# Microbial Enantioselective Ester Hydrolysis for the Preparation of Optically Active 4,1-Benzoxazepine-3-acetic Acid Derivatives as Squalene Synthase Inhibitors[1]

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Microbial enantioselective ester hydrolysis for the preparation of optically active (3*R*,5*S*)-(−)-5-phenyl-4,1-benzoxazepine-3-acetic acid derivatives as potent squalene synthase inhibitors was investigated. *Pseudomonas diminuta* and *Pseudomonas taetrolens* hydrolyzed the racemic ethyl ester of the 5-(2-chlorophenyl) analogue to yield the (−)-carboxylic acid with excellent enantiomeric excess (>99% ee). We found that the (−)-enantiomer was an active inhibitor. Bulkiness of the ester moiety did not affect the enantioselectivity but did affect reactivity. The racemic ethyl ester of the 5-(2-methoxyphenyl) analogue, 5-(2,3-dimethoxyphenyl) analogue and 5-(2,4-dimethoxyphenyl) analogue were also hydrolyzed with *Pseudomonas taetrolens* to afford enantiomerically pure (−)-carboxylic acids in large scale. As another route to (3*R*,5*S*)-(−)-7-chloro-5-(2,3-dimethoxyphenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic acid [(−)-1c], the earlier intermediate (−)-2-amino-5-chloro-α-(2,3-dimethoxyphenyl)benzyl alcohol [(−)-12] was successfully obtained by asymmetric hydrolysis of (±)-5-chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl acetate with *Pseudomonas* sp. S-13 with >99% ee in kilogram scale followed by alkaline treatment. The product (−)-12 was converted to (−)-1c without racemization.

**Key words** squalene synthase; 4,1-benzoxazepine-3-acetic acid; asymmetric hydrolysis; *Pseudomonas taetrolens*; *Pseudomonas* sp. S-13; hypocholestemic agent

In previous papers,[1] we have reported the syntheses of a series of 4,1-benzoxazepine-3-acetic acid derivatives, which led to the identification of potent squalene synthase inhibitors, such as (±)-**1a**—**d** (Fig. 1). These compounds were first prepared as racemates, as shown in Chart 1. Racemic aminoalcohols (±)-**2** which were obtained by reduction of 2-aminobenzophenones, were treated with aldehydes and sodium cyano borohydride (NaBH₃CN) to afford the alkylated compounds (±)-**3**. After condensation of (±)-**3** with fumaric acid chloride monoethyl ester **4**, intramolecular Michael addition of the resulting amides (±)-**5** afforded ethyl 4,1-benzoxazepine-3-acetates (±)-**6**. In this reaction, the thermodynamically stable 3,5-*trans*-isomers were obtained, which were hydrolyzed to give the carboxylic acids (±)-**1**. To clarify which optical isomer is the more active inhibitor, optical resolution of compound (±)-**1c** was carried out *via* chromatographic separation of the diastereomeric ʟ-amino acid ester amide derivatives. We found the (−)-(3*R*,5*S*)-**1c** exhibited remarkably potent inhibition of both rat and human HepG2 enzymes, while the (+)-(3*S*,5*R*)-**1c** exhibited modest inhibition.[1b] However, since this optical resolution method was not appropriate for large scale synthesis, an efficient and

practical process for producing the optically active isomer was required in order to obtain quantities sufficient for extended biological studies.

Kinetic asymmetric resolution with microorganisms has received considerable attention because of its high enantioselectivity.[2] Since there are currently a great number of differ-



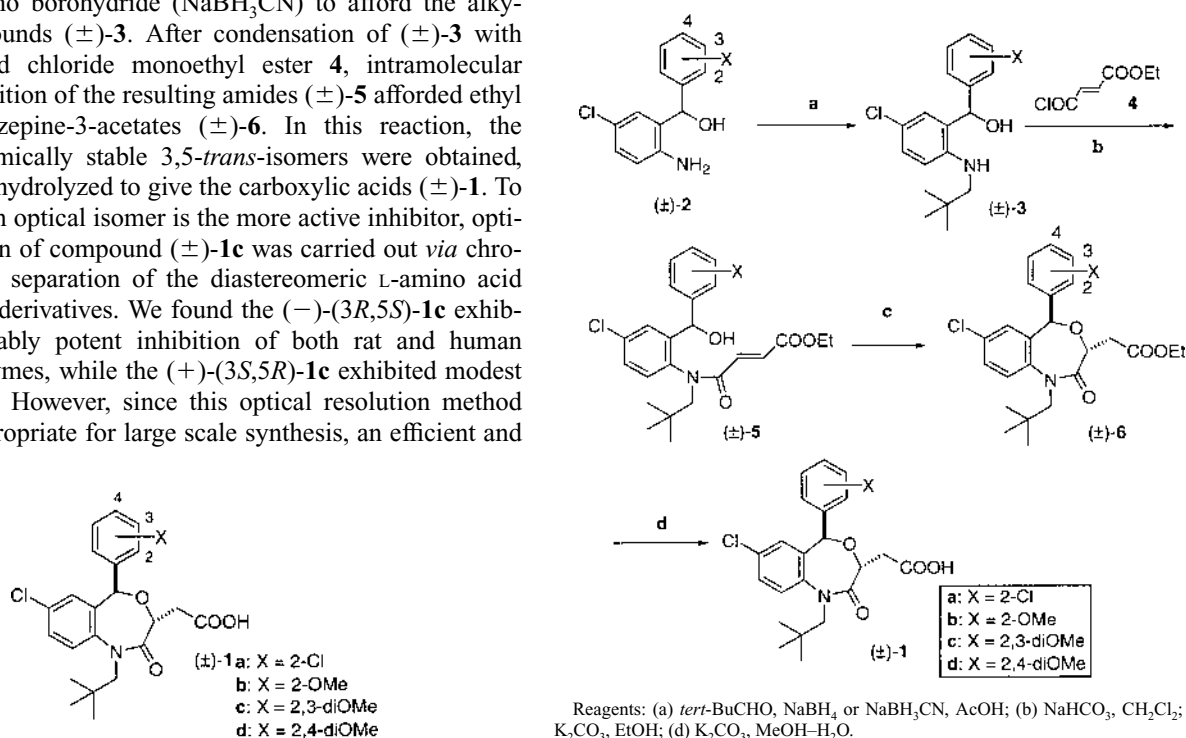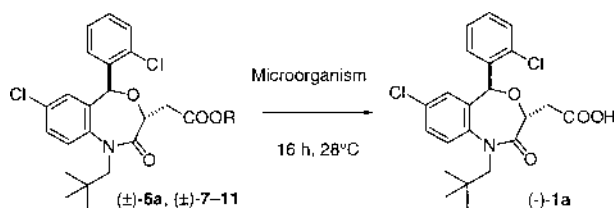Fig. 1. Structures of 4,1-Benzoxazepine-3-acetic Acid Derivatives



Reagents: (a) *tert*-BuCHO, NaBH₄ or NaBH₃CN, AcOH; (b) NaHCO₃, CH₂Cl₂; (c) K₂CO₃, EtOH; (d) K₂CO₃, MeOH–H₂O.

