Biocatalysis

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Engineering Rieske Non-Heme Iron Oxygenases for the Asymmetric Dihydroxylation of Alkenes

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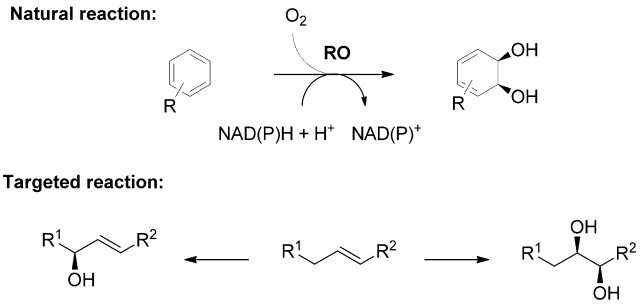
Dedicated to Professor Franz Lingens on the occasion of his 90th birthday

Abstract: *The asymmetric dihydroxylation of olefins is of special interest due to the facile transformation of the chiral diol products into valuable derivatives. Rieske non-heme iron oxygenases (ROs) represent promising biocatalysts for this reaction as they can be engineered to efficiently catalyze the selective mono- and dihydroxylation of various olefins. The introduction of a single point mutation improved selectivities (≥95%) and conversions (>99%) towards selected alkenes. By modifying the size of one active site amino acid side chain, we were able to modulate the regio- and stereoselectivity of these enzymes. For distinct substrates, mutants displayed altered regioselectivities or even favored opposite enantiomers compared to the wild-type ROs, offering a sustainable approach for the oxyfunctionalization of a wide variety of structurally different olefins.*

The stereo- and regioselective oxidative functionalization of olefins is amongst the most challenging reactions in organic chemistry and much effort has been made to develop selective methodologies that cover a broad range of substrates. Metal-catalyzed asymmetric dihydroxylations (AD) of alkenes provide both stereo- and regiospecific *cis*-diol moieties representing key intermediates in organic synthesis. One of the most widely applied techniques for the preparation of enantiomerically pure *cis*-diols is the Sharpless dihydroxylation. Using catalytic amounts of osmium(VIII) oxide in combination with a secondary oxidant and a chiral cinchona alkaloid ligand, various functionalized and non-functionalized olefins of different classes can be converted into their corresponding diols yielding good to excellent stereoselectivities for five of the six alkenes (mono-, *gem*-di-, *trans*-di-, tri- and tetra-substituted alkenes).[1] However, next to their toxicity, these metal catalysts can also lead to byproduct formation due to overoxidation and cleavage of the diol products.[1,2]

Analogously to chemical strategies, nature has evolved highly selective biocatalysts shown to catalyze a wide variety of challenging oxidation reactions. Nevertheless, the diversity of accessible *cis*-diols is limited by enzyme availability. Despite the vast number of characterized hydroxylating enzymes, only Rieske non-heme iron oxygenases (ROs) display the remarkable ability to stereoselectively introduce two hydroxy groups in one enzymatic step.[3] During the oxidation reaction, molecular oxygen is bound side-on to the catalytic non-heme iron in the active site of the enzyme leading to the formation of vicinal *cis*-diols with high stereospecificity.[4] In nature, this catalytic machinery is used for the attack of unactivated arene ring systems, making ROs key enzymes in the degradation of aromatic hydrocarbons (Scheme 1).[5–7] Like the well-known P450 monooxygenases,



Scheme 1. RO-catalyzed dihydroxylation of their natural arene substrates is performed with excellent stereoselectivities. During the reaction, O_2 is incorporated by using electrons derived from NAD(P)H. R = aryl or alkyl, R^1,R^2 = aryl, alkyl or cyclic.

these versatile biocatalysts display a relaxed substrate specificity and furthermore can catalyze various other oxidation reactions including monohydroxylations, dealkylations, desaturations, epoxidations and oxidative cyclizations.[3,8–10] Thereof, the regio- and stereospecific allylic hydroxylation of C–H bonds is of particular interest as this reaction can chemically only be reliably achieved by stoichiometric amounts of SeO_2.[11]

