DOI: 10.1002/ejoc.200600699

Exploring the Synthetic Applicability of a Cyanobacterium Nitrilase as Catalyst for Nitrile Hydrolysis

Chandrani Mukherjee,[a] Dunming Zhu,[a] Edward R. Biehl,[a] and Ling Hua*[a]

Keywords: Enzyme catalysis / Nitrilase / Hydrolysis / Cyanobacterium / Cyano carboxylic acid / β-Hydroxy carboxylic acid

The substrate specificity and synthetic applicability of the nitrilase from cyanobacterium *Synechocystis* sp. strain PCC 6803 have been examined. This nitrilase catalyzed the hydrolysis of both aromatic and aliphatic nitriles to the corresponding acids in high yields. Furthermore, the stereoselective hydrolysis of phenyl-substituted β-hydroxy nitriles to (*S*)-enriched β-hydroxy carboxylic acids and selective hydrolysis of α,ω-dinitriles with five or less methylene groups to ω-cyano carboxylic acids have been achieved. This suggested that nitrilase from *Synechocystis* sp. PCC 6803 could be a useful enzyme catalyst for the "green" nitrile hydrolysis.

Introduction

Organic nitriles are readily available and useful precursors for production of a wide variety of carboxylic acids. Chemical procedures to convert nitriles to carboxylic acids usually require strong acids or bases and high temperature, thus producing unwanted byproducts and large amount of inorganic wastes. This prevents the straightforward preparation of many important carboxylic acids from the corresponding nitriles. For example, β-hydroxy carboxylic acids and their derivatives are precursors of β-blockers, 1,3-amino alcohols[1–3] and copolyesters for film, fiber, molding and coating applications.[4] A straightforward approach to β-hydroxy carboxylic acids is hydrolysis of β-hydroxy nitriles, which are readily accessible by the cyanization of α-haloketones with sodium cyanide followed by $NaBH_4$ reduction[5] or ring-opening of epoxides by sodium cyanide.[6] However, chemical hydrolysis often results in the undesirable elimination of OH for nitriles with β-hydroxy group, yielding unsaturated by-products.[7] Cyano carboxylic acids are also important intermediates for a variety of applications. Because dinitriles are readily available both on bench scale and in technical quantities, selective hydrolysis of dinitriles to cyano carboxylic acids is particularly interesting. However, selective chemical hydrolysis of dinitriles is virtually impossible.[8] To solve these problems, nitrilase-catalyzed hydrolysis became an attractive route because it offers tremendous advantages such as mild and green reaction conditions, high yield of desired products and high chemo-, regio- and stereoselectivity.[7–10] Therefore, great efforts have been made to search for new nitrilases, and many nitrilases have been biochemically characterized.[11–21] However, the synthetic applicability of these nitrilases has barely explored.[22]

Recently, Stolz and co-workers have cloned a nitrilase from cyanobacterium *Synechocystis* sp. strain PCC 6803 by genome mining.[16] Bertholet assay, i.e. quantitation of the amount of ammonia released during hydrolysis, showed that this nitrilase was active toward both aromatic and aliphatic nitriles, and was tolerant of organic solvents. The enzymatic conversion of hydrophobic nitriles in aqueous solution can be limited by the low bioavailability of the hydrophobic substrates. This limitation may be overcome by inclusion of organic solvents to the reaction medium. The organic solvent tolerance as well as the possible broad substrate range of nitrilase from *Synechocystis* sp. PCC 6803 inspired us to explore its synthetic applicability. Therefore, the substrate specificity of this enzyme has been evaluated over the hydrolysis of a wide range of nitrile substrates. Especially the stereoselective hydrolysis of β-hydroxy nitriles to β-hydroxy carboxylic acids and selective hydrolysis of dinitriles to cyano carboxylic acids have been studied, as these transformations are difficult to be achieved by chemical methods.

Result and Discussion

The nitrilase gene from cyanobacterium *Synechocystis* sp. strain PCC 6803 was cloned and over-expressed in *E. coli* and the encoded protein was purified from cell-free extract as described in the Experimental Section. The substrate specificity of this nitrilase was then studied by treating nitrile substrates with the purified enzyme in potassium phosphate buffer. The conversion was measured by GC or HPLC analysis, and the products were isolated and charac-

[a] Department of Chemistry, Southern Methodist University, Dallas, Texas 75275, USA

terized by NMR spectroscopy. The results are summarized in Tables 1–3.