Chart 1

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Table 1. Asymmetric Hydrolysis of (±)-Ethyl (3,5-*trans*)-7-Chloro-5-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetate [(±)-**6a**] with Various Microorganisms



| Microorganism | Product | | |
| --- | --- | --- | --- |
| | No. | Conversion rate[a] (%) | Optical purity[a] (% ee) |
| *Pseudomonas diminuta* IFO 13182 | (−)-**1a** | 27.8 | >99 |
| *Pseudomonas taetrolens* IFO 12691 | (−)-**1a** | 26.7 | >99 |
| *Pseudomonas vesicularis* IFO 12165 | (−)-**1a** | 9.0 | >99 |
| *Pseudomonas aeruginosa* IFO 3923 | (+)-**1a** | 3.1 | >99 |
| *Bacillus subtilis* IFO 3026 | (+)-**1a** | 12.9 | >99 |

*a*) Determined by HPLC analysis [column: Ultron ES-OUM (Shinwa Chemical Industries, Ltd.); mobile phase: 20 mM $KH_2PO_4$ (pH 3.5)–$CH_3CN$ (75 : 25)].

ent readily available microorganisms for this purpose, it should be possible to find a microorganism suitable for a desired transformation by means of random screening. This prompted us to examine optical resolution using microorganisms,[3] and we describe here the microbial asymmetric hydrolysis of racemic 4,1-benzoxazepine-3-acetic acid ethyl esters (±)-**6a**—**d**. We also report the kinetic resolution of an earlier synthetic intermediate, 2-amino-α–phenylbenzylalcohol derivative (±)-**13**.

### Results and Discussion

**Microbial Asymmetric Hydrolysis of Racemic Esters (±)-6a—d** Initially over 400 strains of bacteria were screened in order to identify those microorganisms that were capable of catalyzing enantioselective hydrolysis of (±)-**6a** and we selected 5 strains of bacteria. The hydrolysis of (±)-**6a** was carried out by incubation for 4.5 h at 28 °C. *Pseudomonas diminuta* IFO 13182, *Pseudomonas taetrolens* IFO 12691, and *Pseudomonas vesicularis* IFO 12165 hydrolyzed the substrate stereospecifically to yield (−)-acid [(−)-**1a**] in excellent enantiomeric excesses (>99% ee), and *Pseudomonas aeruginosa* IFO 3923 and *Bacillus subtilis* IFO 3026 hydrolyzed the substrate to yield pure (+)-acid [(+)-**1a**] in high enantiomeric excess (Table 1).[4] The products (−)-**1a** and (+)-**1a** were isolated, and evaluated for inhibition of squalene synthase prepared from rat liver and human hepatoma (HepG2) cells according to the method of Cohen *et al.* with slight modification.[5] Compound (−)-**1a** exhibited more potent inhibition of both rat and human HepG2 enzymes than racemate (±)-**1a**, while (+)-**1a** exhibited weak inhibition of rat enzyme (Table 2). The above results indicate that the compound (−)-**1a** is an eutomer.

Next, we examined the effect of the ester moiety on enantioselectivity as well as reactivity with *Pseudomonas diminuta* IFO 13182 and *Pseudomonas taetrolens* IFO 12691, which smoothly produced the eutomer (−)-**1a**. All reactions of ethyl, methyl, isopropyl, butyl, phenyl and benzyl esters [(±)-**6a**, (±)-**7**—**11**] with these microorganisms for 16 h at 28 °C yielded (−)-**1a** with high optical purities (>99% ee). Small esters, ethyl ester (±)-**6a** and methyl ester (±)-**7**, were more reactive than other bulky esters [(±)-**8**—**11**] (Table

Table 2. Squalene Synthase Inhibitory Activities and Optical Rotations of (±)-, (−)-, and (+)-(3,5-*trans*)-7-Chloro-5-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid **1a**



| Compound | $IC_{50}$ (μM)[a] | | $[\alpha]_D$ in MeOH |
| --- | --- | --- | --- |
| | Rat enzyme | HepG2 enzyme | |
| (±)-**1a** | 0.072[b] | 0.024[b] | |
| (−)-**1a** | 0.042 | 0.016 | −252.2° (c=0.50) |
| (+)-**1a** | >10 (2.8)[c] | NT[d] | +244.8° (c=0.52) |

*a*) $IC_{50}$ values were determined by a single experiment run in duplicate. *b*) References 1*a*, *b*. *c*) % Inhibition at $10^{-5}$ M. *d*) Not tested.

3).[4]

Based on the results obtained above, we applied these microbial reactions to compounds with other substituents on the 5-phenyl group for large scale synthesis. The ethyl esters (±)-**6b**—**d** (200—400 g) hydrolysed with *Pseudomonas taetrolens* IFO 12691 afforded (−)-**1b**—**d** with excellent enantioselectivities (>99% ee) and reasonable yields (23—37%) (Table 4).[4]

The compounds obtained here were evaluated for inhibition of squalene synthase prepared from HepG2 cells. These (−)-isomers were shown to be eutomers, since compounds (−)-**1b**—**d** were more potent than the corresponding racemates (Table 5). Optical rotation of (−)-**1c** prepared here [−246° (c=0.39, MeOH)] was consistent with that of the authentic sample (−)-(3R,5S)-**1c** [−247° (c=0.43, MeOH)] reported in a previous paper.[1b] Since the absolute configuration of (−)-**1c** was determined to be 3R,5S, the stereochemistry of the other eutomers (−)-**1a**, **b**, **d** should also be 3R,5S.

