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Asymmetric synthesis and biological evaluation of the enantiomeric isomers of the immunosuppressive FTY720-phosphate

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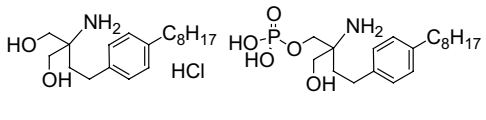
Abstract—A practical asymmetric synthesis of both enantiomers of the immunosuppressive FTY720-phosphate (**2**) was accomplished, and the enantiomers were pharmacologically evaluated. Several lipases showed considerable activity and enantioselectivity for *O*-acylation of *N*-acetyl FTY720 (**3**) or *N*-benzyloxycarbonyl FTY720 (**7**) in combination with vinyl acetate or benzyl vinyl carbonate as the acyl donors. The synthesis using the lipase-catalyzed acylation as the key step produced the enantiomerically pure (>99.5% ee) enantiomers of **2** in multigram quantities. (*S*)-Isomer of **2** had more potent binding affinities to $S1P_{1,3,4,5}$ and inhibitory activity on lymphocyte migration toward S1P than (*R*)-**2**, suggesting that (*S*)-isomer of **2** is responsible for the immunosuppressive activity after administration of **1**. Severe bradycardia was observed in anesthetized rats when (*S*)-**2** was administered intravenously, while (*R*)-**2** had no clear effect on heart rate up to 0.3 mg/kg.

1. Introduction

FTY720 (**1,** Fig. 1) is an immunosuppressive compound,[1,2] which is efficacious in various models of autoimmune diseases and transplantation.[3] Recently, it was reported that **1** was effective to prevent graft rejection in transplanted kidneys in humans.[4] Although the detail of the immunosuppressive mechanism is unclear, it was suggested that the decrease of the number of lymphocytes in peripheral blood and lymph relates the immunosuppressive activity.[5,6] Making the mechanism of action clear is important for drug development and understanding a new aspect of immunological system.



1 (FTY720) **2** (FTY720-phosphate)

Figure 1. Structures of an immunosuppressant FTY720 (**1**) and the active form, FTY720-phosphate (**2**).

Sphingosine-1-phosphate (S1P) is ubiquitous in the body, and influences multiple physiological systems[7] such as endothelial integrity[8] and coronary artery blood flow[9] by binding to its G-protein coupled receptors ($S1P_{1-5}$). One of hydroxyl group of **1** is phosphorylated in vivo to form FTY720-phosphate (**2**, Fig. 1) acting as an agonist for four S1P receptors ($S1P_{1,3,4,5}$) out of five ($S1P_{1-5}$).[10,11] It was reported that **2** downregulates $S1P_1$ after its agonistic effect to the receptor, and the temporary pharmacological $S1P_1$-null state in lymphocytes is one of the mechanism of lymphopenia caused by administration of **1**.[12] While **1** was well tolerated in Phase I clinical trials, a transient bradycardia was reported as an adverse event.[13] The transient heart rate changes caused by a close analog of **2** disappeared in $S1P_3$-deletant mice,[14] strongly suggesting that the bradycardia relates to the agonistic activity of **2** to $S1P_3$.

Compound **1** was designed to delete the asymmetric centers of its lead compound, myriocin,[15] having three asymmetric carbons; however, the phosphorylation of **1** into the active form **2** makes an asymmetric carbon again. The optically pure forms of **2** are essential to

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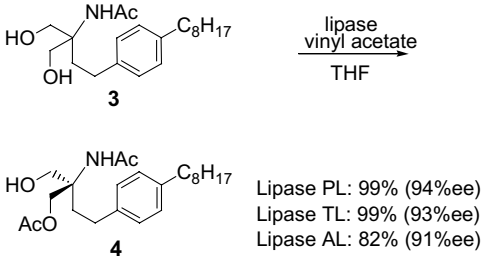
investigate the mechanisms of action and bradycardia of **2**. A preparation of enantiomers of **2** by a chiral HPLC separation of the fully protected **2** and a formal asymmetric synthesis via a serine-derived oxazolidine were reported recently.[16] Although the method was scrupulous, it should be inadequate for a multigram scale synthesis of the both enantiomers of **2** because the long scheme was required for synthesis of each enantiomer. In this paper, we describe a shorter and highly practical asymmetric synthesis of both enantiomers of **2** using an asymmetric acylation by lipase as the key step.

2. Chemistry

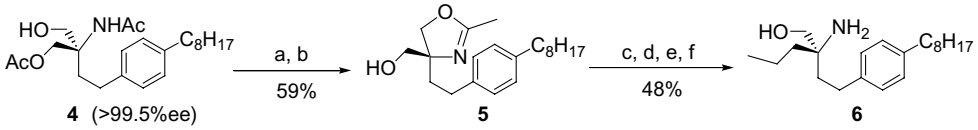
Although asymmetric acylation of 2,2-disubstituted propane-1,3-diol such as **1** has scarcely reported, asymmetric acylation of 2-mono-substituted propane-1,3-diols by acyl donor such as vinyl acetate using enzymes was investigated well.[17,18] Once the enantioselectivity of the acylation was achieved, it does not make much difference in terms of efficient synthesis of both enantiomers which alcohol is acylated by enzymes. It incited us to try enantioselective *O*-acylation of *N*-protected **1** by lipase. We first tried *O*-acetylation of compound **3**, *N*-acetylated **1**, to make clear the possibility of asymmetric acylation for 2,2-disubstituted propane-1,3-diol. Interestingly, Lipase PL, TL, and AL acetylated **3** into diacetate (*R*)-**4** in excellent chemical yields and considerable optical yields (Scheme 1) in spite of the bulkiness of the substituents, acetylamino and 2-(4-octylphenyl)ethyl, at the C-2 position. The configuration of **4** was determined by deriving it into the known compound **6** (Scheme 2). Although the enantiomeric excess of **6** derived from enantiomerically pure **4** was 43.6% due to the migration of acetyl group during cyclization of **4**, the major component of **6** had the *R* configuration showing that the configuration of **4** was *R*. These results suggested that lipase catalyzing acylation would be a powerful method to synthesize the enantiomeric isomers of **2**; however, the possibility of racemization on 2-aminopropane-1,3-diol monophosphates such as **2** was unclear at the beginning of this research, especially under acidic or basic conditions required to cleave *N*- and *O*-acetyl groups. During the study to synthesize racemic **2**, we had also noticed that neither extraction nor chromatography was effective to purify **2** due to its poor solubility. Therefore, we tried to change *N*-acetyl groups of **4** into carbobenzyloxy groups (Cbz) in order to make it easier to cleave the acyl groups and purify the resultant **2**.



