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Enantioselective Oxidation of Aldehydes Catalyzed by Alcohol Dehydrogenase**

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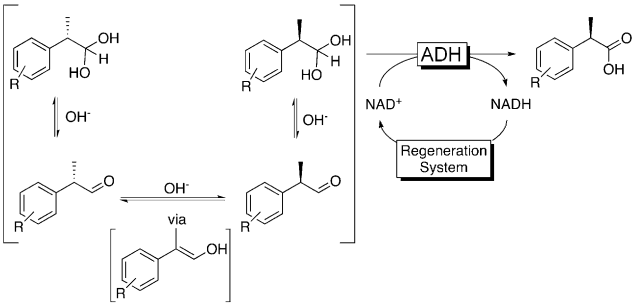
Alcohol dehydrogenases (ADH) are enjoying increasing interest as versatile and selective biocatalysts in the context of both academic research and industrial implementation.[1] The stereospecific reduction of prochiral ketones is an area of application most attractive to preparative organic chemists.[2] In contrast, the oxidation of alcohols is less popular because chirality is mostly destroyed rather than generated.[3] Furthermore, ADH-catalyzed oxidation is limited to the conversion of primary or secondary alcohols into the corresponding aldehydes and ketones. The few biocatalytic examples reporting on the complete oxidation of alcohols to the corresponding carboxylic acids focused on either whole cells[4] or the combination of an ADH with an aldehyde dehydrogenase (AldDH).[5] Simplifying the reaction scheme to ideally only one biocatalyst would greatly increase the preparative value of the biocatalytic synthesis of carboxylic acids from alcohols. In principle, the ADH-mediated oxidation of aldehydes is well-known as the dismutation activity of some ADHs.[6] Quite surprisingly, however, this reactivity has been largely considered a curiosity and preparative applications are, to the best of our knowledge, yet unknown.

Hence we became interested in evaluating the scope of the ADH-catalyzed oxidation of aldehydes yielding the corresponding carboxylic acids. As a model reaction we choose the oxidation of (racemic) profen aldehydes. Profens (2-methyl-arylpropionates), efficient nonsteroidal antiinflammatory drugs, are generally marketed as racemates. With the growing demand for enantiomerically pure, active pharmaceutical ingredients, routes to enantiomerically pure profens are being increasingly investigated.[7] To circumvent the intrinsic drawbacks of the kinetic resolution of racemic profens, enantioselective routes are gaining relevance.[8]

We envisaged an oxidative dynamic kinetic resolution (DKR) as shown in Scheme 1. Compared to the previously reported reductive DKRs of profen aldehydes yielding enantiopure profen alcohols[8a–c] this approach produces the desired product directly without further chemical oxidation.



Scheme 1. The oxidative dynamic kinetic resolution of profen aldehydes. ADH: alcohol dehydrogenase.

To evaluate whether aldehyde oxidation is a common activity amongst ADHs or rather an exception, we screened the proprietary c-LEcta ADH collection, comprising 70 ADHs from diverse origins recombinantly expressed in *Escherichia coli* for the oxidation of racemic 2-phenyl propionaldehyde.[9] Regeneration of catalytic amounts of $NAD(P)^+$ was achieved by using an H_2O-forming NAD(P)H-oxidase (NOX) from *Lactobacillus sanfanciscensis*.[10] To ensure efficient regeneration of $NAD(P)^+$ and to compensate for the comparably poor stability of this enzyme, it was applied at more than 100-fold excess (based on activity).[11] Out of the 70 ADHs screened, 9 showed significant accumulation of the desired acid (Table 1). Both *R*- and one *S*-specific ADHs were identified.[9] Notably, there was a significant background activity of the *E. coli* host enzymes with modest *R* selectivity. Possibly, endogeneous aldehyde dehydrogenases or ADHs exhibiting "dismutase activity" accounted for this.

