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Preparation of both enantiomers of methyl 3-benzoyloxypentanoate by enzyme-catalysed hydrolysis of corresponding racemic nitrile and amide

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Abstract

Rhodococcus rhodochrous IFO 15564 enantioselectively hydrolysed racemic 3-benzoyloxypentanenitrile and 3-benzoyloxypentanamide to afford (*R*)-amide and (*S*)-carboxylic acid with high enantiomeric excess (> 90%). In this reaction, both enantiomers of the starting nitrile were converted to the amide by nitrile hydratase, and amidase-catalysed enantioselective hydrolysis of the amide was responsible for the kinetic resolution. The lack of enantioselectivity of the nitrile hydratase toward the racemic nitrile forms a marked contrast to the case of previously reported highly enantioselective conversion of prochiral 3-benzoyloxypentanedinitrile by this enzyme. Since (*R*)-amide could be hydrolysed chemically to (*R*)-carboxylic acid without any loss of its ee, the present microbial kinetic resolution serves as an effective method for preparing both enantiomers of synthetically useful 3-hydroxypentanoic acid derivatives.

Keywords: *Rhodococcus rhodochrous*; Amidase; Nitrile hydratase; Kinetic resolution; 3-Hydroxypentanoate derivatives

1. Introduction

Optically active 3-hydroxypentanoic acid and its esters are versatile starting materials for natural product synthesis [1,2], and various methods for their preparation have been developed so far. Asymmetric hydrogenation of 3-oxopentanoic acid esters by using ruthenium complex [3,4] or by means of biocatalysis [5–11] has been most extensively studied for this purpose. Other practical methods include enantioselective 3-hydroxylation of pentanoic acid mediated by microorganisms [12] and degradation of poly(3-hydroxybutanoate-co-3-hydroxypentanoate) esters of bacterial origin [13,14].

Enzyme catalysed kinetic resolution of racemic 3-hydroxyalkanoic acid derivatives is also an effective method for preparing their enantiomerically enriched counterparts, particularly when both of the antipodes are synthetically useful. Our recent success on microbial asymmetric hydrolysis of nitriles and amides prompted us to investigate the problem on the kinetic resolution of racemic 3-hydroxypentanenitrile derivatives.

In our previous reports we showed that an enzyme system of a microorganism could perform kinetic resolution of racemic 2-arylpro-

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panenitriles [15] as well as asymmetric hydrolysis of prochiral 3-substituted pentanedinitriles [16] and a disubstituted propanedinitrile [17]. The microorganism, whose catalytic activity was found through our screening, has been erroneously identified as the *Rhodococcus rhodochrous (butanica)* ATCC 21197 strain. However, the strain was later found to be independent from the ATCC 21197 strain, and is now registered at the Institute for Fermentation, Osaka, Japan as *R. rhodochrous* IFO 15564. The strain has potent nitrile hydratase activity and relatively low amidase activity, while no nitrilase activity is detected. The two enzymes are simultaneously induced by the addition of ϵ-caprolactam to the cultivation medium.

In the previous studies on the asymmetric hydrolysis of 3-substituted pentanedinitriles [16,18], it was found that the degree of enantioselectivity depended strongly on the structure of 3-substituent. In our study [16], hydrolysis of 3-benzoyloxypentanedinitrile (**1**) gave the best result, affording the corresponding cyanocarboxylic acid **2** in optically pure state (Fig. 1). Based on this result, we first selected 3-benzoyloxypentanenitrile (**3**) as the substrate of microbial hydrolysis in the present study.

