J|A|C|S
COMMUNICATIONS

Published on Web 07/10/2002

An Enzyme Library Approach to Biocatalysis: Development of Nitrilases for Enantioselective Production of Carboxylic Acid Derivatives

Grace DeSantis,* Zuolin Zhu, William A. Greenberg, Kelvin Wong, Jenny Chaplin, Sarah R. Hanson, Bob Farwell, Lawrence W. Nicholson,[1] Cynthia L. Rand,[1] David P. Weiner, Dan E. Robertson, and Mark J. Burk*

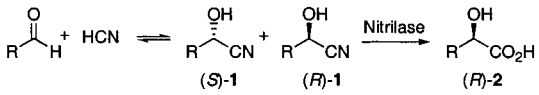
Diversa Corporation, 4955 Directors Place, San Diego, California 92121

Received February 20, 2002

Biocatalytic processes can offer unique advantages in transformations that are challenging to accomplish through conventional chemical methods.[2] Nitrilases (EC 3.5.5.1) catalyze the mild hydrolytic conversion of organonitriles directly to the corresponding carboxylic acids.[3] Fewer than 15 microbially derived nitrilases have been characterized and reported to date.[3,4] Several nitrilases previously have been explored for the preparation of single-enantiomer carboxylic acids, although little progress has been made in the development of nitrilases as viable synthetic tools. We now report the discovery of a large and diverse set of nitrilases and herein demonstrate the utility of this nitrilase library for identifying enzymes that catalyze efficient enantioselective production of valuable hydroxy carboxylic acid derivatives.

In an effort to access the most diversified range of enzymes that can be found in Nature, we create large genomic libraries by extracting DNA directly from environmental samples that have been collected from varying global habitats.[5] We have established a variety of methods to identify novel activities through screening these libraries.[6] Using this approach, we now have discovered and characterized over 200 new nitrilases.[7] All nitrilases were defined as unique at the sequence level and were shown to possess the conserved catalytic triad Glu-Lys-Cys which is characteristic for this enzyme class.[8] Each nitrilase in our library was overexpressed and stored as a lyophilized cell lysate to facilitate rapid evaluation of the library for particular biocatalytic functions.

Our initial investigations focused upon the efficacy of nitrilases for production of α-hydroxy acids **2** through hydrolysis of cyanohydrins **1**. Cyanohydrins are well-documented to racemize under basic conditions through reversible loss of HCN.[9] Thus, a dynamic kinetic resolution (DKR) process is possible whereby an enzyme selectively hydrolyzes only one enantiomer of **1**, affording **2** in 100% theoretical yield and with high levels of enantiomeric purity.



One important application of nitrilases involves commercial production of (*R*)-mandelic acid from mandelonitrile.[4d,10] Mandelic acid and derivatives find broad use as intermediates and resolving agents for the production of many pharmaceutical and agricultural products.[11] However, the few known nitrilases derived from cultured organisms have not been found useful for efficient and selective hydrolysis of analogous substrates.

We first screened our nitrilase library for activity and enantioselectivity in the hydrolysis of mandelonitrile (**3a**, Ar = phenyl) to mandelic acid. Preliminary results revealed that 27 enzymes afforded mandelic acid in >90% ee. One enzyme (nitrilase I) was studied in greater detail and was found to be very active for hydrolysis of mandelonitrile. Under standard conditions using 25 mM **3a**, and 0.12 mg/mL enzyme in 10% MeOH (v/v) 0.1 M phosphate buffer at 37 °C and pH 8, (*R*)-mandelic acid was formed quantitatively within 10 min and with 98% ee. To confirm synthetic utility, the reaction was performed using 1.0 g of **3a** (50 mM) and 9 mg of nitrilase (0.06 mg/mL nitrilase I); after 3 h (*R*)-mandelic acid was isolated in high yield (0.93 g, 86%) and with 98% ee.