**Preparation of 2-Amino-α-phenylbenzylalcohol Derivative (−)-2c** Our next approach to obtain the chiral compound **1** was by optical resolution of an earlier synthetic in-

Table 3. Asymmetric Hydrolysis of Various Esters with *Pseudomonas taetrolens* IFO 12691 and *Pseudomonas diminuta* IFO 13182



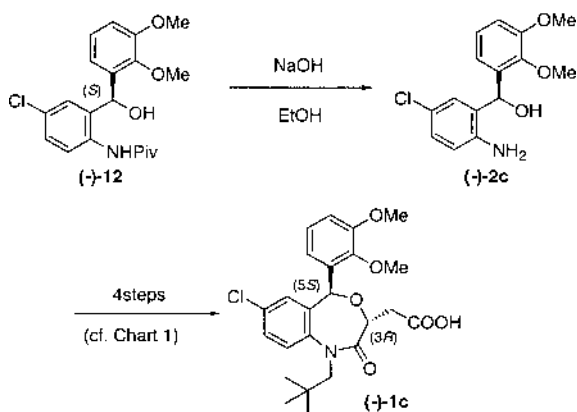|  Substrate | | *Pseudomonas taetrolens* IFO 12691 | | *Pseudomonas diminuta* IFO 13182 | |
| R | No. | Conversion rate[a] (%) | Optical purity[a] (% ee) | Conversion rate[a] (%) | Optical purity[a] (% ee) |
|---|---|---|---|---|---|
| Methyl | (±)-**7** | 43.6 | >99 | 48.2 | >99 |
| Ethyl | (±)-**6a** | 45.6 | >99 | 45.0 | >99 |
| Isopropyl | (±)-**8** | 10.4 | >99 | 14.6 | >99 |
| *n*-Butyl | (±)-**9** | 4.1 | >99 | 8.9 | >99 |
| Phenyl | (±)-**10** | 24.7 | >99 | 32.0 | >99 |
| Benzyl | (±)-**11** | 0.7 | >99 | 6.5 | >99 |

*a*) Determined by HPLC analysis [column: Ultron ES-OUM (Shinwa Chemical Industries, Ltd.); mobile phase: 20 mM $KH_2PO_4$ (pH 3.5)–$CH_3CN$ (75 : 25)].

Table 4. Asymmetric Hydrolysis of 4,1-Benzoxazepine Derivatives by *Pseudomonas taetrolens* IFO 12691
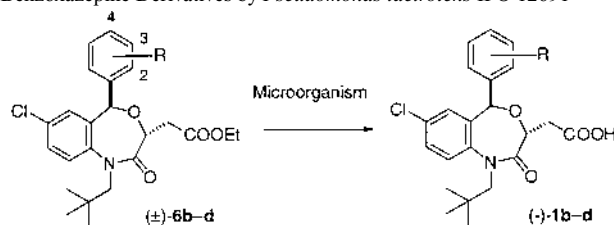


|  Substrate | | | Condition | Product | | | |
| R | No. | Amount | | No. | Conversion rates[a] (%) | Optical purity[a] (% ee) | Yield |
|---|---|---|---|---|---|---|---|
| 2-OMe | (±)-**6b** | 200 g | 54 h, 28 °C | (−)-**1b** | 45.0 | >99 | 44 g (23%) |
| 2,3-diOMe | (±)-**6c** | 200 g | 42 h, 28 °C | (−)-**1c** | 44.2 | >99 | 67 g (35%) |
| 2,3-diOMe | (±)-**6c** | 400 g | 48 h, 28 °C | (−)-**1c** | 44.7 | >99 | 138 g (37%) |
| 2,4-diOMe | (±)-**6d** | 220 g | 72 h, 28 °C | (−)-**1d** | 43.3 | >99 | 76 g (37%) |

*a*) Determined by HPLC analysis [column: Ultron ES-OUM (Shinwa Chemical Industries, Ltd.); mobile phase: 20 mM $KH_2PO_4$ (pH 3.5)–$CH_3CN$ (75 : 25)].
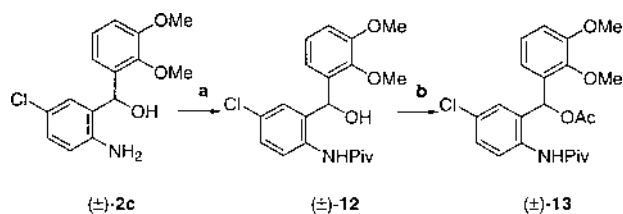


Reagents: (a) PivCl, NaHCO₃, DMAP; (b) Ac₂O, DMAP, Pyridine. Piv=Pivaloyl.

Chart 2
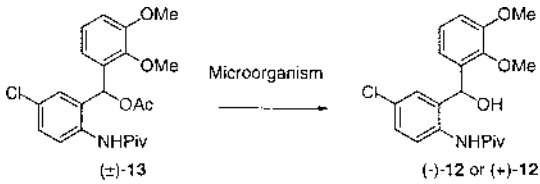
termediate, since this is more efficient and economical, and we decided to examine the preparation of chiral 2-amino-5-chloro-$\alpha$-(2,3-dimethoxyphenyl)benzyl alcohol **2c**.[6] We chose 5-chloro-$\alpha$-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl acetate (±)-**13** as a substrate for microbial asymmetric hydrolysis.

Racemic 2-amino-5-chloro-$\alpha$-(2,3-dimethoxyphenyl)benzyl alcohol (±)-**2c** was converted to *N*-pivaloyl derivative (±)-**12**, which was reacted with acetic anhydride to provide the benzyl acetate derivative (±)-**13** (Chart 2). Asymmetric hydrolysis of the compound (±)-**13** was carried out using 3

Table 5. Squalene Synthase Inhibitory Activities and Optical Rotations of (−)- and (±)-(3,5-*trans*)-7-Chloro-5-phenyl-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid [(−)- and (±)-**1a—d**]
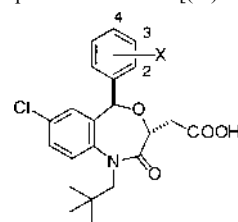


|  Compounds | | IC₅₀ (μM)[a] HepG2 enzyme | $[\alpha]_D$ in MeOH |
| No. | X | | |
|---|---|---|---|
| (−)-**1b** | 2-OMe | 0.018 | −246° (*c*=0.42) |
| (−)-**1c** | 2,3-diOMe | 0.011[b] | −246° (*c*=0.39) |
| (−)-**1d** | 2,4-diOMe | 0.0086 | −233° (*c*=0.41) |
| (±)-**1b**[c] | 2-OMe | 0.023 | |
| (±)-**1c**[c] | 2,3-diOMe | 0.018 | |
| (±)-**1d**[c] | 2,4-diOMe | 0.020 | |

*a*) IC₅₀ values were determined by a single experiment run in duplicate.  *b*) The IC₅₀ value of (−)-(3*R*,5*S*)-**1c** reported in the reference 1*b* was 0.013 μM.  *c*) Reference 1*b*.