As shown in Table 1, the nitrilase from *Synechocystis* sp. PCC 6803 was active toward aromatic nitriles such as benzonitrile, with benzoic acid being isolated in 88% yield. This nitrilase effectively converted phenylacetonitrile to phenylacetic acid. The α substituent of phenylacetonitriles greatly affected the enzyme activity. The α-methyl group only slightly decreased the activity as shown in Entry 4 in Table 1, but the α-hydroxy group as in mandelonitrile greatly decreased the enzyme activity (Entry 7), and this nitrilase is not active for 2-phenylbutyronitrile (α-ethyl group, Entry 6). This indicated that both electronic and steric factors of the α substituent played an important role in determining the enzyme activity toward the hydrolysis of phenylacetonitrile derivatives. It can also be seen that the nitrilase from *Synechocystis* sp. PCC 6803 effectively catalyzed the hydrolysis of aliphatic nitriles to corresponding carboxylic acids in greater than 90% yields irrespective of their chain length and structure.

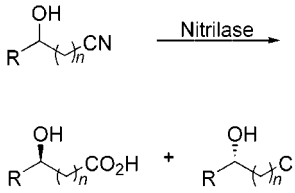
Table 1. Hydrolysis of aromatic and aliphatic nitriles catalyzed by the nitrilase from *Synechocystis* sp. PCC 6803.

Entry	Nitrile	Conversion [%][a]	Yield [%][b]
1	benzonitrile	100	88
2	phenylacetonitrile	100	97
3	3-phenylpropionitrile	100	94
4	2-phenylpropionitrile	99	89[c]
5	4-phenylbutyronitrile	100	97
6	2-phenylbutyronitrile	0	0
7	mandelonitrile	29	25[c]
8	(methylthio)acetonitrile	100	96
9	butyronitrile	92	90
10	valeronitrile	100	99
11	hexanenitrile	100	97
12	heptanenitrile	100	99
13	3-butenenitrile	100	95
14	2-butenenitrile	100	98

[a] Conversions were determined by GC, HPLC analysis (Entries 1–7) or ^{1}H NMR (Entries 8–14) of the crude products. [b] Isolated yields, all carboxylic acid products were characterized by comparing the ^{1}H and ^{13}C NMR spectroscopic data with those in the literature or standard samples. [c] Products were racemic as determined by chiral HPLC or GC analysis, no significant extent of degradation of mandelonitrile to HCN and benzaldehyde was observed.

As mentioned in the Introduction, the stereoselective hydrolysis of β-hydroxy nitriles to β-hydroxy carboxylic acids is difficult to be achieved by chemical methods. The nitrilase from *Synechocystis* sp. PCC 6803 was thus tested for the hydrolysis of several β-hydroxy nitriles (Table 2). Interestingly, this nitrilase catalyzed the enantioselective hydrolysis of β-hydroxy nitriles to give (*S*)-enriched β-hydroxy carboxylic acids, although the enzyme was not enantioselective for the hydrolysis of mandelonitrile (Entry 7 in Table 1). The observed stereo-discrimination in the hydrolysis of β-hydroxy nitriles was interesting because it was in contrast to the normal observation that a chiral carbon atom at the β position to the reaction center would be recognized with much more difficulty than the one at the α position.[23,24] It appeared that the substituents at *para* position of aromatic β-hydroxy nitriles exerted some effects on the enantioselectivity. The nitrilase from *Synechocystis* sp. PCC 6803 efficiently catalyzed the hydrolysis of 3-hydroxy-3-(2-naphthyl)propionitrile and 3-hydroxy-4,4-dimethylvaleronitrile, but racemic acid products were obtained. This enzyme was also active toward the hydrolysis of γ-hydroxy nitrile but with less enantioselectivity (Entry 9 in Table 2).

Table 2. Hydrolysis of β- and γ-hydroxy nitriles catalyzed by the nitrilase from *Synechocystis* sp. PCC 6803.



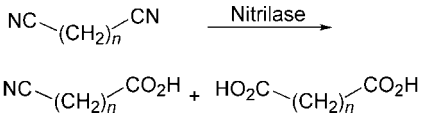
Entry	R	*n*	Yield [%][a]	*ee* [%][b]
1	phenyl	1	51	46
2	4-fluorophenyl	1	28	61
3	4-chlorophenyl	1	30	65
4	4-bromophenyl	1	50	23
5	4-acetylphenyl	1	56	29
6	4-methoxyphenyl	1	48	32
7	2-naphthyl	1	93	2
8	*tert*-butyl	1	93	0
9	phenyl	2	52	11

[a] All carboxylic acid products were characterized by comparing their ^{1}H and ^{13}C NMR spectroscopic data with those in the literature or standard sample. [b] The *ee* values were determined by chiral HPLC or GC.

