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Bioorganic & Medicinal Chemistry Letters 13 (2003) 229–236

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis of Non-Competitive Inhibitors of Sphingomyelinases with Significant Activity

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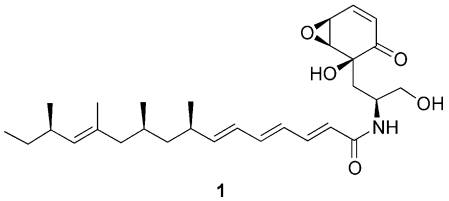
Received 2 August 2002; accepted 9 October 2002

Abstract—A series of short-chain analogues of *N*-palmitoylsphingosine-1-phosphate, modified by replacement of the phosphate and the long alkenyl side chain with hydrolytically stable difluoromethylene phosphonate and phenyl, respectively, were prepared to study the structure–activity relationship for inhibition of sphingomyelinase. The study revealed that inhibition is highly dependent upon the stereochemistry of the asymmetric centers of the acylamino moiety, and resulted in identification of a non-competitive inhibitor with the same level of inhibitory activity of schyphostatin, the most potent of the few known small molecular inhibitors of sphingomyelinase.

Introduction

Sphingomyelin is a major structural component of the cell membrane. The sphingomyelin metabolites, ceramide, sphingosine, and sphingosine-1-phosphate, have become the focus of extensive research due to their possible role as lipid second messengers. The primary sphingomyelin metabolite, ceramide, is generated through the action of either a lysosomal acid sphingomyelinase (A-SMase) or a membrane-bound neutral sphingomyelinase (N-SMase) in a so-called sphingomyeline cycle.[1] The generated ceramide is believed to play a pivotal role in regulation of cell growth and differentiation, cell cycle arrest, and apoptosis as the lipid messenger.[1,2] However, direct links between SMases and specific signaling systems have not yet been fully established. To establish a clear picture of the metabolic links, the synthesis of specific SMase inhibitors is strongly required. Additionally, the SMase inhibitor is expected to have some clinical values, because ceramide generation following sphingomyelin hydrolysis might be implicated in pathological states such as inflammation and AIDS.[1–3]

While several modest inhibitors are known,[1] few inhibitors of significant activity against SMases are reported. Natural product schyphostatin **1**, recently isolated from a constituent of *Trichopeziza mollissima*, was found to be a competitive inhibitor towards N-SMase with IC_{50} of 1.0 μM.[4] This natural product is the most potent of the few known small molecule inhibitors of N-SMase.[5]



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In a search for a novel structural class of SMase inhibitors, we have been interested in the synthesis and biological evaluation of a hydrololyticaly stable analogue **2** of sphingomyelin, in which the oxygen of the phosphoester linkage is replaced by a difluoromethylene (CF_2) unit. The particular utility of replacement of a phosphoric ester oxygen of *p*-Tyr in a peptidyl framework with a CF_2-unit has been demonstrated in the synthesis of useful biochemical tools for studying the mechanism of tyrosine-kinase-mediated signal transductions,[6,7] since the resulting phosphonate analogue

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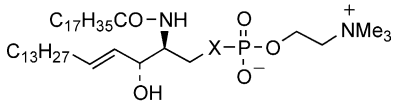
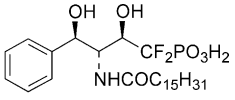
PII: S0960-894X(02)00888-0

mimics accurately the parental phosphate in their isosterical and isopolar properties.[8] However, the strategy has not been applied to synthesis of a hydrolytically stable analogue of sphingomyelin, which might be useful to create SMase inhibitors valuable for studying the ceramide-mediated signaling cascade.



During the studies directed towards synthesis and biological evaluation of **2** and the related analogues, we have found that a short-chain analogue of *N*-palmitoyl sphingosin-1-phosphate, modified by replacement of the phosphate moiety and the long alkenyl side chain with hydrolytically stable difluoromethylene phosphonic acid and phenyl, respectively, as illustrated in the structure **3**, non-competitively inhibited Mg^{2+}-dependent N-SMase from bovine brain microsomes with IC_{50} of 400 μM.[9,10] This compound **3** had the ability to suppress tumor necrosis factor (TNF) α-induced apoptosis of PC-12 neurons at a low concentration of 0.1 μM.[9] In our continuous research for novel SMase inhibitors of significant activity, we have pursued the structure–activity relationship (SAR) of **3**. In this paper, we describe that the inhibitory activity is highly dependent upon the stereochemistry of the asymmetric center of the acylamino moiety and the studies identified a non-competitive inhibitor with the same level of inhibitory activity of schyphostatin.



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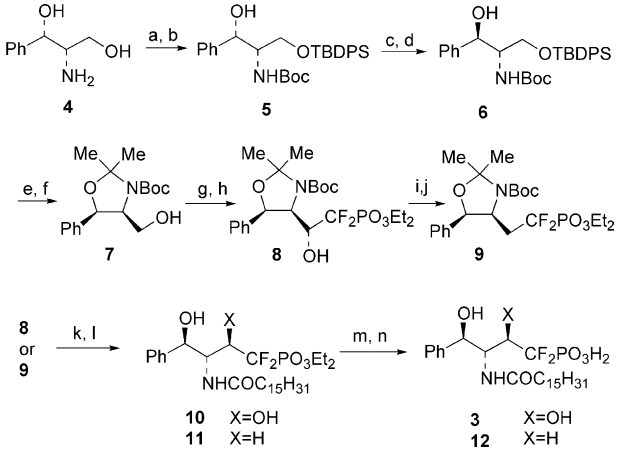
Results and Discussion

Our SAR-study of **3** focussed on examination of the effects of the hydroxy group (α-OH) adjacent to the difluoromethylene as well as the stereochemistry of the asymmetric centers of the acylamino alcohol moiety on the inhibitory activity. Previously, the synthesis of **3** was achieved from (*S*)-Gerner aldehydes through a multistep sequence.[9] To synthesize a series of the analogous compounds with different stereochemistry more readily, commercially available (1*S*,2*S*)-2-amino-1-phenyl-1,3-propanediol **4**[11] was chosen as the starting material in the present study (Schemes 1 and 3).

