DOI: 10.1002/adsc.200700067

Nitrilase-Catalyzed Selective Hydrolysis of Dinitriles and Green Access to the Cyanocarboxylic Acids of Pharmaceutical Importance

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

[a] Department of Chemistry, Southern Methodist University, Dallas, TX 75275, USA
Fax: (+1)-214-768-4089; e-mail: lhua@smu.edu

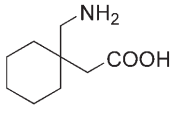
Received: January 25, 2007; Revised: May 8, 2007

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

Abstract: To further explore its synthetic applications, the nitrilase bll6402 from *Bradyrhizobium japonicum* strain USDA110 has been examined toward the hydrolysis of various dinitriles. It has been found that nitrilase bll6402 effectively hydrolyzed α,ω-dinitriles to ω-cyanocarboxylic acids, and the selectivity was independent of the substrate chain length. This feature is distinct from all the known nitrilases of various sources. Nitrilase bll6402 was thus applied to the synthesis of 1-cyanocycloalkaneacetic acids, the useful precursors for the synthesis of gabapentin and its analogues.

Keywords: cyanocarboxylic acids; dinitriles; enzyme catalysis; hydrolysis; nitrilase

The chemical hydrolysis of nitriles to carboxylic acids typically requires drastic conditions such as strong bases, acids and/or elevated temperature, thus producing unwanted by-products and/or large amounts of inorganic wastes. Nitrilase-catalyzed hydrolysis of nitriles offers a "greener" alternative,[1] because this eco-friendly biotransformation allows the clean and mild synthesis of carboxylic acids with high yield and selectivity.[2–15] Cyanocarboxylic acids are important intermediates for a variety of applications. For example, 1-cyanocyclohexaneacetic acid is a useful precursor for the synthesis of gabapentin.[16–18]



Gabapentin

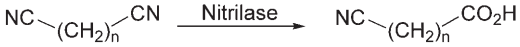
An attractive approach to the synthesis of this type of important compounds is the selective hydrolysis of dinitriles to cyanocarboxylic acids. However, the selective chemical hydrolysis of dinitriles is virtually impossible. Therefore, nitrilase-catalyzed selective hydrolysis of dinitriles to cyanocarboxylic acids becomes particularly appealing to synthetic chemists. In this context, DiCosimo et al. have reported that aliphatic dinitriles were selectively hydrolyzed to cyanocarboxylic acids by the heat-treated resting cells of *Acidovorax facilis* 72W.[15] Effenberger and Osswald have demonstrated that a nitrilase from *Arabidopsis thaliana* catalyzed the selective hydrolysis of α,ω-dinitriles to ω-cyanocarboxylic acids.[11] Meth-Cohn and Wang have studied the selective hydrolysis of a variety of dinitriles using *Rhodococcus* sp. AJ270, a nitrile hydratase/amidase-containing organism.[19] In all cases, it has been found that the selectivity was dependent on the chain length of aliphatic dinitriles. The cyanobacterium *Synechocystis* sp. strain PCC 6803 also harbors a nitrilase which shows chain length-dependent selectivity toward the hydrolysis of aliphatic dinitriles.[20] Selective hydrolysis (desymmetrization) of 3-substituted glutaronitriles has also been achieved with whole cell biocatalysts.[21–23] Li et al. have reported the enantioselective hydrolysis of α,α-disubstituted malononitriles by *Rhodococcus* sp. CGMCC 0497 strain having both nitrile hydratase and amidase activity to give (*R*)-α,α-disubstituted malonamic acids.[24] The selective hydrolysis of aromatic dinitriles has been observed in some early studies.[25,26]

In our effort to search for effective nitrilase catalysts, a nitrilase gene (bll6402) from *Bradyrhizobium japonicum* strain USDA110 has been cloned and expressed in *E. coli*. The encoded protein has been purified and has shown highest activity towards the hydrolysis of mandelonitrile.[27] In addition, this nitrilase catalyzes the enantioselective hydrolysis of β-hydroxy nitriles to (*S*)-enriched β-hydroxy carboxylic acids.[28] To further explore its synthetic application, this enzyme catalyst has been studied toward the hydrolysis of a series of α,ω-dinitriles. The nitrilase bll6402 was produced by following our previously reported

protocol.[27] The dinitriles were treated with the enzyme in potassium phosphate buffer (100 mM, pH 7.2) for 24 hours. The products were isolated and characterized as described in the Supporting Information. The results are presented in Table 1.