In analogy to the generally accepted mechanism of ADH-catalyzed alcohol oxidation, we assumed that aldehyde oxidation proceeds via the aldehyde hydrate form of the aldehyde. Hence, we suspected an influence of pH on the rate and equilibrium of the aldehyde hydrate formation. Indeed, raising the pH generally increased the ratio of acid over alcohol formed (Table 1). Interestingly, however, the initial rates of the reactions were hardly influenced by the pH value,

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Table 1: Results from the initial ADH screening for the oxidative turnover of 2-phenyl propionaldehyde.[a]

Entry[b]	A_{spec} [U mL^{-1}][c]		Acid/alcohol[d]		*ee* [%][e]	
	pH 7	pH 9	pH 7	pH 9	pH 7	pH 9
R-specific ADHs						
1	0.1	0.04	0.6	1.6	72	53
2	0.7	0.7	3.6	5.3	50	47
3	0.6	0.7	3.9	7.5	46	49
4	0.4	0.2	2.4	9.6	37	47
5	0.7	0.2	2.1	2.4	23	21
6	0.7	0.6	1.9	2.8	81	81
7[f]	2.2	2.1	3.2	5.4	94	90
8	0.1	0.04	1.3	>10	39	45
S-specific ADHs						
9[g]	0.4	0.2	>10	>10	77	46
control[h]	0.02	0.04	>10	>10	45 (*R*)	40 (*R*)

[a] Conditions: 50 mM Tris/HCl buffer (30 °C, pH 7 or pH 9, 2 mM $MgCl_2$); 1% (v/v) DMSO; c(2-phenylpropionaldehyde)$_0$ = 5 mM, $c(NAD^+) = c(NADP^+)$ = 1 mM, crude NADH oxidase (NOX) extract. [b] Each entry represents a different ADH recombinantly expressed in *E. coli*. [c] Specific activity of crude cell extracts determined after 3 h. [d] For values exceeding 10, only the acid was detected by HPLC analysis. [e] *ee* value of the acid (2-phenyl propionic acid) was determined by HPLC on a chiral stationary phase. [f] Corresponds to *E. coli* ADH dkgB (2,5-diketo-D-gluconate reductase B, GenBank: BAL37507.1). [g] For the sequence of ADH-9 please refer to the Supporting Information. [h] *E. coli* cells containing the empty expression vector under otherwise identical conditions.

which was unexpected considering the general preference of ADHs for alkaline media in oxidative direction.[12] Possibly, this trend is counteracted by the decreasing NOX activity under alkaline conditions.[9,10] Nevertheless, NMR experiments corroborated that already at neutral pH the concentration of the aldehyde hydrate of 2-phenyl propionaldehyde was sufficiently high.[9]

Encouraged by these results, we further investigated ADH-9. A protein-engineering campaign was initiated to increase the activity and enantioselectivity of ADH-9, resulting in an improved variant (ADH-9V1, which was also purified).[9,13] To further understand the mechanistic origin for acid selectivity we compared the Michaelis–Menten parameters of ADH-1 (exhibiting the lowest acid selectivity) with those of ADH-9 (exhibiting high acid selectivity) and ADH-9V1 (Table 2). The most striking difference between ADH-9 and the other enzymes investigated lies in the very poor affinity towards the reduced nicotinamide cofactor (NADH), which delivers a kinetic argument for the higher acid selectivity of ADH-9 relative to that of ADH-1 and ADH-9V1, respectively.

Table 2: Comparison of the kinetic parameters of (un)selective ADHs.[a]

	K_M [mM]		Selectivity[b]
	NAD^+	NADH	
ADH-1	0.059	0.054	0.6
ADH-9	0.26	13.3	4.55
ADH-9-V1	0.12	0.37	1.38

[a] Conditions: 50 mM Tris/HCl buffer (30 °C, pH 7, 2 mM $MgCl_2$); 20% (v/v) DMSO; c(2-phenylpropionaldehyde)$_0$ = 5 mM, $c(NAD^+)$ = 1.5 mM. [b] Selectivity = [acid]/[alcohol] (Table 1).

We hypothesize that the ratio of $K_M(NAD^+)$ over K_M(NADH) could represent a simple diagnostic value to predict the suitability of an ADH for aldehyde oxidation. Further investigations to support this are currently underway.

However, even if the chemoselectivity of a given ADH is not exclusive, application of an efficient $NAD(P)^+$ regeneration system may be suitable to shift the disproportionation reaction into the desired oxidation direction. To validate this hypothesis we compared the conversion of 2-phenylpropionaldehyde with ADH-9 and ADH-9V1 (the latter as a crude extract as well as a purified enzyme, to exclude potential contributions of *E. coli* proteins) in the presence and absence of a NAD^+ regeneration system (Figure 1). In the absence of a NAD^+ regeneration system the "natural" chemoselectivity of the ADHs resulted in the formation of trace amounts of alcohol in the case of ADH-9 and an almost equimolar ratio of acid and alcohol in the case of ADH-9V1 (both as a crude extract and a purified enzyme). If however, under the same conditions NOX was added for cofactor regeneration, only trace amounts of alcohol were observed after a reaction time of 40 h resulting in almost exclusive acid selectivity with both enzymes. Hence, we conclude that even an ADH with "unfavorable" acid selectivity can be used for the oxidation of aldehydes, provided efficient NAD^+ regeneration is performed.