2. Results and discussion

When (±)-**3** was incubated with the grown cells of *R. rhodochrous*, the substrate quickly disappeared, and the formation of two products, the corresponding amide **4** and carboxylic acid **5**, was observed. After 10.5 h incubation and subsequent methylation of carboxylic acid **5**,

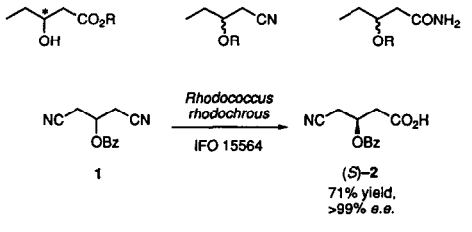
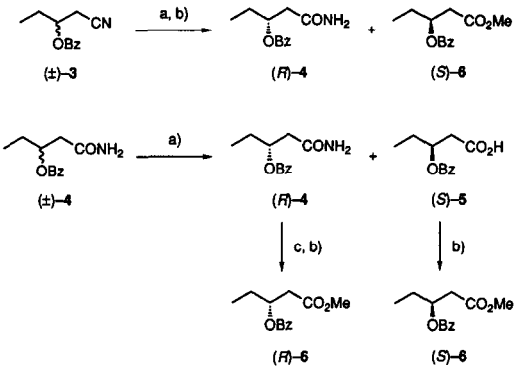


Fig. 1. Hydrolysis of nitriles and amides by *R. rhodochrous*.



a) *Rhodococcus rhodochrous* IFO 15564; b) CH_2N_2 / Et_2O; c) $NaNO_2$, H^+

Fig. 2. Hydrolysis of (±)-3-benzoyloxypentanenitrile and the corresponding amide.

amide **4** and methyl ester **6** were obtained in 35% and 39% yield, respectively (Fig. 2). The enantiomeric excess (ee) of the products was determined by HPLC analyses using a column with a chiral stationery phase to be 96% for amide **4** and 55% for ester **6**.

This result indicated that nitrile **3** was hydrated to the corresponding amide **4** in an enantiomerically non-selective manner by the action of nitrile hydratase and that the amide underwent kinetic resolution by the amidase. Accordingly, the racemic amide (±)-**4** was employed as the substrate. As expected, kinetic resolution of **4** proceeded smoothly during 11.5 h incubation to give the ester (+)-**6** in 25% yield with 14% ee, and the enantiomerically pure form of the amide (−)-**4** was recovered in 27% yield.

The absolute configuration of the products was determined as follows. An authentic sample of (*R*)-(−)-**6** was prepared from methyl (*R*)-3-hydroxypentanoate [12]. Since its sign of rotation was opposite to that of the present sample of microbial product (+)-**6**, the absolute configuration of the carboxylic acid **5** was determined to be (*S*). On the other hand, the amide (−)-**4** afforded (−)-**6** via chemical hydrolysis and subsequent esterification, which indicated the absolute configuration of **4** to be (*R*).

Although the hydrolysis of amide **4** proceeded in a stereoselective manner, two prob-

lems arose from the result: the low total recovery (52%) and the low ee (14%) of ester **6**. To optimise the incubation conditions, the time course of hydrolysis was monitored by an HPLC analysis. The result is shown in Fig. 3. An undesired side product, benzoic acid was detected and its amount increased with prolonged incubation time. Incubation for 24 h hydrolysed 40% of the total benzoate functionality.

Apparently, the participation of this alternative hydrolytic activity of *R. rhodochrous* toward ester group lowered the combined yield of amide **4** and carboxylic acid **5**. Then the question was whether an independent esterase was really present and worked on benzoate or the amidase itself was responsible for the hydrolysis of ester group. Accordingly, control experiments were carried out by using *R. rhodochrous* without amidase activity.

The cells of *R. rhodochrous* that showed neither nitrile hydratase nor amidase activity were obtained by cultivation in a medium without ϵ-caprolactam. When (±)-**4** was incubated with the cells for 10 h, consumption of 15% of the substrate was confirmed by HPLC analysis. However, expected production of benzoic acid was not detected at all. On the contrary, incubation of (±)-**5** for 10 h under the same conditions led to hydrolysis of 20% of the substrate to release the corresponding amount of benzoic acid. These experiments revealed that lowering