Table 6. Asymmetric Hydrolysis of (±)-5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Acetate [(±)-13] with Microorganisms



| Microorganism | Condition | Product | | | |
|---|---|---|---|---|---|
| | | No. | Conversion rate[a] (%) | Optical purity[a] (% ee) | $[\alpha]_D$ In MeOH |
| *Streptomyces* sp. 121-39 | 48 h, 28 °C | (−)-12 | 49 | 88 | −51.7[ob] |
| *Pseudomonas* sp. S-13 | 24 h, 28 °C | (−)-12 | 35 | >98 | (c=0.64) |
| *Bacillus subtilis* IFO 14117 | 24 h, 28 °C | (+)-12 | 49 | >99 | +50.1[oc] |
| | | | | | (c=0.46) |

*a*) Determined by HPLC analysis [column: CHIRALCEL OD (Daicel Chemical Industries, Ltd.); mobile phase: hexane–iso-PrOH (9 : 1)]. *b*) Value of optically pure (−)-**12** obtained after recrystallization (tree times) from MeOH–H₂O (see Experimental section). *c*) Value of optically pure (+)-**12** obtained after recrystallization from MeOH–H₂O (see Experimental section). Piv=Pivaloyl.
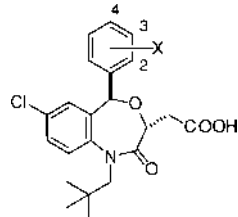


Chart 3. Conversion of (−)-**12** to (−)-**1c**
Piv=Pivaloyl.

Table 7. Physicochemical Data of (−)- and (+)-(3,5-*trans*)-7-Chloro-5-phenyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid Derivatives [(−)- and (+)-**1a**—**d**]



| Compd. | X | mp (°C) | Formula | Analysis (%) Calcd (Found) | | |
|---|---|---|---|---|---|---|
| | | | | C | H | N |
| (−)-**1a** | 2-Cl | 242—245 | $C_{22}H_{23}Cl_2NO_4$ | 60.56 (60.32 | 5.31 5.51 | 3.21 2.82) |
| (+)-**1a** | 2-Cl | 241—245 | $C_{22}H_{23}Cl_2NO_4$ ·1/4H₂O | 59.94 (60.19 | 5.37 5.47 | 3.18 2.97) |
| (−)-**1b** | 2-OMe | 176—180 | $C_{23}H_{26}ClNO_5$ ·3/2H₂O | 60.19 (60.05 | 6.37 5.88 | 3.05 3.22) |
| (−)-**1c** | 2,3-diOMe | 230—233 | $C_{24}H_{28}ClNO_6$ | 62.19 (62.40 | 6.22 6.11 | 2.97 3.03) |
| (−)-**1d** | 2,4-diOMe | 234—235 | $C_{24}H_{28}ClNO_6$ | 62.40 (62.39 | 6.11 6.20 | 3.03 2.81) |

strains selected from about. 400 strains of bacteria (Table 6).[4] The reaction with *Streptomyces* sp. 121-39 for 48 h at 28 °C and with *Pseudomonas* sp. S-13 for 24 h at 28 °C yielded (−)-alcohol [(−)-**12**] in 88% ee and >98% ee, respectively. *Bacillus subtilis* IFO 14117 hydrolyzed the substrate to yield (+)-alcohol [(+)-**12**] in a high enantiomeric ratio (>99% ee).

Although hydrolysis using *Pseudomonas* sp. S-13 yielded (−)-**12** with a high degree of stereoselectivity, this method was not satisfactory because of a low conversion rate (35%), probably due to foaming in the culture inhibiting the microbial reaction. In fact, when large scale hydrolysis [15 kg of (±)-**13**] was carried out using *Pseudomonas* sp. S-13 for 24 h at 28 °C with the addition of an appropriate amount of antifoaming agent (Actocol®) to the culture, the conversion rate was improved to 44% (99% ee).

In order to determine the stereochemistry of the product (−)-**12**, (−)-**12** was converted to (−)-**1c** (Chart 3). Removal of the pivaloyl group of (−)-**12** by alkaline hydrolysis afforded the aminoalcohol (−)-**2c** and conversion of (−)-**2c** to (−)-**1c** was carried out according to a method reported previously (*cf.* Chart 1).[1] Thus compound (−)-**12** was shown to have the (*S*)-configuration by its conversion into (−)-**1c**, the stereochemistry of which was previously determined to be (3*R*,5*S*).[1b] As determined by HPLC analysis, the optical pu-

rity of (−)-**1c** was greater than 99% ee and conversion of (−)-**12** to (−)-**1c** was achieved without racemization.

## Conclusions

In this study, we have demonstrated the preparation *via* microbial hydrolysis of optically active 4,1-benzoxazepine-3-acetic acid derivatives [(−)-**1a**—**d**] as potent squalene synthase inhibitors. We investigated optical resolution at the final step as well as at an earlier step in the synthesis route of (−)-**1a**—**d**, and found that both of the final intermediates (±)-**6a**—**d** and the early intermediate (±)-**13** were hydrolyzed enantioselectively by the treatment with *Pseudomonas*, *Bacillus*, and *Streptomyces*. The microbial hydrolyses described here were found to be efficient and practical for large scale preparation of optically active 4,1-benzoxazepine-3-acetic acid derivatives [(−)-**1a**—**d**], and in particular the most potent squalene synthase inhibitor (3*R*,5*S*)-(−)-**1c**.

## Experimental

All melting points were determined on a Yanagimoto micro melting point apparatus and are uncorrected. Proton nuclear magnetic resonance ($^1$H-NMR) spectra were recorded on a Varian GEMINI-300 (300 MHz) spectrometer (with tetramethylsilane as an internal standard). Infrared (IR) absorption spectra were recorded on a Shimazu FT-IR-8200PC. Optical rotations were determined in the indicated solvents on a JASCO DIP-370 polarimeter. TLC analyses were carried out on Merck Kieselgel 60 F$_{254}$ plates. Elemental analyses were carried out by Takeda Analytical Laboratories, Ltd., and are within ±0.4% of the theoretical values unless otherwise noted. For column chromatography, Merck Kieselgel 60 (70—230 mesh ASTM) was used. Yields were not maximized. The following abbreviations are used: s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad.

**Microorganisms and Materials** *Pseudomonas taetrolens* IFO 12691, *Pseudomonas diminuta* IFO 13182, *Pseudomonas aeruginosa* IFO 3923, *Pseudomonas vesicularis* IFO 12165, *Bacillus subtilis* IFO 3026, and *Bacillus subtilis* IFO 14117 were purchased from the Institute for Fermentation, Osaka. *Pseudomonas* sp. S-13 and *Streptomyces* sp. 121-39 were isolated from soils and identified in Discovery Research Division. Trypticase Soy Broth was purchased from Becton Dickinson. Ion exchange resin IRA-68 was supplied by Rohm and Haas Co. Actocol® was supplied by Takeda Chemical Industries. Ceramic filter was purchased from Toshiba Ceramics. Cation PB-40 was purchased from NOF Corporation.

