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A New Enzymatic Synthesis of (*R*)-γ-Chloro-β-Hydroxybutyronitrile

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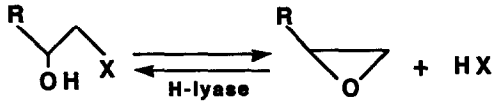
Abstract: A new enzymatic synthesis of (*R*)-γ-chloro-β-hydroxybutyronitrile from epichlorohydrin or 1,3-dichloro-2-propanol using halohydrin hydrogen-halide-lyase purified from a recombinant *Escherichia coli* that carried the enzyme gene of *Corynebacterium* sp. strain N-1074 was described.

INTRODUCTION

Halohydrin hydrogen-halide-lyase (H-lyase) catalyzes the interconversion of halohydrins to epoxides and hydrogen halide.



R=alkyl group, X=Cl or Br

Recently we found the occurrence of two kinds of the enzyme in *Corynebacterium* sp. strain N-1074.[1,2] The expression of the two cloned enzymes (H-lyases A and B) was accomplished in *Escherichia coli*[3]. Using the two cloned enzymes purified from the recombinants, we investigated their enzymatic properties in detail.[4,5] The resulting epichlorohydrin **1** from prochiral 1,3-dichloro-2-propanol **2** by H-lyase A was almost racemate, whereas the formation of **2** catalyzed by H-lyase B was considerably *R*-enantioselective. Further investigations of the two enzymes led us to discover a new catalytic function, the formation of β-hydroxynitriles from epoxides and cyanide, catalyzed by the enzymes. We earlier demonstrated the synthesis of some β-hydroxynitriles using H-lyase A.[6] However, the resulting products were almost racemate (unpublished data). Further attempts have also been performed to synthesize optically active β-hydroxynitriles using H-lyase B, since the enzyme exhibited enantioselectivity in the conversion of **2** to **1**.

(*R*)-γ-chloro-β-hydroxybutyronitrile **3** represents the central intermediates for the synthesis of L-carnitine[7] which plays an important function in mitochondrial fatty acid oxidation[8]. In the present paper we report a new method for the synthesis of **3** using H-lyase B.

RESULTS AND DISCUSSION

Fig. 1 shows typical results of the conversion of **1** to **3** in the presence of KCN by using H-lyase B (scheme 1).

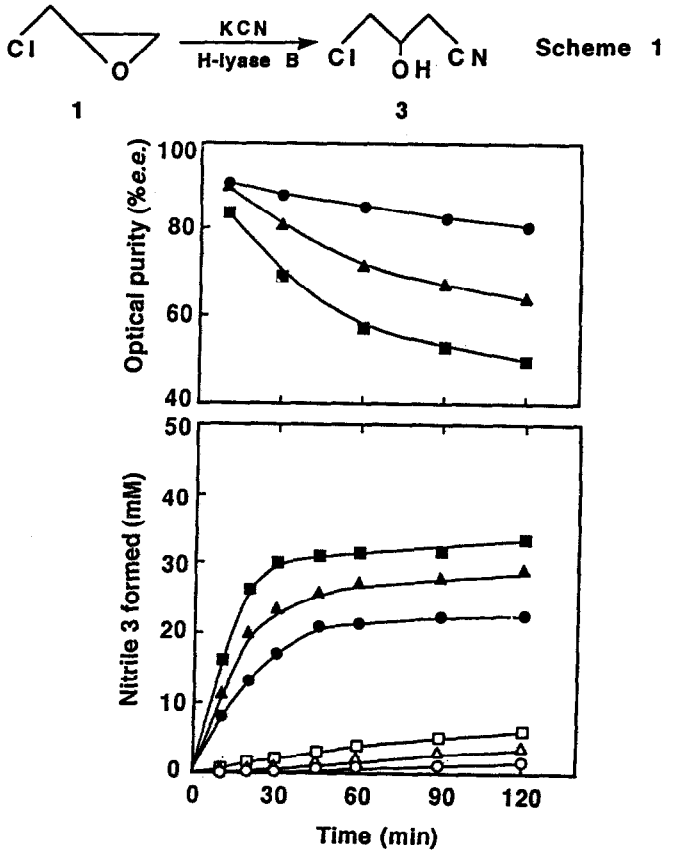


Fig. 1 Typical time course of the conversion of **1** to **3**
The reaction was carried out in 10 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.0) containing 50 mM **1** and 100 mM (●,○), 250 mM (▲,△) or 500 mM (■,□) KCN in the presence of 0.43 mg of H-lyase B (closed symbols) or in the absence of the enzyme (open symbols) at 20°C. The optical purity of **3** formed was determined by HPLC analysis of its (*R*)-MTPA ester.