However, in contrast to P450 enzymes, which have been extensively studied over the last decades, far less is known about ROs to date. Most of the research focused on the dihydroxylation of aromatic hydrocarbons for the bioremediation of environmental pollutants.[12] Hudlicky and Boyd were among the first to discover the potential of ROs as biocatalysts for the preparation of oxyfunctionalized aromatics in organic synthesis.[13,14]

Although displaying a broad substrate scope, the conversion of unnatural substrates by ROs has only started to receive a growing interest within recent years and limited data is available for the RO-catalyzed oxidation of alkenes. To explore the oxidative potential of this group of enzymes, three

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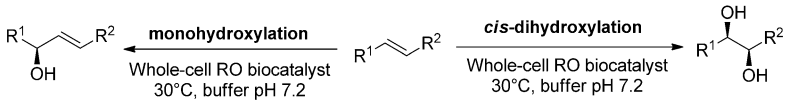
different ROs were assayed for their ability to hydroxylate aliphatic C=C double bonds. Naphthalene- (NDO), benzene- (BDO) and cumene (CDO) dioxygenases from different *Pseudomonas* strains were selected as they are amongst the RO enzymes that have already been reported to catalyze the oxidation of several olefins.[15–19] However, it has been shown that their application in organic synthesis is restricted by their poor stereoselectivities and activities especially for linear alkenes.[19] To overcome these limitations, we introduced changes in the active-site topology of RO catalysts. With this set of wild-type (WT) enzymes and evolved variants, we focused on the oxyfunctionalization of a wide range of structurally diverse aliphatic compounds including linear and cyclic arene-substituted alkenes, cycloalkenes as well as different terpenes (Scheme 2).

Similar to P450 monooxygenases, ROs are complex enzymes that utilize a reductase and a ferredoxin for electron transfer from NAD(P)H to the terminal oxygenase (subunits α and β). Due to their cofactor dependency and multicomponent nature, the substrate scope of NDO, BDO and CDO was examined in whole-cell biotransformations using 0.2 g mL^{-1} (*cww*) resting *E. coli* cells supplemented with glucose for in situ NAD(P)H regeneration. In in vivo biotransformations, a substrate panel of seven mono- and polyenes was investigated consisting of compounds **1** to (*R*)-**7** (Scheme 2). To evaluate RO-catalyzed AD of different alkene types against heavy-metal catalysts, the substrates studied (10 mM) comprised mono- (**1**, **2**, **5**), *gem*-di- (**5**, **7**), *cis*-di- (**3**, **4**) and tri-substituted alkenes (**5**, **6**, **7**).

The majority of olefins from our selected substrate panel could be converted by NDO, BDO and CDO (Table 1). In addition to the corresponding diol products, monohydroxylations for substrates **3**, **4**, **5** and **7** were found yielding products **3a**, **4a**, **5a**, **7a** and **7b** (Scheme 2). Consistent with previous reports from literature, monohydroxylation reactions were observed to be dependent on both the aliphatic compound as well as the allylic position.[18] Furthermore, it is of interest to note that in contrast to Sharpless AD,[20] dihydroxylation of **5** did not only occur at the more electron rich C=C double bond yielding product **5c** (>95% *ee*, Table 2). In addition, products **5b** and **5d** could be obtained using RO enzymes with stereoselectivities up to >95% (Table 2). Likewise, RO-catalyzed allylic hydroxylation of **5** afforded exclusively (*Z*)-**5a** instead of the (*E*)-isomer obtained in the SeO_2-mediated oxidation of **5** (Figure S16 in the Supporting Information).[21]

