

## Hydrolysis of Nitriles Using an Immobilized Nitrilase: Applications to the Synthesis of Methionine Hydroxy Analogue Derivatives

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Mild and selective hydrolysis of a large range of nitriles leading to carboxylic acids was achieved under neutral conditions by an immobilized and genetically modified enzyme preparation from *Alcaligenes faecalis* ATCC8750. This immobilized nitrilase has been shown to be an effective catalyst for the stereoselective hydrolysis of mandelonitrile **1a** to *R*-(–)-mandelic acid **1c**. This method is particularly useful for the production of hydroxy analogues of methionine derivatives **2c–4c** that could have an interest in cattle feeding and for the transformation of compounds containing other acid- or base-sensitive groups **3a–10a**. A series of aliphatic dinitriles **11a–15a** was hydrolyzed to the corresponding cyano acids. The suitability of the immobilized catalyst as a robust and versatile biocatalyst is discussed, and models to account for the stereoselectivity of the enzymic hydrolysis have been proposed.

**KEYWORDS:** Biotransformation; enzymes; hydrolases; methionine hydroxy analogue derivatives; dinitriles

### INTRODUCTION

During the past 15–20 years, there has been an enormous increase in the use of enzymes as catalysts for organic synthesis (1). In particular, hydrolytic enzymes (2) (e.g., lipases, esterases, and acylases) have proven to be particularly useful catalysts for the preparation of homochiral molecules. There are two major reasons for the widespread use of these catalysts by a variety of laboratories: (i) the enzymes operate on wide range of substrates with very high stereoselectivity and (ii) they are robust and extremely easy to use. Furthermore, the replacement of water by organic solvent (3) has expended the range of reactions that can be affected (e.g., esterification and peptide bond formation). In the same context, chemical transformation of nitriles into carboxylic acids or amides is of synthetic importance owing to the ease with which the desired organic nitrile usually can be obtained. Thus, organonitriles can be readily prepared by a number of methods including the addition of cyanide ion to alkyl halides (4), the Strecker reaction (5), the Sandmeyer reaction (6), the reaction of aryl halides with copper cyanide (7), the reaction of ketone with tosylmethyl isocyanide (8), and the dehydration of amides (9). However,

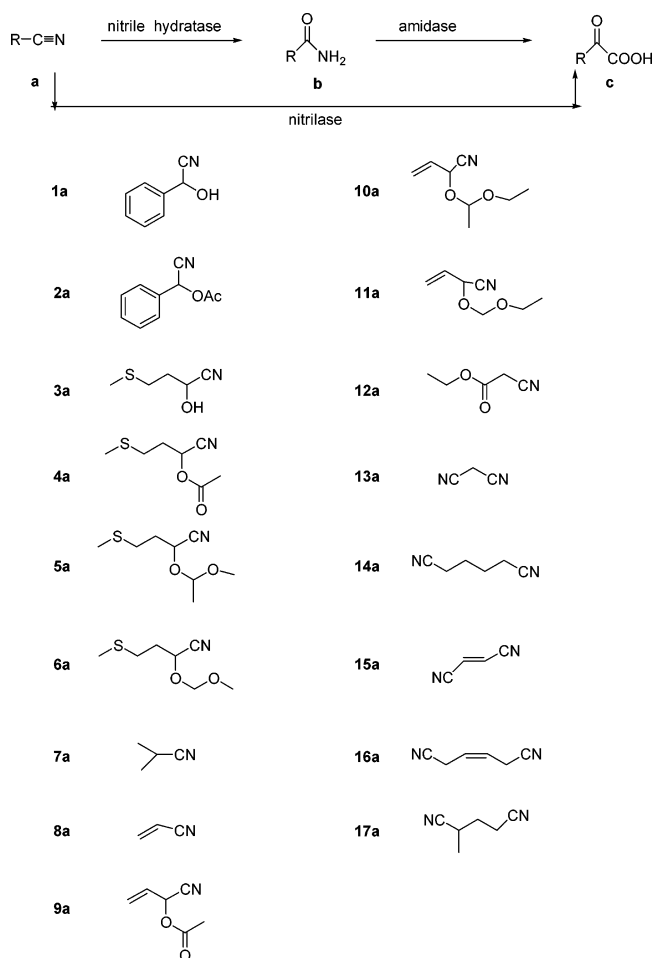
the chemical hydrolysis of nitriles usually requires rather harsh conditions. Such media are often incompatible with other hydrolyzable or acid/base sensitive groups within the molecule, and in these cases neutral hydrolytic conditions would be a clear advantage. Chemical hydration under neutral conditions has been conducted, albeit with varying degree of success. Often relatively expensive and elaborate reagent systems are required, for example, platinum (10), palladium (11), and cobalt complexes (12). Furthermore, elevated reaction temperatures are usually necessary. However, the preparations of hydrophilic cross-linked polymers that are carrying carbonyl groups of the 4-piperidone type catalyze the hydration of  $\alpha$ -aminonitriles to  $\alpha$ -amino acids have been reported (13, 14). Moreover, borates are an efficient method for the hydration of  $\alpha$ -hydroxynitriles to  $\alpha$ -hydroxyacids (15). Biocatalysis plays an increasingly important role in synthetic organic chemistry due to the very mild conditions normally required and the usually high chemo-, regio-, and stereoselectivity with which such transformations take place. Moreover, the continuous development of genetic methodologies has permitted the achievement of controlled and highly productive engineered microorganisms. In this respect, the ability of enzymes to hydrolyze nitriles, in particular, employed as whole microorganisms, is well-established (16–18), and a range of both aromatic and aliphatic nitriles as well as dinitriles has been successfully hydrolyzed (19–21). Optically active products have also been obtained (22, 23). Indeed, in addition to operating

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**Figure 1.** Reaction and structures of substrates tested with the immobilized nitrilase from *A. faecalis* ATCC8750.

under mild conditions, enzyme-catalyzed hydrolysis offers the potential for carrying out (chemo-, regio-, stereo-) transformations that are difficult to achieve nonenzymatically (24). The mechanism of these enzyme systems has been investigated (25–27), and two distinct pathways are believed to be operating: first, the stepwise hydrolysis of the nitrile (a) into an amide (b) via a nitrile hydratase followed by transformation of the latter into a carboxylic acid (c) by an amidase, and second, the direct hydrolysis of the nitrile via a nitrilase (Figure 1).

Research studies on the mechanisms of the enzymatic hydrolysis were essentially concentrated toward the nitriles hydratases. Thus, several nitrile hydratases from *Rhodococcus* (28), *Corynebacterium* sp. (29), *Brevibacterium* R312 (30), and *Pseudomonas chlororaphis* B23 (31) were isolated and purified. It results from the investigations carried out by Yamada et al. that these enzymes would contain in their active site of the enzyme a prosthetic group, the pyroloquinoline quinone (PQQ) (31), as well as an iron atom (32) or cobalt (28). In the case of the nitrilases, the mechanism defined since 1964 per Hook et al. (33) described an analogy with the traditional chemical hydrolysis of the nitrile function in basic medium.

The nitrilase of *A. faecalis* ATCC8750 was purified from cell-free extract 29.0-fold in a yield of 17.9% by Yamamoto et al. (34, 35) who examined the ability of the enzyme to catalyze the hydrolysis of various nitriles. The enzyme appears to have strict substrate specificity for aromatic hydroxy nitriles derived from mandelonitrile but showed no activity for other hydroxy nitriles such as hydroxy acetonitrile, lactonitrile, and 2-hydroxy-4-phenylbutyronitrile.