Scheme 1. Acetylation of **3**, *N*-acetylated **1**, using lipases and vinyl acetate.

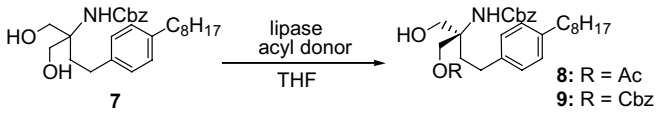
We then tried acetylation of **7**, *N*-benzyloxycarbonated **1**, to replace the *N*-acetyl group of **3** with benzyloxycarbonyl, which would be cleaved easily. The most suitable lipases for the enantioselective *O*-acetylation of **7** were Lipase PL and AL, which gave 95% ee of the product in 96% yield (Table 1). Although some other lipases (Lipase AS, PS, AK, M10, F-AP15, AYS, and G50) and PPL (porcine pancreas lipase) were ineffective like Lipase SL, our successive screening of lipase revealed that immobilized lipase (Toyobo Co.) and Novozyme 435 had excellent catalytic activities. However, they would be too active to get higher optical yield. These results prompted us to investigate *O*-benzylcarbonation because both of *N*- and *O*-benzyloxycarbonyl groups could be removed by hydrogenolysis. The result of screening is shown in Table 1. While the conversion was low in the case of dibenzyl dicarbonate and dibenzyl carbonate, benzyl vinyl carbonate,[19] and acetone *O*-(benzyloxy)carbonyloxime[20] showed good reactivity. Between them, benzyl vinyl carbonate was better for this reaction because *O*-(benzyloxy)carbonyloxime in combination with Lipase PL or TL gave *O*-isopropylideneaminooxycarbonyl derivative of **7** as a by-product, which was difficult to be removed by a silica gel column chromatography from the product. The immobilized lipase (Toyobo Co.) gave the best result, which was quantitative conversion accompanied with 97% ee.

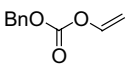
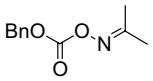
The synthesis of (*S*)-enantiomer of **2** was carried out as shown in Scheme 3. Compound **7**, which was derived by quantitative *N*-benzyloxycarbonation of **1**, was *O*-benzylcarbonated by benzyl vinyl carbonate using the immobilized lipase to give **9**. The reaction gave similar chemical and optical yields in multigram quantity (5.10 g of **7**) to them of the screening scale. The chiral alcohol **9** was phosphorylated into the fully protected intermediate **10** by a phosphoramidite with 1*H*-tetrazole followed by 3-chloroperbenzoic acid oxidation. Hydrogenolysis of **10** followed by washing in methanol gave 99.5% ee



Scheme 2. Determination of absolute configuration by deriving **4** to a reported compound **6**. Reagents and conditions: (a) MsCl, Et_3N, CH_2Cl_2; (b) 0.1 M NaOH, acetone; (c) (i) $(COCl)_2$, DMSO, CH_2Cl_2, (ii) Et_3N; (d) Ph_3PEtBr, *n*-BuLi, Et_2O; (e) H_2, Pd/C, EtOH; (f) concd HCl, EtOH.

Table 1. Results of lipase screening using various acyl donors



Acyl donor (equiv)	Vinyl acetate (excess)	 (5 equiv)	 (10 equiv)	$(BnOCO)_2O$ (5 equiv)	$(BnO)_2CO$ (10 equiv)
Lipase PL	96 (95 ee)	70 (91 ee)	81 (89 ee)	23	Trace[a]
Lipase TL	97 (92 ee)	84 (87 ee)	25 (83 ee)	31	Trace
Lipase AL	96 (95 ee)	3	16	14	—[b]
Lipase QL	24	NR[c]	NR	NR	—
Lipase QLM	40	NR	NR	NR	—
Lipase SL	NR	NR	NR	—	—
Immobilized lipase	95[d] (89 ee)	99 (97 ee)	99 (91 ee)	33	—
Novozyme 435	95 (89 ee)	73 (49 ee)	Trace	NR	—

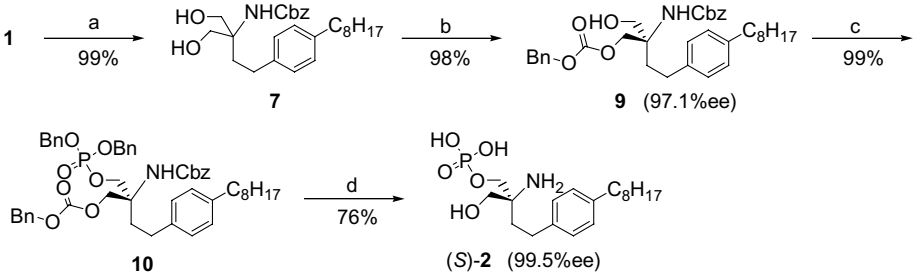
Lipases were screened at room temperature overnight. For the primary screening, 0.1 mmol of **7** was used. The results are shown as percent of conversion determined by LC/MS. For the detailed screening to determine the yields and enantiomeric excesses (ee), 1.0 mmol of **7** was used. The results are shown as isolated yields (%) followed by ee (%) determined by HPLC (Chiralcel OJ-RH, CH_3CN/H_2O) in parentheses.