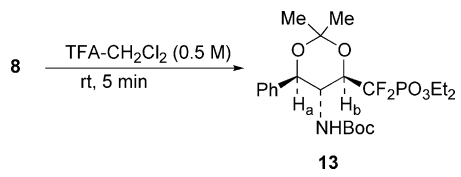
tert-Butyloxycarbonylation of **4**, followed by selective silylation with *tert*-butyldiphenylsilyl chloride (TBDPSCl) gave the *threo*-amino alcohol derivative **5** in a virtually quantitative yield. The Mitsunobu inversion of the secondary hydroxyl with 4-nitrobenzoic acid, followed by reductive removal of the benzoate gave *erythro*-amino alcohol derivative **6** in 77% yield. Both amino alcohol derivatives **5** and **6** were used for synthesis of the analogous compounds of **3**.

In an attempt to identify the minimum structural requirements for the activity of **3**, we first synthesized the desoxy derivative **12** from **6**. *N*,*O*-Acetalization of **6**, followed by desilylation gave **7** in 60% yield. The alcohol **7** was oxidized with the Dess–Martin periodinane; the resulting aldehyde was treated with lithium salts of diethyl difluoromethylphosphonate ($LiCF_2PO_3Et_2$) at −78 °C in THF to give the adduct **8**, $[\alpha]_D^{25}$ −21.6 (*c* 1.1, MeOH), in 60% yield (vide infra). Deoxygenation of α-OH in **8** was achieved via the corresponding phenylthionocarbonate in two steps by the Martin procedure[12] to give **9**, $[\alpha]_D^{25}$ −12.9 (c 1.0, $CHCl_3$), in 55% yield. The compounds **8** and **9** were transformed into **3**, $[\alpha]_D^{25}$ −19.5 (*c* 1.1, MeOH), and the desoxy derivative **12**, $[\alpha]_D^{25}$ −9.16 (*c* 1.1, MeOH), respectively, through removal of the *N*,*O*-acetal and the Boc groups, and subsequent *N*-palmitoylation and hydrolysis of the esters via **10** and **11** as shown in Scheme 1.

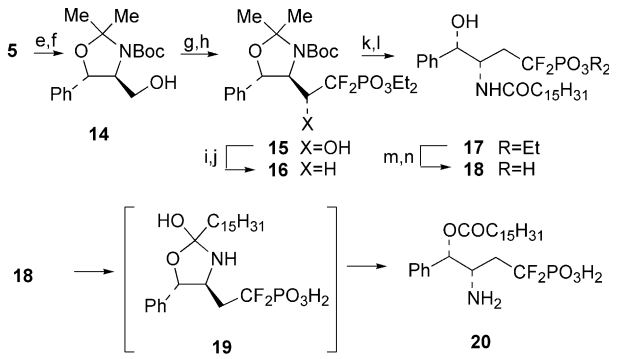
Our previous report described that the reaction between $LiCF_2PO_3Et_2$ and the aldehyde derived from **7** proceeded with low diastereoselectivity on the basis of the multiplicity of the ^{19}F NMR spectrum of the adduct **8**.[9] However, careful reexamination of the NMR (^1H, ^{13}C, ^{31}P, ^{19}F) spectrum of **8** as well as its ring-transformation to the acetonide **13** under the conditions of Dondoni[13] revealed that the configuration of α-OH of the adduct **8** is homogeneous, and was determined to be *R* on the basis of the observed strong correlation between H_a and H_b in the NOESY spectrum (500 MHz, $CDCl_3$) of the acetonide **13** (Scheme 2). The stereochemistry of **8** is



Scheme 1. Reagents and conditions: (a) Boc_2O, MeOH; (b) TBDPSCl, imidazole, DMF; (c) 4-nitrobenzoic acid, diisopropylazodicarboxylate, Ph_3P, THF; (d) DIBAL-H, THF. −20 °C; (e) DMP, *p*-TsOH, benzene; (f) TBAF, THF; (g) Dess–Martin periodinane, CH_2Cl_2; (h) $LiCF_2PO_3Et_2$, THF, −78 °C; (i) *n*-BuLi, ClC(S)OPh, THF, −78 °C; (j) *n*-Bu_3SnH, AIBN, toluene, 90 °C; (k) 3M HCl, EtOAc; (l) palmitoyl chloride, DMAP, Et_3N; (m) TMSBr, CH_2Cl_2; (n) MeOH.



Scheme 2. Ring-transformation of **8**.