As shown in Table 1, nitrilase bll6402 efficiently catalyzed the selective hydrolysis of α,ω-dinitriles to exclusively afford ω-cyanocarboxylic acids. The selectivity of this enzyme was not dependent on the chain length of the α,ω-dinitriles. When the ω-cyanocarboxylic acids were treated with this nitrilase under the same conditions, no reaction was detected. This was different from the results obtained with nitrilases from *Arabodopsis thaliana*, *Acidovorax facilis* 72W and cyanobacterium *Synechocystis* sp. strain PCC 6803, in which di-acids were obtained as the chain length increased.[11,15,20]

Table 1. Selective hydrolysis of dinitriles catalyzed by nitrilase bll6402.

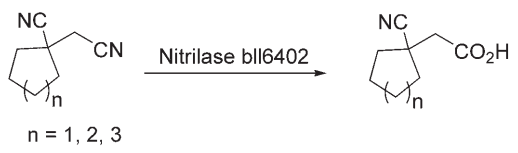


Dinitrile[a]	Yield [%][b]
α,α-Dimethylmalononitrile	93
Fumaronitrile	84
Succinonitrile	97
Glutaronitrile	93
1,4-Dicyanobutane	97
1,5-Dicyanopentane	90
1,6-Dicyanohexane	88
Sebeconitrile	88

[a] The substrate concentrations were 24.4–51.2 mM. 8 mg (10.8 U) of nitrilase were used.
[b] In all cases, no starting dinitrile was detected by TLC analysis of the reaction mixture after 24 h.

Since 1-cyanocycloalkaneacetic acids are useful precursors for the synthesis of gabapentin and its analogues,[16,17] nitrilase bll6402 was used for the preparation of 1-cyanocycloalkaneacetic acids such as 1-cyanocyclopentaneacetic acid, 1-cyanocyclohexaneacetic acid and 1-cyanocycloheptaneacetic acid, as shown in Scheme 1.

1-Cyanocycloalkaneacetonitiles were prepared by a modified procedure from the literature.[29,30] When 1-cyanocyclopentaneacetonitrile ***or*** 1-cyanocyclohexaneacetonitrile was treated with the nitrilase in potassium phosphate buffer, the corresponding cyanocarboxylic acids were isolated in 92 and 88% yields, respectively. For 1-cyanocycloheptaneacetonitrile, the reaction was not completed even after 48 hours. 1-Cyanocycloheptaneacetic acid was obtained in 55% yield with 33% of substrate recovery. This might result from the low solubility of 1-cyanocycloheptaneacetonitrile in aqueous buffer. Therefore, nitrilase bll6402 was a useful catalyst for the preparation of the precursors of gabapentin and its analogues *via* regioselective hydrolysis of 1-cyanocycloalkaneacetonitiles.



Scheme 1. Preparation of 1-cyanocycloalkaneacetic acids.

In conclusion, the nitrilase bll6402 from *Bradyrhizobium japonicum* strain USDA110 not only efficiently hydrolyzes α- and β-hydroxynitriles as reported in our previous studies,[27,28] but also selectively hydrolyzes α,ω-dinitriles to give ω-cyanocarboxylic acids exclusively. The selectivity in the hydrolysis of α,ω-dinitriles was independent of the substrate chain length, a distinct feature from the known nitrilases of various sources.[11,15,20] Nitrilase bll6402 was then applied to the synthesis of 1-cyanocycloalkaneacetic acids, the useful precursors for the synthesis of gabapentin and its analogues. It has thus been demonstrated that nitrilase bll6402 is a versatile biocatalyst for the useful transformation of nitriles to carboxylic acids.

Experimental Section

Hydrolysis of Dinitriles

Hydrolysis of dinitriles was carried out as follows (using 1,4-dicyanobutane as an example): To a solution of 1,4-dicyanobutane (200 mg, 1.85 mmol) in potassium phosphate buffer (50 mL, 100 mM, pH 7.2), enzyme (8 mg, 10.8 U, 1 U was defined as the enzyme which hydrolyzed 1 μmol of phenylacetonitrile per min at 30 °C) was added and the reaction mixture was incubated at 30 °C for 24 h. No starting nitrile was detected by TLC analysis. The mixture was acidified with 1 N HCl solution to pH ~5, saturated with NaCl and extracted with ethyl acetate. The organic extract was dried over anhydrous sodium sulfate. Removal of solvent provided the crude product, which was further purified by preparative TLC using ethyl acetate-hexane as eluting solvent. 5-Cyanopentanoic acid (yield: 227 mg) was obtained as colorless oil.[11,15] and characterized as described in the Supporting Information.