[a] The product was less than 3%.

[b] The reaction was not tried.

[c] The product was not detected.

[d] The reaction was done at 0 °C.



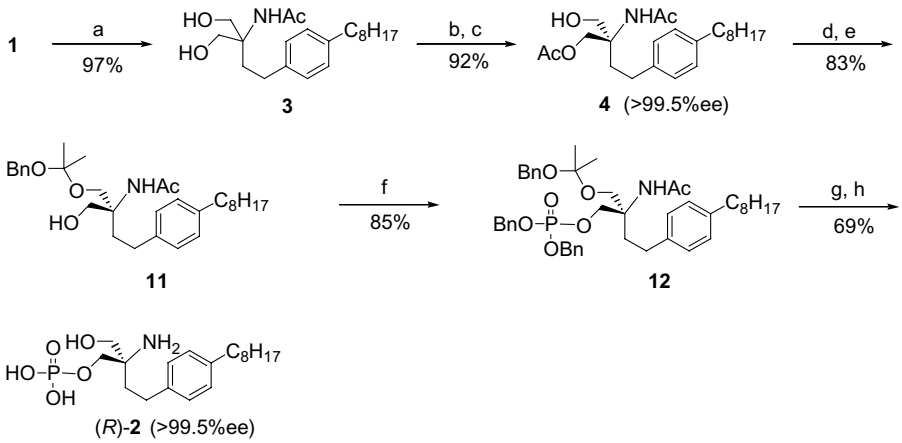
Scheme 3. Synthesis of (*S*)-**2**. Reagents and conditions: (a) ClCOOBn, $NaHCO_3$, $CHCl_3/H_2O$; (b) $BnOCOOCH{=}CH_2$, immobilized lipase, THF; (c) (i) $(BnO)_2PN(iso\text{-}Pr)_2$, 1*H*-tetrazole, CH_2Cl_2/CH_3CN, (ii) *m*-CPBA; (d) (i) H_2, Pd/C, MeOH, (ii) wash in MeOH.

of (*S*)-enantiomer of **2**. Any method for quantitation of the enantiomeric excess of **2** had not been reported[16] because general chiral selectors of HPLC column such as cellulose or crown ether derivatives were ineffective to separate the enantiomers of **2** due to the low solubility and its unique structure including amine, phosphate, alcohol, and highly lipophilic groups. However, our successive screening of HPLC column revealed that both enantiomers of **2** were completely separable on a Sumichiral® OA-7000 HPLC column (CH_3CN/pH 3 phosphate buffer), which has β-cyclodextrin as the chiral selector. We confirmed that the treatment of (*S*)-**2** in 1 M HCl or 2 M NaOH at room temperature for 12 h did not change the enantiomeric excess.

The (*R*)-enantiomer of **2** was synthesized as shown in Scheme 4. Acetylation of compound **3** by Lipase PL gave 92% ee of the product, which was purified by recrystallization to give optically pure compound **4**. The remaining alcohol was protected by 2-benzyloxy-1-propene as an acetal,[21] which would be removed by hydrogenolysis, then *O*-acetyl group was cleaved to give an alcohol **11**. Phosphorylation followed by removal of the protecting groups yielded optically pure (*R*)-**2**. In the last acidifying step, adjusting the pH of the reaction mixture to around 1 was important to get a free form of (*R*)-**2** unlike the method where pH was adjusted to 7.[16] The reported method gave a mono-sodium salt of (*R*)-**2** in our case.[22]

3. Biology

To confirm which enantiomer is responsible for the activity of compound **2**, and investigate their S1P receptor selectivity, both enantiomers were tested against human S1P receptors expressed in CHO ($S1P_{1,2,4}$) or RH7777 ($S1P_{3,5}$) cells in competitive ligand binding assay using [^{32}P]S1P as the ligand (Table 2). As reported results for *rac*-**2**,[10,11,16] both enantiomers had no ability



Scheme 4. Synthesis of (*R*)-**2**. Reagents and conditions: (a) Ac_2O, $NaHCO_3$, $CHCl_3/H_2O$; (b) vinyl acetate, Lipase PL, THF; (c) recrystallization; (d) $CH_2{=}C(CH_3)OBn$, $PdCl_2(COD)$, benzene; (e) K_2CO_3, MeOH/acetone/H_2O; (f) (i) $(BnO)_2PN(iso\text{-}Pr)_2$, 1*H*-tetrazole, CH_2Cl_2/CH_3CN, (ii) *m*-CPBA; (g) H_2, Pd/C, MeOH; (h) (i) 2 M NaOH, MeOH, (ii) 1 M HCl.

Table 2. Binding affinities ($K_i \pm$ SEM, nM) to S1P receptors

Compound	$S1P_1$	$S1P_2$	$S1P_3$	$S1P_4$	$S1P_5$
S1P	1.2 ± 0.04	3.4 ± 0.18	5.4 ± 0.23	2.7 ± 0.28	0.92 ± 0.05
rac-**2**	4.1 ± 0.63	>1000	13 ± 1.6	37 ± 2.1	4.2 ± 0.71
(*R*)-**2**	277 ± 29.0	>1000	586 ± 66.5	105 ± 16.1	121 ± 9.5
(*S*)-**2**	2.1 ± 0.12	>1000	5.9 ± 0.26	23 ± 2.2	2.2 ± 0.25

K_i Values were determined by competition of [^{32}P]-S1P binding to stably transfected CHO ($S1P_{1,2,4}$) or RH7777 ($S1P_{3,5}$) cells expressing the indicated S1P receptors. Data represent the mean ± SE of three experiments.

