.4). [b] Conversion and *ee* are shown in percent. [c] TOF: turnover frequency. [d] n.d.: not determined since the *ee* was not improved.

[4] a) I. Dreveny, K. Gruber, A. Glieder, A. Thompson, C. Kratky, *Structure* **2001**, *9*, 803 – 815; b) I. Dreveny, C. Kratky, K. Gruber, *Protein Sci.* **2002**, *11*, 292 – 300.
[5] R. K. Tikare, WO 0242244A, **2004** [*Chem. Abstr.* **2002**, *136*, 401540].
[6] A. Matsuyama, I. Takase, Y. Ueda, Y. Kobayashi, US 5429935, **1995** [*Chem. Abstr.* **1995**, *123*, 141884].
[7] a) A. Schummer, H. Yu, H. Simon, *Tetrahedron* **1991**, *47*, 9019 – 9034; b) H. Yu, H. Simon, *Tetrahedron* **1991**, *47*, 9035 – 9052.
[8] M. Kottenhahn, K. Drauz (Degussa AG), US 5616727, **1997** [*Chem. Abstr.* **1997**, *126*, 305791].
[9] a) H. U. Blaser, E. Schmidt, *Asymmetric Catalysis on Industrial Scale: Challenges, Approaches and Solutions*, VCH, Weinheim, **2004**, pp. 91 – 103; b) M. Studer, S. Burkhardt, A. F. Indolese, H.-U. Blaser, *Chem. Commun.* **2000**, 1327 – 1328.
[10] a) O. May, P. T. Nguyen, F. H. Arnold, *Nat. Biotechnol.* **2000**, *18*, 317 – 320; b) K. E. Jaeger, T. Eggert, *Curr. Opin. Biotechnol.* **2004**, *15*, 305 – 313; c) B. Lingen, J. Groetzinger, D. Kolter, M.-R. Kula, M. Pohl, *Protein Eng.* **2002**, *15*, 585 – 593.
[11] a) H. Ayguen, S. Wojczewski, S. Rosmus (Biospring GmbH), DE 10251547, **2004** [*Chem. Abstr.* **2002**, *140*, 401356]; b) R. Weis, P. Poechlauer, R. Bona, R. Luiten, M. Wubbolts, H. Schwab, A. Glieder, *J. Mol. Catal. B* **2004**, *29*, 211 – 218.
[12] B. Steipe, *Curr. Top. Microbiol. Immunol.* **1999**, *243*, 55 – 86.
[13] H. Buhler, F. Effenberger, S. Forster, J. Roos, H. Wajant, *ChemBioChem* **2003**, *4*, 211 – 216.
[14] M. Chen-Goodspeed, M. A. Sogorb, F. Wu, F. M. Raushel, *Biochemistry* **2001**, *40*, 1332 – 1339; M. T. Reetz, F. Daligault, B. Brunner, H. Hinrichs, A. Deege, *Angew. Chem.* **2004**, *116*, 4170 – 4173; *Angew. Chem. Int. Ed.* **2004**, *43*, 4078 – 4081.
[15] J. P. Gerrits, W. F. Willeman, A. J. J. Straathof, J. J. Heijnen, J. Brussee, A. van der Gen, *J. Mol. Catal. B* **2001**, *15*, 111 – 121.
[16] L. M. van Langen, R. P. Selassa, F. van Rantwijk, R. A. Sheldon, *Org. Lett.* **2005**, *7*, 327 – 329.
[17] a) U. T. Bornscheuer, *Curr. Opin. Biotechnol.* **2002**, *13*, 543 – 547; b) M. T. Reetz, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 5716 – 5722; c) D. Rotticci, J. C. Rotticci-Mulder, S. Denman, T. Norin, K. Hult, *ChemBioChem* **2001**, *2*, 766 – 770.

