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PRODUCTION OF (*S*)-4-CHLORO-3-HYDROXYBUTYRONITRILE USING MICROBIAL RESOLUTION

Toshio SUZUKI,* Hideaki IDOGAKI and Naoya KASAI*
Research Laboratories of DAISO Co., Ltd. 9 Otakasu-Cho, Amagasaki, Hyogo 660, Japan

Abstract: A new production procedure of (*S*)-4-chloro-3-hydroxybutyronitrile was developed using microbial resolution. The resting cells of *Pseudomonas* sp. OS-K-29 preferentially converted *(R)*-4-chloro-3-hydroxybutyronitrile to the corresponding 1,2-diol by the dehalogenating activity so that *(S)*-4-chloro-3-hydroxybutyronitrile (94.5 %ee) was obtained from the racemate in 40 % yield at the microbial resolution step.
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Cyanohydrins have always been important as both chemicals and reagents in organic syntheses. In particular, during the syntheses of chiral drugs and physiolosically active compounds, optically active cyanohydrins have been useful as a precursor for chiral carboxylic acids and alcohols.[1] Therefore, the stereoselecvtive syntheses of optically active cyanohydrins using chemical and enzymatic methods were developed over the past few years. In the case of the chemical methods, the diastereoselective addition of trimethylsilyl cyanide to aldehydes[2] and the enantioselective addition of trimethylsilyl cyanade to aldehydes in the presence of chiral catalysts[3] are known. With respect to the enzymatic preparation of chiral cyanohydrins, the oxynitrilase-catalyzed enantioselective addition of HCN to aldehydes and ketones, and the enantioselective hydrolysis and esterification of cyanohydrins with lipase have been reported.[4] However, there have been little preparation of β-hydroxycyanohydrins.

Recently, the chemical preparation of optically active 4-chloro-3-hydroxybutyronitrile (BN), which is a kind of chlorocyanohydrin, from optically active epichlorohydrin[5,6] as a starting material has been developed.[7,8] In particular, *(R)*-BN is found to be useful as a precursor of L-carnitine,[9] which plays an important role in the human metabolism and transport of long chain fatty acids on the mitochondrial membrane.[10] On the other hand, *(S)*-BN is used as a key structural feature in the synthesis of the inhibitors of HMG-CoA reductase,[11,12] which is the rate limiting enzyme in cholesterol biosynthesis.[13] Also, the 1α,25-dihydroxyvitamin D_3, namely, the physiologically active form of vitamin D, which plays a vital role in the immune system,[14] can be prepared using *(S)*-BN.[15] Moreover, optically active BN is easily transformed into biologically interesting compounds such as 4-amino-3-hydroxybutyric acid (GABOB)[16-18] and 4-hydroxypyrrolidone through 4-chloro-3-hydroxybutanoate.[19,20] Based on these points, optically active BN is considered to be an important key substance for the syntheses of the chiral pharmaceuticals. Most of all, in the case of the biochemical production of optically active BN, the preparation of *(R)*-BN from racemic epichlorohydrin and KCN using the bacterial dehalogenating enzyme is known,[21] but no generation of *(S)*-BN have been observed.

More recently, we have developed a novel generation of *(S)*-BN using a bacterium, *Pseudomonas* sp. OS-K-29. This strain could stereoselectively assimilate *(R)*-2,3-dichloro-1-propanol *via* 3-chloro-1,2-propanediol and glycerol, so that it gave *(S)*-2,3-dichloro-1-propanol with high optical purity.[5,22] In addition, we found that this strain had the dehalogenating enzyme which catalyzed the reaction involved in transforming several halohydrins such as propylene chlorohydrin, butylene chlorohydrin, 3-chloro-1,2-propanediol and 2,3-dichloro-1-propanol to the corresponding diols with the release of chloride ion.[22] Therefore, we applied this activity to the optical resolution of racemic BN as the important C4 chiral synthon. In this paper, we discuss the production of *(S)*-BN using resting cells of *Pseudomonas* sp. OS-K-29 and the possibility of its microbial resolution during the mass production of *(S)*-BN.