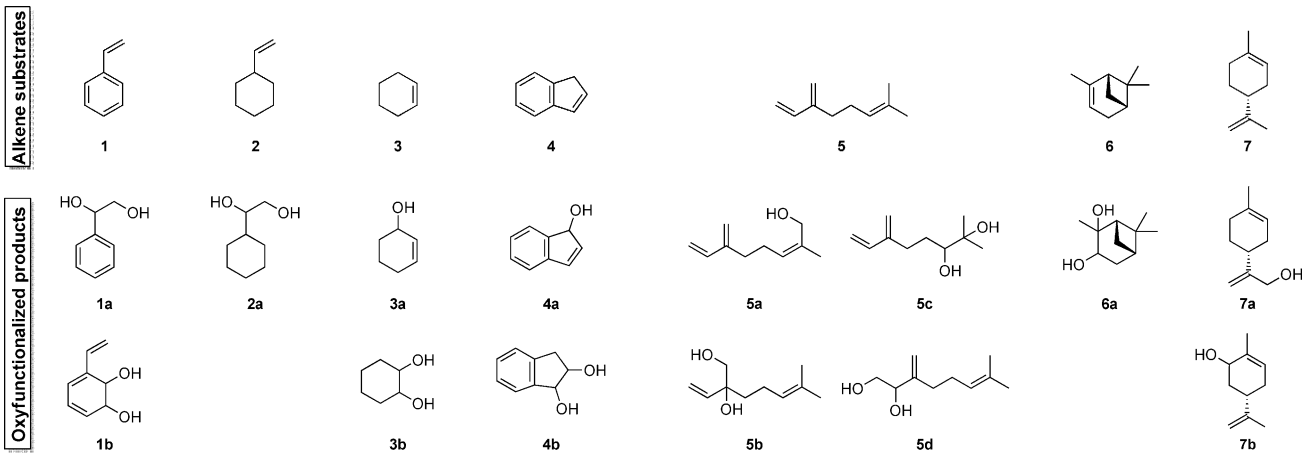
However, unlike described for their natural substrates, enzymes displayed low activities (<50%) and modest

Table 1: Product formations for NDO, BDO and CDO wild-type (WT) compared to CDO mutant M232A.



Olefin	Product formation [%][a]			
	NDO WT	BDO WT	CDO WT	CDO M232A
1	>99 (100 **1a**)	90±7 (59:41 **1a**:**1b**)	71±6 (0.3:99.7 **1a**:**1b**)	97±10 (92:8 **1a**:**1b**)
2	20±3	<1	44±6	93±5
3[b]	27±5	59±6	36±4	36±4
4[c]	87±10 (38:62 **4a**:**4b**)	>99 (10:90 **4a**:**4b**)	80±7 (64:36 **4a**:**4b**)	80±11 (56:44 **4a**:**4b**)
5	34±11 (12:41:3:44 **5a**:**5b**:**5c**:**5d**)	5.3±0.1 (56:14:14:15 **5a**:**5b**:**5c**:**5d**)	29±6 (48:18:18:17 **5a**:**5b**:**5c**:**5d**)	>99 (71:3:21:4 **5a**:**5b**:**5c**:**5d**)
6	–	–	–	33±7
(*R*)-**7**	n.d.	<1	46±10	>99

[a] Product formations were determined by GC-FID analysis after 24 h. Standard deviations also include variations in the expression level as triplicates from at least two different expression batches were used for the determination of product formations. [b] Cyclohexanone formation resulting from isomerization of **3a** was added to the total amount of **3a**. [c] Traces of 1-indanol were detected (1–2%) resulting from the RO-catalyzed oxidation of indane trace impurities present in **4**. –: not detected. n.d.: not determined. Values for BDO M220A and the CDO NDT library variants are available in the Supporting Information.



Scheme 2. Oxyfunctionalization of alkenes (**1**–**7**) with ROs and the observed mono- and dihydroxylated products (**1a**, **1b**—**7a**, **7b**).

Table 2: Stereoselectivities for NDO, BDO and CDO WT compared to CDO mutant M232A.