Recently, the purified nitrilase from ATC8750 was genetically modified and immobilized on alumina. This immobilized catalyst that showed an excellent turnover was used in an industrial process of production of the hydroxy analogue of methionine (36).

The aim of this work is to demonstrate the suitability and the robustness of this immobilized catalyst toward a wide range of nitriles. First of all, we examined nitrile precursors of mandelic acid **1c**, *O*-acetyl mandelic acid **2c**, a hydroxy analogue of methionine (**3c**), and a hydroxy analogue of methionine derivatives (**4c–6c**) in which the hydrophobic groups (*O*-acetyl or *O*-alkyl) have cropped up in the alcohol function (Figure 1). These compounds should be interesting in the field of cattle feeding.

Indeed, (±)-2-hydroxy-4-methylthiobutanoic acid **1c** (methionine hydroxy analogue call HMB hereafter) is the only known instance in which an amino acid has been successfully replaced by an analogue in nutrition. This compound is produced industrially in very high quantities because of its important interest in poultry feeding. In cattle feeding, the effectiveness of administering free HMB has been questioned in a study on nutrition in lactating dairy cows (37). Rumen microbes either altered or degraded 99% of the HMB since recovery of the analogue in duodenal digesta is less than 1% of the amount fed for both the acid form and the calcium salt (38). However, it has been showed that the insertion of a hydrophobic group in the acid function facilities stomach crossing (39, 40). Recently, this concept was also envisaged by the use of oligopeptides of HMB (41).

Second and to generalize the use of this catalyst, the immobilized nitrilase was used for the hydrolysis of nitriles bearing acido or baso labile groups (7a–12a) and dinitriles (13a–17a) (Figure 1).

Moreover, this work is completed on a several gram scale of substrates, thus authorizing an industrial extrapolation.

## MATERIALS AND METHODS

Unless otherwise stated, all reagents were obtained from commercial suppliers and used without further purification. NMR spectra were recorded on a Bruker DRX 400 MHz spectrometer (400 MHz <sup>1</sup>H and 100.6 MHz <sup>13</sup>C). Chemical shift (δ) is expressed in ppm downfield from tetramethylsilane and reported as δ<sub>H</sub>, δ<sub>C</sub>, number of equivalent nuclei (by integration), and multiplicity (s = singlet, d = doublet, q = quartet, m = multiplet, br = broad). IR spectra were determined on a Bruker IFS 25 spectrophotometer. Mass spectra (*m/z*) were recorded on JEOL JMS-DX 300. All HPLC were run using a Varian chromatograph with an UV detector set at 210 nm. HPLC was achieved using a Nucleosil RP C18 (5 μm, 100 Å). Determination of ee was run using an HP1050 chromatograph with an UV detector set at 210 nm. Separation of enantiomers was achieved using a chiral column CHIRALCEL OD (25 cm, Baker, Deventer, The Netherlands) eluted at 0.9 mL/min with Heptan-2-propanol 90:10 (v/v), 0.1% (v/v) H<sub>3</sub>PO<sub>4</sub>. The pH 7.3 phosphate buffer solution used for the biotransformation was prepared by using a 120 mmol dm<sup>-3</sup> solution of KH<sub>2</sub>PO<sub>4</sub> and K<sub>2</sub>HPO<sub>4</sub>.

**Preparation of (±)-2-Acetoxymandelonitrile 2a.** To a round-bottomed flask was added 100 mL of dichloromethane, 15 g (0.113 mol) of commercial mandelonitrile **1a** and 17.3 g (0.17 mol) of acetic anhydride. The mixture was agitated a few minutes at ambient temperature. Then, 2 g (0.025 mol) of acetyl chloride was added to the reaction medium. The mixture was carried to backward flow (70 °C) during 20 h. At the ambient temperature, 100 mL of saturated Na<sub>2</sub>CO<sub>3</sub> solution was added with precaution under agitation. The solution was separated, and the organic phase was evaporated. A total of 100 mL of ether was then added to the oil. The ether phase was washed with water until neutral pH and dried with Na<sub>2</sub>SO<sub>4</sub>. Yield of (±)-2-acetoxymandelonitrile: 86%. <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> ppm: 2.16 (s,

3H, CH<sub>3</sub>CO), 6.69 (s, 1H, CH (OAc)CN), 7.52–7.61 (m, 5H, aromatic CH). <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> ppm: 21.03 (CH<sub>3</sub>CO), 63.69 (CH(OAc)CN), 117.9 (CN), 128.61 (C-2,6), 129.9 (C-3,5), 130.9 (C-4), 136.26 (C-1), 169.55 (CO<sub>2</sub>CH<sub>3</sub>). IR (CHCl<sub>3</sub>) cm<sup>-1</sup>: 2245, 1750. Mass FAB<sup>+</sup> (NBA): *m/e* (relative intensity) 176 (20), 149 (5), 116 (70).

**2-Hydroxy-4-methylthiobutanenitrile (3a) 97% w/w.** <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 1.67 (td, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>), 1.79 (s, 3H, CH<sub>3</sub>-S), 2.26 (t, 2H, CH<sub>2</sub>-S-CH<sub>2</sub>), 4.31 (td, 1H, HO-CH-CN), 6.54 (d, 1H, OH); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 15.28 (CH<sub>3</sub>-S), 29.13 (CH<sub>2</sub>-S-CH<sub>2</sub>), 35.18 (S-CH<sub>2</sub>-CH<sub>2</sub>), 59.40 (HO-CH-CN), 121.76 (CN); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 3300–3500, 2175; mass FAB<sup>+</sup> (GT), *m/e* (relative intensity) 132 (25), 131 (20), 115 (100), 105 (35), 61 (45).

**Preparation of 2-Acetoxy-4-methylthiobutanenitrile (4a).** To 2-acetoxy-but-3-enenitrile (Aldrich) (2.5 g, 20 mmol) at –10 °C was added carefully methanethiol (1.6 mL, 28 mmol) with a syringe through a septum. Then, the solution was stirred for 10 min under UV irradiation while returning to 20 °C. Next, the temperature was raised to 20 °C, and 4.0 mL (4 mmol) of the triethylborane solution (Aldrich) (1 M in hexane) was added dropwise with a syringe. The reaction was then stirred at 20 °C for 4 h under UV irradiation. After being stirred for 4 h, the system was purged with nitrogen, and the reaction mixture was washed with 30 mL of aqueous solution. The aqueous solution was extracted with ether (3 × 50 mL). The ether was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness to give the compound **4a** (3.35 g, 19.4 mmol); yield, 97%; <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 2.03 (td, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>) 2.07 (s, 3H, CH<sub>3</sub>-S), 2.12 (s, 3H, CH<sub>3</sub>-CO<sub>2</sub>), 2.58 (t, 2H, CH<sub>2</sub>-S-CH<sub>2</sub>), 5.48 (t, 1H, CH (OAc)CN); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 15.25 (CH<sub>3</sub>-S), 20.93 (CH<sub>3</sub>-CO<sub>2</sub>), 28.83 (CH<sub>2</sub>-S-CH<sub>2</sub>), 31.76 (S-CH<sub>2</sub>-CH<sub>2</sub>), 60.86 (CH(OAc)CN), 118.1 (CN), 170.1 (CH<sub>3</sub>CO<sub>2</sub>); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2145, 1745; mass FAB<sup>+</sup> (NBA), *m/e* (relative intensity) 174 (55), 149 (45), 131 (65), 105 (35), 61 (75).