Production of optically active *(S)*-BN by resting cells of *Pseudomonas* sp. OS-K-29

The production of *(S)*-BN was carried out as follows: the resting cells of *Pseudomonas* sp. OS-K29 were prepared in a 5-*l* jar fermenter (Model KMJ-5B, Mitsuwa Rikagaku Co., Ltd., Japan) with 2.5 *l* of a nutrient medium containing 1 %(w/v) each of polypeptone, yeast extract and glycerol (pH 7.2). The cultivation was done under aerobic conditions; agitation, 500 rpm; aeration, 1 *l*/min; temperature, 30 °C. Fifty milliliters of the seed culture cultivated in a 300-ml Erlenmeyer flask containing 60 ml of the nutrient medium was inoculated into 2.5 *l* of the above nutrient medium. After a 20hr cultivation, 65 g of the wet cells was harvested by centrifuge (26,000 g, 5-10 °C), washed twice with 500 ml of 2 mM potassium phosphate buffer (pH 7.2) and used as the resting cells. One gram (wet weight) of the resting cells was suspended in a 300-ml Erlenmeyer flask containing 50 ml of a resolution solution containing 0.02 %(w/v) $Na_2HPO_4 \cdot 12H_2O$, 0.02 %(w/v) K_2HPO_4, 0.04 %(w/v) $NaH_2PO_4 \cdot 2H_2O$, 0.05 %(w/v) $MgSO_4 \cdot 7H_2O$, 1% (v/v) racemic BN[8] and 1%(w/v) $CaCO_3$ as a neutralizing agent, and the resolution reaction was done with vigorous shaking at 30 °C. The measurement of the residual amount of BN[23] and the identification of the products of the resolution reaction were carried out by gas chromatography[24] and the analysis with GC/MS[19] was also done. The optical purity and configuration of the residual BN and the corresponding diol were measured by gas chromatography.[25] Ninty-six hours later, the resolution reaction was stopped. At this time the residual amount of BN and its optical purity were estimated to be 40 % and 94.5 %ee, respectively. Its configuration was found to be the *(S)* form. On the other hand, the product was identified to be the corresponding diol, that is, *(R)*-3,4-dihydroxybutyronitrile (42.2 %ee).

In order to develop the practical and industrial production of *(S)*-BN, we examined the large scale production of *(S)*-BN and the stability of the stereoselective dehalogenating activity of the resting cells. The reaction was carried out in a 5-*l* jar fermenter with 2.5 *l* of the above resolution solution without $CaCO_3$ containing 1 %(v/v) BN, and 50 g (wet weight) of the resting cells were used as a catalyst. The concentration of the resting cells, namely, optical density (OD) at 660 nm was estimated to be about 10. The conditions were as follows: agitation, 200 rpm; temperature, 30 °C; pH was controlled at 6.7 with 25 %(w/w) NaOH (7.9 N) instead of $CaCO_3$. The amounts of added NaOH were monitored every hour using a computer (Model BX3, NEC, Japan). The degradation activity for BN was increased two fold (0.03 mg/ml OD hr), but the stereoselectivity was low, compared to that in an Erlenmeyer flask. Therefore, in order to obtain *(S)*-BN of more than 94.5 %ee, the final yield was decreased to 30% from 40% at the microbial resolution step. On the other hand, the ratio of the amounts (moles) of degraded BN to added NaOH was calculated to be about 1 (Fig. 1). This result suggested that the amount of releasing chloride ion was equal to that of degraded BN.

The recovery and purification of the obtained *(S)*-BN after the reaction were as follows: the reaction solution (2.5 *l*) was centrifuged to remove the resting cells. The resulting supernatant was concentrated to 500 ml, extracted twice with an equal volume of ethyl acetate, dried with anhydrous $MgSO_4$ and evaporated *in vacuo* to remove the solvent. *(S)*-BN in the obtained syrup was purified by distillation *in vacuo* (86 °C/ 0.3 mmHg). Finally, 5 g of *(S)*-BN (94.5 %ee) from 30 g of the racemate was obtained in a yield of 16 %. The specific rotation of the obtained *(S)*-BN was estimated to be $[\alpha]_D^{22} = -16.8$ (*c* 1.25, CH_3OH), confirming that the configuration was *S* form. Moreover, the possibility of multiple batches for *(S)*-BN production with the resting cells was examined. The repeated reactions were carried out in the 5-*l* jar fermenter with 2.5 *l* of the above resolution solution under the same conditions described above, and 1 % (v/v) of racemic BN was used as the substrate. Figure 2 shows the time course of the repeated resolution reactions. The degradation activity was found to be stable so the resolution reactions were repeated at least 10 times for 730h. The yield of (*S*)-BN obtained was constant at the respective reaction conditions. (30 %, 94.5 %ee).