Scheme 3. Reagents and conditions are refereed to those shown in Scheme 1.

consistent with the fact that the reaction proceeds through the Felkin–Ahn transition state from the less-hindered face of the carbonyl.[14]

To examine the influence of the asymmetric center at the benzylic position on the activity, we next attempted to synthesize the *threo*-isomer **18** (Scheme 3). *threo*-Amino alcohol derivative **5** was transformed into the *N*,*O*-acetal **14**[15] in a similar sequence to the synthesis of alcohol **7**. The aldehyde derived from **14** was condensed with $LiCF_2PO_3Et_2$ in THF at −78 °C to give **15** stereoselectively (98% *de*) in 74% yield.[16] The adduct **15** was deoxygenated via the corresponding phenylthionocarbonate to give **16** in 97% yield, which was manipulated to **18** via **17** in good overall yield as shown in Scheme 3. While the compound **18** was isolated as good crystals, its structure was too unstable to be evaluated for biological activity; the palmitoyl group completely migrated to the neighboring hydroxyl to give **20** within 24 h on standing at room temperature. The facile *N*→*O* acyl migration may proceed by the phosphonic acid-catalyzed formation of **19**, followed by selective cleavage of the C–N bond.[17,18]

Our initial design for the SMase inhibitor **3** was based on the substrate analogues.[9] Therefore the asymmetric center of the acylamino moiety of the analogues was defined as the same stereochemistry to that of the natural sphingomyelin. However, a preliminary study on the compound **3** revealed that the mode of inhibition for Mg^{2+}-dependent N-SMase from bovine brain microsomes was non-competitive.[9] The analysis indicates the binding site of **3** not to be the catalytic site, and prompted us to examine influence of the stereochemistry of the asymmetric center of the acylamino moiety on the inhibition. For this purpose, we synthesized the enantiomers (*ent*-**3**, and *ent*-**12**) of **3** and **12** from (1*R*,2*R*)-2-amino-1-phenyl-1,3-propanediol (*ent*-**4**)[11] through exactly the same sequence to that for synthesis of **3** and **12**.

All compounds thus prepared were assessed for inhibition of N-SMase from *Bacillus cereus* as well as N-SMase from bovine brain microsomes in comparison with **3**. While all compounds had no effect on the N-SMase from *Bacillus cereus,* these compounds were good inhibitors for Mg^{2+}-dependent N-SMase from bovine brain microsomes (Table 1). The desoxy derivative **12** showed $IC_{50} = 181$ μM and was found to be two-fold more potent than our initial inhibitor **3**. The results clearly demonstrated that α-OH in **3** is not critical for inhibition of the N-SMase.

Inhibition of the N-SMase significantly increases when the asymmetric center of the acylamino moiety was modified to the unnatural configuration (Table 1). The *ent*-**3** showed $IC_{50} = 99$ μM and ca. 4-fold more potent than **3**. This trend was more striking with the desoxy derivatives (**12** vs *ent*-**12**). The *ent*-**12** was found to be ca. 60-fold more potent than **12**. The IC_{50} and K_i of *ent*-**12** were determined to be 3.3 and 1.6 μM, respectively. The results reveal that the stereochemistry of the asymmetric center of the acylamino moiety is critical for interaction with the enzyme. The mode of inhibition for *ent*-**12** was determined to be non-competitive by using Lineweaver–Burk plot analysis (Fig. 1). The data are results from one of two experiments that produced very similar. The *ent*-**12** is also a good inhibitor of A-SMase from bovine brain lysosomes, showing 48% inhibition at a low concentration of 3.3 μM. This result shows that the inhibitor has almost identical inhibitory potency for both N- and A-SMases, while the IC_{50} values of schyphostatin for N- and A-SMases are 1.0 and 49 μM, respectively.[4]

In summary, we have identified a new non-competitive inhibitor (*ent*-**12**), which is strongly active against

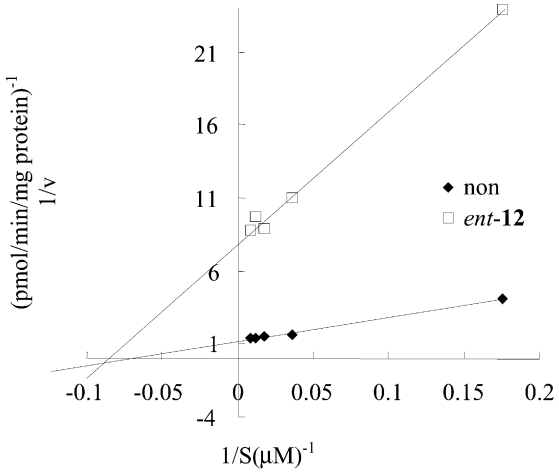


Figure 1. Lineweaver–Burk plots for inhibition of the N-SMase with *ent*-**12**.

Table 1. Inhibitory activity of *N*-palumitoyl sphingosin-1-phosphate analogues for N-SMase from bovine brain microsomes

Compd[a]	IC_{50}, (μM)[b]	K_i (μM)[c]
3	377	253
12	181	Nd[d]
***ent*-3**	99	Nd[d]
***ent*-12**	3.3	1.6

[a]All compounds had no effect on N-SMase from *Bacillus cereus* at the IC_{50} concentration.
[b]IC_{50} values were determined using four different inhibitor concentrations and represent the mean of two independent experiments.
[c]Determined by the Dixon-plots analysis.
[d]Not determined.

Mg^{2+}-dependent N-SMase from bovine brain microsomes, but inactive for N-SMase from *Bacillus cereus*. The N-SMase selectivity of this inhibitor will be an important therapeutic strategy for various cytokine-related and ischemic diseases since the activation of N-SMase is linked to the receptors of TNF-α and IL-1β and ischemic stress.[2] However, *ent*-**12**, differently from schyphostatin, inhibits A-SMase in the same potency. TNF-α also activates A-SMase to produce ceramide.[20] But, the role of A-SMase in apoptosis induced by various stimuli remains an open issue. It is conceivable that stress signaling via the SM pathway is initiated by hydrolysis of the plasma membrane SM by N-SMase and later achieved by the activation of A-SMase. Therefore, at present, design of the inhibitor(s) having higher selectivity for N-SMase will be needed. But, the utility of an agent having the ability to inhibit both N-SMase and A-SMase in the same potency remains not to be bypassed.