**Preparation of 2-(1-Ethoxyethoxy)-4-methylthiobutanenitrile (5a).** (±)-2-(1-Ethoxyethoxy)-but-3-enenitrile was prepared as described by ref 47 yield, 88%; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ<sub>H</sub> 1.15 (td, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.29 (dd, 3H, CH<sub>3</sub>-CH), 3.50 (qd, 2H, O-CH<sub>2</sub>-CH<sub>3</sub>), 4.92 (q, 1H, -O-CH (CH<sub>3</sub>)-O), 4.82–4.97 (m, 1H, O-CH-CN), 5.35–5.55 (m, 2H, CH<sub>2</sub>=CH), 5.72–5.81 (m, 1H, CH<sub>2</sub>=CH); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ<sub>C</sub> 15.34 (CH<sub>3</sub>-CH<sub>2</sub>-O), 20.03 (CH<sub>3</sub>-CH), 58.50 (CH<sub>3</sub>-CH<sub>2</sub>-O), 63.27 (O-CH-CN), 99.80 (-O-CH(CH<sub>3</sub>)-O), 117.24 (CN), 120.47 (CH<sub>2</sub>=CH), 131.06 (CH<sub>2</sub>=CH); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2950, 2184, 1635; mass FAB<sup>+</sup> (NBA), *m/e* (relative intensity) 154 (35), 73 (100), 43 (44).

To (±)-2-(1-ethoxyethoxy)-but-3-enenitrile (**8**) (3.2 g, 20 mmol) at –10 °C was added carefully methanethiol (2.0 mL, 36 mmol) with a syringe through a septum. Then, the solution was stirred during 10 min under UV irradiation while returning to 20 °C. Next, the temperature was raised to 20 °C and 2.0 mL (2 mmol) of the triethylborane solution Aldrich (1 M in hexane) was added slowly dropwise with a syringe. The reaction was then stirred at 20 °C for 3 h under UV irradiation. After being stirred for 3 h, the system was purged with nitrogen, and the reaction mixture was washed with 30 mL of aqueous solution. The aqueous solution was extracted with ether (3 × 50 mL). The ether was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness to give the compound **5a** (2.92 g, 14.4 mmol): yield, 72%; <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 1.23 (td, 3H, CH<sub>3</sub>-CH<sub>2</sub>-O), 1.55 (dd, 3H, CH<sub>3</sub>-CH), 2.07 (m, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>), 2.11 (s, 3H, CH<sub>3</sub>-S), 2.65 (m, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>), 3.51 (m, 2H, O-CH<sub>2</sub>-CH<sub>3</sub>), 4.68 (m, 1H, O-CH-CN), 4.90 (m, 1H, O-CH(CH<sub>3</sub>)-O); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 15.67 (CH<sub>3</sub>-S), 15.89 (CH<sub>3</sub>-CH<sub>2</sub>-O), 21.23 (CH<sub>3</sub>-CH), 27.40 (S-CH<sub>2</sub>-CH<sub>2</sub>), 33.72 (CH<sub>3</sub>-S-CH<sub>2</sub>), 57.2 (CH<sub>3</sub>-CH<sub>2</sub>-O), 60.65 (O-CH-CN), 99.49 (O-CH(CH<sub>3</sub>)-O), 119.13 (CN); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2950, 2145; mass FAB<sup>+</sup> (NBA), *m/e* (relative intensity) 204 (5), 188 (5), 158 (15), 149 (25), 73 (100), 61 (30).

**Preparation of (±)-2-Methoxymethoxy-4-methylthiobutanenitrile (6a).** (±)-2-Methoxymethoxy-but-3-enenitrile was prepared as described by ref 48: yield, 63%; <sup>1</sup>H NMR (CDCl<sub>3</sub>) δ<sub>H</sub> 3.36 (s, 3H, CH<sub>3</sub>-O), 4.71–4.82 (ABq, 2H, O-CH<sub>2</sub>-O), 4.86 (td, 1H, O-CH-CN), 5.40–5.60 (dd, 2H, CH<sub>2</sub>=CH), 5.75–5.85 (m, 1H, CH<sub>2</sub>=CH); <sup>13</sup>C NMR (CDCl<sub>3</sub>) δ<sub>C</sub> 56.54 (CH<sub>3</sub>-O), 65.49 (O-CH-CN), 95.51 (O-CH<sub>2</sub>-O), 117.06 (CN), 121.28 (CH<sub>2</sub>=CH), 130.36 (CH<sub>2</sub>=CH); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2985, 2174, 1665; mass FAB<sup>+</sup> (NBA), *m/e* (relative intensity) 126 (absent), 57 (98), 55 (100), 43 (96).

To (±)-2-methoxymethoxy-but-3-enenitrile previously prepared (0.20 g, 1.6 mmol) at –10 °C was added carefully 0.25 mL (2.5 mmol) of methanethiol with a syringe through a septum. The solution was stirred at temperature for 10 min under UV irradiation. Next, the temperature was raised to 20 °C, and 2.0 mL (2 mmol) of the triethylborane solution from Aldrich (1 M in hexane) was added slowly dropwise with a syringe. The reaction was then stirred at 20 °C for 3 h under UV irradiation. After being stirred for 3 h, the system was purged with nitrogen, and the reaction mixture was washed with 20 mL of aqueous solution. The aqueous solution was extracted with ether (3 × 20 mL). The ether was dried over Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness to give compound **4a** (0.26 g, 1.52 mmol): yield, 95%; <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 2.05 (td, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>), 2.11 (s, 3H, CH<sub>3</sub>-S-CH<sub>2</sub>), 2.59 (t, 2H, S-CH<sub>2</sub>-CH<sub>2</sub>), 3.34 (s, 3H, CH<sub>3</sub>-O), 4.67 (td, 1H, O-CH-CN), 4.73 (s, 2H, O-CH<sub>2</sub>-O); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 15.30 (CH<sub>3</sub>-S), 29.08 (S-CH<sub>2</sub>-CH<sub>2</sub>), 33.34 (S-CH<sub>2</sub>-CH<sub>2</sub>), 56.46 (CH<sub>3</sub>-O), 64.49 (O-CH-CN), 96.50 (O-CH<sub>2</sub>-O), 119.74 (CN); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2950, 2145; mass FAB<sup>+</sup> (NBA), *m/e* (relative intensity) 175 (absent), 149 (95), 45 (100).

**Preparation and Immobilization of the Enzyme.** The immobilized enzyme was courteously provided by Adisseo. Preparation and immobilization are described in the Adisseo U.S. Patent 6180359 (39). The batch used in this study had a nitrilase activity of 3600 μmol/h per mg of dry catalyst (substrate, 2-hydroxy-4-methylthiobutanenitrile).

**General Procedure for the Enzymatic Hydrolysis of Nitriles.** The substrates were suspended in potassium phosphate buffer (120 mmol dm<sup>-3</sup>, pH 7.3). The immobilized enzyme system was added, and the reaction was shaken at 200 rpm, 30 °C. The reaction was terminated by filtration of the biocatalyst. The aqueous filtrate was acidified (pH 1.5 with HCl 10 N) and extracted with ethyl acetate or ether. The combined extracts were dried (Na<sub>2</sub>SO<sub>4</sub>) and filtered, and the filtrate was subjected to rotary evaporation to afford any unchanged nitrile and/or acid product.