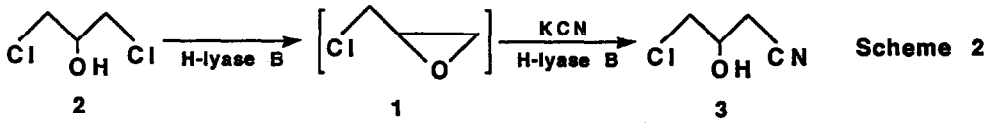
The optical purity and absolute configuration of the resulting **3** were determined by HPLC analysis of its (*R*)-MTPA ester. The resulting **3** was considerably *R*-rich, but elongation of the reaction time led to a decrease

in optical purity. The decrease was more prominent by increasing KCN concentration in the reaction mixture. The decrease in optical purity seems to be due to a nonenzymatic formation of **3**, since a spontaneous formation of **3** was observed under the reaction conditions even in the absence of the enzyme.

The enzyme followed Michaelis-Menten kinetics in the reaction. The apparent *K*m values for **1** and cyanide were calculated to be 8.06 mM and 15.0 mM, respectively. The *V*max was calculated to be 27.8 μmol/min/mg protein.

When **3** (50 mM) was incubated in 10 ml of Tris-H_2SO_4 buffer (pH 8.0) with 4.3 mg of the enzyme at 20°C for 2 hours, no formation of **1** and cyanide was observed. Thus, the enzyme did not catalyze the reverse reaction, the conversion of **3** to **1**.

Synthesis of **3** from **2** was also attempted, since the enzyme can catalyze the reversible conversion of **1** to **2** (scheme 2).



2 **1** **3** **Scheme 2**

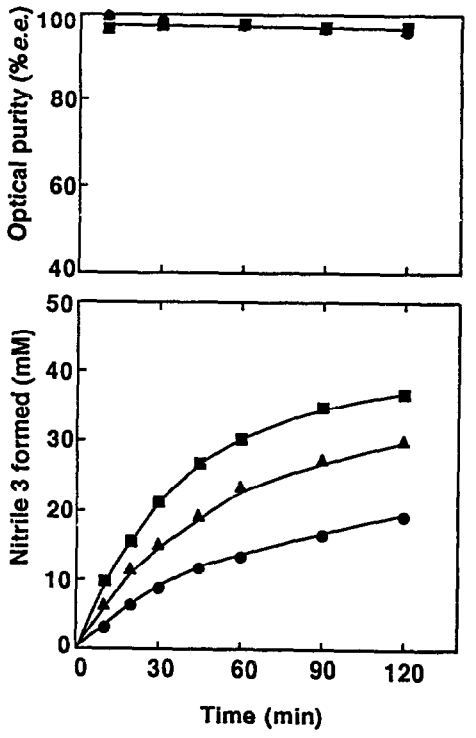


Fig. 2 Typical time course of the conversion of **2** to **3**
The reaction was carried out in 10 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.0) containing 50 mM **2** with H-lyase B (0.43 mg) at 20°C in the presence of 100 mM (●), 250 mM (▲) or 500 mM (■) KCN. The optical purity of **3** formed was determined by HPLC analysis of its (*R*)-MTPA ester.

Fig. 2 shows typical results of the conversion of **2** to **3**. In this case, the optical purity of the resulting **3** barely decreased even though a concentration of KCN in the reaction mixture increased. No formation of **3** in the absence of the enzyme was observed. When 50 mM **2** was incubated in 100 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.0) containing 500 mM KCN and H-lyase B (4.3 mg) at 20°C for 2 hours, formation of 38 mM **3** was observed. Silicagel chromatography of the product extracted from the reaction mixture gave (*R*)-**3** with 95.2%*e.e.* in 65.3% yield. This satisfactory result may be due to the synergistic effects of the enantioselectivity for the interconversion of **2** to **1** and that for the irreversible conversion of **1** to **3**.

H-lyase B would also be a useful catalyst for the synthesis of other optically active β-hydroxynitriles. We are presently attempting to synthesize some useful optically active β-hydroxynitriles using the enzyme.

EXPERIMENTAL PART

^1H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ using tetramethylsilane as an internal standard with a JEOL JNM GX-270 spectrometer. Infrared and mass spectra were recorded with a Perkin Elmer 1710 FT-IR and a Hitachi M-80 spectrometer. Rotations were measured with a JASCO DIP-360 polarimeter.