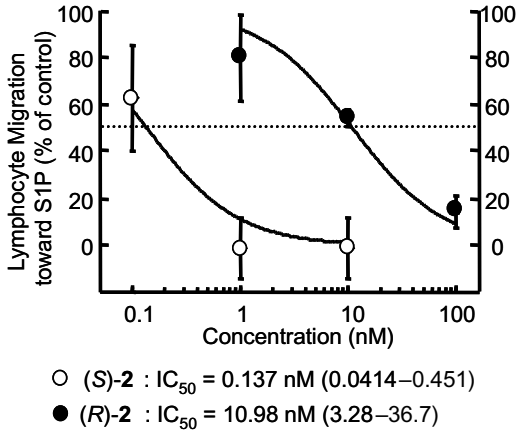


Figure 2. Inhibitory activity of (*S*)- and (*R*)-**2** on lymphocyte migration toward 10 nM of S1P. IC_{50} values are followed by 95% confidential limits.

to bind $S1P_2$. While (*R*)-**2** bound to $S1P_{1,3,4,5}$ only at high concentrations, (*S*)-**2** had the ability to bind them in nanomolar or subnanomolar ranges. Figure 2 shows the inhibitory activity of both enantiomers of **2** on T-lymphocyte chemotaxis toward S1P. It was reported that peripheral lymphocytes taken from FTY720-treated mice failed to migrate in response to S1P, and that this activity could explain the blocking effect of **1** on lymphocyte egress.[12] (*S*)-Isomer of **2** inhibited T cell chemotactic response to 10 nM of S1P completely at 1 nM or higher concentrations (IC_{50} = 0.137 nM). On the other hand, (*R*)-**2** required approximately 100 times higher concentration to inhibit it (IC_{50} = 11.0 nM). These data are show that (*S*)-isomer not only binds to S1P receptors strongly but also is responsible for decreasing lymphocytes in blood and lymph. Accordingly, it was confirmed that (*S*)-isomer is the active form of **2**. This result was supported by our previous studies,[2,23] in which pro-(*S*) hydroxymethyl between two hydroxymethyl groups of **1** was essential for the immunosuppressive activity. As to the bradycardia, we evaluated both enantiomers of **2** in anesthetized Sprague-Dawley rats.[24] After intravenous bolus administration of (*S*)-**2**, the marked decrease of heart rate was observed at doses of 0.003 or 0.03 mg/kg in a dose dependent manner (Table 3). In contrast to the severe bradycardia induced by (*S*)-isomer, (*R*)-**2** had no clear effect on heart rate up to a dose of 0.3 mg/kg. This result revealed not only that (*S*)-isomer is closely related to bradycardia induced by administration of **1**, also that S1P/S1P receptors are significant to regulate the heart rate. In a preliminary attempt to identify the ratio of (*R*)- and (*S*)-**2** in rat serum after oral administration of **1** (1 mg/kg, 8 h), (*S*)-**2** was the only isomer detected by

Table 3. Bradycardia induced by enantiomers of **2** in anesthetized rats

Compound	Dose (mg/kg)	Heart rate (%)
(*S*)-**2**	0.003	46.6 ± 13.0
(*S*)-**2**	0.03	16.4 ± 3.9
(*R*)-**2**	0.3	91.8 ± 1.7
Vehicle		97.1 ± 2.1

Heart rate was monitored on an electrocardiogram. Data were taken one minute after intravenous administration of the test compound, and expressed as the percentage against before administration. Results are the mean ± SE of three animals (Sprague-Dawley rats).

LC/MS.[25] In the future, the more detail of the bioactive mechanisms of **1** would be made clear by using the enantiomerically pure form of **2**.

4. Conclusion

A practical asymmetric synthesis of both enantiomers of the immunosuppressive FTY720-phosphate (**2**) was accomplished using the lipase-catalyzed acylation as the key step. (*S*)-Isomer of **2** had more potent binding affinities to $S1P_{1,3,4,5}$ and inhibitory activity on lymphocyte migration toward S1P than (*R*)-**2**, suggesting that (*S*)-isomer of **2** is responsible for the immunosuppressive activity after administration of **1**. Severe bradycardia was observed in anesthetized rats when (*S*)-**2** was administered intravenously, while (*R*)-**2** had no clear effect on heart rate up to 0.3 mg/kg. The more detail of the bioactive mechanisms of **1** would be made clear by using the enantiomerically pure form of **2**.

5. Experimental

5.1. Chemistry

Lipase PL, TL, AL, QL, QLM, and SL were obtained from Meito Sangyo Co., Ltd. Immobilized lipase (Toyobo) and PPL were purchased from Nacalai tesque. Novozyme 435 and other lipases (lipase AS, PS, AK, M10, F-AP15, AYS, and G50) were purchased from Sigma-Aldrich Japan K. K. and Wako Pure Chemical Industries, Ltd, respectively. Silica gel column chromatography was performed on Fuji Silysia PSQ100B. NMR spectra were recorded on a Bruker AVANCE 400 (400 MHz for proton and 162 MHz for phosphorus) spectrometer. Chemical shifts are expressed in ppm relative to TMS as internal standard for proton and H_3PO_4 for phosphorus, respectively. Mass spectra were measured in a combination with a Waters Alliance 2795 HPLC system (0.05% TFA in CH_3CN/0.05% TFA in water), a Chromolith SpeedROD RP-18e column, and a Micromass ZQ (ESI) spectrometer. Melting points were obtained on a Buchi 535 melting point apparatus and are uncorrected. Optical rotations were determined on a JASCO digital polarimeter DIP-360 in a 10 cm path length cell. Elemental analyses were performed on a Yanako CHN coder MT-5.