Olefin	Stereoselectivity *ee* or *de* [%][a]			
	NDO WT	BDO WT	CDO WT	CDO M232A
1	80±1 (*R*-**1a**)	8±2 (*R*-**1a**)	43±3 (*R*-**1a**)	95±1 (*R*-**1a**)
2	>98 (*R*-**2a**)	n.d.	91±3 (*R*-**2a**)	>98 (*R*-**2a**)
3	65±2 (*R*-**3a**)	90±1 (*R*-**3a**)	74±1 (*R*-**3a**)	28±1 (*R*-**3a**)
4	71±4 (*S*-**4a**)	45±1 (*S*-**4a**)	10±1 (*R*-**4a**)	87±1 (*S*-**4a**)
	76±1 (1*R*,2*S*-**4b**)	42±1 (1*R*,2*S*-**4b**)	38±1 (1*S*,2*R*-**4b**)	54±4 (1*R*,2*S*-**4b**)
5	>95 (n.d. **5b**)	n.d.	78 (n.d. **5b**)	>95 (n.d. **5b**)
	n.d.		>95 (*R*-**5c**)	>95 (*R*-**5c**)
	>95 (n.d. **5d**)		47 (n.d. **5d**)	n.d.
6	–	–	–	>95 (1*S*,2*S*,3*R*,5*S*-**6a**)
(*R*)-**7**	n.d.	n.d.	>98 (1*R*,5*S*-**7b**)	>98 (1*R*,5*S*-**7b**)

[a] Enantiomeric and diastereomeric excess values were determined by GC-FID or HPLC analyses using a chiral stationary phase. –: not detected, n.d.: not determined. Values for BDO M220A and the CDO NDT library variants are available in the Supporting Information.

stereoselectivities (< 80 % *ee*, Table 1 and Table 2) for several of the tested olefins. Especially the sterically demanding terpene **6** proved to be a challenging substrate for RO-catalyzed oxyfunctionalization and no conversion could be detected with NDO, BDO or CDO.

Although closely related (46–78 % similarity of the α-subunits containing the active site), we observed that the three enzymes displayed a strong bias with respect to substrate specificity as well as selectivity. Caused by variations in the active-site topology of NDO, BDO and CDO that influence enzyme characteristics, selectivities and activities for the different alkene types were strongly dependent on the RO employed. One of the most striking examples for differing selectivities was observed for the oxidation of **1**. NDO catalyzed the dihydroxylation exclusively at the terminal alkene C=C double bond as already reported in the literature.[16] In contrast, *cis*-dihydrodiol **1b** was formed along with alkene-1,2-diol **1a** with BDO and CDO as biocatalysts. While CDO showed excellent regioselectivity for the aromatic moiety of the substrate (0.3:99.7 **1a**:**1b**), a mix of **1a** and **1b** (59:41) was found for biotransformations employing BDO. All enzymes favored the (*R*)-enantiomer of **1a** with NDO having the highest stereoselectivity (80 % *ee*), however, *ee* values obtained for CDO (43 % *ee*) and BDO (8 % *ee*) were rather low.

As our emphasis has been placed on the selective oxyfunctionalization of alkenes, we were interested in the identification of amino acid residues essential for RO selectivity, enabling us to design efficient biocatalysts for olefin oxidation. Due to observed variations, we assumed that tested substrates might have different orientations in the active sites of CDO, NDO and BDO, caused by diversely shaped substrate binding pockets. Therefore, a comparison of the active sites of the three enzymes by structural and sequence alignments was performed. Examining all amino acids within 8 Å of the catalytic iron center, only those residues were considered for mutagenesis that differed between ROs in structurally equivalent positions. Interestingly, one of the selected residues, a methionine pointing into the active site, was not only present in CDO (M232) and BDO (M220), but also at the corresponding position of another member of this family, toluene dioxygenase from *P. putida* F1 (M220).[22] As all three enzymes catalyzed the dihydroxylation of the aromatic ring, we assumed this methionine residue to play a role in the positioning and orientation of **1** in the substrate binding pocket. We thus decided to replace methionine residues in BDO and CDO by the corresponding alanine found in NDO (Figure S22).