The aqueous portion was then basified (pH 10, 2 mol dm<sup>-3</sup> NaOH) and extracted with ethyl acetate or ether. The combined extracts were dried (Na<sub>2</sub>SO<sub>4</sub>), filtered, and subjected to rotary evaporation to afford the amide. Amounts of substrate catalyst and buffer used as well as reaction times and purification techniques are given in **Table 4**. Yields, <sup>1</sup>H and <sup>13</sup>C NMR, IR, and mass spectrometry data for each individual reaction are given.

**Enzymatic Hydrolysis of Mandelonitrile (1a).** Substrate concentrations were 8.0 mmol dm<sup>-3</sup>. White crystals were isolated and shown by <sup>1</sup>H NMR spectroscopy to be mandelic acid **1c**: yield, 99%; <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 5.06 (s, 1H, CH (OH)CO<sub>2</sub>H), 5.88 (s, 1H, OH), 7.25–7.52 (m, 5H, CH aromatic), 12.54 (s, 1H, CO<sub>2</sub>H); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 73.26 (CH(OH)CN), 127.51 (C-2,6), 128.52 (C-4), 128.92 (C-3,5), 141.07 (C-1), 175.01 (CO<sub>2</sub>H); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2500–3500, 1743; mass FAB<sup>-</sup> (NBA), *m/e* (relative intensity) 152 (100), 122 (20), 92 (5).

Enantiomeric excess (chiral HPLC): 61%. Retention time (S)-(+)-mandelic acid 8.99 min, (R)-(–)-mandelic acid 10.95 min.

**Enzymatic Hydrolysis of O-Acetyl Mandelonitrile (2a).** Substrate concentration of 8.0 mmol dm<sup>-3</sup>. White crystals were isolated and shown by <sup>1</sup>H NMR spectroscopy to be a mixture of O-acetyl mandelic acid **6c** (83%) and mandelic acid **5c** (16%): <sup>1</sup>H NMR (DMSO *d*<sub>6</sub>) δ<sub>H</sub> 2.13 (s, 3H, CH<sub>3</sub>CO<sub>2</sub>), 5.83 (s, 1H, CH (OAc)CO<sub>2</sub>H), 7.38–7.51 (m, 5H, CH aromatic), 13.26 (s, 1H, CO<sub>2</sub>H); <sup>13</sup>C NMR (DMSO *d*<sub>6</sub>) δ<sub>C</sub> 21.32 (CH<sub>3</sub>CO), 75.07 (CH(OAc)CO<sub>2</sub>H), 128.53 (C-2,6), 129.51 (C-3,5), 129.84 (C-4), 135.22 (C-1), 170.75 (CO<sub>2</sub>CH<sub>3</sub>), 170.75 (CO<sub>2</sub>H); IR (CHCl<sub>3</sub>) cm<sup>-1</sup> 2500–3500, 1734, 1725; mass FAB<sup>-</sup> (NBA), *m/e* (relative intensity) 193 (40), 153 (70), 149 (40); enantiomeric excess (chiral HPLC), 0%; retention time (S)-(+)-mandelic acid, 8.99 min, (R)-(–)-mandelic acid, 10.95 min, (S)-(+)-2 acetoxy-mandelic acid, 6.59 min, and (R)-(–)-2-acetoxy-mandelic acid 7.12 min.

**Enzymatic Hydrolysis of 2-Hydroxy-4-methylthiobutanenitrile (3a).** HPLC analysis had indicated that all the starting material had been converted into a single more polar product (water/acetonitrile 80:20 (v/v)/0.05% (v/v) trifluoroacetic acid; λ = 210 nm; flow = 1 mL/min; column: Nucleosil RP C18 (5 μm, 100 Å). After recovery, a liquid was isolated (11.1 g, 74 mmol) and shown by <sup>1</sup>H NMR spectroscopy to be 2-hydroxy-4-methylthiobutanoic acid **1c**: yield,

99%;  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.78 (s, 3H,  $\text{CH}_3\text{-S}$ ), 1.56–1.68 (m, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 2.26 (t, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 3.79 ( $\text{CH(OH)CO}_2\text{H}$ ), 11.74 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  15.38 ( $\text{CH}_3\text{-S}$ ), 30.08 ( $\text{CH}_3\text{-S-CH}_2$ ), 34.32 ( $\text{S-CH}_2\text{-CH}_2$ ), 69.34 ( $\text{CH(OH)CO}_2\text{H}$ ), 176.40 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1725; mass  $\text{FAB}^-$  (NBA),  $m/e$  (relative intensity) 149 (100), 122 (5), 46 (15).

**Enzymatic Hydrolysis of 2-Acetoxy-4-methylthiobutanenitrile (4a).** HPLC analysis had indicated that all the starting material had been converted into two more polar products (water/acetonitrile 80:20 (v/v)/0.05% (v/v) trifluoroacetic acid;  $\lambda = 210$  nm; flow = 1 mL/min; column: Nucleosil RP C18 (5  $\mu\text{m}$ , 100  $\text{\AA}$ ). A liquid was isolated and shown by  $^1\text{H}$  NMR spectroscopy to be a mixture of 2-acetoxy-4-methylthiobutanoic acid **4c** (82%) and 2-hydroxy-4-methylthiobutanoic acid **3c** (6%):  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.75 (m, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 1.79 (s, 3H,  $\text{CH}_3\text{CO}_2$ ), 1.84 (s, 3H,  $\text{CH}_3\text{-S}$ ), 2.28 (t, 2H,  $\text{CH}_3\text{-S-CH}_2$ ), 4.69 (td, 1H,  $\text{CH(OAc)CN}$ ), 11.9 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  15.24 ( $\text{CH}_3\text{-S}$ ), 21.17 ( $\text{CH}_3\text{-CO}$ ), 29.73 ( $\text{CH}_3\text{-S-CH}_2$ ), 30.96 ( $\text{S-CH}_2\text{-CH}_2$ ), 71.35 ( $\text{CH(OAc)CN}$ ), 170.77 ( $\text{CH}_3\text{-CO}_2$ ), 171.86 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1745, 1720.; mass  $\text{FAB}^-$  (NBA),  $m/e$  (relative intensity) 191 (100), 149 (70), 59 (25).

**Enzymatic Hydrolysis of 2-(1-Ethoxyethoxy)-4-methylthiobutanenitrile (5a).** HPLC analysis had indicated that all the starting material had been converted into a single more polar product (water/acetonitrile 95:5 (v/v)/0.05% (v/v) trifluoroacetic acid;  $\lambda = 210$  nm; flow = 1 mL/min; column: Nucleosil RP C18 (5  $\mu\text{m}$ , 100  $\text{\AA}$ ). A liquid was isolated (4.54 g, 20.4 mmol) and shown by  $^1\text{H}$  NMR spectroscopy to be 2-(1-ethoxyethoxy)-4-methylthiobutanoic acid **5c**: yield, 93%;  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.09 (td, 3H,  $\text{CH}_3\text{-CH}_2\text{-O}$ ), 1.18 (dd, 3H,  $\text{CH}_3\text{-CH}$ ), 1.72–1.84 (m, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 2.10 (s, 3H,  $\text{CH}_3\text{-S}$ ), 2.52 (t, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 3.51 (m, 2H,  $\text{O-CH}_2\text{-CH}_3$ ), 3.84 (m, 1H,  $\text{O-CH-CO}_2\text{H}$ ), 4.72 (qd, 1H,  $\text{O-CH(CH}_3\text{)-O}$ ), 11.54 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  15.42 ( $\text{CH}_3\text{-S}$ ), 16.1 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 21.32 ( $\text{CH}_3\text{-CH}$ ), 30.07 ( $\text{S-CH}_2\text{-CH}_2$ ), 33.89 ( $\text{S-CH}_2\text{-CH}_2$ ), 62.00 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 77.06 ( $\text{CH(CO}_2\text{H)-O}$ ), 99.49 ( $\text{O-CH(CH}_3\text{)-O}$ ), 176.42 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1725; mass  $\text{FAB}^-$  (NBA),  $m/e$  (relative intensity) 221 (100), 152 (40), 122 (30), 46 (65).