The amounts of **1**, **2** and **3** were measured by gas-liquid chromathography (GLC). GLC was performed with a Shimadzu GC-7A system equipped with a flame ionization detector with a capillary column of ULBON HR-1 (Chromatopacking Center). The optical purity of **3** was determined by HPLC analysis of the (*R*)-MTPA ester of **3**. HPLC was performed with a Shimadzu LC-5A system with a PARTISIL-5 (25 cm x 4.6 mm I.D.; Gasukuro Kogyo) column by using hexane-*iso*-propanol (98.5:1.5 v/v) as eluent at the flow rate of 1 ml/min.The detector was set to 254 nm.

The amount of cyanide ion was determined from the absorbance at 575 nm using *N*-chlorosuccinimide-succinimide reagent and barbituric acid-pyridine reagent.[9]

H-lyase B purified from *Escherichia coli* JM109/pST111 that carried its gene from *Corynebacterium* sp. stain N-1074 was used in this study.[5]

Chemical synthesis of **3**

The reference compound **3** was synthesized from 2-hydroxy-3-chloropropyl *p*-toluenesulfonate and potassium cyanide.[10] 2-Hydroxy-3-chloropropyl *p*-toluenesulfonate was prepared from epichlorohydrin and p-toluenesulfonic acid.[11] Purification by vacuum distillation (b.p. 110°C/6 mmHg) gave **3** as a colorless liquid (lit.[12] b.p. 134-136°C/13 mmHg). δ_H ($CDCl_3$) 2.73 (2H, m), 3.50 (1H, s), 3.65 (2H, d), 4.20 (1H, m). δ_C ($CDCl_3$) 23.3, 47.3, 61.6, 98.4. (*R*)-**3** was also synthesized by the same way using (*R*)-epichlorohydrin for the determination of absolute configuration of the product formed with the enzyme. ^1H- and ^{13}C-NMR spectra of (*R*)-**3** (lit.[7] δ_H ($CDCl_3$) 2.68(1H, dd) 2.74 (1H, dd), 3.17 (1H, bs), 3.64 (2H, d), 4.19 (1H, brquin),. δ_C ($CDCl_3$) 23.2, 47.3, 67.2, 116.8) were almost the same as those of racemic **3**.

(*R*)-MTPA ester of **3**

To **3** (0.3 mmol) dissolved in a mixture of dry pyridine and carbon tetrachloride (1 ml, 1/1) (*R*)-MTPA chloride (0.6 mmol) was added dropwise with stirring on ice bath. After reaction for 5 hours at room

temperature, diethyl ether (15 ml) was added to the reaction mixture. The solution was washed with 1 N hydrochloric acid, saturated sodium hydrogencarbonate and water. After drying and removing the solvent in vacuo, the (*R*)-MTPA ester was purified by TLC (*n*-pentane-dichloromethane 1:1 v/v). The purification did not influence *e.e.* of the resulting (*R*)-MTPA ester.

In the case of time course experiments of enzymatic reactions, the (*R*)-MTPA ester was prepared routinely as follows. **3** was extracted from 1 ml of the reaction mixture with 3 ml of ethylacetrate. After drying and removing the solvent *in vacuo*, 0.1 ml of carbon tetrachloride, 0.1 ml of pyridine and 2 drops of (*R*)-MTPA were added to the residure. The (*R*)-MTPA ester was purifed as described above.

Enzyme catalyzed synthesis of **3**

Time course experiments of the enzymatic reactions were carried out with the following procedures.

Synthesis of **3** from **1**

To **1** (46.3 mg, 0.5 mmol) and potassium cyanide (65.2 - 326 mg, 1 - 5 mmol) dissolved in 10 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.0) H-lyase B (0.43 mg) was added, and the mixture was incubated at 20°C.

Synthesis of **3** from **2**

In this case, the reaction was carried out under the same conditions as above except that **2** was used instead of **1**.

Preparative experiments of the synthesis of **3** from **2** were carried out using the following procedures: To **2** (0.645 g, 5 mmol) and potassium cyanide (3.26 g, 50 mmol) dissolved in 100 ml of 0.1 M Tris-H_2SO_4 buffer (pH 8.0) H-lyase B (4.3 mg) was added, and the mixture was incubated at 20°C for 2 hours. Formed **3** was extracted with ethyl acetate (3 x 50 ml), dried over sodium sulfate anhydrous and evaporated *in vacuo*. The product was purified by column chromatograpy on silica gel with dichloromethane. Pure **3** (390 mg) was obtained. $[\alpha]_D^{25}$ +17.2 (*c* 1 in methanol), *e.e.* 95.2% (HPLC analysis of the (*R*)-MTPA ester). ^1H- and ^{13}C-NMR spectra of the product was in agreement with those of chemical synthesized **3**.

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