In the cases of dinitriles, although selective hydrolysis to monocarboxylic acids is virtually impossible to be achieved by chemical methods, a few reports on biocatalytic selective hydrolysis of dinitriles have appeared.[8–10] Because ω-cyano carboxylic acids are useful precursors to bioactive lactam derivatives[9] and Bertholet assay showed that for some aliphatic dinitriles only one mol of ammonia was released per mol of dinitrile under the action of the nitrilase from *Synechocystis* sp. PCC 6803,[16] we have examined the possibility of utilizing this nitrilase to synthesize ω-cyano carboxylic acids from α,ω-dinitriles. Therefore, α,ω-dinitriles were treated with the nitrilase from *Synechocystis* sp. PCC 6803 in potassium phosphate buffer at 30 °C for 12 h. The products were isolated and characterized by ^{1}H and ^{13}C NMR and the results are presented in Table 3. For α,ω-dinitriles with three or less methylene carbon atoms that separated the two cyano groups, including dimethylmalononitrile and fumaronitrile, ω-cyano carboxylic acids were isolated in excellent yields (>94%). It was interesting to note that this nitrilase was not active toward malononitrile although it catalyzed the hydrolysis of the dimethyl-substituted counterpart, indicating that the substrate size played an important role in determining the nitrilase activity. When two cyano groups were separated by four or five methylene groups, there was a loss of selectivity and a mixture of mono- and di-acids was obtained. If the chain length increased to six or more methylene groups, the selectivity was

completely lost and di-acids were isolated in very high yields. This indicated that the selectivity of this nitrilase-catalyzed hydrolysis of α,ω-dinitriles was dependent on the chain length of substrates.

Table 3. Hydrolysis of α,ω-dinitriles catalyzed by the nitrilase from *Synechocystis* sp. PCC 6803.



Entry	Dinitrile (*n*)	Conversion [%][a]	Yield [%][b] Mono-acid	Di-acid
1	dimethylmalononitrile	100	94	0
2	fumaronitrile	100	97	0
3	2	100	99	0
4	3	100	99	0
5	4	100	81	15
6	5	100	60	35
7	6	100	0	98
8	8	100	0	99

[a] Conversions were determined by ^{1}H NMR of the crude products. [b] Isolated yields, all carboxylic acid products were characterized by comparing the ^{1}H and ^{13}C NMR spectroscopic data with those in the literature or standard samples.

For the α,ω-dinitriles with four or five methylene groups, a question was raised whether the ω-cyano carboxylic acid could be isolated as sole product within a shorter period of reaction time. To answer this question, α,ω-dinitriles with *n* being 3, 4, 5 and 6 were hydrolyzed by the nitrilase from *Synechocystis* sp. PCC 6803 for different periods. The results are summarized in Figure 1. From these results it can be seen that 1,3-dicyanopropane was selectively hydrolyzed to 4-cyanobutyric acid even within extended reaction time (24 h). ω-Cyano carboxylic acids were obtained in high yields from the hydrolysis of α,ω-dinitriles with four or five methylene groups when reaction times were shorter than 6 or 2 h, respectively, although mixtures of mono- and di-acids were isolated when the reactions were allowed to run for longer time. This suggested that the nitrilase from *Synechocystis* sp. PCC 6803 selectively hydrolyzed one cyano group in 1,4-dicyanobutane and 1,5-dicyanopentane and the second cyano group was hydrolyzed after the complete hydrolysis of the first one. For 1,6-dicyanohexane, the differentiation between these two steps became smaller, because it was difficult to isolate mono-acid in high yield and a mixture of mono- and di-acids was obtained even within 2 h of reaction time.