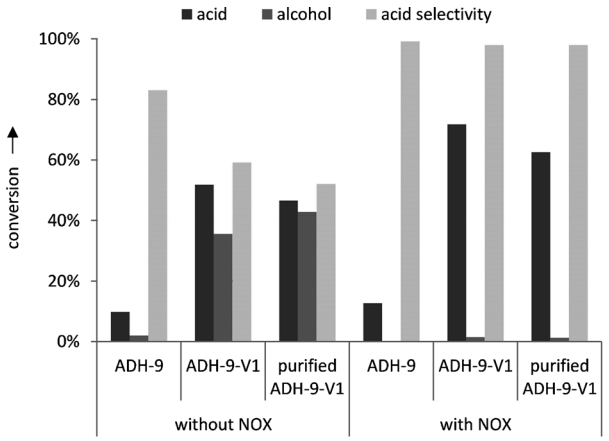


Figure 1. Comparison of acid/alcohol selectivity of ADH-9 and ADH-9V1 in the absence (left) and presence (right) of an NAD^+ regeneration system. Conditions: 50 mM Tris/HCl buffer (30 °C, pH 7, 2 mM $MgCl_2$); 20% (v/v) DMSO; T = 30 °C; c(2-phenylpropionaldehyde)$_0$ = 5 mM, $c(NAD^+)$ = 1 mM, c(ADH) = c(NOX) = 1 mg mL^{-1}; t = 40 h.

A typical time-course for the conversion of racemic 2-phenylpropionaldehyde using ADH-9V1 is given in Figure 2. The starting aldehyde was consumed within the first 5 h yielding the desired (*S*)-2-phenylpropionic acid in greater than 90% yield and 88% *ee*. Trace amounts of 2-phenylpropionyl alcohol formed at the beginning of the reaction (<10% conversion) were slowly consumed over time, presumably via the aldehyde to give the acid.

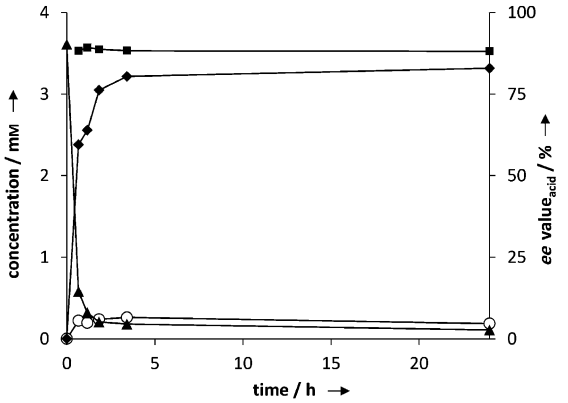
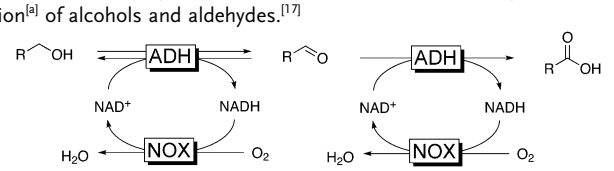
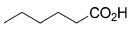
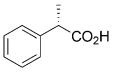
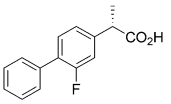


Figure 2. Typical time-course of the ADH-9-catalyzed DKR of 2-phenylpropionaldehyde. Conditions: see Figure 1. (◆): 2-phenylpropionic acid, (■): *ee* value; (○): 2-phenylpropionyl alcohol; (▲): 2-phenylpropionaldehyde. The *ee* value of the product was 88% throughout the reaction.[17]

Table 3: Preliminary substrate scope of the ADH-9V1-catalyzed oxidation[a] of alcohols and aldehydes.[17]



Product	Product [mM] (conv. [%])[b] starting from alcohol	aldehyde
	1.4 ± 0.2 (73)	1.39 ± 0.01 (>99)
	1.36 ± 0.04 (70)	1.00 ± 0.06 (67)
	0.61 ± 0.03 (37)	2.28 ± 0.03 (95)
	0.39 ± 0.02 (9)	2.43 ± 0.09 (94)
	n.d.	0.72 ± 0.07 (65)

[a] Conditions: 50 mM Tris/HCl buffer (30°C, pH 7, 2 mM $MgCl_2$); 20% (v/v) DMSO; $T = 30$°C; c(2-phenylpropionaldehyde)$_0 = 5$ mM, $c(NAD^+) = 1$ mM, c(ADH) = 20 mg mL^{-1}, c(NOX) = 10 mg mL^{-1}, values given represent the average of two independent experiments each; $t = 21$ h. [b] Conversion calculated based on the concentrations determined after 21 h. [c] *ee* value depended on the biocatalyst concentration.[9] [d] *ee* value of the acid: 56% *ee*. n.d. = not determined.

