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Tetrahedron: Asymmetry
Tetrahedron: *Asymmetry* 18 (2007) 1888–1892

A mild biosynthesis of lactones via enantioselective hydrolysis of hydroxynitriles

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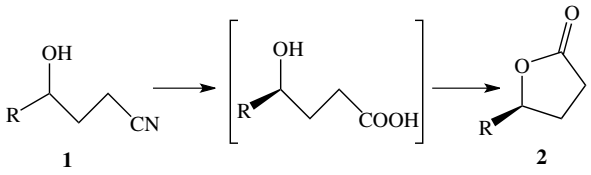
Received 3 May 2007; accepted 30 July 2007

Abstract—We have developed a biocatalytic method to produce lactones and related compounds via the enzymatic hydrolysis of γ- and β-hydroxynitriles. The synthesis is a mild, general, and environmentally friendly way to enantioselectively hydrolyze nitriles with commercially available nitrilase enzymes. The synthesis of four pheromones is demonstrated via a one-step method.

1. Introduction

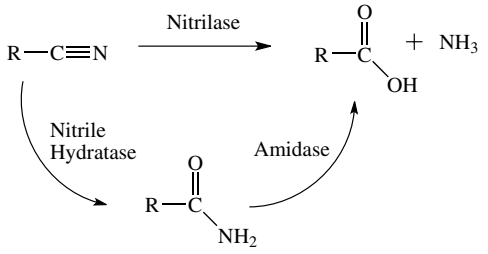
Many components of natural flavors and fragrances, insect pheromones, and building blocks for the synthesis of natural products and pharmaceuticals are γ-butyrolactones.[1] The physiological functionality of these lactones is usually dependent on the enantiomeric purity of the lactone,[1] implying that an enantioselective synthesis is important when preparing the desired stereoisomers. The hydrolysis of 4-hydroxynitriles to the corresponding hydroxyacid and subsequent lactonization is a method of producing these biologically active compounds (Eq. 1) via a one-step process.



1 **2** (1)

	-R
1a	Et
1b	Bu
1c	C_5H_{11}
1d	C_8H_{17}
1e	$CH_2CH(CH_3)_2$
1f	$CH(CH_3)_2$

Chemical hydrolysis of nitriles to carboxylic acids requires extremely harsh conditions usually involving reflux in concentrated acid or base for extended reaction times at high temperatures.[2] The microbial hydrolysis of hydroxynitriles that produces lactones in good yield under mild conditions has been developed. Initial investigations in our lab used *Rhodococcus rhodochrous* whole cells,[3] which include three types of hydrolytic enzymes: nitrilase, nitrile hydratase, and amidase (Scheme 1).



Scheme 1. Enzymes used for hydrolysis of nitriles.

When nitrilases became commercially available, our emphasis moved to the direct hydrolysis of γ-hydroxynitriles to hydroxyacids, and the subsequent internal esterification to a lactone (Eq. 1). The benefits of using biotransformations in performing this chemistry include mild, environmentally friendly conditions (pH 6 or 7 and temperatures ranging from 15 °C to 35 °C), clean reactions due to the selectivity of the nitrilase enzyme, and a general synthesis method. This work shows that nitrilases can be

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doi:10.1016/j.tetasy.2007.07.034

enantioselective in their hydrolysis.[4] The enantioselective hydrolysis of a γ-hydroxynitrile with a straight side chain served as a straightforward model (see Table 1). However, we also wanted to determine the optimum conditions for this and other analogs. We also investigated the reactivity of branched side chains of γ-hydroxynitriles with nitrilases. Two straight side chain β-hydroxynitriles were investigated to determine if having the stereogenic center closer to the reacting nitrile group had a significant effect.

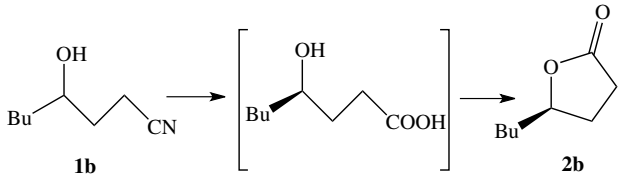
2. Results and discussion

The γ-hydroxynitrile precursors were synthesized using our general nitrile enolate-epoxide ring opening reaction;[5] these substrates were used extensively in this study.