After altering their active site topology by site-directed mutagenesis, both RO variants showed indeed a dramatic shift in regioselectivity towards the terminal C=C double bond (Figure 1 and Table S2). For CDO M232A and BDO M220A, product formation of alkene-1,2-diol **1a** was increased to 92 % compared to 0.3 % and 59 % for CDO and BDO, respectively. Docking analyses supported our hypothesis of different substrate positions in the active sites of WT and mutant enzymes. Depending on the shape of the chiral binding pocket, either the aromatic ring (CDO WT) or the vinyl group (CDO M232A) of **1** is oriented close to the catalytic iron center (Figure S24). The change in regioselectivity was accompanied by a significant increase in stereospecificity. For CDO M232A, the *ee* for (*R*)-**1a** improved from 43 % to 95 %, giving the highest stereoselectivity reported so far for the dihydroxylation of a linear aliphatic substrate by ROs. Moreover, the single amino acid substitution of methionine to alanine also increased the stereoselectivity of BDO M220A for **1a** by about 8-fold to 67 %.

In addition to **1**, the panel of aliphatic compounds was re-examined with CDO M232A and BDO M220A variants.

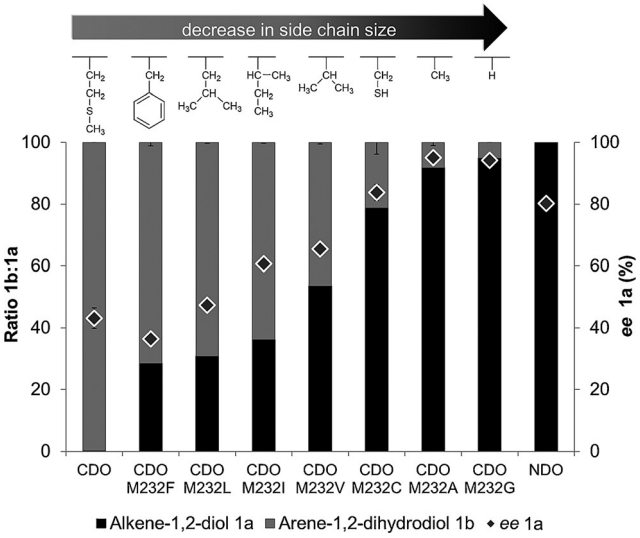


Figure 1. Product distribution for RO-catalyzed conversion of styrene **1** yielding alkene-1,2-diol **1a** (black) and arene-1,2-dihydrodiol **1b** (gray).

Compared to WT enzymes, mutants displayed not only altered selectivities but also changes in activity for several of the investigated compounds. Using CDO M232A as biocatalyst, conversions of **2**, **5** and (*R*)-**7** were enhanced significantly. Remarkably, CDO WT already catalyzed the oxidation of **2** with 44% product formation, even though it has been reported in literature that non-conjugated linear alkenes are generally found to be poor substrates for *cis*-dihydroxylation by ROs.[23] With CDO M232A, the conversion was increased additionally by about 2-fold, resulting in 93% product formation and an excellent stereoselectivity (>98% *ee*). Furthermore, the formation of **7b** in the CDO M232A-catalyzed allylic oxidation of (*R*)-**7** was improved from 46% to >99% while maintaining a *de* value >98% for (1*R*, 5*S*)-**7b**. Enhanced substrate oxidation up to 7-fold was also achieved for the linear polyalkene **5** by exchanging methionine to alanine in CDO M232A and BDO M220 A (Table 1 and Table S2).

Compared to minor changes in selectivity in the formation of diol **2a**, a significant alteration in stereospecificity was detected for CDO M232A and BDO M220A during oxyfunctionalization of **4**. The arene-substituted cyclic alkene was converted by WT and mutant ROs yielding diol **4b** and the monohydroxylated product **4a**. Interestingly, for both products CDO favored opposite enantiomers compared to NDO and BDO albeit only low selectivities were obtained for (*R*)-**4a** and (1*S*,2*R*)-**4b** (Table 2). The single amino acid substitution in the active site of CDO, however, reversed selectivity, yielding (*S*)-**4a** with an improved enantiomeric excess of 87%. Likewise, the increase in *ee* to 54% for diol **4b** was accompanied by a change in absolute configuration from (1*S*,2*R*)-**4b** to (1*R*,2*S*)-**4b**, turning CDO and CDO M232A into enantiocomplementary catalysts for the oxidation of **4**. An altered stereospecificity was also observed for the BDO variant M220A. Yet, improvement of the enantiomeric excess from 45% to 82% was only present for (*S*)-**4a** while the stereoselectivity for (1*R*, 2*S*)-**4b** decreased to 33% (Table S6).