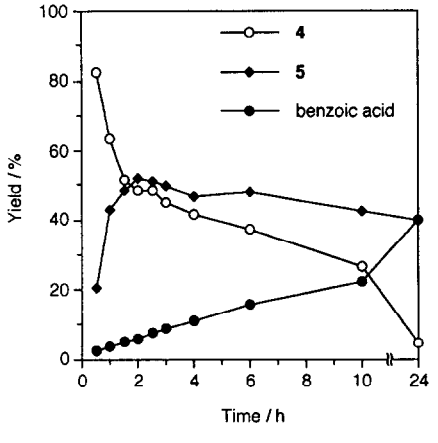
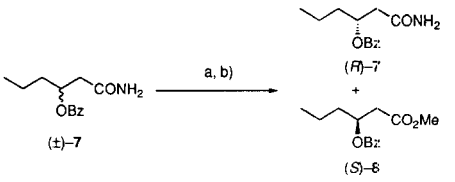


Fig. 3. Time course of hydrolysis.



a) *R. rhodochrous* IFO 15564; b) CH_2N_2 / Et_2O

Fig. 4. Hydrolysis of (±)-3-benzoyloxyhexanamide.

the yields of the desired products was caused by two side reactions, i.e., non-hydrolytic decomposition of **4** by an unidentified path and hydrolysis of the benzoate group of **5** by a constitutive esterase. It was obvious that the loss of products was the result of the unnecessarily prolonged incubation time.

When the incubation was stopped within a short period (2 h), both (*R*)-**4** (45% yield, > 99% ee) and (*S*)-**6** (48% yield, 91% ee) were efficiently obtained. The resulting (*R*)-**4** could be converted to (*R*)-**6** in 75% yield without any loss of its ee. In this way, both enantiomers of 3-hydroxypentanoate of high ee became available as their suitably protected forms, starting from readily available racemic substrates.

One carbon homologated analogue **7** also worked as the substrate (Fig. 4). In this case, however, the microbial hydrolysis became slower to give (*R*)-amide **7** (47% yield, 79% ee) and (*S*)-acid, which was isolated as its corresponding methyl ester (*S*)-**8** (38% yield, 94% ee). Prolonged incubation caused further hydrolysis of the benzoate moiety.

In the present hydrolysis, both enantiomers of nitrile **3** were converted to amide **4** by nitrile hydratase, and amidase-catalysed enantioselective hydrolysis of **4** was responsible for the kinetic resolution (Fig. 5). The lack of enantioselectivity of the nitrile hydratase toward **3** is a marked contrast to the case of the conversion of the prochiral dinitrile **1**, where only the pro-(*S*) cyano group was selectively hydrated to give the final product, (*S*)-cyano-carboxylic acid **2** [16]. In the latter case, there was detected no product originated from the hydration of pro-(*R*) cyano group. The difference in enantioselectiv-

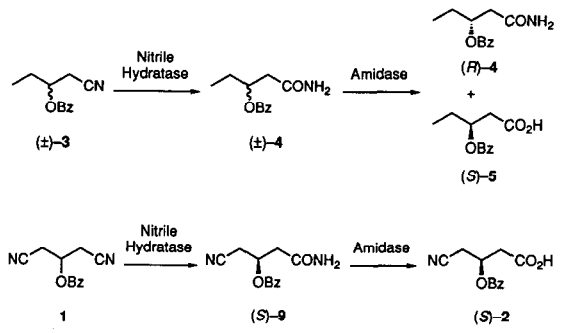


Fig. 5. Enantioselectivity of nitrile hydratase and amidase.

ity of the nitrile hydratase toward the two substrates (**1** and **3**) with close structural similarity is noteworthy. Only the displacement of a cyano group of **1** to a methyl group of **3** brings about loss of enantioselectivity of the enzyme. It is a very interesting problem how a specific amino acid residue of the nitrile hydratase controls the enantioselectivity. It is suggested that an effective hydrogen bonding to ω-cyano group of **1** will control the spatial arrangement of the substrate in the active site.