The yield and optical purity of the acid product clearly corroborate the assumed dynamic kinetic resolution of the racemic starting material (Scheme 1). Interestingly, when we used purified ADH-9V1 we observed an inverse correlation between the enantiomeric purity of the acid product and the catalyst concentration applied.[9,14] Hence, lowering the concentration of purified ADH-9V1 from 20 to 0.5 mg mL^{-1} (i.e. decreasing the initial acid formation rate from 7.4 to 0.19 mM h^{-1}) increased the *ee* value of the product from 52 to 97% *ee*. We attribute this to a comparably low racemization rate of the aldehyde under the given reaction conditions as had also been determined previously by Galletti et al.[8b] Hence the oxidation rate even of the nonpreferred enantiomer exceedsthe rate of racemization. Potential solutions such as lowering the pH value of the reaction mixture as well as application of resins catalyzing the racemization[15] are currently under investigation.

Next, we investigated the scope of this oxidation approach. Starting from the corresponding alcohol it could represent an interesting biocatalytic alternative to "classical" chemical oxidations, particularly considering that oxygen serves as terminal electron acceptor producing water as the sole by-product. Table 3 demonstrates that both the oxidative dynamic kinetic resolution of aldehydes as well as the "through oxidation" starting from primary alcohols is feasible with a broad range of substrates.

Finally, it should be mentioned here that the current setup was somewhat limited by the poor overlap of operational windows of the production enzyme (ADH) and regeneration enzyme (NOX) as well as by the poor stability of NOX under the reaction conditions.[9] Applying a substrate-coupled approach would significantly simplify the reaction system to only one biocatalyst. Hence, we also tested the oxidative DKR of flurbiprofen aldehyde using acetone as a sacrificial cosubstrate. Gratifyingly, also this setup was successful for the chemoselective conversion using ADH-9. Already in the presence of one equivalent of acetone more than 90% of the flurbiprofen aldehyde was transformed into the acid, whereas in the presence of two equivalents the conversion was complete.[9] In any case, the corresponding alcohol was not detected. ADH-catalyzed Oppenauer oxidations, unless performed using special cosubstrates[16] are hampered by the unfavorable equilibrium of the reaction. Thus, significant molar surpluses are generally applied to shift the equilibrium. This was not the case in the aldehyde oxidation proposed here, which we attribute to the thermodynamic and kinetic irreversibility of the acid formation. Performing the oxidation of, for example, flurbiprofenaldehyde on a preparative (1 g) scale was successful, although the modest yield of the reaction (46%) implies that significant improvements can be made in the experimental procedure. Especially the high reactivity and poor solubility of the aldehyde starting materials need to be addressed. Experiments further exploring the scope of this highly simplified reaction setup are currently underway in our labs.

Overall, we report that also "old dogs" such as ADHs, widely used and well-characterized as catalysts for chemical redox chemistry, can be used for new tricks such as the proposed oxidative DKR. The results presented suggest that this activity may be present in a broad range of (commercially available) ADHs. Compared to the established methods, our approach is simple and has great potential for "greener" syntheses of, for example, profens.

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[1] a) K. Faber, *Biotransformations in Organic Chemistry*, 6th ed., Springer, Berlin, **2011**; b) K. Faber, S. M. Glueck, B. Seisser, W. Kroutil in *Enzyme Technologies: Metagenomics, Evolution, Biocatalysis and Biosynthesis* (Eds.: W. K. Yeh, H. C. Yang, J. R. McCarty), Wiley, Hoboken, **2010**, pp. 199 – 248; c) S. M. A. De Wildeman, T. Sonke, H. E. Schoemaker, O. May, *Acc. Chem. Res.* **2007**, *40*, 1260 – 1266; d) F. Hollmann, I. W. C. E. Arends, D. Holtmann, *Green Chem.* **2011**, *13*, 2285 – 2313; e) F. Hollmann, I. W. C. E. Arends, K. Buehler, A. Schallmey, B. Buhler, *Green Chem.* **2011**, *13*, 226 – 265; f) S. Wenda, S. Illner, A. Mell, U. Kragl, *Green Chem.* **2011**, *13*, 3007 – 3047; g) A. Liese, K. Seelbach, C. Wandrey, *Industrial Biotransformations*, Wiley-VCH, Weinheim, **2006**.