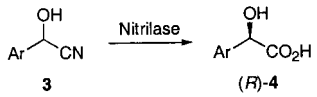


Table 1. Nitrilase I-Catalyzed Production of Mandelic Acid Derivatives and Analogues **4** under DKR Conditions[a]

entry	Ar in **4**	specific activity[b]	TOF[c]	% ee[d]
1	C_6H_5	50	28	98
2	2-Cl-C_6H_5	3	1.7	97
3	2-Br-C_6H_5	10	5.6	96
4	2-Me-C_6H_5	9	5.1	95
5	3-Cl-C_6H_5	6	3.4	98
6	3-Br-C_6H_5	3	1.7	99
7	4-F-C_6H_5	21	11.8	99
8	1-naphthyl	5	2.8	95
9	2-naphthyl	5	2.8	98
10	3-pyridyl	33	18.6	97
11	3-thienyl	30	16.8	95

[a] Reactions were conducted under standard conditions (see text). Reaction time for complete conversion to **4** was 1−3 h. Entries 8−9 were conducted at pH 9 and 5 mM substrate concentration. [b] Specific activities were measured at 5 min transformation timepoints and are expressed as μmol mg^{-1} min^{-1}. [c] TOF = turnover frequency, mol product/mol catalyst/s. [d] Enantioselectivites were determined by chiral HPLC analysis. Hydroxy acids were isolated, and absolute configurations were determined to be (*R*) in all cases.

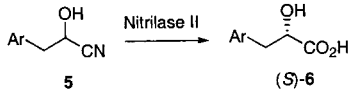
We next explored the substrate scope of nitrilase I. As shown in Table 1, a broad range of mandelic acid derivatives as well as aromatic and heteroaromatic analogues (**4**) may be prepared through this method. Nitrilase I tolerates aromatic ring substituents in the ortho-, meta-, and para-positions of mandelonitrile derivatives, and products of type **4** were produced with high enantioselectivities. Other larger aromatic groups such as 1-naphthyl and 2-naphthyl also are accommodated within the active site, again affording the acids **4** with high selectivity (Table 1, entries 8−9). Finally, 3-pyridyl and 3-thienyl analogues of mandelic acid were prepared readily using this process (Table 1, entries 10−11). To our knowledge, this is the first reported demonstration of a nitrilase that affords a range of mandelic acid derivatives and heteroaromatic

* To whom correspondence should be addressed. E-mail: gdesantis@diversa.com; mburk@diversa.com.

10.1021/ja0259842 CCC: $22.00

analogues of type **4**. High activity on the more sterically encumbered ortho-substituted and 1-naphthyl derivatives is particularly noteworthy.

We next examined the preparation of aryllactic acid derivatives **6** through hydrolysis of the corresponding cyanohydrins **5**. Phenyllactic acid and derivatives serve as versatile building blocks for the preparation of numerous biologically active compounds.[11] Upon screening our nitrilase library against the parent cyanohydrin **5a** (Ar = phenyl), we found several enzymes that provided **6a** with high enantiomeric excess. One enzyme, nitrilase II, was further characterized. A reaction using 2 mg of nitrilase (0.016 mg/mL nitrilase II) allowed complete transformation of 1.0 g of **5a** (50 mM) within 6 h and afforded (*S*)-phenyllactic acid (**6a**), which was isolated in high yield (0.95 g, 84%) and with 96% ee. The highest enantioselectivity previously reported for biocatalytic conversion of **5** to **6** was 75% ee achieved through a whole cell transformation using a *Pseudomonas* strain.[12]



5 (*S*)-**6**

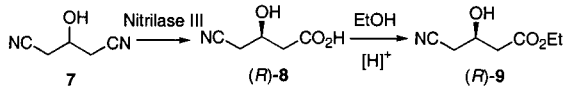
Table 2. Nitrilase II-Catalyzed Production of Aryllactic Acid Derivatives and Analogues **6** under DKR Conditions[a]

entry	Ar in **6**	specific activity[b]	TOF[c]	% ee[d]
1	C_6H_5	25	16	96
2	2-Me-C_6H_5	160	100	95
3	2-Br-C_6H_5	121	76	95
4	2-F-C_6H_5	155	97	91
5	3-Me-C_6H_5	21	13	95
6	3-F-C_6H_5	22	14	99
7	1-naphthyl	64	40	96
8	2-pyridyl	10.5	6.6	99
9	3-pyridyl	11.6	7.2	97
10	2-thienyl	3.4	2.1	96
11	3-thienyl	2.3	1.4	97

