DOI: 10.1002/adsc.200700057

Branched-Chain Keto Acid Decarboxylase from *Lactococcus lactis* (KdcA), a Valuable Thiamine Diphosphate-Dependent Enzyme for Asymmetric C–C Bond Formation

Dörte Gocke,[a] Cong Luan Nguyen,[a] Martina Pohl,[a,c,*] Thomas Stillger,[b] Lydia Walter,[b] and Michael Müller[b]

[a] Institute of Molecular Enzyme Technology, Heinrich-Heine University of Düsseldorf, Research Center Jülich, 52426 Jülich, Germany
Fax: (+49)-2461-61-2490; e-mail: ma.pohl@fz-juelich.de
[b] Institute of Pharmaceutical Sciences, Albert-Ludwigs-University of Freiburg, Albertstr. 25, 79104 Freiburg, Germany
Fax: (+49)-761-203-6351
[c] Both groups contributed equally to the publication

Received: January 24, 2007; Revised: April 3, 2007

Supporting information for this article is available on the WWW under http://asc.wiley-vch.de/home/.

Abstract: The thiamine diphosphate-dependent, branched-chain 2-keto acid decarboxylase from *Lactococcus lactis* sup. *cremoris* B1157 (KdcA) is a new valuable enzyme for the synthesis of chiral 2-hydroxy ketones. The gene was cloned and the enzyme was expressed as an N-terminal hexahistidine fusion protein in *Escherichia coli*. It has a broad substrate range for the decarboxylation reaction including linear and branched-chain aliphatic and aromatic keto acids as well as phenyl pyruvate and indole-3-pyruvate. The dimeric structure of recombinant KdcA is in contrast to the tetrameric structure of other 2-keto acid decarboxylases. The enzyme is stable between pH 5 and 7 with a pH optimum of pH 6–7 for the decarboxylation reaction. While KdcA is sufficiently stable up to 40 °C it rapidly looses activity at higher temperatures. In this work the carboligase activity of KdcA is demonstrated for the first time. The enzyme shows an exceptionally broad substrate range and, most strikingly, it catalyzes the carboligation of different aromatic aldehydes as well as CH-acidic aldehydes such as phenylacetaldehyde and indole-3-acetaldehyde with aliphatic aldehydes such as acetaldehyde, propanal, and cyclopropanecarbaldehyde, yielding chiral 2-hydroxy ketones in high enantiomeric excess. Noteworthy, the donor-acceptor selectivity is strongly influenced by the nature of the respective substrate combination.

Keywords: benzaldehyde lyase; benzoylformate decarboxylase; bioorganic chemistry; biotransformations; C–C coupling; pyruvate decarboxylase

Introduction

Thiamine diphosphate-dependent enzymes such as pyruvate decarboxylase (PDC, E.C. 4.1.1.1), benzoylformate decarboxylase (BFD, E.C. 4.1.1.7) and benzaldehyde lyase (BAL, E.C. 4.1.2.38) have been intensively studied with respect to their carboligation activity, offering an easy access to chiral 2-hydroxy ketones from aldehydes.[1–8] In order to enlarge the range of accessible 2-hydroxy ketones, we studied the carboligase properties of a recently described branched-chain keto acid decarboxylase (E.C. 4.1.1.72). Two highly homologous enzymes have been found in different *Lactococcus lactis* strains: *Lactococcus lactis* sup. *cremoris* B1157[9,10] (KdcA) and *Lactococcus lactis* IFPL730[11] (Kivd). These enzymes are involved in the process of cheese ripening due to their decarboxylation activity of 2-keto acids which are formed through transamination of the corresponding branched-chain amino acids.[9] Recently the substrate binding site of KdcA has been probed by site-directed mutagenesis studies based on a homology model using the structure of pyruvate decarboxylase from *Zymomonas mobilis* as a template.[12]

In this study we investigated the carboligase properties of KdcA for the first time. Compared to other decarboxylases, KdcA has a broader substrate range and accepts besides acetaldehyde several aliphatic aldehydes such as propanal, butanal, isobutyraldehyde and cyclopropanecarbaldehyde as donor and/or acceptor in the enzyme-catalyzed acyloin condensation. Moreover, KdcA catalyzes the carboligation of eno-

lizable CH-acidic aldehydes such as indole-3-acetaldehyde and phenylacetaldehyde. The substrate range of the decarboxylase and the carboligase reaction, the pH and temperature-dependent stability and activity, as well as the stereoselectivity of KdcA are reported.

Results and Discussion

Cloning, Overexpression and Purification

The coding gene (*kdca*, gene bank CAG34226) was cloned and overexpressed in *E. coli* as an N-terminal hexahistidine fusion protein. Addition of the His-tag led to an N-terminal elongation of the protein by 23 amino acids: MGSS**HHHHHH**SSGLVPRGSHMAS.

A 15-L fed-batch fermentation of the recombinant *E. coli* strain resulted in 720 kU (decarboxylase activity) KdcA in 1.2 kg cells. The enzyme was purified by immobilized metal chelate chromatography yielding 1.7 g KdcA from 1.2 kg cells.

Determination of the Native Molecular Weight

The native molecular weight of the recombinant KdcA was determined by size-exclusion chromatography to afford a molecular weight of about 146 kDa (for log Mr/Kav plot see Supporting Information). As the calculated size of the monomeric subunit is 63.337 kDa, the observed data correlate best with a dimeric structure of KdcA in the native state. This is in contrast to other tetrameric 2-keto acid decarboxylases, such as PDC[13,14] and BFD.[15] However, other ThDP-dependent enzymes are known to be active as a dimer, such as acetohydroxy acid synthase (AHAS)[16] and transketolase (TK).[17] Meanwhile the three-dimensional structure of KdcA has been determined proving the dimeric structure of the enzyme (unpublished results).

Decarboxylase Activity

The investigation of the substrate range of decarboxylation is of interest to deduce information about the acyl donor spectrum for carboligase activity of KdcA. If a respective 2-keto acid is a substrate for the enzyme, the binding of the corresponding aldehyde to the C-2 atom of ThDP located in the active center is most likely. In the case of carboligation the ThDP-bound aldehyde is ligated to a second acceptor aldehyde molecule. Therefore, the aldehydes which are products of the decarboxylation reaction can, in principle, be used at least as acyl donors for the carboligation reaction.

KdcA has an exceptionally broad substrate range for the decarboxylation reaction as determined by Smit et al.[10] and Yep et al.[12] (Table 1). In contrast to other decarboxylases like PDC or BFD, KdcA accepts different aliphatic branched-chain as well as enolizable 2-keto acids such as phenyl pyruvate and indole 3-pyruvate. Although the natural branched-chain aliphatic substrate **1** as well as its analogue **2** (Table 1)

Table 1. Substrate range and kinetic data of the decarboxylation reaction.[a] Data are compared with the literature: Smit et al. used crude extracts of KdcA without any tag, Yep et al. used a purified variant with a C-terminal hexahistidine tag and own data were obtained with a purified variant containing an N-terminal hexahistidine tag.