5.2. Lipase screening

Unless otherwise stated, all primary screening reactions were done at room temperature in 0.1 mmol scale. A part of reaction solution was diluted with DMSO and analyzed by LC/MS to determine the rate of conversion. Lipases showing considerable activity were further tested in 1.0 mmol scale to determine the isolated yields and enantiomeric excess (ee). At the end of reaction, the reaction mixture was passed through a Celite pad, concentrated, and purified by a silica gel column chromatography. The ee values were determined by HPLC using a Chiralcel OJ-RH (CH_3CN/H_2O). All lipase catalyzed products had *R* configuration (a shorter retention time).

5.3. *N*-[1,1-Bis(hydroxymethyl)-3-(4-octylphenyl)propyl]-acetamide (3)

A mixture of **1** (10.0 g, 29.1 mmol), chloroform (800 mL), and saturated sodium bicarbonate (500 mL) was stirred vigorously. After adding of acetic anhydride (3.29 mL, 34.9 mmol) to the mixture eight times at every 30 min, the organic layer was separated, washed with brine, and dried over $MgSO_4$. The solution was evaporated, and the residue was crystallized in diethyl ether to give 9.82 g (97%) of **3** as a white crystalline powder. ^{1}H NMR ($CDCl_3$): δ 7.10 (4H, s), 5.75 (1H, s), 3.88 (1H, d, J = 8.6 Hz), 3.86 (1H, d, J = 8.6 Hz), 3.65–3.60 (4H, m), 2.64–2.61 (2H, m), 2.56 (2H, t, J = 7.8 Hz), 1.98–1.94 (2H, m), 1.93 (3H, s), 1.58 (2H, quint, J = 7.4 Hz), 1.33–1.24 (10H, m), 0.88 (3H, t, J = 6.8 Hz). MS (ESI): *m*/*z* 350 (M+H), 372 (M+Na).

5.4. (*R*)-2-Acetamido-2-hydroxymethyl-4-(4-octylphenyl)-butyl acetate (4)

A mixture of **3** (9.82 g, 28.1 mmol), THF (130 mL), vinyl acetate (130 mL), and Lipase PL (4.91 g) was stirred at room temperature for 140 min. The mixture was passed through a Celite pad and concentrated to give 94% ee of **4**. Crystallization in diethyl ether and hexane afforded 10.2 g (93%) of enantiomerically pure (>99.5% ee) **4** as a white crystalline powder. ^{1}H NMR ($CDCl_3$): δ 7.10 (4H, s), 5.70 (1H, s), 4.47 (1H, t, J = 7.0 Hz), 4.38 (1H, d, J = 11.4 Hz), 4.18 (1H, d, J = 11.4 Hz), 3.77 (1H, dd, J = 12.2, 7.4 Hz), 3.72 (1H, dd, J = 12.0, 6.8 Hz), 2.70–2.62 (1H, m), 2.59–2.52 (1H, m), 2.56 (2H, t, J = 7.8 Hz), 2.18–2.10 (1H, m), 2.11 (3H, s), 1.97–1.90 (1H, m), 1.93 (3H, s), 1.58 (2H, quint, J = 7.2 Hz), 1.33–1.24 (10H, m), 0.88 (3H, t, J = 6.8 Hz). MS (ESI): *m*/*z* 392 (M+H), 414 (M+Na). $[\alpha]_D^{24}$ −0.9 (*c* 1.01, $CHCl_3$).

5.5. (*S*)-[2-Methyl-4-[2-(4-octylphenyl)ethyl]oxazolin-4-yl] methanol (5)

To a mixture of **4** (3.0 g, 7.62 mmol), Et_3N (1.16 mL, 8.38 mmol) in CH_2Cl_2 (25 mL) was added methanesulfonyl chloride (0.619 mL, 8.00 mmol) at 0 °C. The mixture was stirred for 15 min, extracted with CH_2Cl_2, and washed with brine. The solution was dried over $MgSO_4$ and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/1) to afford acetate of **5** (1.82 g, 64%) as a colorless oil. To a solution of this intermediate in acetone (50 mL) was added 0.25 M NaOH (19.2 mL, 4.80 mmol) at 0 °C. The mixture was stirred at 0 °C for 1 h, and concentrated to remove acetone. The aqueous residue was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 1/4) to afford **5** (1.47 g, 92%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.08 (4H, s), 4.22 (1H, d, J = 8.4 Hz), 4.06 (1H, d, J = 8.4 Hz), 3.68 (1H, br d, J = 11.0 Hz), 3.44 (1H, dd, J = 11.0, 7.8 Hz), 2.61–2.49 (4H, m), 2.10–2.04 (1H, m), 2.02 (3H, s), 1.88 (1H, ddd, J = 13.6, 11.4, 5.8 Hz), 1.75 (1H, ddd, J = 13.6,

11.0, 5.8 Hz), 1.58 (2H, quint, $J = 7.5$ Hz), 1.34–1.23 (10H, m), 0.87 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 332 (M+H). $[\alpha]_D^{24}$ +15.9 (*c* 0.57, $CHCl_3$).