[a] Reaction conditions as in Table 1, except 0.016 mg/mL nitrilase was used. Full conversion to **6** was observed within 6 h. [b−d] See Table 1. The absolute configuration was determined to be (*S*) for phenyllactic acid and entries 2−11 were assigned (*S*) based upon identical chiral HPLC peak elution order.

Ortho and meta substituents appear to be tolerated well by nitrilase II, with ortho substituted derivatives surprisingly being converted with higher rates relative to the parent substrate **5a**. Novel heteroaromatic derivatives, such as 2-pyridyl-, 3-pyridyl, 2-thienyl-, and 3-thienyllactic acids, were prepared with high conversions and enantioselectivities (Table 2, entries 8−11). Unexpectedly, para substituents greatly lowered the rates of these reactions, with full conversion taking over two weeks under these conditions (data not shown).

The final transformation that we examined was desymmetrization of the readily available prochiral substrate 3-hydroxyglutaronitrile (**7**)[13] to afford hydroxy acid (*R*)-**8** which, once esterified to (*R*)-**9**, is an intermediate used in the manufacture of the cholesterol-lowering drug Lipitor. Previously reported attempts to use enzymes for this process were unsuccessful, and **8** was produced with low selectivity (highest: 22% ee) and the undesired (*S*)-configuration.[14]



7 (*R*)-**8** (*R*)-**9**

We screened our nitrilase library and have discovered four unique enzymes that provided the required product (*R*)-**8** with high conversion (>95%) and >90% ee. Using one of the (*R*)-specific nitrilases, this process was operated on a 1.0 g scale (240 mM **7**, 30 mg enzyme, 22 °C, pH 7) and after 22 h, (*R*)-**8** was isolated in 98% yield and 95% ee. Interestingly, the same screening program also identified 22 nitrilases that afford the opposite enantiomer (*S*)-**8** with 90−98% ee. Thus, our extensive screen of biodiversity has uncovered enzymes that provide ready access to either enantiomer of the intermediate **8** with high enantioselectivities. Our discovery of the first enzymes that furnish (*R*)-**8** underscores the advantage of having access to a large and diverse library of nitrilases.

By plumbing our environmental genomic libraries created from uncultured DNA, we have discovered a large array of novel nitrilases. This study has revealed nitrilases that furnish mandelic and aryllactic acid derivatives, as well as either enantiomer of 4-cyano-3-hydroxybutyric acid in high yield and enantiomeric excess. A more detailed survey of substrate scope associated with our nitrilase library is underway and will be reported in due course.

Acknowledgment. We thank B. Morgan, J. McQuaid, K. Keegan, I. Saliba, and N. Farid for assisting with preliminary enzyme screening, and P. Kretz, E. Chi, A. Milan, M. Miller, D. Wyborski, and I. Andruszkiewicz for enzyme discovery. T. Richardson, M. Podar, and L. Waters are gratefully acknowledged for bioinformatic analysis. We thank D. Baptista, J. Gemsch, and L. Bibbs for sequencing, R. Melkus and X. Tan for subcloning, P. Chen for analytical assistance, and A. Flordeliza, D. DiMasi and A. Vasavada for expression. We thank K. Avery (Dow) for discussion on substrate synthesis. Special thanks goes to Mark Madden for providing initial inspiration for the nitrilase program, and to Jay Short for valuable discussions and support.