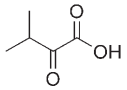
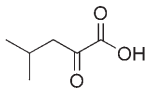
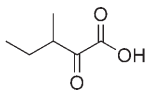
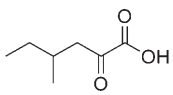
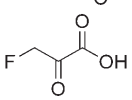
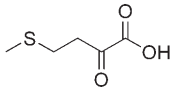
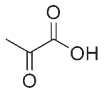
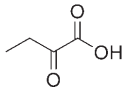
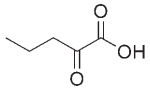
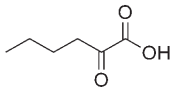
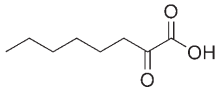
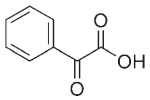
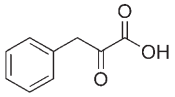
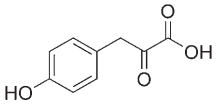
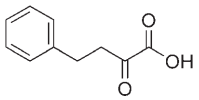
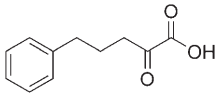
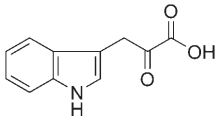
2-Keto acid		Relative activity [%]: own data compared to [Smit et al.][10] (Yep et al.)[12]	Kinetic data: own data (Yep et al.)[12] V_{max} [U/mg]	K_M [mM]
1		100 [100] (100)	181.64 ± 0.21 (47.3)	5.02 ± 0.21 (2.8)
2		26.3 [31] (100)	34.3 ± 0.2 (48.2)	0.264 ± 0.011 (3.7)
3		38.1 [27.8]	n.d.	n.d.
4		19	n.d.	n.d.
5		0	n.d.	n.d.

Table 1. (Continued)

2-Keto acid		Relative activity [%]: own data compared to [Smit et al.][10] (Yep et al.)[12]	Kinetic data: own data (Yep et al.)[12] V_{max} [U/mg]	K_M [mM]
6		(18)	n.d. (8.6)	n.d. (1.3)
7		1.2 [1.3]	3.67 ± 0.31	29.77 ± 6.44
8		9.3 [7.3]	n.d.	n.d.
9		15 [19] (20.6)	n.d. (9.8)	n.d. (1.3)
10		13 [25.3] (26.5)	n.d. (12.8)	n.d. (0.6)
11		0	n.d.	n.d.
12		8.4 (15.2)	n.d. (7.2)	n.d. (7.5)
13		8.6 [7] (56.3)	15.69 ± 0.21 (26.6)	0.127 ± 0.007 (0.21)
14		(6)	n.d. (2.9)	n.d. (0.63)
15		1.6	n.d.	n.d.
16		1.1	n.d.	n.d.
17		0.85 [3.5]	1.55 ± 0.6	0.234 ± 0.024

[a] *Conditions:* Activity towards different 2-ketoacids was measured using the coupled continuous assay. All substrates were applied in a concentration of 30 mM, except **17**, which was applied with 1 mM. Relative activities for **2**, **13**, and **17** have been calculated based on the maximal velocities.

show the highest reaction velocities, the K_M values are significantly higher than those of linear aliphatic 2-keto acids, phenyl pyruvate, and indole-3-pyruvate.

Kinetic constants were determined for the physiological substrate 3-methyl-2-oxobutanoic acid (**1**), 4-methyl-2-oxopentanoic acid (**2**) as well as phenyl pyruvate (**13**) and indole-3-pyruvate (**17**) (Table 1). With substrates **1** and **2** hyperbolic v/[S] plots have been observed up to 50 mM. With phenyl pyruvate the v/[S] plot is hyperbolic up to 12 mM (Figure 1 a) followed by a progressive decay of activity up to 30 mM and a subsequent rapid complete loss of activity (not shown). In accordance with the observation of Yep et al.[12] KdcA exhibits an extremely low K_M value for phenyl pyruvate (0.13 mM), which is about 40-times lower compared to the natural substrate **1**. However,

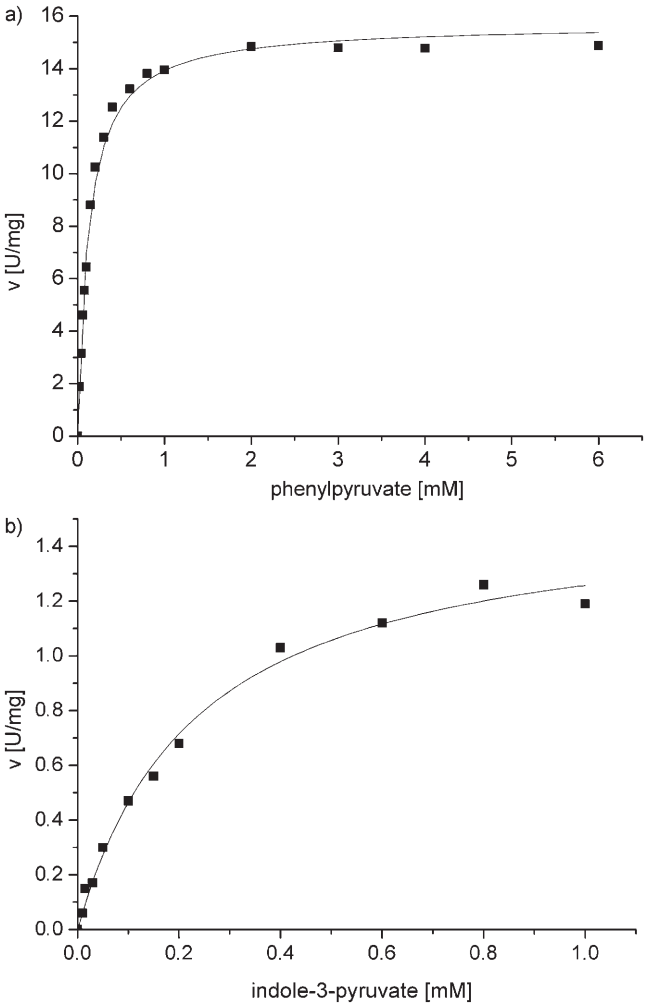


Figure 1. v/[S]-plot of the KdcA-catalyzed decarboxylation of (*top*) phenyl pyruvate (**13**) and (*bottom*) indole-3-pyruvate (**17**). Buffer: 50 mM potassium phosphate, 2.5 mM $MgSO_4$, 0.1 mM ThDP, pH 6.8, 30 °C. Data have been obtained in triplicate using the coupled decarboxylase assay.

the maximal velocity in the presence of phenyl pyruvate is only about 9 % of the velocity obtained with **1**. Our data correlate well with those obtained by Smit et al.[10] who investigated KdcA without any hexahistidine tag (Table 1). However, we could not reproduce the high relative activity of 56.3 % towards phenyl pyruvate compared to the natural substrate **1** which was found by Yep et al. with a KdcA-variant carrying a C-terminal hexahistidine tag (Table 1).