Experimental

General. All reactions were carried out under nitrogen atmosphere. Optical rotations were recorded on a JASCO DIP-360 digital polarimeter under standard conditions. NMR data were obtained on a Bruker DPX 400 or a Varian Mercury-300 BB using $CDCl_3$ or CD_3OD as a solvent. ^{13}C NMR (100 MHz) and ^{31}P NMR (162 MHz) were taken with broad-band ^1H decoupling. The chemical shift data for each signal on ^1H NMR (400 or 300 MHz) are expressed as relative ppm from $CHCl_3$ (δ 7.26) or CH_3OH (δ 3.30). The chemical shifts of ^{13}C are reported relative to $CDCl_3$ (δ 77.0) or CD_3OD (δ 49.0). The chemical shifts of ^{31}P are recorded relative to external 85% H_3PO_4. ^{19}F NMR spectra (376 MHz) were measured using benzotrifluoride (BTF) as an internal reference. IR spectra were recorded on a JASCO FTIR-620 spectrometer. Mass spectra were measured on a Finnigan TSQ-700 or a VG Auto Spec E spectrometer.

***tert*-Butyl (1*S*,2*S*)-1-({[*tert*-butyl(diphenyl)silyl]oxy}methyl)-2-hydroxy-2-phenylethylcarbamate (5).** To a stirred solution of **4**[11] (25.0 g, 150 mmol) in MeOH (75 mL) was added Boc_2O (37.9 mL, 165 mmol) under ice-cooling. The mixture was stirred for 30 min, then the solvent was evaporated. The residue was dissolved in DMF (700 mL) and cooled to 0 °C. To the stirred solution, a solution of imidazole (22.5g, 330 mmol) in DMF (260 mL) and a solution of TBDPSCl (49.5 g, 180 mmol) in DMF (260 mL) were successively added. After being stirred at room temperature for 12 h, and heated at 60 °C for 1.5 h, the mixture was poured onto sat. $NaHCO_3$ and extracted with ether. The extracts were washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The residue was chromatographed on silica gel (hexane/EtOAc = 10:1) to give **5** (75 g, 95%) as crystals: mp 93–95 °C; $[\alpha]_D^{20}$ +26.8 (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.74–7.59 (4H, m), 7.48–7.28 (11H, m), 5.19–5.09 (1H, m), 5.06–4.99 (1H, m), 3.90–3.68 (3H, m), 1.37 (9H, s), 1.10 (9H, s); IR (KBr) 3437, 2930, 2857, 1693, 1497, 1170, 1113 cm^{-1}; MS (EI) *m*/*z* 506 ($M^+ + 1$), 392. Anal. calcd for $C_{30}H_{39}NO_4Si$: C, 71.25; H, 7.77; N, 2.77. Found: 71.47; H, 7.80; N, 2.39.

***tert*-Butyl (1*R*,2*R*)-1-({[*tert*-butyl(diphenyl)silyl]oxy}methyl)-2-hydroxy-2-phenylethylcarbamate (*ent*-5).** This compound was prepared from *ent*-**4**[11] in an analogous manner for that of the preparation of **5**. The spectroscopic data of *ent*-**5** were identical with those of **5** except for the specific rotation: $[\alpha]_D^{25}$ −26.3 (*c* 1.0, $CHCl_3$).

***tert*-Butyl (1*S*,2*R*)-1-({[tert-butyl(diphenyl)silyl]oxy}methyl-2-hydroxy-2-phenylethylcarbamate (6).** To a stirred solution of **5** (50.7 g, 100 mmol), 4-nitrobenzoic acid (20.5 g, 120 mmol) and triphenyl phosphine (34.5 g,120 mmol) in THF (1000 mL) was added diisopropyl azodicarboxylate (23.6 mL, 120 mmol) under ice cooling. After being stirred at room temperature for 12 h, the volatile components of the mixture were removed in vacuo. The residue was portioned to ether and hexane, and then the crystallized material was filtered. The filtrates were evaporated to give an oil (40.6 g), which was dissolved in CH_2Cl_2 (560 mL) and treated with DIBAL (260 mL of 0.95 M hexane solution) at −20 °C for 1.5 h. The reaction was quenched with MeOH and saturated potassium sodium tartrate and the mixture was extracted with $CHCl_3$. The extracts were dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica gel (hexane/EtOAc = 1:1) to give **6** (22.0 g, 77%) as an oil. $[\alpha]_D^{25}$ +6.03 (*c* 0.9, $CHCl_3$); ^1H NMR ($CDCl_3$, 300 MHz) δ 7.73–7.28 (15H, m), 5.34–5.24 (1H, m), 4.99–4.94 (1H, m), 3.98–3.88 (1H, m), 3.79–3.64 (2H, m), 1.44 (9H, s), 1.06 (9H, s); MS (EI) *m*/*z* 506 ($M^+ + 1$).

***tert*-Butyl (1*R*,2*S*)-1-({[*tert*-butyl(diphenyl)silyl]oxy}methyl-2-hydroxy-2-phenylethylcarbamate (*ent*-6).** This compound was prepared from *ent*-**5** in an analogous manner for that of the preparation of **6**. The spectroscopic data of *ent*-**6** were identical with those of **6** except for the specific rotation: $[\alpha]_D^{25}$ −5.98 (*c* 1.06, $CHCl_3$).