The stereoselectivity of the amidase toward 3-benzoyloxy-4-cyanobutanamide (**9**) was also investigated. Since only the (*S*)-cyano-amide **9** was formed as an intermediate in the hydrolysis of prochiral dinitrile **1**, it was not clear whether the amidase might hydrolyse its antipode (*R*)-**9** or not. To elucidate this, racemic cyano-amide (±)-**9** was subjected to microbial transformation. After 2 h, the reaction afforded the (*S*)-cyano-carboxylic acid **2** in 36% yield (isolated as its corresponding methyl ester, 65% optical purity) together with 6% recovery of the substrate. A very similar result ((*S*)-**2** in 36% yield, 83% ee from (±)-**9**) has been reported on the transformation by *Brevibacterium* sp. R 312 [19]. The result suggested that the amidase should also be (*S*)-selective in some degree. The moderate yield of (*S*)-**2** may be due to conversion of **9** to the corresponding diamide by the nitrile hydratase.

In conclusion, the stereoselectivity of nitrile hydratase differed with the structure of ω-position of the substrates (a cyano group in **1** and a methyl group in **3**), while the amidase exhibited similar enantioselectivity toward both amides (**4** and **9**).

3. Experimental

IR spectra were recorded as thin films for oils or in KBr discs for solids on a Jasco IRA-202 spectrometer. ^{1}H-NMR spectra were measured in chloroform-d with tetramethylsilane as the internal standard at 270 MHz on a JEOL JNM EX-270 spectrometer, or at 400 MHz on a JEOL JNM GX-400 spectrometer and JEOL α-400 spectrometer, unless otherwise stated. Optical rotation values were recorded on a Jasco DIP-360 polarimeter with chloroform as the solvent. HPLC analyses were performed with a Shimadzu LC-6A chromatograph and a Shimadzu SPD-6A detector. Wako gel B-5F and silica gel 60 K070-WH (70–230 mesh) from Katayama Chemical Co. were used for preparative TLC and column chromatography, respectively. Peptone and yeast extract were purchased from Kyokuto Pharmaceutical Co. and used for the incubation of the microorganism.

3.1. Cultivation of Rhodococcus rhodochrous IFO 15564

To a sterilised medium (pH 7.2, 100 ml) containing glucose (15 g/l), KH_2PO_4 (0.4 g/l), K_2HPO_4 (1.2 g/l), $MgSO_4 \cdot 7H_2O$ (0.5 g/l), yeast extract (1.0 g/l) and peptone (5.0 g/l) in a 500 ml Erlenmeyer flask with two internal projections was added a solution of ϵ-caprolactam (0.1 g) and $FeSO_4 \cdot 7H_2O$ (0.03 g) in deionised water (5 ml) via a sterilised membrane filter (0.20 μm). A loopful of *Rhodococcus rhodochrous* IFO 15564 was inoculated and the flask was shaken at 30°C on a gyrorotary shaker for 2 days. The grown cells (ca. 3 g in wet weight) were harvested by centrifugation (2000 *g* for 15 min at 4°C), washed with 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0) and col-

lected by centrifugation (2000 *g* for 15 min at 4°C).

3.2. Microbial hydrolysis of racemic nitrile and amides

3.2.1. Hydrolysis of 3-benzoyloxypentanenitrile (3)

To the wet cells (1 g) suspended in 0.1 M KH_2PO_4–Na_2HPO_4 buffer (pH 6.0, 50 ml) was added (±)-**3** (53.9 mg, 0.27 mmol), and the mixture was stirred at 30°C for 10.5 h. The reaction mixture was filtered through a pad of Celite, and the filtrate was saturated with NaCl, adjusted to pH 2 with 2 M hydrochloric acid and extracted with ethyl acetate. The organic extracts were combined, dried over Na_2SO_4 and concentrated in vacuo. The residue was treated with excess ethereal diazomethane solution. After removal of the solvent, the residue was chromatographed on silica gel (hexane/ethyl acetate = 9/1–1/2) to give (*R*)-3-benzoyloxypentanamide (**4**) (20.5 mg, 35%) and methyl (*S*)-3-benzoyloxypentanoate (**6**) (24.2 mg, 39%).