Thus it seemed that the nitrilase from *Synechocystis* sp. PCC 6803 was not able to catalysize the hydrolysis of ω-cyano carboxylic acids with *n* being 3 or less. To evaluate the validity of this reasoning, several ω-cyano carboxylic acids were tested as the substrates of this nitrilase. As expected, this nitrilase was not active toward $(CH_3)_2C(CN)$-COOH, CNCH=CHCOOH, and $CN(CH_2)_nCOOH$ (n = 2 or 3), while the other two ω-cyano carboxylic acids $CN(CH_2)_nCOOH$ (n = 4 or 5) were hydrolyzed to di-acids

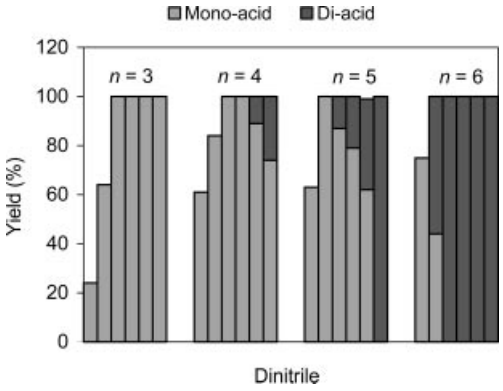


Figure 1. Time-dependent studies on the hydrolysis of $CN(CH_2)_nCN$ catalyzed by the nitrilase from *Synechocystis* sp. PCC 6803. The reaction times were 1, 2, 4, 6, 12, 24 h (from left to right for each substrate).

in 40 and 70% yields, respectively, when they were treated with the enzyme at 30 °C for 12 h. Therefore, the nitrilase from *Synechocystis* sp. PCC 6803 selectively hydrolyzed α,ω-dinitriles [$CN(CH_2)_nCN$] with *n* being 3 or less to give ω-cyano carboxylic acids exclusively. When *n* was 4 or 5, ω-cyano carboxylic acids could be obtained as sole products by controlling reaction time. Selective hydrolysis of α,ω-dinitriles [$CN(CH_2)_nCN$] with *n* being 6 or more could not be achieved, and di-acids were isolated in high yields.

Conclusions

The nitrilase from cyanobacterium *Synechocystis* sp. strain PCC 6803 catalyzed the hydrolysis of both aromatic and aliphatic nitriles to the corresponding acids in high yields. Recently, a few nitrilases have been reported to show nitrile hydratase activity, i.e. certain amount of amide products were obtained.[24,25] Amide products were not detected in the nitrile hydrolysis catalyzed by the nitrilase from *Synechocystis* sp. PCC 6803. Moreover, phenyl-substituted β-hydroxy nitriles were stereoselectively hydrolyzed to (*S*)-enriched β-hydroxy carboxylic acids under the action of the nitrilase from *Synechocystis* sp. PCC 6803. Selective hydrolysis of α,ω-dinitriles with five or less methylene groups to ω-cyano carboxylic acids have also been achieved with this nitrilase as the catalyst. This demonstrated the synthetic applicability of the nitrilase from *Synechocystis* sp. PCC 6803 as a useful enzyme catalyst for the "green" nitrile hydrolysis.

Experimental Section

General Remarks: The GC analysis was performed with a Hewlett–Packard 5890 series II plus gas chromatograph. The HPLC analysis was performed with an Agilent 1100 series high-performance liquid chromatography system. ^{1}H and ^{13}C NMR spectra were recorded with a 400 MHz Bruker AVANCE DRX-400 Multinuclear NMR spectrometer. β-Hydroxy nitriles and β-hydroxy carboxylic acids were prepared by following literature procedures.[5,23,26] All the other nitriles and acid standards were purchased from Aldrich.

Table 4. Details of the chiral HPLC and GC analysis of β-hydroxy carboxylic acids.

Entry	β-Hydroxy carboxylic acid	Method[a]	Retention time $t_{(S)}$ [min]	$T_{(R)}$ [min]
1	3-hydroxy-3-phenylpropionic acid	A	12.5	17.3
2	3-(*p*-fluorophenyl)-3-hydroxypropionic acid	A	11.1	14.7
3	3-(*p*-chlorophenyl)-3-hydroxypropionic acid	A	12.1	16.5
4	3-(*p*-bromophenyl)-3-hydroxypropionic acid	A	12.4	16.9
5	3-(*p*-acetylphenyl)-3-hydroxypropionic acid	B	17.4	20.9
6	3-hydroxy-3-(*p*-methoxyphenyl)propionic acid	B	13.8	20.9
7	3-hydroxy-3-naphthylpropionic acid[b]	B	20.6	32.5
8	3-hydroxy-4,4-dimethylpentanoic acid	C	34.3	34.9
9	4-hydroxy-4-phenylbutyric acid[b]	A	14.3	15.1

[a] (A) HPLC, column: Whelk O-1; flow rate: 1 mL/min; solvent: hexane (0.1% HOAc): 2-propanol(0.1% HOAc) = 95:5. (B) HPLC, column: Whelk O-1; flow rate: 1 mL/min; solvent: hexane (0.1% HOAc)/2-propanol (0.1% HOAc) = 90:10. (C) GC, column: Chirasil DEX-CB; temperature: 60 °C for 2 min, 1 °C/min, 100 °C for 10 min. The acid was converted to methyl ester prior to GC analysis. [b] The low *ee* of the samples resulted in difficulty in determining their absolute configurations, so they were assigned on the basis of correlation of the retention time with other samples in the Table.