**Enzymatic Hydrolysis of 2-Methoxymethoxy-4-methylthiobutanenitrile (6a).** HPLC analysis indicated that all the starting material had been converted into a single more polar product (water/acetonitrile 80:20 (v/v)/0.05% (v/v) trifluoroacetic acid;  $\lambda = 210$  nm; flow = 1 mL/min; column: Nucleosil RP C18 (5  $\mu\text{m}$ , 100  $\text{\AA}$ ). A liquid was isolated (2.98 g, 15.5 mmol) and shown by  $^1\text{H}$  NMR spectroscopy to be 2-methoxymethoxy-4-methylthiobutanoic acid **6c**: yield, 70%;  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.96 (m, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 2.05 (s, 3H,  $\text{CH}_3\text{-S-CH}_2$ ), 2.59 (t, 2H,  $\text{S-CH}_2\text{-CH}_2$ ), 3.31 (s, 3H,  $\text{CH}_3\text{-O}$ ), 3.91 (m, 1H,  $\text{O-CH-CO}_2\text{H}$ ), 4.60–4.72 (dd, 2H,  $\text{O-CH}_2\text{-O}$ ), 11.32 ( $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  15.37 ( $\text{CH}_3\text{-S}$ ), 30.80 ( $\text{CH}_3\text{-S-CH}_2$ ), 33.60 ( $\text{S-CH}_2\text{-CH}_2$ ), 55.74 ( $\text{CH}_3\text{-O}$ ), 76.55 ( $\text{O-CH-CO}_2\text{H}$ ), 95.44 ( $\text{O-CH}_2\text{-O}$ ), 176.13 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1720; mass  $\text{FAB}^-$  (NBA),  $m/e$  (relative intensity) 193 (70), 149 (55), 115 (20), 46 (100).

**Enzymatic Hydrolysis of Isobutanenitrile 7a.** Yield in isobutanenitrile acid = 71% **7c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.05 (d, 6H,  $(\text{CH}_3)_2\text{-CH}$ ), 2.40 (h, 1H,  $(\text{CH}_3)_2\text{-CH}$ ), 11.74 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  19.61 ( $(\text{CH}_3)_2\text{-CH}$ ), 33.90 ( $(\text{CH}_3)_2\text{-CH}$ ), 178.7 (s, 1H,  $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1715; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 87 (100), 79 (20).

**Enzymatic Hydrolysis of Acrylonitrile (8a).** Yield in acrylic acid = 93% **8c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  5.90–6.13 (m, 2H,  $\text{CH}_2=\text{CH}$ ), 6.32 (dd, 1H,  $\text{CH}_2=\text{CH}$ ), 12.43 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  RMN (DMSO  $d_6$ )  $\delta_{\text{C}}$  130.7 ( $\text{CH}_2=\text{CH}$ ), 131.45 ( $\text{CH}_2=\text{CH}$ ), 171.16 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1695; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 71 (70), 53 (20). NMR analysis revealed 0.8% of acrylamide.

**Enzymatic Hydrolysis of ( $\pm$ )-2-Acetoxy-3-butenenitrile 9a.** Yield after extraction = 92% of ( $\pm$ )-2-acetoxybut-3-enoic acid **9c**: NMR analysis showed 6% of ( $\pm$ )-2-hydroxybut-3-enoic acid;  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  2.01 (s, 3H,  $\text{CH}_3\text{-CO}_2$ ), 4.87–5.25 (m, 3H,  $\text{CH}_2=\text{CH-CH}$ ), 5.93–6.01 (m, 1H,  $\text{CH}_2=\text{CH-CH}$ ), 12.33 ( $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  21.77 ( $\text{CH}_3\text{-CO}_2$ ), 76.69 ( $\text{CH}_2=\text{CH-CH}$ ), 115.12 ( $\text{CH}_2=\text{CH}$ ), 135.59 ( $\text{CH}_2=\text{CH}$ ), 171.24 ( $\text{CH}_3\text{-CO}_2$ ), 176.86 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1740, 1715; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 143 (100), 59 (30).

**Enzymatic Hydrolysis of ( $\pm$ )-2-(1-Ethoxyethoxy)-but-3-enenitrile 10a.** Yield after extraction = 86% of ( $\pm$ )-2-(1-ethoxyethoxy)-but-3-enoic acid **9c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.06 (t, 3H,  $\text{CH}_3\text{-CH}_2\text{-O}$ ), 1.19 (d, 3H,  $\text{CH}_3\text{-CH}$ ), 3.40–3.55 (m, 2H,  $\text{CH}_3\text{-CH}_2\text{-O}$ ), 4.32 (m, 1H,  $\text{CH}_2=\text{CH-CH}$ ), 4.75–4.66 (qd, 1H,  $\text{O-CH-O}$ ), 4.95–5.2 (m, 2H,  $\text{CH}_2=\text{CH-}$ ), 5.78–5.91 (m, 1H,  $\text{CH}_2=\text{CH}$ ), 12.32 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  15.94 and 16.06 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 20.75 and 21.15 ( $\text{CH}_3\text{-CH}$ ), 60.24 and 61.10 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 78.61 and 78.92 ( $\text{CH}_2=\text{CH-CH}$ ), 98.31 and 98.87 ( $\text{O-CH-O}$ ), 113.86 and 115.0 ( $\text{CH}_2=\text{CH-}$ ), 138.25 and 138.56 ( $\text{CH}_2=\text{CH}$ ), 174.56 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1725; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 173 (100), 43 (35).

**Enzymatic Hydrolysis of ( $\pm$ )-2-Methoxymethoxy-but-3-enenitrile 11a.** Yield after extraction = 80% in ( $\pm$ )-2-methoxymethoxy-but-3-enoic acid **11c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  3.30–3.95 (m, 3H,  $\text{CH}_3\text{-O}$ ), 4.37 (m, 1H,  $\text{CH}_2=\text{CH-CH}$ ), 4.62–4.71 (dd,  $\text{O-CH}_2\text{-O}$ ), 5.07–5.23 (m, 2H,  $\text{CH}_2=\text{CH-}$ ), 5.91–5.98 (m, 1H,  $\text{CH}_2=\text{CH}$ ), 11.47 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  55.58 ( $\text{CH}_3\text{-O}$ ), 78.87 ( $\text{CH}_2=\text{CH-CH}$ ), 94.62 ( $\text{O-CH}_2\text{-O}$ ), 114.38 ( $\text{CH}_2=\text{CH-}$ ), 138.11 ( $\text{CH}_2=\text{CH}$ ), 173.67 (s, 1H,  $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1725; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 193 (100).