(*R*)-**4**: colourless solid; $[\alpha]_D^{19}$ −1.5 (*c* 1.03, $CHCl_3$); IR ν_{max} 3400, 3200, 1715, 1665, 1625, 1450, 1430, 1410, 1360, 1270, 1220, 1180, 1110, 1070, 970, 850, 710 cm^{-1}; ^{1}H-NMR (270 MHz) δ 1.00 (t, J = 7.3 Hz, 3H), 1.84 (dq, J = 6.6 and 7.3 Hz, 2H), 2.59 (dd, J = 5.6, 14.9 Hz, 1H), 2.69 (dd, J = 6.6, 14.9 Hz, 1H), 5.32–5.41 (m, 1H), 5.65 (br, 1H), 5.87 (br, 1H), 7.42–7.61 (m, 3H), 8.01–8.05 (m, 2H); Anal. Found: C, 65.27; H, 6.96; N, 6.37%. Calcd for $C_{12}H_{15}NO_3$: C, 65.14; H, 6.83; N, 6.33%. HPLC analysis (Daicel CHIRALCEL OJ column, 25 cm × 4.6 mm; solvent, hexane/2-propanol = 9/1; flow rate, 0.2 ml/min; detection at 254 nm) 96% ee. Retention time, 57 min (*S*) and 62 min (*R*).

(*S*)-**6**: colourless oil; $[\alpha]_D^{18}$ +7.9 (*c* 1.21, $CHCl_3$); IR ν_{max} 1740, 1720, 1605, 1585, 1450, 1440, 1320, 1270, 1200, 1175, 1110, 1070, 1030, 970, 715 cm^{-1}; ^{1}H-NMR (270 MHz) δ 0.99 (t, J = 7.3 Hz, 3H), 1.80 (dq, J = 6.6 and 7.3 Hz, 2H), 2.67 (dd, J = 5.6 and 15.3 Hz, 1H), 2.77 (dd, J = 7.3 and 15.3 Hz, 1H), 3.66 (s, 3H), 5.37–5.47 (m, 1H), 7.41–7.59 (m, 3H), 8.01–8.05 (m, 2H); Anal. Found: C, 66.10; H, 7.03%. Calcd for $C_{13}H_{16}O_4$: C, 66.09; H, 6.83%. HPLC analysis (Daicel CHIRALCEL OB column, 25 cm × 4.6 mm; solvent, hexane/2-propanol = 50/1; flow rate, 0.5 ml/min; detection at 254 nm) 55% ee. Retention time, 33 min (*R*) and 43 min (*S*).

3.2.2. Hydrolysis of 3-benzoyloxypentanamide (4)

Racemic 3-benzoyloxypentanamide (**4**) (51.8 mg, 0.23 mmol) was incubated with *R. rhodochrous* (1 g) in the phosphate buffer (50 ml) for 11.5 h. After the same workup procedure as described above, (*R*)-**4** (14.2 mg, 27%) and (*S*)-**6** (13.7 mg, 25%) were obtained.

(*R*)-**4**: $[\alpha]_D^{18}$ −1.1 (*c* 1.11, $CHCl_3$); HPLC analysis, > 99% ee.

(*S*)-**6**: $[\alpha]_D^{18}$ +1.6 (*c* 0.95, $CHCl_3$); HPLC analysis, 14% ee.

The same reaction was carried out with the incubation period shortened to 2 h.

(*R*)-**4**: 45% yield. HPLC analysis, > 99% ee.

(*S*)-**6**: 48% yield. $[\alpha]_D^{20}$ +10.6 (*c* 0.26, $CHCl_3$); HPLC analysis, 91% ee.

The time course of hydrolysis was monitored by HPLC analysis of the reaction mixture (GL Sciences Inertsil ODS-2 column, 25 cm × 4.6 mm; solvent, 10 mM H_3PO_4–KH_2PO_4 buffer (pH 2.8)/acetonitrile = 3/2; flow rate, 0.8 ml/min; detection at 254 nm). Retention time, 7 min (benzoic acid), 8 min (**4**) and 17 min (**5**).