Expression of Nitrilase Gene in *E. coli*: The nitrilase gene from cyanobacterium *Synechocystis* sp. strain PCC 6803 (NCBI accession number D64005) was cloned by following the literature procedure.[16] This nitrilase gene was cloned into pET15b expression vector at the *Nco* I/*Bam*H I sites and the plasmid DNA containing this nitrilase gene was transformed into Rosetta2(DE3)pLysS *E. coli* strain (Novagen). Overnight pre-cultures were diluted into LB containing 100 μg/mL of ampicillin and 34 μg/mL of chloramphenicol, the cells were induced with 0.1 mM of IPTG when optical density at 595 nm was 0.6. The bacterial cultures were incubated at 30 °C on an orbital shaker at 180 rpm for another 4 h. The cells were harvested.

Preparation of Cell-free Extract and Purification of Nitrilase Enzyme: The cultures of *E. coli* Rosetta2(DE3)pLysS were harvested by centrifugation. The cell pellet was re-suspended in potassium phosphate lysis buffer (10 mM, pH 7.2, 1 mM DTT), and the cell was lyzed by homogenizer. The cell-free extract was mixed with equal volume of PEI solution (0.25% polyethyleneimine MW 40K-60K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids.[27] After centrifugation the supernatant was precipitated with 30% ammonium sulfate. The resulting precipitate was collected after centrifugation and dissolved in potassium phosphate buffer (10 mM, pH 7.2, 1 mM DTT). The lysate was desalted by gel filtration into potassium phosphate buffer (10 mM, pH 7.2, 1 mM DTT), and resulting enzyme solution was lyophilized and used for enzymatic reactions.

Typical Procedure for the Enzymatic Hydrolysis of Nitriles: To a solution of nitriles (0.5–1.0 mmol) in potassium phosphate buffer (5 mL, 100 mM, pH 7.2), the enzyme from *Synechocystis* sp. PCC 6803 (2–3 mg) was added. The reaction mixture was incubated at 30 °C for 12 h or the specified period. The mixture was acidified with hydrochloric acid (1 N) to pH ca. 5, saturated with NaCl and then extracted with ethyl acetate. The organic layer was separated and dried with anhydrous sodium sulfate. Removal of solvent provided the crude products that were further purified by preparative TLC using ethyl acetate/hexane as eluting solvents. The products were characterized by comparing the ^{1}H and ^{13}C NMR spectroscopic data with those in the literature or standard samples.[8,9,23,24,26,28] For β-hydroxy carboxylic acids, the *ee* value was measured by chiral HPLC or GC analysis (Table 4) and the absolute configuration was determined by comparing the retention time with those of standard samples.[23,26] For 3-hydroxy-4,4-dimethylpentanoic acid, the acid was converted to methyl ester by treating with freshly prepared diazomethane, and the resulting methyl ester was subjected to chiral GC analysis.