5.6. (*R*)-2-Amino-2-[2-(4-octylphenyl)ethyl]pentanol (6)

To a solution of oxalyl chloride (0.748 mL, 8.58 mmol) and DMSO (0.915 mL, 12.9 mmol) in CH_2Cl_2 (20 mL) was added a solution of **5** (1.43 g, 4.29 mmol) in CH_2Cl_2 (15 mL) at −70 °C. After stirring the mixture for 2 h at the same temperature, Et_3N (4.46 mL, 32.2 mmol) was added. The mixture was stirred at −55 °C for 30 min, diluted with saturated NH_4Cl, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated to afford an aldehyde (1.43 g, quant.) as a yellow oil. To a suspension of (ethyl)triphenylphosphonium bromide (3.19 g, 8.58 mmol) in Et_2O (20 mL) was added 1.6 M *n*-BuLi in hexanes (4.69 mL, 7.50 mmol), and the mixture was stirred for 1 h. A solution of the aldehyde obtained above in Et_2O (15 mL) was added to the mixture, and the mixture was stirred for 14 h. After adding saturated $NaHCO_3$, the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 4/1) to afford (*S*)-[2-methyl-4-[2-(4-octylphenyl)ethyl]-4-(1-propenyl)oxazoline (791 mg, 54%) as a yellow oil. A mixture of the product and 10% Pd/C (100 mg) in EtOH (25 mL) was stirred under hydrogen atmosphere for 7 h, passed through a Celite pad, and concentrated. The residue was dissolved in EtOH (15 mL) and concentrated HCl (5 mL), and the mixture was heated at 70 °C for 30 min, and concentrated. After adding saturated $NaHCO_3$, the mixture was extracted with EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated to afford **6** (506 mg, 63%) as a white powder. ^{1}H NMR ($CDCl_3$): δ 7.09 (4H, s), 3.36 (2H, s), 2.56 (4H, t, $J = 8.1$ Hz), 1.64–1.56 (6H, m), 1.47–1.43 (1H, m), 1.36–1.26 (14H, m), 0.95 (3H, t, $J = 6.9$ Hz), 0.88 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 320 (M+H). $[\alpha]_D^{24}$ +0.81 (*c* 0.50, $CHCl_3$). The retention time of *N*-3,5-dinitrobenzoyl derivative of **6** was identical with that of (*R*)-**6** (shorter retention time on Chiralcel OD (hexane/EtOH), 43.6% ee).[23]

5.7. 2-(Benzyloxycarbonyl)amino-2-[2-(4-octylphenyl)-ethyl]propane-1,3-diol (7)

A mixture of **1** (4.03 g, 11.7 mmol) and benzyl chloroformate (5.98 mL, 41.9 mmol) in $CHCl_3$ (500 mL) and saturated $NaHCO_3$ (250 mL) was stirred at room temperature for 18 h. The organic layer was separated, washed with brine, and dried over $MgSO_4$. The solution was concentrated, and the residue was crystallized in Et_2O to afford **7** (5.10 g, 99%) as a white powder. ^{1}H NMR ($CDCl_3$): δ 7.37–7.31 (5H, m), 7.07 (4H, s), 5.27 (1H, s), 5.09 (2H, s), 3.91 (2H, dd, $J = 11.6$, 6.0 Hz), 3.68 (2H, dd, $J = 11.6$, 6.8 Hz), 3.13 (2H, br s), 2.60–2.56 (2H, m), 2.55 (2H, t, $J = 8.0$ Hz), 1.92–1.88 (2H, m), 1.61–1.56 (2H, m), 1.33–1.26 (10H, m), 0.88 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 442 (M+H), 464 (M+Na).

5.8. (*R*)-Acetic acid 2-(benzyloxycarbonyl)amino-2-hydroxymethyl-4-(4-octylphenyl)butyl ester (8)

A mixture of **7** (442 mg, 1.0 mmol), lipase PL (220 mg), vinyl acetate (10 mL), and THF (10 mL) was stirred at room temperature for 5 h. The mixture was passed through a Celite pad, and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 4/1) to afford **8** (462 mg, 96%, 95.3% ee by HPLC (Chiralcel OJ-RH, CH_3CN/H_2O, shorter retention time)) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.39–7.31 (5H, m), 7.07 (2H, d, $J = 8.2$ Hz), 7.05 (2H, d, $J = 8.2$ Hz), 5.10 (1H, s), 5.09 (2H, s), 4.36 (1H, d, $J = 11.4$ Hz), 4.19 (1H, d, $J = 11.4$ Hz), 3.76 (1H, dd, $J = 12.0$, 7.6 Hz), 3.70 (1H, dd, $J = 12.0$, 6.8 Hz), 3.46 (1H, br s), 2.62–2.49 (2H, m), 2.55 (2H, t, $J = 7.8$ Hz), 2.15–2.08 (1H, m), 2.08 (3H, s), 1.94–1.87 (1H, m), 1.58 (2H, quint, $J = 7.4$ Hz), 1.34–1.24 (10H, m), 0.88 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 484 (M+H), 506 (M+Na). $[\alpha]_D^{24}$ −3.27 (*c* 0.94, $CHCl_3$).

5.9. (*R*)-2-(Benzyloxycarbonyl)amino-2-(benzyloxycarbonyloxy)methyl-4-(4-octylphenyl)butanol (9)

A mixture of **7** (5.10 g, 11.5 mmol), benzyl vinyl carbonate (5.12 g, 28.8 mmol), immobilized lipase (Toyobo, 5.10 g), and THF (90 mL) was stirred at room temperature for 16 h. The mixture was passed through a Celite pad, and concentrated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 95/5–8/2) to afford **9** (6.52 g, 99%, 97.1% ee by HPLC (Chiralcel OJ-RH, CH_3CN/H_2O, shorter retention time)) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.38–7.30 (10H, m), 7.06 (2H, d, $J = 8.2$ Hz), 7.04 (2H, d, $J = 8.2$ Hz), 5.16 (2H, s), 5.11 (1H, s), 5.07 (2H, s), 4.41 (1H, d, $J = 11.0$ Hz), 4.30 (1H, d, $J = 11.0$ Hz), 3.79 (1H, dd, $J = 12.0$, 7.2 Hz), 3.73 (1H, dd, $J = 12.0$, 7.2 Hz), 3.40 (1H, br s), 2.62–2.49 (2H, m), 2.54 (2H, t, $J = 7.6$ Hz), 2.10 (1H, ddd, $J = 14.0$, 11.4, 5.2 Hz), 1.89 (1H, ddd, $J = 14.0$, 11.8, 5.6 Hz), 1.57 (2H, quint, $J = 7.2$ Hz), 1.34–1.23 (10H, m), 0.88 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 576 (M+H), 598 (M+Na). $[\alpha]_D^{24}$ −2.34 (*c* 0.96, $CHCl_3$).