In the case of indole-3-pyruvate kinetic investigation has been hampered by the low solubility (about 1.3 mM) and the strong absorbance of the substrate in aqueous buffer. However, as the K_M value is also very low (0.23 mM), saturation is nearly achieved at 1 mM (Figure 1 b). Compared to 3-methyl-2-oxobutanoic acid (**1**) the KdcA shows 0.85 % relative activity with indole-3-pyruvate.

Optimal Cofactor Concentration

As in all ThDP-dependent enzymes the cofactors in KdcA are bound non-covalently to the active site. The main contribution to the binding arise from the coordinative interaction of the diphosphate moiety *via* Mg^{2+} to the protein as well as from hydrophobic and ionic interactions between protein site chains and the thiazole and pyrimidine rings of ThDP.

For stability of the holoenzyme most ThDP-dependent enzymes require the addition of cofactors to the buffer. In case of KdcA the addition of 2.5 mM $MgSO_4$ and 0.1 mM ThDP to the buffer is sufficient to keep the enzyme stable and active.

pH-Dependent Activity and Stability

KdcA shows a pH optimum of pH 6–7 for the decarboxylation of **1** (Figure 2). This activity optimum overlaps very well with the stability optimum of KdcA in potassium phosphate buffer, where no loss of activity can be detected at pH 5–7 within 60 h. Rapid inactivation occurs at pH 4 (< 2 h) and slower inactivation at pH 8 (half-life: 40 h).

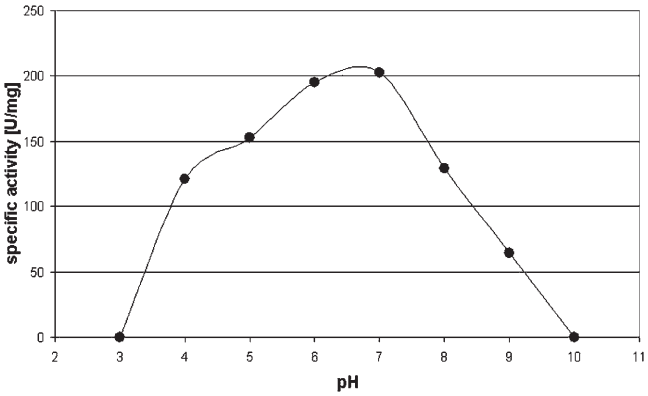


Figure 2. Determination of the pH optimum of the KdcA-catalyzed decarboxylation of 3-methyl-2-oxo-butanoic acid (**1**). Data were obtained using the coupled decarboxylase assay.

Temperature Optimum for Activity and Stability

Under initial rate conditions (90 sec), the temperature optimum was observed at 50 °C. Further increases in temperature resulted in a fast decay of activity with a midpoint of thermal inactivation (Tm) at about 62 °C (Figure 3).

From these data, the activation energy of the decarboxylase reaction was calculated from a $\ln V_{max}/[1/T]$ plot in the range of 25–40 °C as 8.5 kJ mol^{-1}, which is relatively low compared to other 2-keto acid decarboxylases. For benzoyl formate decarboxylase from

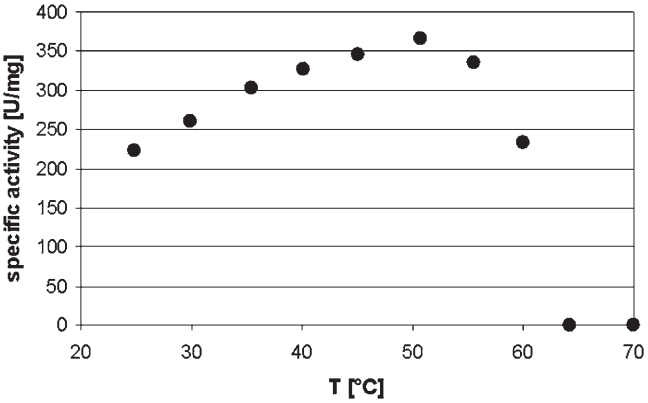


Figure 3. Determination of the temperature optimum and the midpoint of thermal inactivation of the KdcA-catalyzed decarboxylation of 3-methyl-2-oxobutanoic (**1**). Data were obtained using the direct decarboxylase assay.

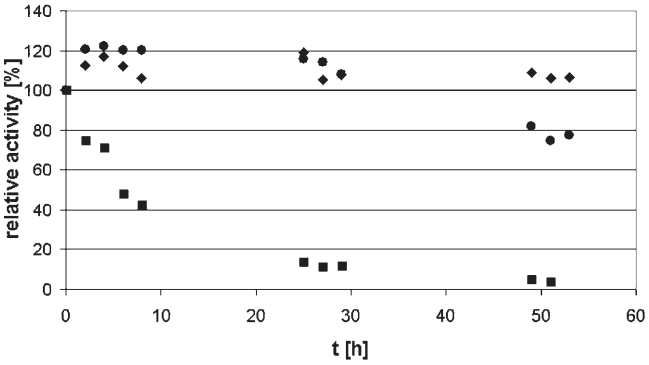


Figure 4. Stability of KdcA in 50 mM potassium phosphate buffer, pH 6.8, 2.5 mM $MgSO_4$, 0.1 mM ThDP (●), in the same buffer plus 20% (v/v) DMSO (◆) and in the same buffer plus 15% (v/v) PEG-400 (■). Activities were related to the starting activity in buffer without additives. The up to 20% higher activity in the case of (●) and (◆) after short time incubation is due to normal fluctuations in decarboxylase activity observed after solution of lyophilized enzyme.

Pseudomonas putida and for pyruvate decarboxylase from *Zymomonas mobilis* activation energies of 38 kJ mol^{-1}[18] and 43 kJ mol^{-1}, respectively, (unpublished) have been determined.

In order to optimize the conditions for the application of KdcA in enzymatic syntheses, the temperature stability of the enzyme in 50 mM potassium phosphate buffer, pH 6.8 in the presence of 2.5 mM $MgSO_4$ and 0.1 mM ThDP was determined. While the enzyme is sufficiently stable up to 40 °C (half-life: 80 h) it rapidly looses activity at higher temperatures (50 °C: half-life 9 h, 55 °C: half-life 4 h).

Organic Solvents

The biotransformation of aromatic aldehydes is often hampered by their low solubility in aqueous systems. Since the addition of either 20% (v/v) DMSO or 15% (v/v) PEG-400 has been successfully applied for BAL- and BFD-catalyzed carboligase reactions,[1,3,19,20] these organic solvents were tested with KdcA. The enzyme is completely stable in the presence of 20% (v/v) DMSO (half-life: 150 h), whereas it is rapidly inactivated in the presence of 15% (v/v) PEG-400 (half-life: 6 h) (Figure 4).