**Enzymatic Hydrolysis of 2-Cyan-O-ethyl Acetate 12a.** Yield after extraction = 94% of 2-carboxyacetic acid **12c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.19 (t, 3H,  $\text{CH}_3\text{-CH}_2\text{-O}$ ), 3.33 (s, 2H,  $\text{HO}_2\text{C-CH}_2\text{-CO}_2\text{Et}$ ), 4.12 (q, 3H,  $\text{CH}_3\text{-CH}_2\text{-O}$ ), 10.71 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  14.66 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 42.34 ( $\text{HO}_2\text{C-CH}_2\text{-CO}_2\text{Et}$ ), 64.47 ( $\text{CH}_3\text{-CH}_2\text{-O}$ ), 167.7 ( $\text{-CO}_2\text{-CH}_2\text{-CH}_3$ ), 168.9 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 1745, 1725; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 131 (65), 104 (40), 87 (100), 41 (25).

**Enzymatic Hydrolysis of Malononitrile 13a.** Yield after extraction = 87% of 2-cyanoacetic acid **13c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  3.57 (s, 2H,  $-\text{CH}_2-$ ), 11.57 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  25.46 ( $\text{CH}_2\text{-CN}$ ), 115.4 ( $\text{CCN}$ ), 165.6 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 2240, 1725; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 84 (100), 46 (60).

**Enzymatic Hydrolysis of Adiponitrile 14a.** Yield after extraction = 90% of 5-cyanopentanoic acid **14c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.60 (m, 2H,  $\text{HO}_2\text{C-CH}_2\text{-CH}_2-$ ), 1.62 (m, 2H,  $\text{NC-CH}_2\text{-CH}_2-$ ), 2.23 (t, 2H,  $-\text{CH}_2\text{-CO}_2\text{H}$ ), 2.51 (t, 2H,  $-\text{CH}_2\text{-CN}$ ), 12.41 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  16.92 ( $\text{NC-CH}_2\text{-CH}_2$ ), 24.92 ( $\text{NC-CH}_2\text{-CH}_2$ ), 25.31 ( $\text{CH}_2\text{-CH}_2\text{-CO}_2\text{H}$ ), 34.95 ( $\text{CH}_2\text{-CO}_2\text{H}$ ), 121.38 (CN), 176.41 (s, 1H,  $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 2245, 1730; mass  $\text{FAB}^-$  (GT);  $m/e$  (relative intensity) 126 (100).

**Enzymatic Hydrolysis of Fumaronitrile 15a.** Yield after extraction = 72% in 3-cyanoacrylic acid **15c** and 28% of 3-cyanoacrylamide **15b**. 3-Cyanoacrylic acid **15c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  5.84 (d, 1H,  $\text{HO}_2\text{C-CH=CH-CN}$ ), 6.54 (d, 1H,  $\text{HO}_2\text{C-CH=CH-CN}$ ), 13.10 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  115.03 ( $\text{HO}_2\text{C-CH=CH-CN}$ ), 119.09 (CN), 153.22 ( $\text{HO}_2\text{C-CH=CH-CN}$ ), 167.13 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 2225, 1695; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 96 (100), 52 (10). 3-Cyanoacrylamide **15b**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  6.56 (d, 1H,  $\text{H}_2\text{NOC-CH=CH-CN}$ ), 7.04 (d, 1H,  $\text{H}_2\text{NOC-CH=CH-CN}$ ), 7.72–7.99 (d, 2H,  $\text{CONH}_2$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  109.54 ( $\text{H}_2\text{NOC-CH=CH-CN}$ ), 117.95 (CN), 144.95 ( $\text{H}_2\text{NOC-CH=CH-CN}$ ), 164.01 ( $\text{CONH}_2$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  3100–3500, 2235, 1695; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 95 (10).

**Enzymatic Hydrolysis of 1,4-Dicyanobut-2-ene 16a.** Yield after extraction = 82% of 5-cyanopent-3-enoic acid **16c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  3.04 (dd, 2H,  $\text{HO}_2\text{C-CH}_2$ ), 3.31 (dd, 2H,  $\text{CH}_2\text{-CN}$ ), 5.51 (td, 1H,  $\text{NC-CH}_2\text{-CH=}$ ), 5.82 (td, 1H,  $\text{H}_2\text{OC-CH}_2\text{-CH=}$ ), 10.51 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  20.34 ( $\text{CH}_2\text{-CN}$ ), 38.14 ( $\text{H}_2\text{OC-CH}_2$ ), 119.44 (CN), 122.21 ( $\text{NC-CH}_2\text{-CH=}$ ), 128.75 ( $\text{H}_2\text{OC-CH}_2\text{-CH=}$ ), 173.44 ( $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 2255, 1725; mass  $\text{FAB}^-$  (GT),  $m/e$  (relative intensity) 124 (100), 80 (25), 46 (30).

**Enzymatic Hydrolysis of 2-Methylglutaronitrile 17a.** Yield after extraction = 91% of 4-cyanopentanoic acid **17c**:  $^1\text{H}$  NMR (DMSO  $d_6$ )  $\delta_{\text{H}}$  1.23 (d, 3H,  $\text{CH}_3\text{-CH}$ ), 1.79 (td, 2H,  $\text{NC-CH(CH}_3\text{)-CH}_2-$ ), 2.35 (td, 2H,  $-\text{CH}_2\text{-CO}_2\text{H}$ ), 2.84 (m, 2H,  $-\text{CH-CN}$ ), 10.89 (s, 1H,  $\text{CO}_2\text{H}$ );  $^{13}\text{C}$  NMR (DMSO  $d_6$ )  $\delta_{\text{C}}$  18.08 ( $\text{CH}_3\text{-CH}$ ), 24.94 ( $\text{NC-CH-}$ ), 29.33 ( $\text{CH}_2\text{-CH}_2\text{-CO}_2\text{H}$ ), 31.89 ( $\text{CH}_2\text{-CO}_2\text{H}$ ), 123.78 (CN), 174.25 (s, 1H,  $\text{CO}_2\text{H}$ ); IR ( $\text{CHCl}_3$ )  $\text{cm}^{-1}$  2500–3500, 2240, 1735; mass  $\text{FAB}^-$  (GT);  $m/e$  (relative intensity) 126 (100), 54 (5).

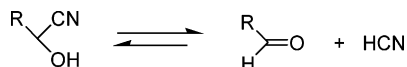


Figure 2. Dissociation equilibrium of cyanohydrins.

## RESULTS AND DISCUSSION

As reported elsewhere (34), the ATCC8750 nitrilase is capable of converting racemic mandelonitrile to *R*-(-)-mandelic acid without the concomitant production of mandelamide indicating that this nitrilase is highly enantioselective for *R*-mandelonitrile. This means that the *S*-mandelonitrile remaining in the reaction was spontaneously racemized by the chemical equilibrium and supplied as substrate. This finding suggested the possibility of establishing a new method for enantiomeric pure  $\alpha$ -hydroxycarboxylic acids.

In the present work, and to evaluate the efficiency of our immobilized biocatalyst, we first tested racemic mandelonitrile **1a**, *O*-acetyl mandelonitrile **2a**, and 2-hydroxy-4-methylthiobutanenitrile **3a** for the production of mandelic acid **1c**, *O*-acetyl mandelic acid **2c**, and HMB **3c**. The *O*-acetyl mandelonitrile was used to avoid the retro conversion of the cyanohydrins. Indeed, studies showed that this nitrilase was inhibited by HCN and the aldehyde resulting from the decomposition of cyanohydrins in aqueous solution (Figure 2). The latter are, respectively, regarded as irreversible and competitive inhibitors of the nitrilase from *Alcaligenes faecalis* ATCC8750 (34).