3.2.3. Hydrolysis of 3-benzoyloxyhexanamide (7)

Racemic 3-benzoyloxyhexanamide (**7**) (169.8 mg, 0.72 mmol) was incubated with *R. rhodochrous* (6 g) in the phosphate buffer (170 ml) for 2.5 h. After extraction and esterification with diazomethane, the crude mixture was separated by silica gel column chromatography (hexane/ethyl acetate = 3/1–1/7) to afford (+)-**7** (80.3 mg, 47%) and (+)-methyl 3-benzoyloxyhexanoate (**8**) (68.6 mg, 38%).

(+)-**7**: colourless solid; $[\alpha]_D^{21}$ +2.7 (*c* 1.28, $CHCl_3$); IR ν_{max} 3420, 3200, 1710, 1660, 1450, 1270, 1180, 1110, 1070, 1030, 710 cm^{-1}; ^{1}H-NMR (270 MHz) δ 0.95 (t, $J = 7.3$ Hz, 3H), 1.36–1.52 (m, 2H), 1.68–1.85 (m, 2H), 2.59 (dd, $J = 5.6$, 14.9 Hz, 1H), 2.69 (dd, $J = 6.6$, 14.9 Hz, 1H), 5.39–5.48 (m, 1H), 5.57 (br, 1H), 5.85 (br, 1H), 7.42–7.60 (m, 3H), 8.01–8.05 (m, 2H); Anal. Found: C, 66.51; H, 6.71; N, 6.62%. Calcd for $C_{13}H_{17}NO_3$: C, 66.36; H, 7.28; N, 5.95%.

(+)-**8**: colourless oil; $[\alpha]_D^{19}$ +5.0 (*c* 1.03, $CHCl_3$); IR ν_{max} 1740, 1720, 1600, 1585, 1450, 1440, 1315, 1270, 1175, 1110, 1070, 1025, 840, 710 cm^{-1}; ^{1}H-NMR (270 MHz) δ 0.95 (t, $J = 7.3$ Hz, 3H), 1.36–1.51 (m, 2H), 1.63–1.86 (m, 2H), 2.66 (dd, $J = 5.6$ and 15.5 Hz, 1H), 2.77 (dd, $J = 7.3$ and 15.5 Hz, 1H), 3.66 (s, 3H), 5.44–5.53 (m, 1H), 7.40–7.59 (m, 3H), 8.01–8.05 (m, 2H); Anal. Found: C, 66.95; H, 7.41%. Calcd for $C_{14}H_{18}O_4$: C, 67.18; H, 7.25%. Its ee was determined to be 94% by ^{1}H-NMR measurement in the presence of tris[3-(trifluoromethylhydroxymethylene)-*d*-camphorato]europium(III) (Eu(tfc)$_3$, 0.4 eq in C_6D_6 at 400 MHz) δ 1.06 and 1.16 (t, $J = 7.3$ Hz, 0.09H and 2.91H, terminal CH_3), 4.12 and 4.25 (s, 2.91H and 0.09H, CO_2CH_3).

3.2.4. *Hydrolysis of 3-benzoyloxy-4-cyanobutanamide (9)*

Racemic 3-benzoyloxy-4-cyanobutanamide (**9**) (202.0 mg, 0.87 mmol) [19] was incubated with *R. rhodochrous* (10 g) in the phosphate buffer (200 ml) for 2 h. The reaction mixture was filtered through a pad of Celite, and the filtrate was adjusted to pH 9 with 2 M aqueous NaOH and extracted with ethyl acetate. The organic extract was washed with brine, dried over Na_2SO_4 and concentrated in vacuo. Upon purification of the residue by preparative TLC (ethyl acetate), a small amount of the substrate (12.0 mg, 6%) was recovered.