Carboligase Activity

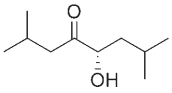
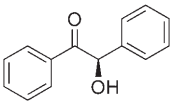
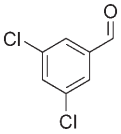
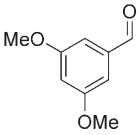
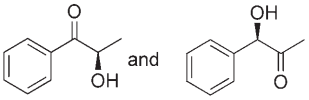
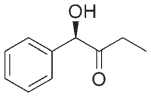
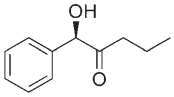
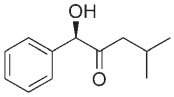
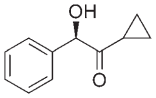
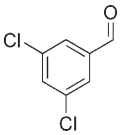
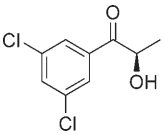
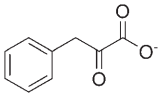
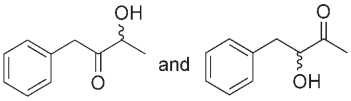
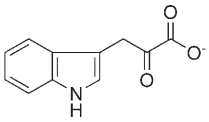
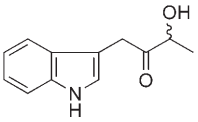
Carboligation of Aliphatic Aldehydes

Since KdcA is involved in the production of branched-chain aliphatic aldehydes, isovaleraldehyde (**18**) and isobutyraldehyde (**19**) were tested at first for carboligation (Table 2, entries 1 and 2). As summarized in Table 2, the enzyme catalyzes the self-carboligation of **18** with a low specific activity of 0.004 U/mg to give (*S*)-5-hydroxy-2,7-dimethyloctan-4-one (**27**) with an enantiomeric excess (*ee*) of 30–47%. BAL catalyzes this reaction with a specific activity of 0.05 U/mg resulting in the formation of (*R*)-**27** (*ee* 80%).[21,22] Thus, although the activity of KdcA is distinctly lower for this transformation it opens the way to both enantiomers of aliphatic acyloins.

Surprisingly, KdcA is not able to catalyze the self-carboligation of isobutyraldehyde (**19**) in detectable amounts, although **19** is the reaction product of the physiological decarboxylation of 3-methyl-2-oxobutanoic acid (**1**) and should therefore fit into the active center. We assumed an inhibition or inactivation of KdcA caused by isobutyraldehyde (**19**). In order to test this hypothesis the enzyme was incubated with increasing concentrations of **19** (5, 20, 40 mM) for 26 h and the decrease of activity of KdcA was followed by measuring the residual decarboxylase activity using the direct decarboxylase assay. The data presented in Figure 5 show a concentration-dependent inactivation of KdcA, which explains the negative results of the carboligation studies with this aldehyde.

To investigate the reversibility of the inactivation, isobutyraldehyde (**19**) was removed from the 40 mM sample after complete inactivation of KdcA using ultrafiltration. The restoration of activity was followed by measuring the decarboxylase activity of the enzyme. After 2 h 7% and after 16 h 30% of the original activity were recovered (data not shown), demonstrating that the inactivation of the enzyme is at least partially reversible. Furthermore, kinetic studies using the direct decarboxylase assay in the presence of 40 mM isobutyraldehyde resulted neither in a decay of V_{max} nor did isobutyraldehyde affect the K_M value for 3-methyl-2-oxobutanoic acid (**1**) (data not shown). We therefore conclude that the observed inactivation

Table 2. Results of KdcA-catalyzed carboligation reactions.

No.	Substrate A	Substrate B	Product(s)	Enantiomeric exess
1	**18** 	-	**27** 	30-47% (*S*)
2	**19** 	-	-	-
3	**20** 	-	**28** 	46% (*R*)
4	**21** 	-	**29** 	>98% (*R*)
5	**22** 	-	-	-
6	**23** 	-	-	-
7	**21** 	**20** 	**30a** and **30b** 	**30a**: (*R*) 93%; **30b**: (*R*) 92%; **30a**/**30b**: 60:40
8	**21** 	**24** 	**31b** 	>98% (*R*)[a]
9	**21** 	**25** 	**32b** 	96.5% (*R*)
10	**21** 	**18** 	**33b** 	88% (*R*)
11	**21** 	**26** 	**34b** 	98% (*R*)[a]
12	**22** 	**20** 	**35a** 	96.5% (*R*)
13	**13** 	**20** 	**36a** and **36b** 	*ee* n.d.; **36a**/**36b**: 80:20
14	**17** 	**20** 		*ee* n.d.

[a] Absolute configuration was determined by comparison of HPLC data and with regard to mechanistic aspects of KdcA catalysis; n.d. = not determined.

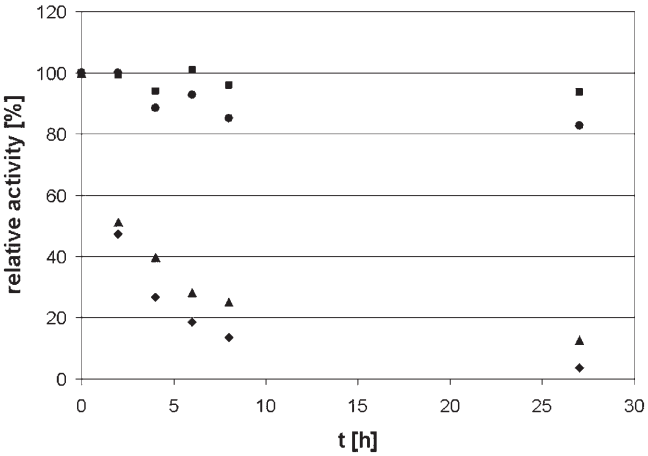


Figure 5. Inactivation of KdcA by isobutyraldehyde (**19**) (■: 0 mM, ●: 5 mM, ▲: 20 mM ◆: 40 mM). After the indicated time intervals 50 μL samples were withdrawn and residual activity was measured by the direct decarboxylase assay.

in the presence of isobutyraldehyde is a slow process and not detectable under initial rate conditions.

Besides the branched-chain aldehydes other aldehydes such as acetaldehyde, propanal and cyclopropanecarbaldehyde were tested as substrates for the carboligase reaction. In all cases carboligase products were observed by means of GC/MS. The specific activity for acetoin formation (0.052 U/mg) is 13-fold higher than the catalytic activity towards the formation of the branched-chain 2-hydroxyketone **27** (Table 2, entry 3). The (*R*)-enantiomer of acetoin (**28**) is formed predominantly (*ee* 46%) as proven by comparison with the product obtained from the PDC (*Saccharomyces cerevisiae*)-catalyzed reaction.

Carboligation of Aromatic Aldehydes

We could show that KdcA catalyzes the self-ligation of benzaldehyde (**21**) to (*R*)-benzoin (**29**) with high enantiomeric excess (>98%), whereas 3,5-dichlorobenzaldehyde (**22**) or 3,5-dimethoxybenzaldehyde (**23**) were not ligated to symmetric benzoin derivatives (Table 2, entries 4–6).