**Asymmetric Hydrolysis of Ethyl (3,5-*trans*)-7-Chloro-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetate [(±)-1a] by Various Microorganisms** *Pseudomonas taetrolens* IFO 12691, *Pseudomonas diminuta* IFO 13182, *Pseudomonas vesicularis* IFO 12165, and *Pseudomonas aeruginosa* IFO 3923 were respectively inoculated into 20 ml of Trypticase Soy Broth in a 200-ml Erlenmeyer flask and grown for 24 h at 28 °C on a rotary shaker. The culture (0.2 ml) was transferred to 20 ml of casein medium (pH 7.0) containing 2% glucose, 2.5% casein, 0.1% KH$_2$PO$_4$, 0.1% NaNO$_3$, and 0.05% MgSO$_4$·7H$_2$O in a 200-ml Erlenmeyer flask. The mixture were incubated for 42 h at 28 °C on a rotary shaker. Solutions of ethyl (3,5-*trans*)-7-chloro-5-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetate [(±)-6a, 20 mg] in dimethyl sulfoxide (DMSO) (2 ml) were added to the culture (20 ml). After incubation for 4.5 h at 28 °C with shaking, the reaction mixtures were extracted with AcOEt. The extracts were analyzed by HPLC to determine hydrolytic conversion rate, optical purity of the hydrolysate. The results are shown in Table 1.

*Bacillus subtilis* IFO 3026 was inoculated into 2 ml of medium containing 1% dextrin, 1% glucose, 1% glycerol, 0.5% polypeptone, 0.5% yeast extract, 0.5% meat extract, 0.3% NaCl, and 0.5% precipitated calcium carbonate in a test tube. At the same time, a solution of (±)-6a (2 mg) in DMSO (50 μl) was added to the medium. The mixture was incubated for 48 h at 28 °C with shaking, the products were extracted with AcOEt. The extract was subjected to HPLC. The results are shown in Table 1.

**Asymmetric Hydrolysis of Various Esters with *Pseudomonas taetrolens* IFO 12691 and *Pseudomonas diminuta* IFO 13182** The methyl, isopropyl, *n*-butyl, and benzyl ester (±)-7—9 and (±)-11 were prepared by the treatment of (±)-1a with iodomethane, isopropyl iodide, butyl bromide, and benzyl bromide. The condensation of (±)-1a and phenol afforded phenyl ester (±)-10.

The various esters (±)-6a or (±)-7—11 (0.5 mg) were dissolved in DMSO (50 μl). The solutions were respectively added to each of the cultures (0.5 ml) of *Pseudomonas taetrolens* IFO 12691 and *Pseudomonas diminuta* IFO 13182. After incubation for 16 h at 28 °C, each reaction mixture was extracted with AcOEt and the extracts were analyzed by HPLC. It was found that all the substrates used were asymmetrically hydrolyzed by each of the cultures to give (−)-1a. The hydrolytic conversion rates and the optical purities of (−)-1a are shown in Table 3.

**Asymmetric Hydrolysis of 4,1-Benzoxazepine Derivatives with *Pseudomonas taetrolens* (IFO 12691). (−)-(3,5-*trans*)-7-Chloro-5-(2,3-dimethoxyphenyl)-1,2,3,5-tetrahydro-1-neopentyl-2-oxo-4,1-benzoxazepine-3-acetic Acid [(−)-1c]** i) *Pseudomonas taetrolens* IFO 12691 was inoculated into 1-l Erlenmeyer flasks containing 200 ml of Trypticase Soy Broth, and grown for 48 h at 28 °C on a rotary shaker. The culture (1.6 l) was transferred to a 200-l fermenter containing 160 l of the casein medium described above. The cultivation was carried out for 42 h at 28 °C with aeration and stirring. (±)-Ethyl (3,5-*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-1,2,3,5-tetrahydro-1-neopentyl-2-oxo-4,1-benzoxazepine-3-acetate [(±)-6c, 200 g, 0.408 mol] was dissolved in DMSO (18 l). This solution was added to the culture (150 l). The mixture was incubated for 48 h at 28 °C with stirring. A portion of the reaction mixture was extracted with AcOEt. The extract was subjected to HPLC analysis. The results are shown in Table 4.

Sodium chloride (17 kg) was added to the above reaction mixture after completion of the reaction and the mixture was adjusted to pH 4 with 2 N HCl and extracted with AcOEt (80 l and then 60 l). The organic layers were combined, washed with 0.5% NaHCO$_3$ (15 l×2) and 2% NaCl (75 l), and concentrated under reduced pressure to give an oil (295 g). This oil was washed with hexane (500 ml and then 200 ml) and suspended in 50% MeOH (30 l). The suspension was adjusted to pH 7 with concentrated HCl and stirred at room temperature for 3 h. This mixture was filtered, and the filtrate was applied to a column of ion exchange resin IRA-68 (1 l, acetate-form). The column was washed with 6 l of 70% MeOH, and then 1 N NaOH/70% MeOH was passed through the column to recover 7 l of a crude fraction. The fraction was adjusted to pH 4 with concentrated HCl (486 ml), diluted with water (3 l), and cooled at 7 °C for 12 h. The precipitate was collected by filtration to give (−)-1c (66.7 g, 0.144 mol, 35%) as colorless powder.

(ii) (±)-6c (400 g, 0.816 mol) was hydrolysed with *Pseudomonas taetrolens* IFO 12691 in the similar manner as described above. After incubation for 48 h at 28 °C, a portion of the reaction mixture was extracted with AcOEt. The extract was subjected to HPLC analysis. The results are shown in Table 4.

After completion of reaction, NaCl (34 kg) was added to the reaction mixture (340 l). The mixture was adjusted to pH 4 with 2 N HCl (8 l) and extracted with AcOEt (170 l). The organic layers were combined, washed with 0.5% NaHCO$_3$ (18 l, twice) and 2% NaCl (100 l), and concentrated under reduced pressure. The residue was suspended in water (2 l), washed with hexane (4 l) and suspended in 50% MeOH (12 l). The suspension was adjusted to pH 7.9 with 10 N NaOH (25 ml). After being stirred for 3 h at 30 °C, this mixture was filtered. The filtrate (12 l) was adjusted to pH 4.1 with 6 N HCl (61 ml), cooled at 6 °C for 14 h, and then filtered to give (−)-1c (138.1 g, 0.299 mol, 37%) as a colorless powder.

**(−)-(3,5-*trans*)-7-Chloro-5-(2,4-dimethoxyphenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid [(−)-1d]** (±)-Ethyl (3,5-*trans*)-7-chloro-5-(2,4-dimethoxyphenyl)-1,2,3,5-tetrahydro-1-neopentyl-2-oxo-4,1-benzoxazepine-3-acetate [(±)-6d, 220 g, 0.449 mol] was dissolved in DMSO (12 l). This solution was added to the culture (150 l) of *Pseudomonas taetrolens* IFO 12691. The incubation was carried out for 72 h at 28 °C. A portion of the reaction mixture was extracted with AcOEt. The extract was subjected to HPLC analysis. The results are shown in Table 4.