***tert*-Butyl (4*S*,5*R*)-4-hyroxymethyl)-2,2-dimethyl-5-phenyl-1,3-oxazolidin-3-carboxylate (7).** A solution of **6** (18.1 g, 36 mmol), 2,2-dimethoxypropane (8.8 mL, 72 mmol) and *p*-TsOH (70 mg, 0.3 mmol) in benzene (300 mL) was heated under reflux for 30 min. Then, the most amounts of benzene were removed under atmospherics pressure during 1 h. The residue was poured to ether (150 mL) and saturated $NaHCO_3$ (150 mL). The phases were separated and the aqueous layer was extracted with ether. The extracts were washed with brine, dried ($MgSO_4$), and evaporated. The residue was dissolved in THF (100 mL) and treated with tetrabutylammonium fluoride (38 g, 150 mmol) at 60 °C for 5 h. THF was removed in vacuo, and the residue was poured to $CHCl_3$ and water. The phases were separated and the aqueous layer was extracted with $CHCl_3$. The combined extracts were washed with brine, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (hexane/EtOAc = 5:1) to give **7** (6.6 g, 60%) as colorless crystals: mp 60–65 °C; $[\alpha]_D^{25}$ −29.0 (*c* 1.0, $CHCl_3$);

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.50–7.25 (5H, m), 5.30 (1H, d, J = 6.5 Hz), 4.20–4.09 (1H, m), 3.60–3.22 (4H, m), 1.73 (3H, s), 1.63 (3H, s), 1.53 (9H, s); MS (EI) m/z 307 (M^+). Anal. calcd for $C_{17}H_{25}NO_4$: C, 66.43; H, 8.20; N, 4.56. Found: C, 66.44; H, 8.19; N, 4.55.

***tert*-Butyl (4*R*,5*S*)-4-hyroxymethyl)-2,2-dimethyl-5-phenyl-1,3-oxazolidin-3-carboxylate (*ent*-7).** This compound was prepared from *ent*-**6** in an analogous manner to that for the preparation of **7**. The spectroscopic data of *ent*-**7** were identical with those of **7**, except for the specific rotation: $[\alpha]_D^{25}$ +29.8 (c 1.06, $CHCl_3$).

1,1-Dimethylethyl-(4*S*,5*R*)-4-{(1*R*)-2-[bis(ethyloxy)phosphoryl]-2,2-difluoro-1-hydroxyethyl}-1,3-oxazolidine-3-carboxylate (8). A solution of **7** (4.1 g, 13.2 mmol) in CH_2Cl_2 (100 mL) was treated with Dess–Martin periodinane (8.4 g, 19.8 mmol) at room temperature for 4 h. The mixture was poured on cold 10% Na_2SO_3 and extracted with ether. The extracts were washed with brine, dried ($MgSO_4$) and concentrated to give an aldehyde (3.6 g) [^{1}H NMR ($CDCl_3$) δ 9.10 (1H, d, J = 2.0 Hz), 7.45–7.29 (5H, m), 5.41 (1H, d, J = 5.5 Hz), 4.95 (1H, dd, J = 5.5, 2.0 Hz), 1.51 (3H, s), 1.39 (3H, s)]. The aldehyde was used for the next reaction without purification. To a stirred solution of $LiCF_2PO_3Et_2$, prepared from $HCF_2PO_3Et_2$ (3.7 g, 23.6 mmol) and LDA (23.6 mmol) in THF (60 mL),[12] was added a solution of the aldehyde in THF (30 mL) at −78 °C. During the addition, the temperature of the reaction mixture was carefully controlled to be below −70 °C. After being stirred at the same temperature for 4 h, the mixture was poured on saturated NH_4Cl and extracted with ether. The extracts were washed with brine, dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica gel to give **8** (3.2 g, 60%) as an oil. $[\alpha]_D^{25}$ −21.6 (c 1.06, MeOH); ^{1}H NMR ($CDCl_3$) δ 7.42–7.26 (5H, m), 5.33 (1H, d, J = 5.1 Hz), 4.60 (1H, broad s), 4.20–3.90 (5H, m), 1.75 (3H, s), 1.69 (3H, s), 1.51 (9H, s), 1.26 (3H, t, J = 7.1 Hz), 1.19 (3H, t, J = 7.1 Hz); ^{13}C NMR ($CDCl_3$) δ 134.6, 128.1, 128.0, 126.8, 117.8 (ddd, J_{CP} = 209.6 Hz, J_{CF} = 266.5, 266.8 Hz), 93.5, 81.0, 67.2, 64.2 (dd, J_{CF} = 6.4, 6.7 Hz), 57.4 (d, J_{CP} = 7.9 Hz), 28.1, 26.2, 24.2, 16.1 (dd, J_{CF} = 5.7, 5.9 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −51.8 ~ −53.6 (1F, m), −65.5 ~ −67.7 (1F, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 7.03 (dd, J_{CF} = 99.0, 98.7 Hz); IR (neat) 3370, 2981, 2935, 1698, 1391, 1257, 1179, 1097, 1057 cm^{-1}; MS (EI) m/z 494 (M^+ + 1), 493 (M^+). HRMS (EI) calcd for $C_{30}H_{51}F_2NO_6P$ (M^+): 493.2039. Found: 493.2027.

1,1-Dimethylethyl-(4*R*,5*S*)-4-{(1*S*)-2-[bis(ethyloxy)phosphoryl]-2,2-difluoro-1-hydroxyethyl}-1,3-oxazolidine-3-carboxylate (*ent*-8). This compound was prepared from *ent*-**7** in an analogous manner to that for the preparation of **8**. The spectroscopic data of *ent*-**8** were identical to those of **8**, except for the specific rotation: $[\alpha]_D^{25}$ +10.2 (c 1.06, $CHCl_3$).