Mixed Carboligation of Aromatic and Aliphatic Aldehydes

As shown above KdcA accepts various aliphatic aldehydes as well as benzaldehyde as acyl donor and acceptor. However, it was unknown whether benzaldehyde derivatives like **22** or **23** could function as selective donors or acceptors for this enzyme. Therefore, we tested the catalytic activity of KdcA with various mixtures of aromatic and aliphatic aldehydes. As demonstrated in Table 2 (entry 7) the mixed carboligation of acetaldehyde and benzaldehyde resulted in the formation of nearly equal amounts of (*R*)-2-hydroxypropiophenone (*ee* 93%) (2-HPP, **30a**) and (*R*)-phenylacetyl carbinol (*ee* 92%) (PAC, **30b**) besides traces of (*R*)-benzoin (**29**) as was deduced from GC/MS, HPLC and NMR. The combination of benzaldehyde with larger aliphatic aldehydes gave remarkable results: the selectivity of KdcA shifted completely to the PAC derivatives **31b–34b**, if propanal (**24**), butanal (**25**), isovaleraldehyde (**18**) or cyclopropanecarbaldehyde (**26**) were applied (Table 2, entry 8–11). This is in contrast to similar BAL-catalyzed reactions, which resulted, for example, in mixtures of the HPP (**32a**) and PAC (**32b**) derivatives using benzaldehyde and propanal as substrates (unpublished results).

Subsequently, 3,5-dichloro- (**22**) and 3,5-dimethoxybenzaldehyde (**23**) were tested in KdcA-catalyzed carboligations together with acetaldehyde. Whereas no reaction occurred with **23** the dichloro derivative **22** is clearly accepted as a donor aldehyde, yielding selectively the 2-HPP derivative **35a** with high enantiomeric excess (*ee* 96.5%) (Table 2, entry 12).

The 2-hydroxy ketones **29–35** are formed with high enantioselectivity (*ee* >88%). The absolute (*R*)-configuration was assigned unambiguously to benzoin (**29**), 2-hydroxypropiophenone (**30a**), phenylacetyl carbinol (**30b**), **32b**, **33b**, and **35a** by using circular dichroism or by comparison with authentic samples. The absolute configuration of the hydroxy ketones **31b** and **34b** is assumed to be (*R*), too, according to a comparison with products obtained from BAL and PDC catalysis.

Carboligation of Phenylacetaldehyde and Indole-3-acetaldehyde with Acetaldehyde

CH-acidic aldehydes such as phenylacetaldehyde and indole-3-acetaldehyde are prone to enolization and thus are difficult substrates for enzymatic[23] and also non-enzymatic transformations. Moreover, indole-3-acetaldehyde is very instable and decomposes rapidly, which renders its direct application in biotransformations almost impossible.[24]

Phenylacetaldehyde is stable and commercially available; however, in aqueous buffer solution an aldol reaction occurs spontaneously. Application of this aldehyde in KdcA-catalyzed carboligations with acetaldehyde gives the 2-hydroxy ketones **36a** and **b** besides significant amounts of the aldol product.

To overcome these problems, we made use of KdcA's ability to decarboxylate the corresponding 2-keto acid of both aldehydes, which offers an easy way to generate these aldehydes *in situ*. Referring to the reaction mechanism, the decarboxylation of a 2-keto

acid results in a reaction intermediate with the corresponding aldehyde bound to the cofactor ThDP. This so-called "activated aldehyde" can react with a further acceptor aldehyde. If the KdcA-catalyzed decarboxylation of phenyl pyruvate (**13**) and indole-3-pyruvate (**17**) is performed in the presence of acetaldehyde the 2-hydroxy ketones **36a**, **b** and **37**, respectively, are formed predominantly in good yield (Table 2, entries 13 and 14).

Conclusions

KdcA is a valuable new biocatalyst for asymmetric C–C bond formation. The enzyme can be produced easily on a large scale in *E. coli* and purified using immobilized metal affinity chromatography. The branched-chain keto acid decarboxylase is stable up to 40 °C at pH 6.8, and also in the presence of water-miscible organic solvents such as 20 % (v/v) DMSO.

The enzyme is able to catalyze the carboligation of a broad range of aldehydes to chiral 2-hydroxy ketones. Most interestingly the selectivity of the products formed in the mixed acyloin synthesis of benzaldehyde and derivatives with different aliphatic acceptor aldehydes strongly depends on the substrate combination. The product composition can be shifted from a selectively formed PAC derivative of the aromatic aldehyde in the presence of large aliphatic aldehydes, over a nearly equal ratio of the HPP and PAC derivatives in the presence of acetaldehyde to a selectively formed HPP derivative in the presence of 3,5-dichlorobenzaldehyde (Table 2). A further important achievement is the selective asymmetric synthesis of 2-hydroxy ketones containing phenylacetaldehyde and indole-3-acetaldehyde. Good results have been obtained by *in situ* production of these enolizable (CH-acidic) aldehydes by enzymatic decarboxylation of the corresponding 2-keto acid in the presence of acetaldehyde, with both reactions being catalyzed by KdcA (Table 2).

Experimental Section

Cloning of KdcA

The KdcA gene was amplified by PCR from the genomic DNA of *Lactococcus lactis* subspecies *cremoris* B1157 which was kindly provided by NIZO food research B. V. (Ede, NL). The strain was grown in M17′ medium and isolation of genomic DNA was performed using the DNA-Tissue Kit (Qiagen). The following primers have been used for cloning:

After digestion of the amplified gene with *EcoR*I the gene was first ligated into the vector pBluescript in order to allow restriction by *Nhe*I and *Eco*RI. Subsequently the *kdca* gene (1.6 kb) was ligated into the vector pET28a (Novagen) carrying already the information for an N-terminal His-tag yielding the final construct pET28a-KdcA-His-N. Transformation of the expression host *E. coli* BL21(DE3) was performed by electroporation. Identity of the gene with the published sequence[10] was confirmed by DNA sequencing (Sequiserve, Germany).

Expression and Purification

A fed-batch cultivation according to the method of Korz et al.[25] was performed in a 40-L Techfors reactor (Infors AG, CH) at 30 °C, pH 7.0. Dissolved oxygen saturation was regulated between 30–40 % saturation by increasing the stirrer speed and the air flow rate and subsequently by a pressure increase during the high oxygen demanding overexpression. After a preliminary growth phase protein expression was induced by the addition of 1.5 mM IPTG at an OD_{600} ~60. From 15 L culture 1.2 kg *E. coli* cells with a specific activity of 21 U/mg in the crude cell extract were gained. Cells were harvested by a separator (Westfalia Separator AG) and stored at −20 °C.

Purification of KdcA to homogeneity (>95 %) was performed by immobilized nickel chelate affinity chromatography followed by size exclusion chromatography using a purification protocol previously developed for benzoyl formate decarboxylase[18] with the following alterations: The pH of all buffers used was set to 6.8. Buffers contained 2.5 mM $MgSO_4$ and 0.1 mM ThDP. Proteins unspecifically bound were eluted with 50 mM imidazole. KdcA was eluted with 250 mM imidazole. After purification the enzyme was either freeze-dried or diluted with 50 % (v/v) glycerol and stored at −20 °C.