2.1. Straight-chain isomers

The initial investigations evaluated six commercially available[†] nitrilase enzymes and their ability to convert enantioselectively 4-hydroxyoctanenitrile **1b** to the corresponding lactone. As seen in Table 1, NIT 1001 gave a racemic product; NIT 1002 and 1003 favored the (*R*)-enantiomer, whereas NIT 1004, 1005, and 1006 were selective for the (*S*)-enantiomer. The (*R*)-enantiomer is the parasitic wasp pheromone.[6]

Table 1. Initial enzymatic lactonization of 4-hydroxyoctanenitrile **1b**–**2b** at 35 °C, 200 rpm, and pH 7



1b **2b**

Enzyme	Reaction time (h)	% Conversion	*R*:*S* ratio	ee	*E*
NIT 1001	11	41	50:50	0	1.0
NIT 1002	16	28	66:34	32	2.2
NIT 1003	16	27	76:24	52	3.8
NIT 1004	1	18	29:71	42	2.7
NIT 1005	11	21	45:55	10	1.3
NIT 1006	48	6	40:60	20	1.5

In kinetic resolutions such as these (involving racemic starting materials), enantioselectivity is a key factor. The formal calculation of enantioselectivity *E* was performed as described by Faber,[7] and he discusses why *E* should approach 20 when excellent kinetic resolution is desired.

This work shows that the most important part of this synthesis was to stop the enzymatic reaction before 50% conversion, as is usually true for kinetic resolutions.[7] Generally, enzymes are more selective for one enantiomer of the racemic reactant. Therefore, the enzyme will first convert this to the product until the more reactive enantiomer is depleted. In order to achieve selective results, we had to make sure that the enzyme did not convert much of the second enantiomer to the product. Stopping the reaction at around 30–40% conversion typically gave adequate enantioselectivity.

From the early experiments NIT 1002, 1003, and 1004 seemed to be the most promising, hence we focused on improving the selectivity of these enzymes by decreasing the temperature from 35 °C to 30 °C and the pH from 7 to 6. We also examined three other substrates with ethyl- **1a**, pentyl- **1c**, and octyl- **1d** R-groups where the (*R*)-enantiomeric lactone products **2a**, **2c**, and **2d** are pheromones of the *Trogoderma* beetle,[8] rice weevil,[9] and rove beetle,[10–12] respectively. The pentyl side chain gave the most selective results, as seen in Table 2. The selectivity was adequate to serve as a synthesis for these important pheromones. Reactions that have an *E* of approximately 10 or higher have been highlighted.

Since we found that in most cases decreasing the temperature to 30 °C increased the selectivity slightly, we tried reducing the temperature further to 15 °C in the hope of obtaining more selective results. In most cases, the temperature actually decreased the *E*-value and extended the reaction times.

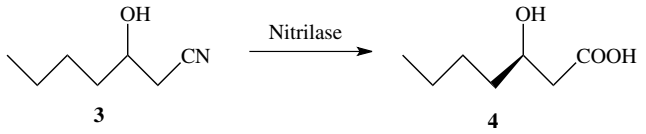
2.2. Chain branching

Next, increasing the selectivity was attempted by using a branched substrate, 4-hydroxy-6-methylheptanenitrile (*R* = isobutyl, Table 3). The branched precursor apparently did not fit into the active site of NIT 1001 and 1006, so it did not convert to the lactone. NIT 1004 was the only enzyme that transformed both the branched and straight-chain substrates.

Another branched side chain analogue, 4-hydroxy-5-methylhexanenitrile, was investigated. Chiral GC or chiral HPLC could not be used to determine the enantioselectivity of the reaction. However, the ability of the enzymes to hydrolyze the nitrile is noteworthy, as seen in Table 4.

2.3. Proximity effect

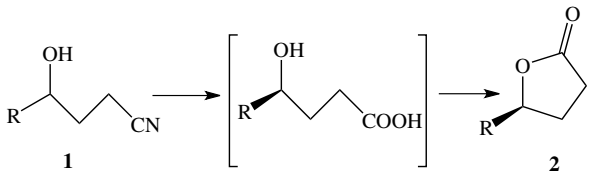
To enhance our study, reactions on β-hydroxynitriles were performed, where the stereogenic center is closer to the reacting nitrile group (Eq. 2). The specific β-hydroxynitrile used was 3-hydroxyheptanenitrile **3**. It should be noted that a lactone does not spontaneously form because of steric influences (a four-membered ring lactone is difficult to form). The results (Table 5) show an improved enantioselectivity using NIT 1002 for hydrolysis.