We compared enantiomeric results of these acids obtained in the same experimental conditions (see Materials and Methods). We found that the immobilized nitrilase produced **1c** with high optical specificity. This study denoted that pH conditions were an important parameter for the optical purity of *R*-(-)-mandelic acid **1c**. When mandelonitrile was incubated at various pHs from 7 to 8.5, optical purity between 60 and 99% ee was obtained. Dissociation of mandelonitrile to the corresponding aldehyde and hydrogen cyanide showed pH dependence, and the racemic reaction itself was faster under alkaline conditions, although the mandelic acid production by the responsible enzyme was accelerated under acidic conditions. As a result, *R*-(-)-mandelic acid with highest optical purity was obtained around pH 8.5 thus confirming the synergic effect of racemization on enantioselectivity. We next examined the production of **2c** and **3c** in the same conditions at pH 7.3 to minimize racemization. When **2a** and **3a** were used as substrates, **2c** and **3c** were produced with 0 versus 60% ee for **1c**. In conclusion, our results for **2a** and **3a** less subject to racemization than **1a** suggested that the aromatic ring was essential for enantioselectivity with this class of substrate. Moreover, it may be suggested that enantioselectivity mechanism is more complicated and that a second interaction by hydrogen bonding between the substrate and the residues at the active enzyme site are also necessary.

Insofar as the absence of enantioselectivity for the conversion of **3a** to **3c** was not a critical point for the access to methionine hydroxy analogues derivatives that are bioavailable in their two forms L and D, we optimized laboratory hydrolysis conditions with **3a** as the reference substrate (see Materials and Methods), and we transposed these conditions for the hydrolysis  $\alpha$ -acetonitrile **4a** and  $\alpha$ -alkoxynitriles **5a–6a**.

The enzyme efficiently attacked cyanohydrin **3a** as shown, for example, in Figure 3, representing the time course of the 2-hydroxy-4-methylthiobutanoic acid-producing reaction by the immobilized nitrilase from *A. faecalis* ATCC8750. In these conditions, the total bioconversion at a 0.1 M scale of **1a** to **1c** was realized in 47 h.

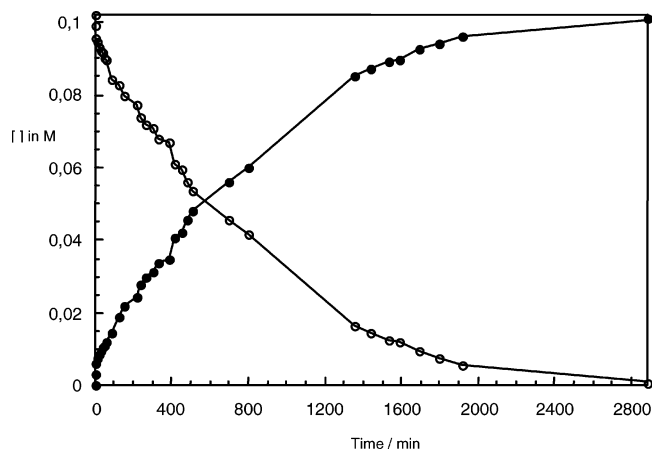


Figure 3. Time course in minutes of the 2-hydroxy-4-methylthiobutanoic acid-producing reaction by the immobilized nitrilase from *A. faecalis* ATCC8750. A reaction mixture (750 mL) containing 75 mmol of **3a** and 120  $\mu$ mol of potassium phosphate buffer (pH 7.3) was incubated (30 °C) with shaking for 47 h, after which the metabolites were analyzed as described in the Materials and Methods (symbols  $\circ$ 3a,  $\bullet$ 3c). Initial concentration 0.1 M, ionic strength 0.01 M, mass ratio of substrate/dry catalyst = 0.55.

Table 1. Enzymatic Hydrolysis of Nitriles **3a–6a**

substrate	incubation time (h)	product(s)	yield <sup>a</sup> (%)
<b>3a</b>	47	<b>3c</b>	99
<b>4a</b>	33	<b>4c, 2c</b>	82, 6
<b>5a</b>	115	<b>5c</b>	93
<b>6a</b>	120	<b>6c</b>	70

<sup>a</sup>Yield obtained after filtration of the biocatalyst, acidification (pH 1.5 with HCl 10 N), extraction with ethyl acetate or ether, and re-extraction of aqueous portion before it was made basic (pH 10 with 2 M NaOH). See Materials and Methods.

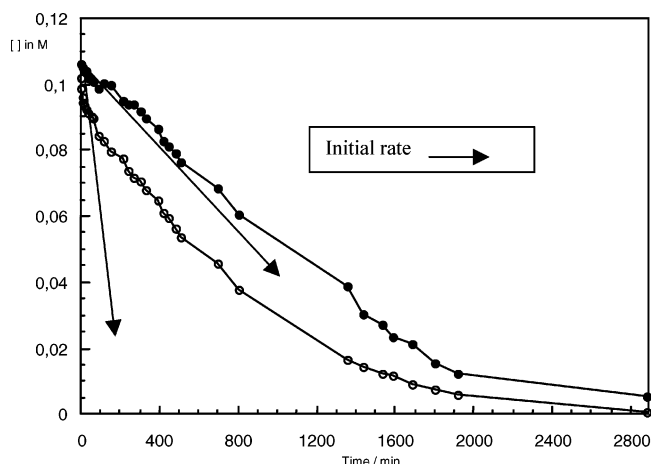
Treatment of raw data ([substrate]/initial rate vs [substrate]) revealed Michaelis–Menten kinetics. In these experimental conditions, the nitrilase appears to have a strict specificity for **3a** estimated to 3600  $\mu$ mol/h/mg of dry immobilized catalyst. This value takes into account that the retrogradation of the substrate at pH 7.3 is in conformity with the previous work (36).

Second, the experimental protocol was transposed to hydrolysis of nitriles **3a–6a**. Both reactions were carried out under the same conditions with 0.1 M concentration of substrate. The results from substrates **3a–6a** are summarized in Table 1.

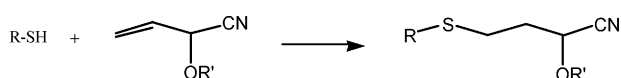
Similarly, (*R,S*)-2-acetoxy-4-methylthiobutyronitrile **4a** was hydrolyzed, albeit more slowly, under the same reaction conditions and gave the corresponding (*R,S*)-2-acetoxy-4-methylthiobutanoic acid **4c** (yield 82%), and HPLC analysis revealed the presence of 6% acid **3c**. The formation of this product is thought to be due to the hydrolysis ester function by an esterase. This result confirms that ester hydrolysis is due to the action of a lipase initially present in the host microorganism.

In a kinetic point of view, the bioconversion rate of **4a** is distinctly lower than **3a** (Figure 4). The ratio of initial rates of bioconversion is close to 5. But this value is certainly underestimated because of the inhibition of HCN and the aldehyde, which decreases nitrilase activity during **3a** hydrolysis.

Upon incubation of **5a** and **6a** with the catalyst, under the usual conditions, the corresponding acids **5c** and **6c** were also obtained. In addition, these reactions have to be run more slowly than **3a** and **4a**. We did not detect the hydrolysis of the ether bond implying that the biocatalyst has no esterase activity.



**Figure 4.** Evolution of nitrile concentrations ○3a, ●4a in the similarly experimental conditions ([nitrile] 0.1 M, pH 7.3, 30 °C, 0.55 g of dry catalyst per g of substrate).



R = alkyl, aryl  
R' = alkyl, aryl, ester

**Figure 5.** Et<sub>3</sub>B induced radical addition of thiol on alkene.

Furthermore, no amide was detected at any time during the reaction, indicating the absence of nitrile hydratase.