The above reaction mixture was purified by the procedure similar to the preparation of the compound (−)-1c [method (i)] to give (−)-1d (76.1 g, 0.165 mol, 37%) as a colorless powder.

**(±)-(3,5-*trans*)-7-Chloro-5-(2-methoxyphenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid [(−)-1b]** (±)-Ethyl (3,5-*trans*)-7-chloro-5-(2-methoxyphenyl)-1,2,3,5-tetrahydro-1-neopentyl-2-oxo-4,1-benzoxazepine-3-acetate [(±)-6b, 200 g, 0.435 mol] was dissolved in DMSO (13.5 l). The solution was added to the culture (170 l) of *Pseudomonas taetrolens* IFO 12691. The incubation was carried out for 54 h at 28 °C. A portion of the reaction mixture was extracted with AcOEt. The extract was subjected to HPLC analysis. The results are shown in Table 4.

After completion of the reaction, NaCl (6 kg) was added to the reaction mixture. The mixture was adjusted to pH 4 with 2 N HCl (4.0 l) and extracted with AcOEt (60 l×2). The organic layers were combined, washed with 2% NaCl (60 l) and 1% NaHCO$_3$ (30 l×2), and concentrated under reduced pressure. The residue was extracted with 3% NaHCO$_3$ (20 l×4) at 0 °C. The aqueous solutions were pooled, adjusted to pH 7 with 63% H$_2$SO$_4$ (1.9 l), and extracted with AcOEt (25 l×2). The organic layers were combined, washed with 0.1 N H$_2$SO$_4$ (25 l×2) and water (25 l×2), and concentrated under reduced pressure. The residue was subjected to a column chromatography on silica gel [eluent: CHCl$_3$ then CHCl$_3$–MeOH (20:1)] and recrystallized from AcOEt to give (−)-1b (43.8 g, 0.101 mol, 23%) as colorless crystals.

**(±)-5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Alcohol [(±)-12]** A mixture of (±)-2c (3 g, 10.2 mmol), NaHCO$_3$ (1.3 g, 15.5 mmol), pivaloyl chloride (1.35 g, 11.2 mmol) and 4-dimethylaminopyridine (DMAP) (0.12 g, 0.982 mmol) in AcOEt (30 ml) was stirred for 1 h at room temperature. The mixture was diluted with AcOEt (50 ml), washed with water (50 ml), 5% KHSO$_4$ (50 ml), saturated NaHCO$_3$ (50 ml) and brine (50 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure. The residue was recrystallized from AcOEt–hexane (1:5) to give (±)-12 (3.6 g, 9.53 mmol, 93%) as colorless prisms. mp 122—123 °C. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3600—3200 (br, OH, NH), 1665 (C=O). $^1$H-NMR (CDCl$_3$) $\delta$: 1.12 (9H, s), 3.90 (3H, s), 3.91 (3H, s), 4.3 (1H, d, J=4.4 Hz), 6.5 (1H, dd, J=1.8, 7.0 Hz), 6.9—7.03 (3H, m), 7.31 (8H, dd, J=2.4, 8.8 Hz), 8.17 (1H, d, J=8.8 Hz), 9.22 (1H, br s). *Anal.* Calcd for C$_{17}$H$_{15}$Cl$_2$NO$_3$: C, 59.97; H,

4.29; N, 3.98. Found: C, 57.79; H, 4.29; N, 4.13.

**(±)-5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Acetate [(±)-13]** A mixture of (±)-**12** (1 g, 2.65 mmol), Ac$_2$O (0.48 g, 3.94 mmol) and DMAP (32 mg, 0.264 mmol) in pyridine (10 ml) was stirred for 1 h at room temperature. The mixture was duluted with AcOEt (100 ml), washed with 1 N HCl (140 ml), saturated NaHCO$_3$ (100 ml), and brine (100 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure. The residue was chromatographed [eluent: hexane–AcOEt (5 : 1)] and crystallized with hexane to give (±)-**13** (0.96 g, 2.29 mmol, 86%) as colorless prisms. mp 111—168 °C. IR $\nu_{max}$ (KBr) cm$^{-1}$: 3364 (NH), 1724, 1685 (C=O). $^1$H-NMR (CDCl$_3$) $\delta$: 1.38 (9H, s), 2.17 (3H, s), 3.34 (3H, s), 3.83 (3H, s), 6.97 (1H, dd, $J$=1.5, 8.4 Hz), 7.11—7.27 (5H, m), 7.8 (1H, d, $J$=8.7 Hz), 9.0 (1H, br s). *Anal.* Calcd for C$_{22}$H$_{26}$ClNO$_5$: C, 62.93; H, 6.24; N, 3.34. Found: C, 62.96; H, 6.14; N, 3.24.

**Asymmetric Hydrolysis of 5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Acetate with Microorganisms. (−)-5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Alcohol [(−)-12]** i) *Streptomyces* sp. 121-39 was grown in a 200-ml Erlenmeyer flask containing 40 ml of a medium consisting of 0.5% glucose, 5% dextrin, 3.5% raw soybean flour and 0.7% CaCO$_3$ for 48 h at 28 °C on a rotary shaker. The culture (10 ml) was transferred to 1-l Erlenmeyer flasks containing 200-ml of the same medium. The cultivation was carried out for 48 h at 28 °C on a rotary shaker. A solution of (±)-**13** (3 g, 7.14 mmol) in *N,N*-dimethylformamide (DMF) (150 ml) was added to the culture (3 l) thus obtained. The mixture was incubated for 48 h at 28 °C with the shaking. A portion of the reaction mixture was extracted with AcOEt. The extract was subjected to HPLC analysis. The conversion rate was 49% and the optical purity of (−)-**12** was 88% ee (Table 6).

The reaction mixture obtained above was extracted with AcOEt (2.0 l). The solvent was removed under reduced pressure. The residue was purified by flash chromatography [eluent: hexane–CH$_2$Cl$_2$–AcOEt (6 : 3 : 1), internal pressure 0.2 kg/cm$^2$], and recrystallized from CH$_2$Cl$_2$–hexane (1 : 5). Crude yield 0.79 g (optical purity 91.8% ee). The crystals were recrystallized from MeOH–H$_2$O (1 : 1). Yield 0.54 g (optical purity 99.2%). The crystals were recrystallized twice from MeOH–H$_2$O (3 : 1) to give (−)-**12** (0.124 g, 0.328 mmol, 100% ee) as colorless needles. mp 107—109 °C. [α]$_D$ −51.7° (*c*=0.64, MeOH). *Anal.* Calcd for C$_{20}$H$_{24}$ClNO$_4$: C, 63.57; H, 6.40; N, 3.71. Found: C, 63.34; H, 6.20; N, 3.51.

ii) *Pseudomonas* sp. S-13 was grown in a 2-l Sakaguchi flask containing 500 ml of Trypticase Soy Broth for 24 h at 28 °C on a reciprocal shaker. The resulting culture was transferred to a 200-l fermenter containing 120 l of a medium consisting of 2% cottonseed meal, 0.25% K$_2$HPO$_4$, 0.5% NaCl, and 0.25% glucose, and the cultivation was carried out for 48 h at 28 °C with aeration and stirring. The culture (24 l) was centrifuged to give 3 l of a cell suspension. A solution of (±)-**13** (30 g, 71.4 mmol) in MeOH (300 ml) was added to the cell suspension. After incubation for 24 h at 28 °C with stirring, a portion of the reaction mixture was extracted with AcOEt and the extract was analyzed by HPLC. The hydrolytic conversion rate was 35% and the optical purity of (−)-**12** was not less than 98% ee (Table 6).