3 **4** (2)

[†] Supplied by BioCatalytics, Inc.

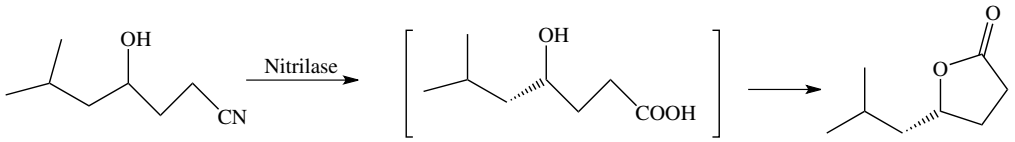
Table 2. Results of nitrilases 1002, 1003, and 1004 with γ-hydroxynitrile substrates with side chains of 2, 4, 5, and 8 carbons at optimal pH, temperature, and 200 rpm



1 **2**

Substrate	Lactone	Enzyme	pH	Time (h)	Temp (°C)	% Conversion to **2**	*R*:*S*	ee	*E*
1a	**2a**	NIT 1002	6	21.5	37–38	15	75:25	50	3.3
1b	**2b**	NIT 1002	6	24	30	40	82:18	64	6.9
1c	**2c**	NIT 1002	7	11.5	30	42	85:15	70	9.3
1d	**2d**	NIT 1002	7	11.25	30	16	79:21	58	4.2
1a	**2a**	NIT 1003	7	4.6	30	47	77:23	54	1.3
1b	**2b**	NIT 1003	6	24	30	26	90:10	80	11.8
1c	**2c**	NIT 1003	7	4.6	30	30	94:6	88	22.6
1d	**2d**	NIT 1003	6	24	30	44	60:40	20	1.7
1a	**2a**	NIT 1004	6	1.75	30	3	31:69	38	2.3
1b	**2b**	NIT 1004	6	1.75	30	6	27:73	46	2.8
1c	**2c**	NIT 1004	6	1.75	30	8	25:75	50	3.1
1d	**2d**	NIT 1004	6	4	30	29	37:63	26	1.9

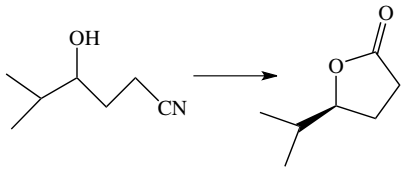
Table 3. Results of nitrilases with substrate **1e** reacting to product **2e** at pH 7 and 200 rpm



1e **2e**

Enzyme	Time (h)	Temp (°C)	% Conversion	*R*:*S* ratio	ee	*E*
NIT 1001	114.5	36	0	—	—	—
NIT 1002	0.5	30	33	74:26	48	3.6
NIT 1003	51	36	28	50:50	0	1
NIT 1004	2	35	27.5	15:85	70	7.3
NIT 1005	27	36	15	47:53	6	1.2
NIT 1006	114.5	36	0	—	—	—

Table 4. Hydrolysis of 4-hydroxy-5-methylhexanenitrile with nitrilases 1002, 1003, and 1004 at pH 7



Enzyme	% Conversion	Temp (°C)	Time (h)
NIT 1002	33	30	1.5
NIT 1003	29	32	19
NIT 1004	32	32	23

Table 5. Reactions of substrate **3** with nitrilases 1002, 1003, and 1004; conversion to product **4**

Enzyme	Reaction time (h)	% Conversion	*R*:*S* ratio	ee	*E*
NIT 1002	5	13	9:91	82	11.4
NIT 1003	30	30	25:75	50	3.7
NIT 1004	18.6	19	70:30	40	2.6

3. Conclusion

The investigation of α-hydroxynitriles (cyanohydrins) was recently communicated.[4b] These substrates undergo highly enantioselective hydrolysis. Their work and our study of γ- and β-hydroxynitriles provide a picture of the potential of these three classes of compounds in enantioselective synthesis. Although the stereogenic center of γ- and β-substrates is distant from the reacting center, significant chiral enantioselection can still be achieved. This reaction can be carried out under much milder reaction (e.g., neutral pH and 30 °C) conditions than typical chemical hydrolysis. More selective enzymes will be developed, but the synthesis of the rice weevil pheromone **2c** using this chemistry is already demonstrated to be a viable procedure ($E > 20$). We hope our publication alerts the chemical community to the potential of these types of reactions.