Decarboxylase Activity

One unit of decarboxylase activity is defined as the amount of KdcA which catalyzes the decarboxylation of 1 μmol 3-methyl-2-oxobutanoic acid (**1**) or another 2-keto acid per minute under standard conditions (pH 6.8, 30 °C).

To investigate the decarboxylase activity of KdcA two continuous decarboxylase assays were used. In the *coupled decarboxylase assay* horse liver alcohol dehydrogenase (HL-ADH) is used to reduce the aldehydes obtained from the KdcA-catalyzed decarboxylation. Thereby NADH is oxidized to NAD^+. The decay of NADH is followed spectrophotometrically for 90 sec at 340 nm.

Assay composition: 700 μL buffer A (50 mM potassium phosphate buffer, pH 6.8, 2.5 mM $MgSO_4$, 0.1 mM ThDP), 100 μL 3-methyl-2-oxobutanoic acid (or another 2-keto acid) 300 mM in buffer A (final concentration 30 mM; except indole-3-pyruvate: 1 mM), 100 μL NADH 2.5 mM in buffer A (final concentration 0.25 mM), 50 μL HL-ADH

LlKdcA_hisN-up: 5′-ATAT*GCTAGC*ATGTATACAGTAGGAGATTAC-3′
*Nhe*I start

LlKdcA_hisN-down: 5′-ATAT*GAATTC*TTATTTATTTTGCTCAGCAAATAA-3′
*EcoR*I stop

(Sigma–Aldrich) 5 Units/mL in buffer A (final concentration 0.25 U/mL). The assay was started by addition of 50 μL KdcA.

Further, a *direct decarboxylase assay* following the direct decay of 3-methyl-2-oxobutanoic acid (**1**) was developed in order to measure KdcA activity under NADH degradating conditions.

Assay composition: 950 μL 3-methyl-2-oxobutanoic acid (**1**) (60 mM in buffer A). The reaction was started by addition of 50 μL KdcA solution. To avoid a background by absorption of isobutyraldehyde, the decay of 3-methyl-2-oxobutanoic acid ($\lambda_{max}=320$ nm) (**1**) was followed at 340 nm ($\varepsilon=0.017\ L\,mmol^{-1}cm^{-1}$).

Kinetic constants were calculated by non-linear regression using Origin 7G SR4 (OriginLab Coop., Northampton, USA). Background activities, measured in the absence of KdcA, were subtracted.

Protein Determination

Protein determination was performed according to Bradford[26] using BSA as a standard.

Determination of Molecular Mass

Size-exclusion chromatography was performed using a Superdex G200 prep grade column [total volume 122 mL (Ø 1.6 cm)] (Amersham) and 50 mM potassium phosphate buffer, pH 6.5, including 2.5 mM $MgSO_4$, 0.1 mM ThDP and 150 mM KCl. The coefficient of available volume [Kav = (Ve−Vo)/(Vt−Vo), Ve: elution volume of the respective protein, Vt: total volume, elution volume of blue dextran) for KdcA and the standard proteins have been determined twice with a standard deviation of 0.2%.

Calibration was performed using ribonuclease A (13.7 kD, Kav = 0.66), chymotrypsinogen A (25 kD, Kav = 0.6), ovalbumin (43 kD; Kav = 0.48), BSA (67 kD, Kav = 0.4), aldolase (158 kD, Kav = 0.29), catalase (232 kD, Kav = 0.27), ferritin (440 kD, Kav = 0.15), and thyroglobulin (669 kD, Kav = 0.07). The Kav coefficient of KdcA was determined as 0.33. Data were plotted as log Mr over Kav resulting in a linear correlation with a $R^2=0.9919$. Flow: 1 mL/min. Sample: 2 mL KdcA (1 mg/mL) in 50 mM potassium phosphate buffer, pH 6.5, including 2.5 mM $MgSO_4$, 0.1 mM ThDP

Determination of pH and Temperature Optima

The pH optimum was measured using the coupled decarboxylase assay. For determination of the temperature optimum the direct decarboxylase assay was used.

Stability Investigations

For investigation of the stability towards pH, temperature, and organic solvents KdcA was incubated under the reaction conditions given in the Figure legends and residual activity was assayed with the coupled decarboxylase assay.

Analytical Performance

The conversion was followed by GC/MS, employing an HP 6890 series GC system fitted with an HP 5973 mass selective detector (Hewlett Packard; column HP-5 MS, 30 m × 250 μm; T_{GC}(injector) = 250 °C, T_{MS}(ion source) = 200 °C, time program (oven): $T_{0min}=60$ °C, $T_{3min}=60$ °C, $T_{14min}=280$ °C (heating rate 20 °C·min^{-1}), $T_{19min}=280$ °C). The enantiomeric excess was determined by chiral GC, employing a Shimadzu GC 2010, fitted with an FS Lipodex D column (50 m × 0.25 mm) and an FID detector, or by chiral HPLC employing an HP 1100 HPLC system (Agilent) fitted with a diode-array detector. NMR spectra were recorded on a Bruker DPX-400. Chemical shifts are reported in ppm relative to $CHCl_3$ (^{1}H NMR: $\delta=7.27$) and $CDCl_3$ (^{13}C NMR: $\delta=77.0$) as internal standards. CD spectra were recorded on a JASCO J-810 spectropolarimeter using acetonitrile as solvent.

Carboligase Activity, Representative Examples for the KdcA-Catalyzed Synthesis of 2-Hydroxy Ketones:

(R)-*1-Hydroxy-1-phenylpentan-2-one* (**32b**)*:* Benzaldehyde (29 mg, 0.27 mmol) was dissolved in a mixture of dimethyl sulfoxide (3 mL) and potassium phosphate buffer [12 mL, 50 mM, pH 6.8, containing $MgSO_4$ (2.5 mM) and ThDP (0.1 mM)]. To this solution 19 mg (0.27 mmol) butanal were added. After addition of KdcA (3.3 mg protein) the reaction was stirred slowly at 30 °C for 104 h. The reaction mixture was extracted with diethyl ether (25 mL) and the organic layer washed with brine and dried over Na_2SO_4. Evaporation of the solvent and purification of the crude product, which contained besides **32b** small amounts of substrates and 5-hydroxyoctan-4-one, by flash column chromatography afforded (*R*)-1-hydroxy-1-phenylpentan-2-one (**32b**) as low-viscous oil; yield: 15.6 mg (32%, 96.5% *ee*). HPLC: (Chiral OM, *n*-hexane/2-propanol, 98:2, 0.5 mL min^{-1}, 40 °C) R_t (*S*) = 30.1 min, R_t (*R*) = 36.5 min; $[\alpha]_D^{23}$: −130.4 (*c* 0.1 g/100 mL, $CDCl_3$); CD (acetonitrile): λ (Δε) [nm] (mol. CD) = 199 (+18.02), 211 (+9.5), 218 (+9.7), 231 (+0.9), 238 (−0.1), 283 (−7.3); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.81$ (t, 3H, $J=7.5$ Hz, CH_3), 1.46–1.65 (m, 2H, CH_2), 2.25–2.41 (m, 2H, CH_2), 4.37 (bs, OH), 5.08 (s, 1H, *CH*OH), 7.3–7.4 (m, 5H, ArH) – contains 2 mol% acyloin; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=13.5$ (CH_3), 17.1, 39.7 (CH_2), 79.7 (CHOH), 127.4, 128.6, 128.9 (CH_{ar}), 138.08 (C_q), 209.4 (C=O); GC-MS: $R_t=9.02$ min; MS (70 eV, EI): *m/z* (%) = 178 (1%) [M$^+$], 107 (100%), 79 (42%).