The reaction mixture obtained above was extracted with AcOEt (3 l). The extract was concentrated under reduced pressure. The residue was purified by flash chromatography [eluent: hexane–isopropyl ether–AcOEt (16 : 3 : 1, v/v), internal pressure 0.2 kg/cm$^2$], and crystallized with *n*-hexane, and the crystals were collected by filtration to give (−)-**12** (6.73 g, 17.8 mmol, 25%) as colorless needles.

(iii) *Pseudomonas* sp. S-13 was grown in a 500-ml Erlenmeyer flask containing 60 ml of Trypticase Soy Broth for 24 h at 28 °C on a rotary shaker. The culture was transferred to 120 l of a medium containing 2% corn steep liquor, 0.25% K$_2$HPO$_4$, 0.5% NaCl, 0.25% sucrose, and Actocol® in a 200-l fermenter, and grown for 24 h at 28 °C with aeration and stirring. The culture (15 l) was transferred to 1500 l of a medium containing 2% corn steep liquor, 0.25% K$_2$HPO$_4$, 0.5% NaCl, 2% sucrose, 3% (NH$_4$)$_2$SO$_4$, and Actocol® [0.005%—0.01% (v/v) of the culture medium] in a 2000-l fermenter. The cultivation was carried out for 45 h at 28 °C with aeration and stirring. A solution of (±)-**13** (15 kg, 35.7 mol) in MeOH (150 l) was mixed with above culture. After incubation for 14 h at 28 °C, a portion of the reaction mixture was extracted with AcOEt. The extract was analyzed by HPLC. The hydrolytic conversion rate was found to be 44% and the optical purities of (−)-**12** and unreacted acetate were found to be 99% ee and 96% ee, respectively.

The reaction mixture was washed with water, acidified (pH 5.0) with H$_2$SO$_4$, and concentrated to 360 l by means of a ceramic filter with a pore diameter of 0.2 μm (Toshiba Ceramics, Japan). After addition of EtOH (540 l), the mixture was stirred for 1 h at 50 °C in order to dissolve (−)-**12**. This mixture was filtered through a ceramic filter with a pore diameter of 0.2 μm. 60% EtOH (1800 l) was added and the mixture was concentrated to 160 l. After addition of Cation PB-40 (6.4 l), the mixture was extracted with AcOEt (300 l). The extract was washed with 0.1 N H$_2$SO$_4$, 3% Na$_2$CO$_3$, and water, and then concentrated to 63 l. The residue was stirred for 1 h with activated carbon (1.2 kg). After filtration, the filtrate was concentrated. The residue was diluted with AcOEt (3 l), and then hexane (103 l) was added for fractional crystallization to give (−)-**12** (5.6 kg, 14.8 mol, 41%). A solution of KOH (221 g) in MeOH (12.3 l) was added to the mother liquor of fractional crystallization, and the hydrolysis reaction was carried out for 30 min. This hydrolysate was crystallized from water (24.6 l) to give (+)-**12** (6.3 kg, 16.7 mol, 47%).

**(R)-5-Chloro-α-(2,3-dimethoxyphenyl)-2-pivaloylaminobenzyl Alcohol [(+)-12]** *Bacillus subtilis* IFO 14117 was grown for 24 h at 28 °C in 40 ml of a medium containing 2% sucrose, 2.5% corn steep liquor, 0.1% KH$_2$PO$_4$, 0.05% (NH$_4$)$_2$SO$_4$ and 0.5% MgSO$_4$ in 200-ml Erlenmeyer flasks on a rotary shaker. The culture (3 ml) was transferred to 1-l Erlenmeyer flasks containing 200 ml of the same medium, and grown for 48 h at 28 °C on a rotary shaker. The culture (2.1 l) was centrifuged, and the cells were suspended in 0.1 M Tris–HCl buffer (pH 7.5) to give 2.1 l of cell suspension. A solution of (±)-**13** (4.2 g, 10.0 mmol) in DMF (105 ml) was added to the cell suspension. After incubation for 24 h at 28 °C with shaking, a portion of this reaction mixture was taken and stirred with AcOEt. The organic layer was analyzed by HPLC. The hydrolytic conversion rate was found to be 49% and the optical purity of (+)-**12** was not less than 99% ee (Table 6).

The reaction mixture obtained above was extracted with AcOEt (2 l), and concentrated under reduced pressure. The residue was purified by flash chromatography [eluent: hexane–isopropyl ether–AcOEt (7 : 2 : 1, v/v), internal pressure: 0.2 kg/cm$^2$] to give crude crystals. Yield 1.5 g (optical purity 99.3% ee). The crude crystals were recrystallized from MeOH–H$_2$O (1 : 2) to give (+)-**12** (0.85 g, 2.25 mmol, 100% ee) as colorless needles. mp 105—106 °C. [α]$_D$ +50.1° (*c*=0.46, MeOH). *Anal.* Calcd for C$_{20}$H$_{24}$ClNO$_4$: C, 63.57; H, 6.40; N, 3.71. Found: C, 63.53; H, 6.47; N, 3.64.

**(−)-2-Amino-5-chloro-α-(2,3-dimethoxyphenyl)benzyl Alcohol [(−)-2c]** A solution of (−)-**12** (0.15 g, 0.397 mmol) and NaOH (0.16 g, 3.97 mmol) in EtOH (2 ml) was refluxed for 3 h. the reaction mixture was diluted with water, extracted with AcOEt (50 ml). The extract was washed with water (50 ml), dried over MgSO$_4$, and then concentrated under reduced pressure. The residue was chromatographed [eluent: hexane–AcOEt (3 : 1)] and recrystallized from hexane–AcOEt (1 : 1) to give (−)-**2c** (0.1 g, 0.340 mmol, 86%) as colorless prisms. mp 128—129 °C. [α]$_D$ −75.9° (*c*=0.47, MeOH). IR $\nu_{max}$ (KBr) cm$^{-1}$: 3600—3200 (br, NH$_2$, OH). $^1$H-NMR (CDCl$_3$) $\delta$: 3.1—3.2 (1H, br), 3.84 (3H, s), 4.1—4.3 (2H, br), 6.03 (1H, s), 6.58 (1H, d, $J$=8.4 Hz), 6.86—6.92 (2H, m), 7.01—7.10 (3H, m). *Anal.* Calcd for C$_{15}$H$_{16}$ClNO$_3$: C, 61.33; H, 5.49; N, 4.77. Found: C, 61.34; H, 5.45; N, 4.67.