4. Experimental

The nitrilase enzymes used were from BioCatalytics, Inc. We made hydroxynitriles **1a–d** and characterized and proved their product purities before (Eq. 2),[5] and the optically active lactones were too (made by conventional techniques using optically active epoxides).[11,12] Compound **1e** is the only new compound reported, and the ^{13}C and ^{1}H NMR spectra data (and HRMS, etc.) have been supplied to prove the identity and purity of it (>95%). All reported enzymatic reactions were performed in duplicate or triplicate.

4.1. General enzymatic procedure

The enzymatic reactions were performed by adding approximately 30 mg of hydroxynitrile to less than 10 mg of nitrilase enzyme in 5 mL of sodium phosphate buffer in a 20 mL vial. The vial was loosely capped and placed in an orbit shaker bath at 200 rpm for various periods of time at the desired temperature. There was some difficulty with the hydroxyacid failing to completely lactonize, but when the reaction mixture was allowed to sit for at least an hour in slightly acidic conditions (pH 3 or 4), more lactonization would occur. The reaction mixture was run through a plug of Florisil with ethyl acetate to remove the enzyme. The product was extracted twice using 1:1 ethyl acetate–diethyl ether and dried over anhydrous $MgSO_4$. The solvents were evaporated. The percent conversion was determined using ^{1}H 400 MHz NMR and integrating the hydrogens at the stereogenic centers of both the reactant and the product. The lactone was isolated by small-scale column chromatography using deactivated silica and dichloromethane. Chiral gas chromatography (Betacyclodextrin column, oven temperature: 100–160 °C) was used to determine the ratio of enantiomers for the γ-butyrolactones. The (*R*)-enantiomer eluted first (this was assumed for **2e**). For the β-hydroxynitriles, the spontaneous lactonization of the β-hydroxyacid did not occur due to ring strain. The enantiomers were separated by high performance liquid chromatography using a chiral column (Chiralcel OD-H). The acid was first derivatized with a chromophore (esterification with benzyl bromide using K_2CO_3 for deprotonation of the acid[13]) and the selectivity was determined by detection of the enantiomers with a UV detector.[14] Table 6 shows the retention times for each product investigated.

4.2. 4-Hydroxy-6-methylheptanenitrile 1f + NIT 1004

In a 20 mL vial, 0.0073 g of NIT 1004 0.0341 g of the 4-hydroxyoctanenitrile, and 5 mL of pH 7 sodium phosphate buffer were combined. The vial was loosely capped and placed in the orbit shaker bath (35 °C, 200 rpm) for 1.5 h. The solution was made slightly acidic by adding 1 M HCl. The reaction mixture was filtered through a plug of Florisil using ethyl acetate and then extracted with 1:1 ethyl acetate–diethyl ether. The organic layers were dried over anhydrous $MgSO_4$ and the solvents were evaporated (0.019 g, 55% crude yield). ^{1}H NMR: δ 3.81 (1H, hydroxynitrile), 4.57 (0.28H, lactone), 22% conversion. The lactone was isolated, and the *R*:*S* ratio was determined through chiral GC (Betadex)-R_t 16.5 min [17% area, (*R*)-enantiomer], 16.8 [83% area, (*S*)-enantiomer].

Table 6. GC and HPLC retention times for γ-lactones and β-hydroxyacid synthesized

Substrate used	Product made	Enantiomer	Retention time (min)
1a	**2a**	(*R*)	41.9
		(*S*)	43.8
1b	**2b**	(*R*)	21.2
		(*S*)	22.1
1c	**2c**	(*R*)	36.8
		(*S*)	38.0
1d	**2d**	(*R*)	49.5
		(*S*)	49.9
1e	**2e**	(*R*)	16.5
		(*S*)	16.8
3	**4**	(*R*)	4.6
		(*S*)	5.8

When the reaction time was 127 h with NIT 1004, 91% pure racemic 4-isobutyl-γ-butyrolactone[15] **2f** was obtained, ^{1}H NMR δ ($CHCl_3$), approximately 0.9 (6H, 2d, $J = 7$ Hz), 1.25–1.9 (5H, m), 2.15–2.3–2.4 (1H, m), 2.5–2.6 (2H, m), 4.5–4.7 (1H, m).[15]