Next to the alteration in selectivity and conversion, the amino acid exchange of methionine to alanine resulted in a CDO variant able to convert terpene **6**. A biocatalytic access to **6a** is of particular interest as osmium(VIII) oxide-catalyzed dihydroxylation of **6** requires elevated temperatures ≥70 °C.[24,25]

In CDO WT productive binding of the substrate might be sterically hindered due to a clash with the bulky methionine side chain at position 232 as biotransformations using CDO M232A gave 33% of **6a** with an excellent stereoselectivity of >95% *de* for the (1*S*,2*S*,3*R*,5*S*)-(+)-isomer.

Considering the strong impact on selectivity, the effect of other amino acid residues at position 232 was examined using the NDT codon degeneracy.[26] From the NDT mutant library, 11 CDO variants (CDO M232F, L, I, V, Y, H, N, D, C, S and G) were tested for conversions of **1** and **4** as changes in regio- and stereoselectivity were most pronounced with these substrates. For compound **1**, the amino acid side chain size at position 232 was shown to directly influence both the regio- and stereoselectivity. In general, smaller amino acid residues favored the formation of **1a** accompanied by increased stereoselectivities (Figure 1 and Figure S6). With substrate **4**, CDO M232H showed a 3-fold enhancement in *ee* to 28% (*R*)-**4a**, whereas a switch in stereoselectivity from (*R*)-**4a** to (*S*)-**4a** was observed for the other CDO variants compared to the WT. As demonstrated for **1a**, the stereoselectivity for (*S*)-**4a** generally increased with smaller amino acid residues (Figure S7). Furthermore, large side chains (F, L, I) gave (1*S*, 2*R*)-**4b** with up to 58% *ee* whereas variants CDO M232V, C, S, and G yielded (1*R*,2*S*)-**4b** (Figure S8).

To proof the applicability of ROs for organic synthesis, semi-preparative scale biotransformations (70 mg) of **6** and (*R*)-**7** were performed with mutant CDO M232A. Without further optimization of the reaction set-up, **6a** and **7b** were isolated in 33% and 38% yield, respectively (35% and 87% conversion), demonstrating that a single point-mutation was sufficient to transform WT CDO into an efficient catalyst.

In summary, we demonstrated that ROs can be evolved for the oxidation of a broad range of olefins. Introduction of a single point-mutation proved sufficient to generate BDO and CDO variants displaying remarkable changes in regio- and stereoselectivity for different aliphatic compounds. The effect of various amino acid residues at this position on selectivity highlights the potential for the engineering of ROs. Of all generated variants, especially CDO M232A gave excellent stereoselectivities (≥95%) and conversion rates >90% for linear alkenes, which have been reported to be challenging substrates for RO-catalyzed oxyfunctionalizations. Furthermore, we demonstrated the preparative oxidation of **6** and (*R*)-**7** yielding **6a** and **7b** in mg scale. We therefore suggest that ROs provide a biocatalytic approach to both the asymmetric *cis*-dihydroxylation and the regio- and stereospecific allylic monohydroxylation of various alkenes, also targeting products difficult to access by Sharpless AD or Riley oxidation.

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Keywords: asymmetric dihydroxylation · biocatalysis · *cis*-diols · protein engineering · rieske non-heme iron oxygenases

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[1] H. C. Kolb, M. S. Van Nieuwenhze, K. B. Sharpless, *Chem. Rev.* **1994**, *94*, 2483–2547.
[2] B. Plietker, M. Niggemann, *Org. Lett.* **2003**, *5*, 3353–3356.
[3] R. E. Parales, S. M. Resnick, *Biocatal. Pharm. Biotechnol. Ind.* **2006**, 299–331.
[4] A. Karlsson, J. V. Parales, R. E. Parales, D. T. Gibson, H. Eklund, S. Ramaswamy, *Science* **2003**, *299*, 1039–1042.
[5] A. K. Haritash, C. P. Kaushik, *J. Hazard. Mater.* **2009**, *169*, 1–15.