**Synthesis of (−)-(3R,5S)-7-Chloro-5-(2-chlorophenyl)-1-neopentyl-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepine-3-acetic Acid [(−)-1c] from (−)-2c** NaBH$_4$ (50 mg, 1.32 mmol) was added to an ice-cooled solution of (−)-**2c** (0.1 g, 0.340 mmol) and pivalaldehyde (40 mg, 0.464 mmol) in AcOH (3 ml). After being stirred for 20 min at room temperature, the mixture was diluted with water (50 ml). The solution was extracted with AcOEt (50 ml, twice). The extracts were washed with saturated NaHCO$_3$ (50 ml) and brine (50 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure. The residue was chromatographed [eluent: hexane–AcOEt (8 : 1)] to give an oil (90 mg, 0.247 mmol, 73%). The oil was dissolved in AcOEt (5 ml). NaHCO$_3$ (0.1 g, 1.19 mmol) was added to the solution, followed by addition of monoethyl fumaryl chloride (50 mg, 0.308 mmol). After being stirred for 40 min at room temperature, the mixture was diluted with AcOEt (50 ml). The solution was washed with water (50 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure. The residue was chromatographed [eluent: hexane–AcOEt (3 : 1)] to give an oil (0.12 g, 0.245 mmol, 99%). The oil was dissolved in EtOH (5 ml). K$_2$CO$_3$ (50 mg, 0.362 mmol) was added to the solution, and the mixture was stirred for 3 h at room temperature. The mixture was diluted with water (50 ml), extracted with AcOEt (50 ml). The extract was washed with water (50 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure to give a colorless powder (90 mg, 0.184 mmol, 75%). The powder was dissolved in EtOH (1 ml), followed by addition of 1 N NaOH (0.2 ml). After being stirred for 30 min at 60 °C, the mixture was diluted with water (50 ml). The solution was acidified with 1 N HCl (0.2 ml), extracted with AcOEt (50 ml). The extract was washed with brine (50 ml), dried over Na$_2$SO$_4$, and then concentrated under reduced pressure. The residue was recrystallized from AcOEt–hexane (1 : 1) to give (−)-**1c** (80 mg, 0.173 mmol, 94%) as colorless

prisms. $[\alpha]_D$ $-250.3°$ ($c=0.41$, MeOH).

**Animals and Materials**   Animals were supplied by Clea, Japan, Inc. *RS*-[2-$^{14}$C]Mevalonolactone and [1-$^3$H]farnesyl pyrophosphate were purchased from New England Nuclear. [2-$^{14}$C]Mevalonic acid was synthesized from [2-$^{14}$C]Mevalonolactone by saponification with potassium hydroxide. [2-$^{14}$C]Sodium acetate was purchased from Amersham. Farnesyl pyrophosphate was synthesized by the method described by V. J. Davisson and coworkers[7] (Nemoto & Co.). HepG2 cells were supplied by The American Type Culture Collection (ATCC). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from GIBCO. Human lipoprotein deficient serum (human LPDS) was purchased from Sigma. All other reagents were supplied by Wako Pure Chemical Industries.

**Preparation of Rat Squalene Synthase**   An Sprague-Dawley (SD) male rat (6-week old) was killed by bleeding, and its liver was excised. About 10 g of the liver was washed with a saline solution cooled with ice, which was then homogenized in 15 ml of an ice-cooled buffer [100 mM potassium phosphate (pH 7.4), 15 mM nicotinamide, 2 mM MgCl$_2$], followed by centrifugation for 20 min at $10000\times\boldsymbol{g}$ (4 °C). The supernatant layer was obtained and subjected to further centrifugation for 90 min at $105000\times\boldsymbol{g}$ (4 °C). The sediment was then suspended in an ice-cooled 100 mM potassium phosphate buffer solution (pH 7.4), which was again subjected to centrifugation for 90 min at $105000\times\boldsymbol{g}$ (4 °C). The sediment thus obtained (microsome fraction) was suspended in an ice-cooled 100 mM potassium phosphate buffer (pH 7.4) (about 40 mg/ml protein concentration, determined using Bicinchoninic acid (BCA) protein assay kit of Pierce Co., Ltd.). This suspension was used as the enzyme solution.

**Preparation of Human Squalene Synthase**   HepG2 cells (about $1\times10^9$ cells) obtained by incubation (37 °C in the presence of 5% CO$_2$) in a DMEM contains 10% FBS, penicillin G (100 units/ml) and streptomycin (10 $\mu$g/ml) were suspended in 10 ml of ice-cooled buffer solution [100 mM potassium phosphate buffer (pH 7.4), 30 mM nicotinamide and 2.5 mM MgCl$_2$]. The cells were crashed by means of ultrasonication (for 30 s, twice). From the sonicate thus obtained, the microsome fraction was obtained by the same procedure as in preparation of rat-derived enzyme, which was suspended in an ice-cooled 100 mM potassium phosphate buffer (pH 7.4) (about 4 mg/ml protein concentration). This suspension was used as the enzyme solution.

**Assay of Squalene Synthase Inhibitory Activity**   Squalene synthase activity was monitored by the formation of [$^3$H]squalene from [1-$^3$H]farnesyl pyrophosphate. Fifty microliters of assay mixture included 5 $\mu$M [1-$^3$H]farnesyl pyrophosphate (25 $\mu$Ci/mol), 1 mM NADPH, 5 mM MgCl$_2$, 6 mM glutathione, 100 mM buffer solution of potassium phosphate (pH 7.4), the test compound dissolved in DMSO (a final concentration of DMSO was 2%) and enzyme solution prepared from rat or HepG2 cells (protein content 0.8 $\mu$g). The assay ran 45 min at 37 °C and stopped by adding 150 $\mu$l of CHCl$_3$–MeOH (1 : 2) containing 0.2% cold squalene as carrier. Aqueous solution of 3 N NaOH (50 $\mu$M) and CHCl$_3$ (50 $\mu$M) were added to the mixture. The chloroform layer containing the reaction mixture having squalene as the principal component and 3 ml of toluene-based liquid scintillator were mixed, and its radioactivity was determined by means of a liquid scintillation counter. The squalene synthase inhibitory activity was expressed in terms of the concentration of the test compound inhibiting by 50% the radioactivity taken into the chloroform layer (IC$_{50}$, molar concentration (M)).

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