In conclusion, we developed a novel generation procedure of (*S*)-BN using the enzymatic method with the resting cells of *Pseudomonas* sp. OS-K-29. This result implies that generation of (*R*)- and (*S*)-BN has developed using the abilities of both stereoselectively assimilating (*R*)-2,3-dichloro-1-propanol and stereoselectively degrading (*R*)-BN of *Pseudomonas* sp. OS-K-29, respectively, because (*S*)-2,3-dichloro-1-propanol is easily converted to (*R*)-BN through (*R*)-epichlorohydrin.[8,9] Interestingly, we showed that this degradation reaction for BN was based on the dehaloganating enzyme with stereoselectivity for the respective enantiomers. In addition, this dehalogenating activity was found to be stable enough for mass production. Based on these results, this method was considered to be efficient and practical for industrial scale use. Production in a larger scale reactor is now in progress.

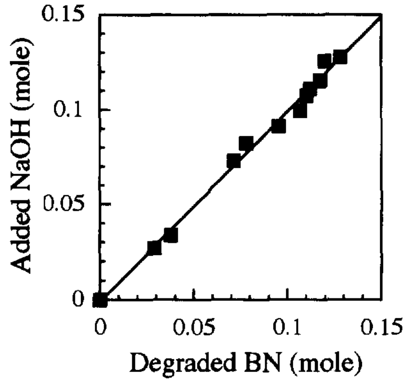

Fig. 1 Stoichiometrical analysis between added NaOH and degraded BN
Resolution reaction with the resting cells was carried out in 2.5 *l* of the resolution solution containing 0.25 mole of BN. pH was controlled at 6.7 with 7.9 N-NaOH.

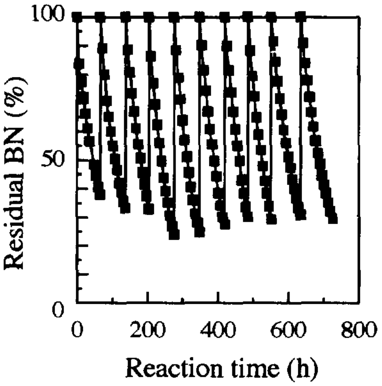

Fig. 2 Time course of repeated resolution reactions
Repeated resolution reactions were carried out in 2.5*l* of the resolution solution containing 1 %(v/v) of BN as a substrate. The resting cells (50 g, wet cells) of *Pseudomonas* sp. OS-K-29 from the nutrient medium were used as a catalyst.

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23. Assay of the degradation reaction was done with a gas chromatograph (Model GC-9A, Shimadzu, Kyoto, Japan) equipped with a PEG 20M-HP (5%, 60/80 mesh, GL Science Co., Ltd., Tokyo, Japan) column (3.2 mm diameter and 1 m length). The conditions of the gas chromatography were as follows: sample size, 1 µl; injection and detection temperature, 240 °C; column temperature, 180 °C; carrier gas, nitrogen; flow rate, 50 ml/min; detector, flame ionization detector (FID).
24. Identification of products by enzymatic reaction was done using a gas chromatograph (Model GC-14A, Shimadzu, Kyoto, Japan) equipped with a capillary column (0.25 mm diameter, 30 m length, TC-1701, GL Science Co., Ltd., Tokyo, Japan). The analytical conditions were as follows: sample size, 1 µl; injection and detection temperature, 250 °C; column temperature, 70 °C for 3 min and 70 to 250 °C at a rate of 10 °C/min; carrier gas, nitrogen; flow rate, 1 ml/min; split ratio, 100:1, detector, FID. GC/MS was done by the chemical ionization (CI) method. The instrument (Model JMS-AX505W, JEOL, Tokyo, Japan) with a capillary column (0.25 mm diameter, 30 m length, TC-1701, GL Science Co., Ltd., Tokyo, Japan) was operated using CH_2Cl_2 as the reagent gas. The conditions were as follows: sample size, 1 µl; injection temperature, 230 °C, column temperature, 200 °C; carrier gas, helium gas; flow rate, 1 ml/min; split ratio, 60:1.
25. Determination of the ee and configuration for BN was done by gas chromatography analysis (Model G-3000, Hitachi, Tokyo, Japan). The conditions were as follows: sample size, 1 µl; column, CHIRALDEX^{R}-G-TA capillary column (0.25 mm diameter, 30 m length, Astec Inc., NJ, USA); column temperature, 150 °C; injection and detector temperatures, 150 °C; detector, FID; carrier gas, nitrogen; flow rate, 1 ml/min; split ratio, 1:100. The retention times for *(S)*- and *(R)*-BN were determined to be 64.1 min and 66.5 min, respectively. In case of the product, that is, 1,2-dihydroxybutyronitrile, determination of the ee and configuration were done by the same gas chromatography analysis of the corresponding trifluoroacetic derivative. The retention times for *(S)*- and *(R)*-1,2-dihydroxybutyronitrile were 113.1 min and 125.3 min, respectively.

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