The nitriles **5a** and **6a** exhibit anomalous behavior when we compared the rates of bioconversion of **3a** and **4a**. This is all the more remarkable since **3a** and **5a–6a** differ only by the presence of the *O*-alkyl group. In an attempt to rationalize the results obtained from all the  $\alpha$ -hydroxynitriles substrates, we propose the two following explanations. First, we rationalize these results on the basis of an increasing steric hindrance close to the nitrile function. Second, we suggest that the nitrilase exhibit high substrate specificity for  $\alpha$ -hydroxynitriles confirming that the  $\alpha$ -hydroxyl group is involved in the activation process.

Thus, these results open unlimited prospects for the synthesis of the hydroxy-analogue of methionine **3c**, which find their applications in agrochemical (42) or pharmaceutical (43) fields or in what concerns us directly nutritionally (36). Moreover, cyanohydrin precursors can be obtained quantitatively in an elegant way by radical addition of thiols (aliphatic or aromatic) on allylic cyanohydrins (**Figure 5**) (44).

In front of these performances and to generalize the use of the immobilized catalyst, we continued the study of the chemoselectivity of the biocatalyst by testing the hydrolysis of nitriles (**7a–12a**). This choice of substrates although non-exhaustive allows nevertheless a sufficiently broad functional sweeping. It can be divided into three classes: (i) aliphatic **7a** and vinyl nitrile **8a**; (ii) allylic nitrile-ester **9a** and nitrile-ether **10a–11a**; and (iii) nitrile-ester **12a**.

Nitriles **7a–12a** were hydrolyzed under operating conditions identical to the hydrolysis of cyanohydrins **3a** to free us from the influence of the experimental conditions on the bioconversions and thus to be able to compare directly the results (**Table 2**).

The hydrolysis of the substrates **7a–12a** are, except for nitrile **7a**, quasi-quantitative in correct residence times. The much slower hydrolysis of the isobutanenitrile joined the results of Yamamoto et al. (34) concerning the difficulty of the nitrilase to transform the aliphatic nitriles. As previously reported, the

**Table 2.** Enzymatic Hydrolysis of Nitriles **7a–12a**

substrate	reaction duration (h)	yield <sup>a</sup> (%)
<b>7a</b>	58	71
<b>8a</b>	9	93
<b>9a</b>	27	92
<b>10a</b>	25	86
<b>11a</b>	47	80
<b>12a</b>	7	94

<sup>a</sup> Yield in extracted acid after total bioconversion of the nitrile (except for the nitrile **7a**) and recovery of the acid. See Materials and Methods.

**Table 3.** Enzymatic Hydrolysis of Nitriles **13a–16a**

substrate	reaction duration (h)	conversion (%)	yield <sup>a</sup> (%)
<b>13a</b>	51	89	87
<b>14a</b>	53	91	90
<b>15a</b>	25	100	72
<b>16a</b>	25	98	82
<b>17a</b>	96	92	91

<sup>a</sup> Yield isolated. Yield in extracted acid after total bioconversion of the nitrile and recovery. See Materials and Methods.

**Table 4.** Experimental Conditions: Amounts of Substrate, Catalyst, and Buffer Used, as well as Reaction Times and Recovery Procedure

sub- strate	amount of substrate (g/mmol)	amount of catalyst (g)	amount of buffer (mL)	reaction time (h)	recovery procedure
<b>1a</b>	(0.54/4)	2.2	500	24	see general
<b>2a</b>	(0.78/4)	3.18	500	24	see general
<b>3a</b>	(9.86/75)	5.4	750	48	HPLC analysis before recovery
<b>4a</b>	(13/75)	7.15	750	48	HPLC analysis before recovery
<b>5a</b>	(4.45/22)	2.45	220	115	HPLC analysis before recovery
<b>6a</b>	(3.80/22)	2.10	220	120	HPLC analysis before recovery
<b>7a</b>	(5.21/75)	2.86	750	58	see general
<b>8a</b>	(4.00/75)	2.30	750	9	see general
<b>9a</b>	(9.40/75)	5.20	750	9	see general
<b>10a</b>	(7.80/50)	4.50	500	26	see general
<b>11a</b>	(3.81/30)	4.5	300	47	see general
<b>12a</b>	(8.50/75)	4.70	500	7	see general
<b>13a</b>	(5.00/75)	4.50	500	51	see general
<b>14a</b>	(8.02/75)	4.50	500	53	see general
<b>15a</b>	(5.90/75)	3.25	750	25	see general
<b>16a</b>	(5.30/75)	3.00	500	25	see general
<b>17a</b>	(8.27/75)	4.56	750	96	see general

biotransformation of nitrile **9a** leads to the privileged formation of the acetylated allylic acid **9c** with a small amounts of the allylic hydroxyacide (<6% molar).

These results testify to the important chemoselectivity of this immobilized biocatalyst. To complete the range of the substrates of the catalyst, we tested symmetrical **13a–16a** and dissymmetrical **17a** dinitriles (**Table 3**). The products resulting from the monohydrolysis thus offer an access to the monocanoacids. The latest one is particularly appreciated in polymer chemistry (48).

NMR <sup>1</sup>H and <sup>13</sup>C analyses revealed on the totality of the undertaken experiments the absence of diacid corresponding to the total hydrolysis of the dinitrile. This finding suggests that the monoacid is not a substrate for the nitrilase. This is certainly due to the predominance of the carboxylate form at pH 7.3 that is unfavorable for the access to the active site of the enzyme. Despite a different length of aliphatic chain, times of biotrans-

formation of nitriles **13a** and **14a** are relatively close. The modest yield obtained at the time for the hydrolysis of the fumaronitrile **15a** is justified by the partial chemical hydration of the nitrile function into amide in the aqueous medium of synthesis selected (pH 7.3; 30 °C). Times of bioconversion are definitely higher than previously thought because of the low solubility of dinitriles in aqueous medium. Among the hydrolysis of the dinitriles **13a–16a**, most remarkable and especially richest of application is that obtained at the time of the hydrolysis of the 2-methylglutaronitrile **17a**. Indeed, its hydrolysis leads exclusively to the cyanopentanoic acid (NC-CH(CH<sub>3</sub>)-CH<sub>2</sub>-CH<sub>2</sub>-CO<sub>2</sub>H). This highly regioselective reaction confirms the discriminatory effect of the steric hindrance near the nitrile function.

**Conclusion.** In summary, we have shown that the immobilized and genetically modified nitrilase system from *A. faecalis* ATCC8750 is a useful catalyst for the chemoselective preparation of  $\alpha$ -hydroxy and  $\alpha$ -hydroxy-substituted carboxylic acids under mild conditions. These carboxylic acids should have an important interest in cattle feeding.

We showed that in addition to its use in synthesis derived from HMB, it can also be of a great help for the preparation of monocynoacids. The results obtained testify to an exemplary chemoselectivity. Concerning the stereoselectivity, several studies are currently underway in our laboratory to confirm the assumptions announced previously. Thus, we try to rationalize the enantioselective exit of the hydrolysis of substrate. Consequently, the number growing of published works on the field testifies in an obvious way of this awakening to the potential applications related to the use of biocatalysts with nitrilase activity. The advent of new methodologies, based on the use of the genetic engineering to the production of enzymes, should make the enzymatic tool even more powerful. It is perfectly conceivable that research of perfectibility, which can result in particular in a better stability of the catalytic species, will allow the nearest use of this type of catalyst in the industrial processes.

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