***tert*-Butyl (4*S*,5*R*)-4-[2-(diethoxyphosphoryl)-2,2-difluoroethyl]-2,2-dimethyl-5-phenyl-1,3-oxazolidine-3-carboxylate (9).** To a stirred solution of **8** (2.55 g, 5.2 mmol) in THF (32 mL) was added *n*-BuLi (4.9 mL of 1.55 M hexane solution) at −78 °C. After being stirred at the same temperature for 15 min, PhOC(S)Cl (1.4 mL, 10.3 mmol) was added. The mixture was stirred at −78 °C for 1 h, then quenched with satd $NaHCO_3$. The mixture was extracted with ether and the extracts were evaporated. The residue was dissolved in toluene (30 mL) and treated with *n*-Bu_3SnH (2.1 mL, 7.8 mmol) and AIBN (10 mg) at 90 °C for 2 h. Toluene was removed in vacuo and the residue was poured between acetonitrile and hexane. The acetonitrile extracts were evaporated. The residue was chromatographed on silica gel (hexane/EtOAc = 5:1) to give **9** (1.37 g, 55%) as an oil. $[\alpha]_D^{25}$ −12.9 (c 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.43–7.21 (5H, m), 5.26 (1H, d, J = 4.6 Hz), 4.58–4.47 (1H, m), 4.17–3.93 (4H, m), 2.20–1.94 (2H, m), 1.58–1.50 (6H, m), 1.49 (9H, s), 1.35–1.12 (6H, m); ^{19}F NMR (376 MHz, $CDCl_3$) δ −49.0 ~ −51.9 (2F, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.91 (t, J_{PF} = 108.2 Hz); IR (neat) 2981, 1699, 1381, 1254, 1176, 1022 cm^{-1}; MS (EI) m/z 478 (M^+ + 1), 376 (M^+–Boc). HRMS (EI) calcd for $C_{17}H_{25}NO_4F_2P$ (M^+–Boc): 375.1411. Found: 375.1407.

***tert*-Butyl (4*R*,5*S*)-4-[2-(diethoxyphosphoryl)-2,2-difluoroethyl]-2,2-dimethyl-5-phenyl-1,3-oxazolidine-3-carboxylate (*ent*-9).** This compound was prepared from *ent*-**8** in an analogous manner to that for the preparation of **9**. The spectroscopic data of *ent*-**9** were identical with those of **9**, except for the specific rotation: $[\alpha]_D^{25}$ +13.9 (c 1.06, $CHCl_3$).

Diethyl (2*R*,3*S*,4*R*)-1,1-difluoro-2,4-dihydroxy-3-(palmitoylamino)-4-phenylbutylphosphonate (10). The compound **8** (1.0 g, 2.1 mmol) was dissolved in EtOAc (2 mL) and treated with 1 mL of 3 M HCl at room temperature for 1 h. The volatile component mixture was removed in vacuo and the residue was acylated with palmitoyl chloride (0.5 mL, 1.65 mmol) in CH_2Cl_2 (7.5 mL) in the presence of Et_3N (0.41 mL, 3 mmol) and DMAP (20 mg, 0.17 mmol) at room temperature for 2 h. The mixture was poured on 1 M HCl (5 mL) and extracted with ether. The extracts were washed with brine, dried ($MgSO_4$) and evaporated. The residue was chromatographed on silica gel (hexane/EtOAc = 1:1) to give **10** (600 mg, 60%) as an oil. $[\alpha]_D^{25}$ +6.1 (c 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.23 (5H, m), 6.58 (1H, d, J = 8.3 Hz), 5.06 (1H, d, J = 5.7 Hz), 4.48–4.40 (1H, m), 4.29–4.08 (5H, m), 2.13–2.05 (2H, m), 1.53–1.43 (2H,m), 1.40–1.15 (31H, m), 0.90 (3H, t, J = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 117.4, 140.8, 128.2 (2 carbons), 127.6, 126.3 (2 carbons), 118.8 (dt, J_{CP} = 207.4 Hz, J_{CF} = 263.1 Hz), 74.4, 68.7-67.7 (m), 65.7, 64.9 (2 carbons, d, J_{CP} = 6.2 Hz), 60.3, 53.0, 36.5, 31.8, 29.6, 29.5, 29.4, 29.3, 29.2, 29.1, 25.4, 22.7 (2 carbons, d, J_{CP} = 16.1 Hz), 16.1 (2 carbons, d, J_{CP} = 4.1 Hz), 15.1, 14.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ -54.9 (1F, ddd, J_{FF} = 307.7 Hz, J_{HF} = 7.5 Hz, J_{PF} = 99.3 Hz), −62.6 (1F, ddd, J_{FF} = 307.7 Hz, J_{HF} = 20.0 Hz, J_{PF} = 103.4 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.77 (dd, J_{PF} = 99.4, 100.2 Hz); IR (neat) 3328, 2924, 2853, 1644, 1522, 1455, 1259, 1027, 701 cm^{-1}; MS (EI) m/z 592 (M^+ + 1), 574 (M^+–OH). HRMS (EI) calcd for $C_{30}H_{53}NO_6F_2P$ (MH^+): 592.3578. Found: 592.3558.

Diethyl (2*S*,3*R*,4*S*)-1,1-difluoro-2,4-dihydroxy-3-(palmitoylamino)-4-phenylbutylphosphonate (*ent*-10). This compound was prepared from *ent*-**9** in an analogous manner to that for the preparation of **10**. The spectroscopic data of *ent*-**10** were identical with those of **10**, except for the specific rotation: $[\alpha]_D^{25}$ −5.1 (*c* 1.0, $CHCl_3$).