[2] a) J. Liang, E. Mundorff, R. Voladri, S. Jenne, L. Gilson, A. Conway, A. Krebber, J. Wong, G. Huisman, S. Truesdell, J. Lalonde, *Org. Process Res. Dev.* **2010**, *14*, 188 – 192; b) J. Liang, J. Lalonde, B. Borup, V. Mitchell, E. Mundorff, N. Trinh, D. A. Kochrekar, R. N. Cherat, G. G. Pai, *Org. Process Res. Dev.* **2010**, *14*, 193 – 198; c) G. W. Huisman, J. Liang, A. Krebber, *Curr. Opin. Chem. Biol.* **2010**, *14*, 122 – 129.

[3] a) T. Orbegozo, I. Lavandera, W. M. F. Fabian, B. Mautner, J. G. de Vries, W. Kroutil, *Tetrahedron* **2009**, *65*, 6805 – 6809; b) C. V. Voss, C. C. Gruber, W. Kroutil, *Angew. Chem.* **2008**, *120*, 753 – 757; *Angew. Chem. Int. Ed.* **2008**, *47*, 741 – 745; c) C. V. Voss, C. C. Gruber, K. Faber, T. Knaus, P. Macheroux, W. Kroutil, *J. Am. Chem. Soc.* **2008**, *130*, 13969 – 13972; d) I. Lavandera, A. Kern, V. Resch, B. Ferreira-Silva, A. Glieder, W. M. F. Fabian, S. de Wildeman, W. Kroutil, *Org. Lett.* **2008**, *10*, 2155 – 2158; e) W. Kroutil, H. Mang, K. Edegger, K. Faber, *Curr. Opin. Chem. Biol.* **2004**, *8*, 120 – 126; f) W. Kroutil, H. Mang, K. Edegger, K. Faber, *Adv. Synth. Catal.* **2004**, *346*, 125 – 142.

[4] a) A. V. Presečki, D. Vasić-Rački, *Proc. Biochem.* **2009**, *44*, 54 – 61; b) R. Gandolfi, K. Cavenago, R. Gualandris, J. V. Sinisterra Gago, F. Molinari, *Proc. Biochem.* **2004**, *39*, 749 – 753; c) F. Molinari, R. Gandolfi, R. Villa, E. Urban, A. Kiener, *Tetrahedron: Asymmetry* **2003**, *14*, 2041 – 2043; d) R. Villa, A. Romano, R. Gandolfi, J. V. Sinisterra Gago, F. Molinari, *Tetrahedron Lett.* **2002**, *43*, 6059 – 6061; e) A. Romano, R. Gandolfi, P. Nitti, M. Rollini, F. Molinari, *J. Mol. Catal. B* **2002**, *17*, 235 – 240; f) R. Gandolfi, A. Borrometi, A. Romano, J. V. Sinisterra Gago, F. Molinari, *Tetrahedron: Asymmetry* **2002**, *13*, 2345 – 2349; g) R. Gandolfi, N. Ferrara, F. Molinari, *Tetrahedron Lett.* **2001**, *42*, 513 – 514.

[5] a) J. Hirano, K. Miyamoto, H. Ohta, *Appl. Microbiol. Biotechnol.* **2008**, *80*, 71 – 78; b) C. H. Wong, J. R. Matos, *J. Org. Chem.* **1985**, *50*, 1992 – 1994.

[6] L. P. Olson, J. Luo, O. Almarsson, T. C. Bruice, *Biochemistry* **1996**, *35*, 9782 – 9791.