(R)-*1-Hydroxy-1-phenylbutan-2-one* (**31b**): HPLC (Chiral OM, *n*-hexane/2-propanol, 95:5, 0.5 mL min^{-1}, 40 °C): *ee* > 98.5%, R_t (*S*) 21.6 min, R_t (*R*) = 25.0 min; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=1.01$ (t, $^3J_{H,H}=7.4$ Hz, 3H, CH_3), 2.29–2.46 (m, 2H, CH_2), 4.3–4.4 (bs, 1H, OH), 5.1 (s, 1H, C*H*OH), 7.3–7.41 (m, 5H, Ar-H) – contains 3 mol% benzaldehyde and acyloin; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=7.6$ (CH_3), 31.1 (CH_2), 79.4 (CHOH), 127.3 (2 Ar), 128.6, 128.9 (CH_{ar}), 138.3 (C_q), 210.1 (C=O); GC-MS $R_t=8.44$ min; MS (70 eV, EI): *m/z* (%) = 164 (0.1%) [M$^+$], 107 (100%), 79 (81%).

(R)-*1-Hydroxy-1-phenyl-4-methylpentan-2-one* (**33b**): Isolated yield: 25%; *ee* = 88%; HPLC, Chiralcel OD-H, *n*-hexane/2-propanol, 98:2, 0.5 mL min^{-1}, 25 °C, R_t (*S*) = 22.9 min, R_t (*R*) = 29.5 min; $[\alpha]_D^{23}$: −256.2 (*c* 0.2 g/100 mL, $CDCl_3$); CD (acetonitrile): λ (Δε) [nm] (mol. CD) = 199 (+20.9), 218 (+11.05), 231 (+0.9), 237 (+0.04), 283 (−8.5); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.75$ (d, 3H, $J=6.5$ Hz, CH_3), 0.89 (d, 3H, $J=6.5$ Hz, CH_3), 2.04–2.31 (m, 3H, CH,

CH_2), 4.39 (d, $J=4.1$ Hz, OH), 5.05 (d, 1H, $J=4.1$, *CH*OH), 7.29–7.41 (m, 5H, ArH) – contains 20 mol% acyloin and substrate; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=22.2$ (CH_3), 22.4 (CH_3), 24.6 (CH), 46.6 (CH_2), 80.0 (CHOH), 127.4, 128.6, 128.9 (CH_{ar}), 137.9 (C_q), 209.0 (C=O); GC-MS: $R_t=$ 9.30 min; MS (70 eV, EI): *m/z* (%)=192 (1%) [M^+], 136 (1%) , 107 (100%), 79 (58%);

(R)*-*2-Cyclopropyl-1-hydroxy-1-phenylethanone* (**34b**): Isolated yield 14%; *ee*=98%; HPLC (Chiracel OD-H, *n*-hexane/2-propanol, 95:5, 0.5 mL min^{-1}, 40 °C): R_t (*S*)= 16.6 min, R_t (*R*)=21.15 min; ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=0.78$–0.86 (m, 1H), 0.94–1.2 (m, 3H), 1.83–1.9 (m, 1H), 4.39 (bs, 1H, OH), 5.27 (s, 1H, C*H*OH), 7.3–7.45 (m, 5H, Ar-H) – contains 20 mol% acyloin; ^{13}C NMR (100 MHz, $CDCl_3$): δ =12.1, 12.7 (CH_2), 17.6 (CH), 80.1 (CHOH), 127.7, 128.6, 128.9 (CH_{ar}), 138.1 (C_q), 209.6 (C=O); GC-MS: $R_t=9.45$ min; MS (70 eV, EI): *m/z* (%)=176 (3%) [M^+], 107 (100%), 79 (71%).

(R)-*1-(3,5-Dichlorophenyl)-2-hydroxypropan-1-one* (**35a**): HPLC (Chiralpak AD, *n*-hexane/2-propanol, 98:2, 0.75 mL min^{-1}, 20 °C): *ee*=96.5%, R_t (*S*)=20.4 min, R_t (*R*)= 25.4 min; CD (acetonitrile): λ (Δε) [nm] (mol. CD)=212 (+4.9), 211 (+9.5), 245 (−2.0), 289 (+1.65); ^{1}H NMR (400 MHz, $CDCl_3$): $\delta=1.46$ (d, 3H, $J=7.1$, CH_3), 3.56 (bs, 1H, OH), 5.07 (q, 1H, $J=7.1$, C*H*OH), 7.61 (t, 1H, $J=2.07$, ArH), 7.78 (d, 2H, $J=2.07$, ArH) – contains 8 mol% 3,5-dichlorobenzaldehyde; ^{13}C NMR (100 MHz, $CDCl_3$): $\delta=21.9$ (CH_3), 69.6 (CHOH), 126.9, 133.5 (CH_{ar}), 135.9, 136.0 (C_q), 200.2 (C=O); GC-MS: $R_t=10.04$ min; MS (70 eV, EI): *m/z* (%)=218 (7%) [M^+], 175 (100%), 147 (43%), 111 (71%).

3-Hydroxy-1-phenylbutan-2-one (**36a**) and *1-phenyl-2-hydroxybutan-3-one* (**36b**): **36a**: yield: 49%; *ee*=not determined; ^{1}H NMR (400 MHz, $CDCl_3$, 300 K): $\delta=1.4$ (d, 3H, $J=7.1$ Hz, CH_3), 3.55 (d, 1H, $J=4.6$ Hz, OH), 3.77 (d, 1H, $J=15.8$ Hz, CH_2), 3.83 (d, 1H, $J=15.8$ Hz, CH_2), 4.3–4.36 (m, 1H, C*H*OH), 7.18–7.34 (m, 5H, ArH) – contains 19 mol% **36b**; ^{13}C NMR (100 MHz, $CDCl_3$, 300 K): $\delta=19.7$ (CH_3), 44.5 (CH_2), 72.2 (CHOH), 127.2, 128.7, 129.4, 133.1, 210.04 (C=O); GC-MS: $R_t=8.6$ min; MS (70 eV, EI): *m/z* (%)=164 (1%) [M^+], 146 (30%), 121 (41%), 103 (38%), 91 (100%).