4.2.1. Preparation of 3-hydroxyheptanenitrile 3.[16,17] Under a nitrogen atmosphere, 10 mL of LDA (2.0 M) was cooled to −78 °C. Anhydrous THF (10.5 mL) was added, immediately followed by acetonitrile (1.1 mL). After stirring for 1 h, valeraldehyde (2.1 mL) was added in 10 mL of anhydrous THF and the reaction continued to stir for 30 min at −78 °C. TMSCl (4 mL) was added dropwise and 10 min later, methanol (5.7 mL) was added. The yellow/white solution was warmed to room temperature overnight. The white precipitate was filtered using ethyl acetate and the THF evaporated. The solid was dissolved in ethyl acetate and washed with saturated NH_4Cl and NaCl.[14] The solvent was removed in vacuo, and the product purified through distillation, bp 60–63 °C (0.25 mm), IR (NaCl disks) 3400 (OH) and 2250 cm^{-1}, ^{1}H NMR ($CDCl_3$) δ 0.93 (t, $J = 7$ Hz, 1H), 1.25–1.5 (m, 4H), 1.55–1.7 (m, 2H), 2.46 (dd, $J = 6$ and 16.4 Hz, 1H), 2.52 (dd, $J = 5$ and 16.5 Hz, 1H), 3.96 (m, 1H), 2.1 (br, OH): ^{13}C NMR ($CDCl_3$) δ 13.9, 22.4, 26.1, 27.5, 36.2, 67.7, and 117.7.[16,17]

4.2.2. 3-Hydroxyheptanoic acid 4.[18,19] Compound **4** was prepared by the general enzymatic procedure. The resulting oil was dissolved in 15 mL of 1:1 EtOAc–ether, and the organic layer was extracted with 5% $NaHCO_3$. The aqueous layer was acidified to pH 3 by the dropwise addition of 1 M HCl, after which it was extracted with 15 mL of 1:1 EtOAc–ether solvent mixture. After drying over Na_2SO_4, the organic layer was evaporated. The resulting organic product was analyzed. ^{1}H NMR ($CDCl_3$) δ 0.91 (t, $J = 7$ Hz, 3H), 1.25–1.6 (m, 6H), 2.4–2.6 (m, 2H), 2.7–3.5 (br, 2OH), 4.0 (m, 1H); IR (NaCl disks) 3650–2700 (COOH) 1715 (–C═O), 2250 (w, –CN). This acid was most often isolated as the ester.[19]

4.2.3. Esterification of 3-hydroxyheptanoic acid 3.[13] 3-Hydroxyheptanoic acid (20 mg prepared from the nitrilase reaction) was dissolved in 1.0 mL of dry DMF. Under nitrogen, K_2CO_3 (0.040 g) and benzyl bromide (20 μL) were added. The white-yellow reaction mixture was stirred for 3 h. Ether (5 mL) and water (5 mL) were then added. After extraction with ether, the organic layer was washed with 15% NaCl and dried over anhydrous $MgSO_4$. The solvent was evaporated giving approximately 10 mg of the ester of **4**.

4.3. Compound characterization

4.3.1. 4-Hydroxy-6-methylheptanenitrile 1e. The clear, colorless oil was purified through distillation (102–103 °C at 1.5 mm Hg); 50–65% yield; IR: 3365 (br), 2249 (–CN), 1665, 1601 1140, 1084, 1051 cm^{-1}; ^1H NMR: δ 3.80 (1H, m), 2.51 (2H, m), 1.91 (1H, s), 1.70 (3H, m), 1.40 (1H, m), 1.25 (1H, m), 0.94 (3H, d, $J = 6.7$ Hz), 0.93 (3H, d, $J = 6.7$ Hz); ^{13}C NMR: δ 13.6, 21.9, 23.2, 24.5, 32.9, 46.4, 67.9, 119.9; TLC: $R_f = 0.30$ in 1:1 hexanes–ethyl acetate; Cap. GC: $t_R = 11.73$ min (29 m capillary column, HP 6890, 50 °C for 4 min then 10 °C/min up to 300 °C for 20 min); MS (EI) m/z 141 (1), 84 (59), 69 (100); 41 (77): HRMS (EI) 141.1154 (calcd) 141.1149 (actual).

Acknowledgements

We would like to acknowledge Pfizer Inc. for donating the orbit shaker bath and the Michigan State University Mass Spectrometry Facility. We gratefully acknowledge support from the National Science Foundation REU program (#0243828), Research Corporation and GlaxoSmithKline, Inc.

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