Diethyl (3*S*,4*R*)-1,1-difluoro-4-hydroxy-3-(palmitoylamino)-4-phenylbutylphosphonate (11). The compound **9** was transformed to **11** in 52% yield in an analogous manner to that for the preparation of **10**. Mp 66–67 °C; $[\alpha]_D^{25}$ −24.5 (*c* 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40–7.23 (5H, m), 6.21 (1H, d, *J* = 7.1 Hz), 5.06–5.02 (1H, m), 4.53 (1H, d, *J* = 2.9 Hz), 4.31–4.23 (1H, m), 4.22–4.00 (4H, m), 2.57–2.32 (1H, m), 2.30–2.09 (3H, m), 1.67–1.55 (2H, m), 1.34–1.17 (30H, m), 0.88 (3H, t, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 140.8, 128.1, 127.3, 125.9, 120.4 (dt, J_{CP} = 214.9, J_{CF} = 261.2 Hz), 75.5, 64.7 (d, J_{CP} = 7.5 Hz), 64.6 (d, J_{CP} = 7.9 Hz), 51.1, 36.6, 31.8, 31.4 (dt, J_{CP} = 16.9, J_{CF} = 19.7 Hz), 29.6, 29.4, 29.3, 29.2, 29.1, 25.5, 22.5, 16.1 (d, J_{CP} = 5.4 Hz), 14.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −44.7 (1F, dddd, J_{HF} = 15.9, 21.1, J_{PF} = 106.9 Hz, J_{FF} = 301.9 Hz), −46.0 (1F, ddt, J_{HF} = 19.0, J_{PF} = 106.9 Hz, J_{FF} = 301.9 Hz); ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.72 (t, J_{PF} = 106.9 Hz); IR (KBr) 3323, 2925, 2854, 1647, 1550, 1261, 1026 cm^{-1}; MS (EI) *m*/*z* 575 (M^+), 557. Anal. calcd for $C_{30}H_{52}NO_5F_2P$: C, 62.59; H, 9.10; N, 2.43. Found: C, 62.52; H, 9.10; N, 2.41.

Diethyl (3*R*,4*S*)-1,1-difluoro-4-hydroxy-3-(palmitoylamino)-4-phenylbutylphosphonate (*ent*-11). This compound (mp 66–67 °C) was prepared from *ent*-**10** in an analogous manner to that for the preparation of **10**. The spectroscopic data of *ent*-**11** were identical with those of **11**, except for the specific rotation $[\alpha]_D^{25}$ +24.3 (*c* 1.3, $CHCl_3$).

(2*R*,3*S*,4*R*) - 1,1 - Difluoro - 2,4 - dihydoxy - 3 - (palmitoylamino)-4-phenylbutylphosphonic acid (3). To a stirred solution of **10** (100 mg, 0.17 mmol) in CH_2Cl_2 (0.34 mL) was added bromotrimethylsilane (0.13 mL, 1.02 mmol). After being stirred at room temperature for 24 h, the volatile components of the mixture were removed in vacuo. The residue was treated with MeOH (1 mL) at room temperature for 2 h. Volatile components of the mixture was removed in vacuo to give **3** (90 mg, 85%) as an amorphous powder. $[\alpha]_D^{25}$ −19.5 (*c* 1.0, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.45–7.23 (5H, m), 5.48–5.38 (1H, m), 4.72 (1H, d, *J* = 8.9 Hz), 4.78–4.61 (1H, m), 4.51 (1H, d, *J* = 8.9 Hz), 2.20–1.85 (1H, m), 1.53–1.43 (1H, m), 1.40–1.15 (31H, m), 0.90 (3H, t, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 175.9, 143.1, 129.1, 128.4, 127.6, 124.0–115.0 (m), 74.2, 54.5, 36.7, 35.3–34.4 (m), 33.1, 30.8, 30.5, 30.0, 26.7, 23.7, 14.4; ^{19}F NMR (376 MHz, CD_3OD) δ −56.2 (1F, ddd, J_{FF} = 305.4 Hz, J_{FP} = 101.5 Hz, J_{HF} = 8.5 Hz), −63.8 (1F, ddd, J_{FF} = 305.4 Hz, J_{FP} = 101.5 Hz, J_{FH} = 20.1 Hz); ^{31}P NMR (121 MHz, CD_3OD) δ 6.53 (t, J_{PF} = 100.0 Hz,); MS (FAB) *m*/*z* 518 (M^+–OH).

(2*S*,3*R*,4*S*) - 1,1 - Difluoro - 2,4 - dihydoxy - 3 - (palmitoylamino)-4-phenylbutylphosphonic acid (*ent*-3). This compound was obtained from *ent*-**10** in an analogous manner to that for the preparation of **3**. The spectroscopic data of *ent*-**3** were identical to those of **3**, except for the specific rotation: $[\alpha]_D^{25}$ +21.0 (*c* 1.0, MeOH).

(3*S*,4*R*)-1,1-Difluoro-4-hydoxy-3-palmitoylamino)-4-phenylbutylphosphonic acid (12). This compound was obtained as crystals (mp 85–86 °C) from **11** in an analogous manner to that for the preparation of **3**. $[\alpha]_D^{25}$ −9.16 (*c* 1.1, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.40 (2H, d, *J* = 7.4 Hz), 7.32 (2H, t, *J* = 7.4 Hz), 7.24 (1H, t, *J* = 7.4 Hz), 4.71 (1H, d, *J* = 6.0 Hz), 4.58–4.49 (1H, m), 2.62–2.27 (2H, m), 2.23–2.09 (2H, m), 1.50–1.38 (2H, m), 1.37–1.05 (24H, m), 0.89 (3H t, *J* = 6.7 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 176.6, 142.7, 129.2, 128.7, 127.7, 121.8 (dt, J_{CP} = 211.4 Hz, J_{CF} = 258.9 Hz), 76.3, 51.4, 36.3, 33.5 (dt, J_{CP} = 15.6, J_{CF} = 19.5 Hz), 33.0, 30.8, 30.7, 30.5, 30.4 (2 carbons), 30.1, 27.0, 23.7, 14.5; ^{19}F NMR (376 MHz, CD_3OD) δ?−50.9 (1F, dddd, J_{HF} = 14.2, 26.7, J_{PF} = 104.5 Hz, J_{FF} = 295.0 Hz), −51.8 (1F, dddd, J_{HF} = 15.7, 24.7 Hz, J_{PF} = 104.5 Hz, J_{FF} = 295.0 Hz), ^{31}P NMR (162 MHz, CD_3OD) δ 4.42 (t, J_{PF} = 104.5 Hz); IR (KBr) 3280, 2924, 2853, 1751, 1657, 1457, 1175, 1023 cm^{-1}; MS (FAB) *m*/*z* 520 (MH^+), 502 (M^+–OH). Anal. calcd for $C_{26}H_{44}NO_5F_2P{\cdot}H_2O$: C, 58.09; H, 8.62; N, 2.61. Found: C, 57.61; H, 8.32; N, 2.67.