Preparation of 1-Cyanocycloalkaneacetonitriles

The 1-cyanocycloalkaneacetonitriles were prepared by a modification of the reported methods (using 1-cyanocyclohexaneacetonitrile as an example):[29–31] Ethyl cyanoacetate (1.0 g, 8.84 mmol) and cyclohexanone (7.10 mmol) were dissolved in anhydrous benzene (10 mL) containing ammonium acetate (1.77 mmol) and glacial acetic acid (0.95 mL). The reaction mixture was refluxed vigorously for 4 h, and the water formed during the reaction was removed with a Dean–Stark condenser placed under the reflux condenser.

Evaporation of the solvent afforded the crude product, which was purified by column chromatography using ethyl acetate/hexane (10/90, v/v) as eluent. The α,β-unsaturated α-cyanoacetate was isolated as a colorless oil in 95% yield.

A solution of the α,β-unsaturated α-cyanoacetate (5 mmol) and NaCN (8.5 mmol) in 90% ethanol (15 mL) was refluxed for 5 h. The resulting dark solution was evaporated, and residue was suspended in water (50 mL) and extracted with dichloromethane. The organic extract was dried over anhydrous Na_2SO_4. Removal of solvents afforded 1-cyanocyclohexaneacetonitrile as a white solid; yield: 0.52 g (70%), which was characterized by ^1H and ^{13}C NMR data (see Supporting Information).[18,30]

Preparation of 1-Cyanocycloalkaneacetic Acids

The general procedure was followed: 1-Cyanocyclopentaneacetonitiles (200 mg, 1.31 mmol) was treated with the nitrilase (10 mg, 13.5 U) in potassium phosphate buffer (50 mL, 100 mM, pH 7.2). The reaction mixture was incubated at 30 °C and the reaction was monitored by ^{13}C NMR. When dinitrile was consumed, the mixture was acidified with 1 N HCl solution to pH ~5. After being saturated with NaCl, the mixture was extracted with ethyl acetate. The organic extract was dried over anhydrous sodium sulfate. Removal of solvent provided the desired product, which was further purified by preparative TLC using ethyl acetate-hexane (35/65, v/v) as eluting solvent.

1-Cyanocyclopentaneacetic acid: Yield: 210 mg (92%); mp 79–81 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.74$ (s, 2H), 2.32–2.35 (m, 2H), 1.90–1.93 (m, 2H), 1.7–1.85 (m, 4H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 175.6$, 124.4, 42.3, 39.9, 38.6, 24.4; anal. calcd. for $C_8H_{11}NO_2$: C 62.73, H 7.24, N 9.14; found: C 62.54, H 7.35, N 8.92.

1-Cyanocyclohexaneacetic acid: Yield: 198 mg (88%); mp 103–105 °C, lit.[16] 102–103 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.64$ (s, 2H), 2.13–2.16 (m, 2H), 1.64–1.78 (m, 5H), 1.35–1.41 (m, 2H), 1.20–1.27 (m, 1H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 175.1$, 122.6, 42.2, 36.6, 35.7, 25.4, 23.1; anal. calcd. for $C_9H_{13}NO_2$: C 64.65, H 7.84, N 8.38; found: C 64.56, H 8.01, N 8.21.

1-Cyanocycloheptaneacetic acid: In this case, the conversion was not complete even after 48 h as monitored by ^{13}C NMR; 65 mg (33%) of starting material was recovered; yield: 123 mg (55%); mp 131–133 °C; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.67$ (s, 2H), 2.13–2.17 (m, 2H), 1.70–1.80 (m, 8H), 1.54 –1.65 (m, 2H); ^{13}C NMR (100.6 MHz, $CDCl_3$): $\delta = 175.3$, 123.6, 44.6, 39.2, 38.2, 28.1, 23.6; anal. calcd. for $C_{10}H_{15}NO_2$: C 66.27, H 8.34, N 7.73; found: C 66.03, H 8.41, N 7.58

Acknowledgements

The authors thank Southern Methodist University and Robert. A. Welch Foundation for financial support.