[6] R. E. Parales, K. S. Ju, *Compr. Biotechnol.* **2011**, 115 – 134.
[7] W. Wegst, U. Tittmann, J. Eberspächer, F. Lingens, *Biochem. J.* **1981**, *194*, 679 – 684.
[8] D. R. Boyd, G. N. Sheldrake, *Nat. Prod. Rep.* **1998**, *15*, 309 – 324.
[9] P. K. Sydor, S. M. Barry, O. M. Odulate, F. Barona-Gomez, S. W. Haynes, C. Corre, L. Song, G. L. Challis, *Nat. Chem.* **2011**, *3*, 388 – 392.
[10] J. Han, S.-Y. Kim, J. Jung, Y. Lim, J.-H. Ahn, S.-I. Kim, H.-G. Hur, *Appl. Environ. Microbiol.* **2005**, *71*, 5354 – 5361.
[11] D. J. C. Constable, P. J. Dunn, J. D. Hayler, G. R. Humphrey, J. L. Leazer, Jr., R. J. Linderman, K. Lorenz, J. Manley, B. A. Pearlman, A. Wells, A. Zaks, T. Y. Zhang, *Green Chem.* **2007**, *9*, 411 – 420.
[12] R. E. Parales, J. D. Haddock, *Curr. Opin. Biotechnol.* **2004**, *15*, 374 – 379.
[13] T. Hudlicky, H. Luna, G. Barbiera, L. D. Kwart, *J. Am. Chem. Soc.* **1988**, *110*, 4735 – 4741.
[14] N. I. Bowers, D. R. Boyd, N. D. Sharma, M. A. Kennedy, G. N. Sheldrake, H. Dalton, *Tetrahedron: Asymmetry* **1998**, *9*, 1831 – 1834.
[15] R. J. Swift, S. F. Carter, D. A. Widdowson, J. R. Mason, D. J. Leak, *Appl. Microbiol. Biotechnol.* **2001**, *55*, 721 – 726.
[16] K. Lee, D. T. Gibson, *J. Bacteriol.* **1996**, *178*, 3353 – 3356.
[17] D. T. Gibson, S. M. Resnick, K. Lee, J. M. Brand, D. S. Torok, L. P. Wackett, M. J. Schocken, B. E. Haigler, *J. Bacteriol.* **1995**, *177*, 2615 – 2621.
[18] W. Takami, T. Yoshida, H. Nojiri, H. Yamane, T. Omori, *J. Gen. Appl. Microbiol.* **1999**, *45*, 69 – 75.
[19] D. Boyd, D. Clarke, M. Cleij, J. T. G. Hamilton, G. N. Sheldrake, *Monatsh. Chem.* **2000**, *685*, 673 – 685.
[20] D. Xu, G. A. Crispino, K. B. Sharpless, *J. Am. Chem. Soc.* **1992**, *114*, 7570 – 7571.
[21] R. Granger, J. Passet, J. Girard, *Phytochemistry* **1972**, *11*, 2301 – 2305.
[22] T. Hudlicky, E. E. Boros, C. H. Boros, *Tetrahedron: Asymmetry* **1993**, *4*, 1365 – 1386.
[23] D. R. Boyd, N. D. Sharma, N. I. Bowers, I. N. Brannigan, M. R. Groocock, J. F. Malone, G. McConville, C. C. R. Allen, *Adv. Synth. Catal.* **2005**, *347*, 1081 – 1089.
[24] E. Erdik, D. Kâhya, T. Daşkapan, *Synth. Commun.* **1998**, *28*, 1 – 7.
[25] R. Ray, D. S. Matteson, *Tetrahedron Lett.* **1980**, *21*, 449 – 450.
[26] M. T. Reetz, D. Kahakeaw, R. Lohmer, *ChemBioChem* **2008**, *9*, 1797 – 1804.

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