Supporting Information Available: Materials and methods and amino acid sequence (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Address: Dow Chemical Co., Midland, MI.
(2) Wong, C.-H.; Whitesides, G. M. *Enzymes in Synthetic Organic Chemistry*, 2nd ed.; Pergamon: New York, 1994. (b) Drauz, K., Waldmann, H., Roberts, S. M., Eds. *Enzyme Catalysis in Organic Synthesis*; VCH: Weinheim, Germany, 2002.
(3) (a) Kobayashi, M.; Shimizu, S. *FEMS Microbiol. Lett*. **1994**, *120*, 217. (b) Bunch, A. W. In *Biotechnology*; Rehm, H.-J., Reed, G., Puhler, A., Stadler, P., Eds.; Wiley−VCH: Weinheim, Germany, 1998; Vol. 8a, Chapter 6, pp 277−324. (c) Wieser, M.; Nagasawa, T. In *Stereoselective Biocatalysis*; Patel, R. N., Ed.; Marcel Dekker: New York, 2000; Chapter 17, pp 461−486.
(4) (a) Harper, D. B. *Int. J. Biochem*. **1985**, *17*, 677. (b) Levy-Schil, S.; Soubrier, F.; Crutz-Le Coq, A. M.; Faucher, D.; Crouzet, J.; Petre, D. *Gene* **1995**, *161*, 15. (c) Yu, F. U.S. Patent 5,872,000, 1999. (d) Ress-Loschke, M.; Friedrich, T.; Hauer, B.; Mattes, R.; Engels, D. PCT Appl. WO 00/23577, April 2000.
(5) For a description of these methods, see: (a) Short, J. M. *Nat. Biotechnol*. **1997**, *15*, 1322. (b) Handelsman, J.; Rondon, M. J.; Brady, S. F.; Clardy, J.; Goodman, R. M. *Chem. Biol*. **1998**, *5*, R245. (c) Henne, A.; Daniel, R.; Schmitz, R. A.; Gottschalk, G. *Appl. Environ. Microbiol*. **1999**, *65*, 3901.
(6) (a) Robertson, D. E.; Mathur, E. J.; Swanson, R. V.; Marrs, B. L.; Short, J. M. *SIM News* **1996**, *46*, 3. (b) Short, J. M. U.S. Patent 5,958,672, 1999. (c) Short, J. M. U.S. Patent 6,030,779, 2000.
(7) For a concise description, see Supporting Information section.
(8) Pace, H.; Brenner, C. *Genome Biol*. **2001**, *2*, 0001.1−0001.9.
(9) Inagaki, M.; Hiratake, J.; Nishioka, T.; Oda, J. *J. Org. Chem.* **1992**, *57*, 5643. (b) van Eikeren, P. U.S. Patent 5,241,087, 1993.
(10) (a) Yamamoto, K.; Oishi, K.; Fujimatsu, I.; Komatsu, K. *Appl. Environ. Microbiol*. **1991**, *57*, 3028. (b) Endo, T.; Tamura, K. U.S. Patent 5,296,373, March, 1994.
(11) Coppola, G. M.; Schuster, H. F. *Chiral α-Hydroxy Acids in Enantioselective Synthesis*; Wiley−VCH: Weinheim, Germany, 1997.
(12) Hashimoto, Y.; Kobayashi, E.; Endo, T.; Nishiyama, M.; Horinouchi, S. *Biosci. Biotechnol. Biochem*. **1996**, *60*, 1279.
(13) Johnson, F.; Panella, J. P.; Carlson, A. A. *J. Org. Chem*. **1962**, *27*, 2241.
(14) (a) Crosby, J. A.; Parratt, J. S.; Turner, N. J. *Tetrahedron: Asymmetry* **1992**, *3*, 1547. (b) Beard, T.; Cohen, M. A.; Parratt, J. S.; Turner, N. J. *Tetrahedron: Asymmetry* **1993**, *4*, 1085. (c) Kakeya, H.; Sakai, N.; Sano, A.; Yokoyama, M.; Sugai, T.; Ohta, H. *Chem. Lett*. **1991**, 1823.

JA0259842