36b: yield: 12%; *ee*=not determined; ^{1}H NMR (400 MHz, $CDCl_3$, 300 K): δ =2.19 (s, 3H, CH_3), 2.87 (dd, 1H, $J=14.2$ Hz, $J=7.5$ Hz, CH_2), 3.12 (dd, 1H, $J=14.2$ Hz, $J=4.6$ Hz, CH_2), 3.52 (d, 1H, $J=5.3$ Hz, OH), 4.38–4.42 (m, 1H, CHOH), 7.18–7.34 (m, 5H, ArH) – contains **36a**; ^{13}C NMR (100 MHz, $CDCl_3$, 300 K): $\delta=25.8$ (CH_3) 39.8 (CH_2) 77.6 (CHOH), 126.8, 128.5, 129.2, 209.4 (C=O); GC-MS: $R_t=8.6$ min; MS (70 eV, EI): *m/z* (%)=164 (1%) [M^+], 146 (30%), 121 (41%), 103 (38%), 91 (100%).

3-Hydroxy-1-(3-indolyl)-butan-2-one (**37a**): Yield: 23%; *ee*=not determined; ^{1}H NMR (400 MHz, $CDCl_3$, 300 K): $\delta=1.46$ (d, 3H, $J=7.1$ Hz, CH_3), 3.43 (d, 1H, $J=4.9$ Hz, OH), 3.96 (s, 2H, CH_2), 4.38–4.45 (m, 1H, *CH*OH), 7.14–7.18 (m, 1H, ArH+1H, NCH), 7.21–7.25 (m, 1H, ArH), 7.39 (d, 1H, ArH), 7.53 (d, 1H, ArH), 8.06–8.19 (bs, 1H, NH); GC-MS: $R_t=12.54$ min; MS (70 eV, EI): *m/z* (%)= 203 (9.5%) [M^+], 130 (100%).

Acknowledgements

Skilful technical assistance of Ilona Frindi-Wosch (enzyme purification and size exclusion chromatography) and Elke Breitling (synthesis of indole-3-acetaldehyde) is gratefully acknowledged. The authors thank Degussa AG for financial support.

References

[1] T. Stillger, M. Pohl, C. Wandrey, A. Liese, *Org. Proc. Res. Develop.* **2006**, *10*, 1172–1177.
[2] M. Knoll, M. Müller, J. Pleiss, M. Pohl, *ChemBioChem* **2006**, *7*, 1928–1934.
[3] A. S. Demir, O. Sesenoglu, P. Dünkelmann, M. Müller, *Org. Lett.* **2003**, *5*, 2047–2050.
[4] P. Dominguez de Maria, T. Stillger, M. Pohl, S. Wallert, K. Drauz, H. Gröger, H. Trauthwein, A. Liese, *J. Mol. Catal. B: Enzym.* **2006**, *38*, 43–47.
[5] P. Siegert, M. J. McLeish, M. Baumann, H. Iding, M. M. Kneen, G. L. Kenyon, M. Pohl, *Protein Eng. Des. Sel.* **2005**, *18*, 345–357.
[6] M. Pohl, B. Lingen, M. Müller, *Chem. Eur. J.* **2002**, *8*, 5288–5295.
[7] A. S. Demir, O. Sesenoglu, E. Eren, B. Hosrik, M. Pohl, E. Janzen, D. Kolter, R. Feldmann, P. Dünkelmann, M. Müller, *Adv. Synth. Catal.* **2002**, *344*, 96–103.
[8] P. Dünkelmann, D. Kolter-Jung, A. Nitsche, A. S. Demir, P. Siegert, B. Lingen, M. Baumann, M. Pohl, M. Müller, *J. Am. Chem. Soc.* **2002**, *124*, 12084–12085.
[9] G. Smit, B. A. Smit, W. J. Engels, *FEMS Microbiol. Rev.* **2005**, *29*, 591–610.
[10] B. A. Smit, J. Vlieg, W. J. M. Engels, L. Meijer, J. T. M. Wouters, G. Smit, *Appl. Environ. Microbiol.* **2005**, *71*, 303–311.
[11] M. de La Plaza, P. Fernandez de Palencia, C. Pelaez, T. Requena, *FEMS Microbiol. Lett.* **2004**, *238*, 367–374.
[12] A. Yep, G. L. Kenyon, M. J. McLeish, *Bioorg. Chem.* **2006**, *34*, 325–336.
[13] P. Arjunan, T. Umland, F. Dyda, S. Swaminathan, W. Furey, M. Sax, B. Farrenkopf, Y. Gao, D. Zhang, F. Jordan, *J. Mol. Biol.* **1996**, *256*, 590–600.
[14] D. Dobritzsch, S. König, G. Schneider, G. Lu, *J. Biol. Chem.* **1998**, *273*, 20196–20204.
[15] M. S. Hasson, A. Muscate, M. J. McLeish, L. S. Polovnikova, J. A. Gerlt, G. L. Kenyon, G. A. Petsko, D. Ringe, *Biochemistry* **1998**, *37*, 9918–9930.
[16] A. K. Chang, R. G. Duggleby, *Biochem. J.* **1997**, *327*, 161–169.
[17] Y. Lindqvist, G. Schneider, U. Ermler, M. Sundstrom, *Embo J.* **1992**, *11*, 2373–2379.
[18] H. Iding, T. Dünnwald, L. Greiner, A. Liese, M. Müller, P. Siegert, J. Grötzinger, A. S. Demir, M. Pohl, *Chem. Eur. J.* **2000**, *6*, 1483–1495.
[19] F. Hildebrand, S. Kühl, M. Pohl, D. Vasic-Racki, M. Müller, C. Wandrey, S. Lütz, *Biotechnol. Bioeng.* **2007**, *96*, 835–843.
[20] P. Dominguez de Maria, T. Stillger, M. Pohl, S. Wallert, K. Drauz, H. Gröger, H. Trauthwein, A. Liese, *J. Mol. Catal. B: Enzym.* **2005**, *38*, 43–47.

[21] E. Janzen, M. Müller, D. Kolter-Jung, M. M. Kneen, M. J. McLeish, M. Pohl, *Bioorg. Chem.* **2006**, *34*, 345–361.
[22] P. Dominguez de Maria, M. Pohl, D. Gocke, H. Gröger, H. Trauthwein, L. Walter, M. Müller, *Eur. J. Org. Chem.* **2007**, in press; DOI 10/1002/ejoc.200600876.
[23] A. Schütz, R. Golbik, K. Tittmann, D. I. Svergun, M. H. Koch, G. Hübner, S. König, *Eur. J. Biochem.* **2003**, *270*, 2322–2331.
[24] J. Koga, T. Adachi, H. Hidaka, *J. Biol. Chem.* **1992**, *267*, 15823–15828.
[25] D. J. Korz, U. Rinas, K. Hellmuth, E. A. Sanders, W.-D. Deckwer, *J. Biotechnol.* **1995**, *39*, 59–65.
[26] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