5.10. (*S*)-2-(Benzyloxycarbonyl)amino-2-(benzyloxycarbonyloxy)methyl-1-(dibenzyl)phosphoryloxy-4-(4-octylphenyl)butane (10)

To a solution of **9** (6.50 g, 11.3 mmol) and 1*H*-tetrazole (953 mg, 13.6 mmol) in CH_2Cl_2 (70 mL) and CH_3CN (70 mL) was added dibenzyl diisopropylphosphoramidite (4.17 mL, 12.4 mmol) at 0 °C. After stirring at 0 °C for 1 h, *m*-CPBA (65% purity, 3.61 g, 13.6 mmol) was added. The mixture was stirred at 0 °C for 20 min, diluted with saturated $NaHCO_3$, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel column chromatography (hexane/EtOAc = 9/1–3/2) to afford **10** (9.39 g, 99%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.35–7.28 (20H, m), 7.04 (2H, d, $J = 7.8$ Hz), 6.97 (2H, d, $J = 7.8$ Hz), 5.12 (3H, br s), 5.04 (2H, s), 5.01 (4H, d, $J = 8.8$ Hz), 4.34 (1H, d, $J = 11.0$ Hz), 4.29 (1H, d, $J = 11.0$ Hz), 4.14 (2H, d, $J = 6.0$ Hz), 2.54 (2H, t, $J = 7.6$ Hz), 2.50–2.41 (2H, m),

2.11–1.94 (2H, m), 1.59–1.54 (2H, m), 1.33–1.25 (10H, m), 0.88 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 858 (M+Na).

5.11. (*S*)-2-Amino-2-(phosphoryloxy)methyl-4-(4-octylphenyl)butanol ((*S*)-2)

A mixture of **10** (9.35 g, 11.2 mmol) and 10% Pd/C (5.0 g) in MeOH (200 mL) was stirred under hydrogen atmosphere for 7 h. The mixture was passed through a Celite pad, and concentrated. The residue was crystallized in $EtOH/H_2O$. The white powder was suspended in MeOH (200 mL), and stirred at room temperature for 20 h. The suspending solid was collected to afford (*S*)-**2** (3.31 g, 76%, 99.5% ee by HPLC (Sumichiral OA-7000, CH_3CN/pH 3 phosphate buffer, slower eluting enantiomer)) as a white powder; mp 198–199 °C. ^{1}H NMR (CD_3OD): δ 7.14 (2H, d, $J = 7.8$ Hz), 7.08 (2H, d, $J = 7.8$ Hz), 4.01 (1H, dd, $J = 11.6$, 5.6 Hz), 3.99 (1H, dd, $J = 11.6$, 5.6 Hz), 3.71 (1H, d, $J = 11.6$ Hz), 3.69 (1H, d, $J = 11.6$ Hz), 2.71–2.60 (2H, m), 2.56 (2H, t, $J = 7.6$ Hz), 2.01–1.92 (2H, m), 1.58 (2H, quint, $J = 7.2$ Hz), 1.34–1.25 (10H, m), 0.89 (3H, t, $J = 6.4$ Hz). ^{31}P NMR (CD_3OD): δ −0.04 (t, $J = 5.5$ Hz). MS (ESI): *m/z* 388 (M+H). $[\alpha]_D^{24}$ +1.26 (*c* <0.1, MeOH. The number of optical rotation was lower reliability due to the poor solubility.). Anal. Calcd for $C_{19}H_{34}NO_5P \cdot 0.5H_2O$: C, 57.56; H, 8.90; N, 3.53. Found: C, 57.69; H, 9.18; N, 3.53.

5.12. (*S*)-*N*-[1-(1-Benzyloxy-1-methylethoxy)methyl-1-hydroxymethyl-3-(4-octylphenyl)propyl]acetamide (11)

A mixture of **4** (10.1 g, 25.8 mmol), dichloro(1,5-cyclooctadiene)palladium(II) (737 mg, 2.58 mmol), and benzyl isopropenyl ether (7.65 g, 51.6 mmol) in benzene (170 mL) was stirred at room temperature for 18 h. The mixture was diluted with EtOAc, washed with saturated $NaHCO_3$ and then brine, and dried over $MgSO_4$. The solution was concentrated, and the residue was purified by silica gel column chromatography (hexane/EtOAc = 9/1–1/1) to afford an intermediate (13.7 g, 98%), (*R*)-2-acetamido-2-(1-benzyloxy-1-methylethoxymethyl)-4-(4-octylphenyl)butyl acetate, as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.34–7.31 (4H, m), 7.29–7.24 (1H, m), 7.08 (4H, s), 5.61 (1H, s), 4.49 (2H, s), 4.38 (1H, d, $J = 11.2$ Hz), 4.36 (1H, d, $J = 11.2$ Hz), 3.66 (1H, d, $J = 9.4$ Hz), 2.62–2.50 (4H, m), 2.36–2.28 (1H, m), 2.11–2.03 (1H, m), 1.93 (3H, s), 1.90 (3H, s), 1.61–1.56 (2H, m), 1.45 (6H, s), 1.33–1.24 (10H, m), 0.88 (3H, t, $J = 7.0$ Hz). MS (ESI): *m/z* 392 ($M-BnOC(CH_3)_2$+H). To a solution of the above intermediate (13.7 g, 25.4 mmol) in acetone (100 mL), water (60 mL), and MeOH (30 mL) was added K_2CO_3 (3.51 g, 25.4 mmol) at 0 °C. The mixture was stirred at room temperature for 11 h, and concentrated to remove organic solvents. The residue was diluted with water, and extracted with EtOAc. The extract was washed with brine, and dried over $MgSO_4$. The solution was concentrated, and the residue was purified by silica gel column chromatography (hexane/EtOAc = 4/1–1/1) to afford **11** (10.6 g, 84%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.36–7.32 (4H, m), 7.29–7.25 (1H, m), 7.07 (4H, s), 5.98 (1H, s), 4.73 (1H, t, J = 6.4 Hz), 4.53 (1H, d, $J = 11.6$ Hz), 4.51 (1H, d, $J = 11.6$ Hz), 3.73 (1H, d, $J = 9.4$ Hz), 3.72 (2H, d, $J = 6.4$ Hz), 3.45 (1H, d, $J = 9.4$ Hz), 2.69–2.62 (1H, m), 2.55 (2H, t, $J = 7.6$ Hz), 2.55–2.47 (1H, m), 2.14 (1H, ddd, $J = 14.0$, 11.6, 5.2 Hz), 1.94–1.85 (1H, m), 1.89 (3H, s), 1.57 (2H, quint, $J = 7.4$ Hz), 1.47 (3H, s), 1.46 (3H, s), 1.33–1.24 (10H, m), 0.88 (3H, t, $J = 6.6$ Hz). MS (ESI): *m/z* 350 ($M-BnOC(CH_3)_2$+H).