[1] C. Thomassigny, F. Sineriz, C. Greck, J.-D. Lou, *Recent Res. Dev. Org. Chem.* **2004**, *8*, 377–400.
[2] W.-H. Ham, C.-Y. Oh, Y.-S. Lee, J.-H. Jeong, *J. Org. Chem.* **2000**, *65*, 8372–8374.
[3] B. Iliev, A. Linden, R. Kunz, H. Heimgartner, *Tetrahedron* **2006**, *62*, 1079–1094.
[4] I. Goodman, in *Concise Encyclopedia of Polymer Science and Engineering* (Ed.: J. I. Kroschwitz), John Wiley & Sons, New York, **1990**, pp. 793–799.
[5] D. N. Ridge, J. W. Hanifin, L. A. Harten, B. D. Johnson, J. Menschik, G. Nicolau, A. E. Sloboda, D. E. Watts, *J. Med. Chem.* **1979**, *22*, 1385–1389.
[6] M. Caron, K. B. Sharpless, *J. Org. Chem.* **1985**, *50*, 1557–1560.
[7] E. C. Hann, A. E. Sigmund, S. K. Fager, F. B. Cooling, J. E. Gavagan, A. Ben-Bassat, S. Chauhan, M. S. Payne, S. M. Hennessey, R. DiCosimo, *Adv. Synth. Catal.* **2003**, *345*, 775–782.
[8] F. Effenberger, S. Osswald, *Synthesis* **2001**, 1866–1872.
[9] J. E. Gavagan, S. K. Fager, R. D. Fallon, P. W. Folsom, F. E. Herkes, A. Eisenberg, E. C. Hann, R. DiCosimo, *J. Org. Chem.* **1998**, *63*, 4792–4801.
[10] M. Kobayashi, T. Nagasawa, H. Yamada, *Eur. J. Biochem./FEBS* **1989**, *182*, 349–356.
[11] C. Kiziak, D. Conradt, A. Stolz, R. Mattes, J. Klein, *Microbiology (Reading, United Kingdom)* **2005**, *151*, 3639–3648.
[12] S. Chauhan, S. Wu, S. Blumerman, R. D. Fallon, J. E. Gavagan, R. DiCosimo, M. S. Payne, *Appl. Microbiol. Biotechnol.* **2003**, *61*, 118–122.
[13] D. E. Robertson, J. A. Chaplin, G. DeSantis, M. Podar, M. Madden, E. Chi, T. Richardson, A. Milan, M. Miller, D. P. Weiner, K. Wong, J. McQuaid, B. Farwell, L. A. Preston, X. Tan, M. A. Snead, M. Keller, E. Mathur, P. L. Kretz, M. J. Burk, J. M. Short, *Appl. Environ. Microbiol.* **2004**, *70*, 2429–2436.
[14] G. DeSantis, K. Wong, B. Farwell, K. Chatman, Z. Zhu, G. Tomlinson, H. Huang, X. Tan, L. Bibbs, P. Chen, K. Kretz, M. J. Burk, *J. Am. Chem. Soc.* **2003**, *125*, 11476–11477.
[15] G. DeSantis, Z. Zhu, W. A. Greenberg, K. Wong, J. Chaplin, S. R. Hanson, B. Farwell, L. W. Nicholson, C. L. Rand, D. P. Weiner, D. E. Robertson, M. J. Burk, *J. Am. Chem. Soc.* **2002**, *124*, 9024–9025.
[16] U. Heinemann, D. Engels, S. Buerger, C. Kiziak, R. Mattes, A. Stolz, *Appl. Environ. Microbiol.* **2003**, *69*, 4359–4366.
[17] S. Levy-Schil, F. Soubrier, A. M. Crutz-Le Coq, D. Faucher, J. Crouzet, D. Petre, *Gene* **1995**, *161*, 15–20.
[18] M. Kobayashi, H. Komeda, N. Yanaka, T. Nagasawa, H. Yamada, *J. Biol. Chem.* **1992**, *267*, 20746–20751.
[19] M. Kobayashi, N. Yanaka, T. Nagasawa, H. Yamada, *Biochemistry* **1992**, *31*, 9000–9007.

[20] T. Nagasawa, J. Mauger, H. Yamada, *Eur. J. Biochem./FEBS* **1990**, *194*, 765–772.
[21] D. M. Stalker, L. D. Malyj, K. E. McBride, *J. Biol. Chem.* **1988**, *263*, 6310–6314.
[22] S. Osswald, H. Wajant, F. Effenberger, *Eur. J. Biochem.* **2002**, *269*, 680–687.
[23] Z.-L. Wu, Z.-Y. Li, *J. Mol. Catal. B* **2003**, *22*, 105–112.
[24] C. Mukherjee, D. Zhu, E. R. Biehl, R. R. Parmar, L. Hua, *Tetrahedron* **2006**, *62*, 6150–6154.
[25] C. Mateo, A. Chmura, S. Rustler, F. van Rantwijk, A. Stolz, R. A. Sheldon, *Tetrahedron: Asymmetry* **2006**, *17*, 320–323.
[26] O. Pamies, J.-E. Backvall, *Adv. Synth. Catal.* **2002**, *344*, 947–952.
[27] P. Milburn, J. Bonnerjea, M. Hoare, P. Dunnill, *Enzyme Microb. Technol.* **1990**, *12*, 527–532.
[28] C. Pouchert, J. Behnke, *Aldrich Library of ^{13}C and ^{1}H FT-NMR Spectra*, Aldrich Chemical Co., **1993**.

Received: August 10, 2006
Published Online: October 5, 2006