The aqueous layer was saturated with NaCl, adjusted to pH 2 with 2 M hydrochloric acid and extracted with ethyl acetate. The organic extract was dried over Na_2SO_4 and concentrated in vacuo to give crude (*S*)-3-benzoyloxy-4-cyanobutanoic acid (**2**). This was converted to its corresponding methyl ester by treatment with diazomethane. Purification by preparative TLC (hexane/ethyl acetate = 2/1) afforded pure methyl (*S*)-3-benzoyloxy-4-cyanobutanoate (78.2 mg, 36%). $[\alpha]_D^{24}$ +30.3 (*c* 1.00, $CHCl_3$), 65% optical purity (lit. $[\alpha]_D^{22}$ +46.6 ($CHCl_3$), > 99% ee [16]). Its IR and ^{1}H-NMR spectra were in accord with those reported previously [19].

3.3. *Chemical conversion of amides to the corresponding esters*

3.3.1. *Conversion of (R)-4 to (R)-6*

The amide (*R*)-**4** (17.7 mg, 0.08 mmol) obtained from the above microbial hydrolysis was dissolved in 2 M hydrochloric acid (2 ml). Excess sodium nitrite was added to the stirred solution in several portions at 60°C during 2.5 h until the substrate was completely consumed. The resulting solution was saturated with sodium chloride, adjusted to pH 2 and extracted with ethyl acetate. The extract was dried over Na_2SO_4 and concentrated in vacuo to afford crude (*R*)-3-benzoyloxypentanoic acid (**5**). This was dissolved in diethyl ether and treated with excess ethereal diazomethane solution. Purification by preparative TLC (hexane/ethyl acetate = 3/1) gave pure (*R*)-**6** (14.2 mg, 75%). $[\alpha]_D^{18}$ −11.0 (*c* 0.99, $CHCl_3$). Its IR and NMR spectra were identical with those of (*S*)-**6**. HPLC analysis, > 99% ee.

3.3.2. *Conversion of (+)-7 to (−)-8*

The amide (+)-**7** (30.2 mg, 0.13 mmol) was converted to (−)-**8** (21.6 mg, 67%) in the same manner as described above. $[\alpha]_D^{19}$ −4.0 (*c* 1.23, $CHCl_3$). Its IR and NMR spectra were identical with those of (+)-**8**. Its ee was determined to be 79% by ^{1}H-NMR measurement in the presence of Eu(tfc)$_3$: δ 1.06 and 1.16 (2.69H and 0.31H), 4.12 and 4.25 (0.31H and 2.69H).

3.4. Determination of absolute configuration

3.4.1. Preparation of (R)-6 from authentic methyl (R)-3-hydroxypentanoate

To a solution of methyl (*R*)-3-hydroxypentanoate (85.0 mg, 0.64 mmol, $[\alpha]_D^{20}$ −16.3 (neat) [12] in dry pyridine (1.5 ml) was added benzoyl chloride (135 mg, 0.96 mmol) at 0°C. After stirring at room temperature for 30 min, the reaction was quenched with excess *N,N*-dimethyl-1,3-propanediamine and the reaction mixture was diluted with water and extracted with ethyl acetate. The extract was sequentially washed with 0.5 M hydrochloric acid, water, saturated aqueous $NaHCO_3$, water, and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (hexane/ethyl acetate = 9/1) to afford (*R*)-**6** (129.4 mg, 85%). $[\alpha]_D^{18}$ −9.0 (*c* 1.11, $CHCl_3$). HPLC analysis, 86% ee.

^{1}H-NMR measurement in the presence of Eu(tfc)$_3$ (0.4 eq in C_6D_6 at 400 MHz): δ 1.51 and 1.65 (t, $J = 7.3$ Hz, 2.79H and 0.21H, terminal CH_3), 4.29 and 4.36 (s, 0.21H and 2.79H, CO_2CH_3). In this measurement, (*R*)-form appeared as follows: terminal CH_3 upfield and CO_2CH_3 downfield. In the case of the measurement of **8**, (−)-form appeared in the same pattern as (*R*)-**6** did (see above). Therefore, it was concluded that (−)-**8** and consequently (+)-**7** had (*R*) configuration and (+)-**8** had (*S*) configuration.

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