5.13. (*R*)-*N*-[1-(Dibenzylphosphoryloxy)methyl-1-(1-benzyloxy-1-methylethoxy)methyl-3-(4-octylphenyl)propyl]-acetamide (12)

To a solution of **11** (10.5 g, 21.1 mmol) and 1*H*-tetrazole (1.77 g, 25.3 mmol) in CH_2Cl_2 (120 mL) and CH_3CN (120 mL) was added dibenzyl diisopropylphosphoramidite (7.80 mL, 23.2 mmol) at 0 °C. After stirring at 0 °C for 1 h, *m*-CPBA (65% purity, 6.72 g, 25.3 mmol) was added. The mixture was stirred at 0 °C for 20 min, diluted with saturated $NaHCO_3$, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and evaporated. The residue was purified by silica gel chromatography (hexane/EtOAc = 9/1–1/1) to afford **12** (13.5 g, 84%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.34–7.21 (15H, m), 7.05 (2H, d, $J = 7.8$ Hz), 7.03 (2H, d, $J = 7.8$ Hz), 5.83 (1H, s), 4.98 (4H, d, $J = 8.4$ Hz), 4.46 (2H, s), 4.25 (1H, dd, $J = 10.0$, 6.4 Hz), 4.21 (1H, dd, $J = 10.0$, 6.4 Hz), 3.70 (1H, d, $J = 9.2$ Hz), 3.61 (1H, d, $J = 9.2$ Hz), 2.55 (2H, t, $J = 7.8$ Hz), 2.52–2.45 (2H, m), 2.22–2.07 (2H, m), 1.82 (3H, s), 1.57 (2H, quint, $J = 7.4$ Hz), 1.40 (3H, s), 1.39 (3H, s), 1.33–1.24 (10H, m), 0.87 (3H, t, $J = 6.8$ Hz). MS (ESI): *m/z* 610 ($M-BnOC(CH_3)_2$+H).

5.14. (*R*)-2-Amino-2-(phosphoryloxy)methyl-4-(4-octylphenyl)butanol ((*R*)-2)

A mixture of **12** (13.2 g, 17.4 mmol) and 10% Pd/C (6.5 g) in MeOH (200 mL) was stirred under hydrogen atmosphere for 9 h. The mixture was passed through a Celite pad, and concentrated. The residue was dissolved in MeOH (20 mL) and 1 M NaOH (60 mL), and refluxed for 10 h. The reaction mixture was cooled to room temperature, and acidified with 1 M HCl to adjust pH 1. The yielded solid was collected, and washed with water to afford (*R*)-**2** (4.67 g, 69%, >99.5% ee by HPLC (Sumichiral OA-7000, CH_3CN/pH 3 phosphate buffer, faster eluting enantiomer)) as a white powder; mp 199–200 °C. ^{1}H and ^{31}P NMR spectra were identical with those of (*S*)-**2**. MS (ESI): *m/z* 388 (M+H). $[\alpha]_D^{24}$ −9.99 (*c* <0.1, MeOH. The number of optical rotation was lower reliability due to the poor solubility.). Anal. Calcd for $C_{19}H_{34}NO_5P \cdot 0.5H_2O$: C, 57.56; H, 8.90; N, 3.53. Found: C, 57.79; H, 9.22; N, 3.56.

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22. Data of mono-sodium salt of **2**: ^{1}H NMR (CD_3OD): δ 7.13 (2H, d, J = 8.0 Hz), 7.07 (2H, d, J = 8.0 Hz), 3.93 (1H, dd, J = 11.4, 7.4 Hz), 3.85 (1H, dd, J = 11.4, 7.4 Hz), 3.65 (1H, d, J = 11.2 Hz), 3.62 (1H, d, J = 11.2 Hz), 2.68–2.60 (2H, m), 2.55 (2H, t, J = 7.6 Hz), 1.89–1.82 (2H, m), 1.61–1.54 (2H, m), 1.34–1.24 (10H, m), 0.89 (3H, t, J = 6.8 Hz). Anal. Calcd for $C_{19}H_{33}NO_5PNa{\cdot}H_2O$: C, 53.39; H, 7.78; N, 3.28. Found: C, 53.08; H, 7.89; N, 3.02.
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25. Our pharmacokinetic study using the enantiomers of **2** will be reported in due course.