[7] a) K. Velonia, I. Smonou, *J. Chem. Soc. Perkin Trans. 1* **2000**, 2283 – 2287; b) R. H. Abeles, H. A. Lee, *J. Biol. Chem.* **1960**, *235*, 1499 – 1503; c) G. T. M. Henehan, S. H. Chang, N. J. Oppenheimer, *Biochemistry* **1995**, *34*, 12294 – 12301; d) G. T. M. Henehan, N. J. Oppenheimer, *Biochemistry* **1993**, *32*, 735 – 738; e) S. Trivić, V. Leskova, G. W. Winston, *Biotechnol. Lett.* **1999**, *21*, 231 – 234; f) B. Mee, D. Kelleher, J. Frias, R. Malone, K. F. Tipton, G. T. M. Henehan, H. J. Windle, *FEBS J.* **2005**, *272*, 1255 – 1264; g) V. Höllrigl, F. Hollmann, A. Kleeb, K. Buehler, A. Schmid, *Appl. Microbiol. Biotechnol.* **2008**, *81*, 263 – 273; h) R. Kourist, P. Dominguez de Maria, K. Miyamoto, *Green Chem.* **2011**, *13*, 2607 – 2618.

[8] a) J. A. Friest, Y. Maezato, S. Broussy, P. Blum, D. B. Berkowitz, *J. Am. Chem. Soc.* **2010**, *132*, 5930 – 5931; b) P. Galletti, E. Emer, G. Gucciardo, A. Quintavalla, M. Pori, D. Giacomini, *Org. Biomol. Chem.* **2010**, *8*, 4117 – 4123; c) D. Giacomini, P. Galletti, A. Quintavalla, G. Gucciardo, F. Paradisi, *Chem. Commun.* **2007**, 4038 – 4040; d) J. Pietruszka, M. Schölzel, *Adv. Synth. Catal.* **2012**, *354*, 751 – 756.

[9] See the Supporting Information.

[10] a) B. R. Riebel, P. Gibbs, W. Wellborn, A. Bommarius, *Adv. Synth. Catal.* **2003**, *345*, 707 – 712; b) B. R. Riebel, P. R. Gibbs, W. B. Wellborn, A. S. Bommarius, *Adv. Synth. Catal.* **2002**, *344*, 1156 – 1168.

[11] Typically, ADHs and NOX were applied in comparable (mass-based) amounts. Based on activity this corresponds to an approximate 1000-fold excess of NAD^+-regeneration activity over aldehyde oxidation activity. Please note: the specific activity of ADH-9V1 on, for example, 2-phenyl propionaldehyde was 6.2 $U\,g^{-1}$, whereas the specific NADH oxidation activity of the NOX preparation was 4.5 $U\,mg^{-1}$.

[12] A. Chang, M. Scheer, A. Grote, I. Schomburg, D. Schomburg, *Nucleic Acids Res.* **2009**, *37*, D588 – D592.

[13] a) M. T. Reetz, *Angew. Chem.* **2011**, *123*, 144 – 182; *Angew. Chem. Int. Ed.* **2011**, *50*, 138 – 174; b) M. T. Reetz, S. Prasad, J. D. Carballeira, Y. Gumulya, M. Bocola, *J. Am. Chem. Soc.* **2010**, *132*, 9144 – 9152; c) M. T. Reetz, J. D. Carballeira, *Nat. Protoc.* **2007**, *2*, 891 – 903; d) M. T. Reetz, M. Bocola, J. D. Carballeira, D. Zha, A. Vogel, *Angew. Chem.* **2005**, *117*, 4264 – 4268; *Angew. Chem. Int. Ed.* **2005**, *44*, 4192 – 4196.

[14] M. Kitamura, M. Tokunaga, R. Noyori, *Tetrahedron* **1993**, *49*, 1853 – 1860.

[15] a) V. Alphand, R. Wohlgemuth, *Curr. Org. Chem.* **2011**, *14*, 1928 – 1965; b) N. Berezina, V. Alphand, R. Furstoss, *Tetrahedron: Asymmetry* **2002**, *13*, 1953 – 1955.

[16] a) T. Orbegozo, J. G. d. Vries, W. Kroutil, *Eur. J. Org. Chem.* **2010**, 3445 – 3448; b) F. Bisogno, E. García-Urdiales, H. Valdés, I. Lavandera, W. Kroutil, D. Suárez, V. Gotor, *Chem. Eur. J.* **2010**, *16*, 11012 – 11019; c) J. H. Schrittwieser, I. Lavandera, B. Seisser, B. Mautner, J. H. L. Spelberg, W. Kroutil, *Tetrahedron: Asymmetry* **2009**, *20*, 483 – 488.

[17] Please note that under the conditions applied in this contribution, mass balances of approximately 60% have been achieved. We attribute this to the adsorption of the hydrophobic (profen) aldehydes to the walls of the reaction vessels, as corroborated by suitable control experiments. Also it should be mentioned here that side products were not detected in any of the experiments reported here.