References

[1] P. T. Anastas, J. C. Warner, *Green Chemistry: Theory and Practice*, Oxford University Press, New York, **1998**, p 152.
[2] U. T. Bornscheuer, R. J. Kazlauskas, *Hydrolases in Organic Synthesis: Regio- and Stereoselective Biotransformations*, Wiley-VCH, Weinheim, **2006**, p 368.
[3] M.-X. Wang, *Top. Catal.* **2005**, *35*, 117–130.
[4] L. Martinkova, V. Mylerova, *Curr. Org. Chem.* **2003**, *7*, 1279–1295.
[5] T. Sugai, T. Yamazaki, M. Yokoyama, H. Ohta, *Biosci. Biotechnol. Biochem.* **1997**, *61*, 1419–1427.
[6] J. A. Chaplin, M. D. Levin, B. Morgan, N. Farid, J. Li, Z. Zhu, J. McQuaid, L. W. Nicholson, C. A. Rand, M. J. Burk, *Tetrahedron: Asymmetry* **2004**, *15*, 2793–2796.
[7] E. C. Hann, A. E. Sigmund, S. K. Fager, F. B. Cooling, J. E. Gavagan, M. G. Bramucci, S. Chauhan, M. S. Payne, R. DiCosimo, *Tetrahedron* **2004**, *60*, 577–581.
[8] 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.
[9] 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.
[10] S. Osswald, H. Wajant, F. Effenberger, *Eur. J. Biochem.* **2002**, *269*, 680–687.
[11] F. Effenberger, S. Osswald, *Synthesis* **2001**, 1866–1872.
[12] F. Effenberger, S. Osswald, *Tetrahedron: Asymmetry* **2001**, *12*, 279–285.
[13] F. Effenberger, S. Osswald, *Tetrahedron: Asymmetry* **2001**, *12*, 2581–2587.
[14] 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.
[15] 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.
[16] R. A. Jennings, D. R. Johnson, R. E. Seamans, J. R. Zeller, (Warner-Lambert Co., USA), *US Patent* 5,319,135, **1994**.
[17] M. P. Burns, J. W. Wong, (Pfizer Products Inc.), *World Patent* WO 2004111256, **2004**.
[18] C. E. Burgos-Lepley, L. R. Thompson, C. O. Kneen, S. A. Osborne, J. S. Bryans, T. Capiris, N. Suman-Chauhan, D. J. Dooley, C. M. Donovan, M. J. Field, M. G. Vartanian, J. J. Kinsora, S. M. Lotarski, A. El-Kattan, K. Walters, M. Cherukury, C. P. Taylor, D. J. Wustrow, J. B. Schwarz, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2333–2336.
[19] O. Meth-Cohn, M.-X. Wang, *J. Chem. Soc., Perkin Trans. 1* **1997**, 3197–3204.
[20] C. Mukherjee, D. Zhu, E. R. Biehl, L. Hua, *Eur. J. Org. Chem.* **2006**, 5238–5242.
[21] H. Kakeya, N. Sakai, A. Sano, M. Yokoyama, T. Sugai, H. Ohta, *Chem. Lett.* **1991**, 1823–1824.
[22] M.-X. Wang, C.-S. Liu, J.-S. Li, O. Meth-Cohn, *Tetrahedron Lett.* **2000**, *41*, 8549–8552.

[23] M.-X. Wang, C.-S. Liu, J.-S. Li, *Tetrahedron: Asymmetry* **2001**, *12*, 3367–3373.
[24] Z.-L. Wu, Z.-Y. Li, *Chem. Commun.* **2003**, 386–387.
[25] M. A. Cohen, J. Sawden, N. J. Turner, *Tetrahedron Lett.* **1990**, *31*, 7223–7226.
[26] C. Bengis-Garber, A. L. Gutman, *Appl. Microbiol. Biotechnol.* **1989**, *32*, 11–16.
[27] D. Zhu, C. Mukherjee, E. R. Biehl, L. Hua, *J. Biotechnol.* **2007**, *129*, 645–650.
[28] S. Kamila, D. Zhu, E. R. Biehl, L. Hua, *Org. Lett.* **2006**, *8*, 4429–4431.
[29] D. Xue, Y.-C. Chen, X. Cui, Q.-W. Wang, J. Zhu, J.-G. Deng, *J. Org. Chem.* **2005**, *70*, 3584–3591.
[30] J. S. New, J. P. Yevich, *Synthesis* **1983**, 388–389.
[31] H. Schaefer, *Liebigs Ann. Chem.* **1965**, *688*, 113–1121.