(3*R*,4*S*)-1,1-dufluoro-4-hydoxy-3-palmitoylamino)-4-phenylbutylphosphonic acid (*ent*-12). This compound was obtained as crystals (mp 85–86 °C) from *ent*-**11** in an analogous manner to that for the preparation of **3**. The spectroscopic data of *ent*-**12** were identical with those of **12**, except for the specific rotation: $[\alpha]_D^{25}$ +9.16 (*c* 1.0, MeOH).

Diethyl {(4*R*,5*R*,6*R*)-5-[(*tert*-butoxycarbonyl)amino]-2,2-dimethyl-6-phenyl-1,3-dioxan-4-yl} (difluoro)methylphosphonate (13). To a stirred solution of **8** (672 mg, 1.36 mmol) in CH_2Cl_2 (4 mL) was added a 0.5 M solution of trifluoroacetic acid in CH_2Cl_2 (45 mL). The mixture was stirred at room temperature for 5 min, then neutralized with satd aq $NaHCO_3$. The phases were separated and the aqueous layer was extracted with $CHCl_3$. The combined organic extracts were dried (Na_2SO_4) and concentrated. The residue was chromatographed on silica gel (hexane/EtOAc = 1:1) to give **13** (638 mg 95%) as an oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.22 (5H, m), 5.39 (1H, d, *J* = 9.0 Hz), 5.10–5.03 (1H, m, H_a), 4.45–4.35 (1H, m), 4.34–4.24 (4H, m), 4.23–4.13 (1H, m, H_b), 1.46–1.21 (21H, m); MS (EI) *m*/*z* 494 ($M^+ + 1$).

***tert*-Butyl (4*S*,5*S*)-4-(hydroxymethyl)-2,2-dimethyl-5-phenyl-1,3-oxazolidine-3-carboxylate (14).** A solution of **5** (26 g, 51 mmol), 2,2-dimethoxypropane (12.6 mL, 104 mmol) and *p*-TsOH (100 mg, 0.51 mmol) in benzene (430 mL) was heated under reflux for 30 min. Then, the most amounts of benzene were removed under atmospheric pressure during 1 h. The residue was portioned to ether (150 mL) and sat aq $NaHCO_3$ (150 mL). The organic extracts were washed with brine, dried ($MgSO_4$), and evaporated. The residue was dissolved in THF (100 mL) and treated with tetrabutylammonium fluoride (235 mL of 1 M solution in THF, 235 mmol) at

50 °C for 12 h. The solvent was removed in vacuo, and the residue was portioned between water and $CHCl_3$. The mixture was extracted with $CHCl_3$. The extracts were washed with brine, dried ($MgSO_4$) and concentrated. The residue was chromatographed on silica gel (hexane:EtOAc = 5:1) to give **14**[15] (12.3 g, 78%) as an oil. $[\alpha]_D^{25}$ +32.0 (*c* 1.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.46–7.26 (5H, m), 4.87–4.69 (1H, m), 3.91-3.65 (3H, m), 1.70 (3H, s), 1.58 (3H, s), 1.52 (9H, s); IR (neat) 3433, 2979, 2935, 1696, 1670, 1403, 1367 cm^{-1}; MS (EI) *m*/*z* 308 ($M^+ + 1$), 292 (M^+–Me). Anal. calcd for $C_{17}H_{25}NO_4$: C, 66.43; H, 8.20; N, 4.56. Found: C, 66.24; H, 8.01; N, 4.44.

***tert*-Butyl (4*S*,5*S*)-4-[(1*R*)-2-(Diethoxyphosphoryl)-2,2-difluoro-1-hydroxyethyl]-2,2-dimethyl-5-phenyl-1,3-oxazolidine-3-carboxylate (15).** The compound **14** (12 g, 39 mmol) was oxidized to the aldehyde [^1H NMR (300 MHz, $CDCl_3$) δ 9.52 (1H, d, *J* = 3.6 Hz), 7.45-7.29 (5H, m), 4.98 (1H, d, *J* = 8.4 Hz), 4.30–4.05 (1H, m), 1.71 (3H, s), 1.52 (3H, s), 1.42 (9H,s)] with Dess–Martin periodinane (28.1 g, 66.3 mmol) in CH_2Cl_2 as described for the synthesis of **8**. The aldehyde was used for the next reaction without purification. To a stirred solution of $LiCF_2PO_3Et_2$, prepared from $HCF_2PO_3Et_2$ (15.5 g, 82 mmol) and LDA (23.6 mmol) in THF (280 mL),[12] was added a solution of the aldehyde in THF (55 mL) at −78 °C. After being stirred at the same temperature for 3 h, the mixture was worked up as described for the synthesis of **8**. The crude was chromatographed on silica gel (hexane/EtOAc = 5:1 to 3:1) to give **15** (13.7 g, 74%) as an oil. $[\alpha]_D^{25}$ −24.0 (*c* 1.0, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.47–7.26 (5H, m), 5.19 (1H, broad s), 5.01 (1H, d, *J* = 8.7 Hz), 4.43–4.19 (5H, m), 1.61 (3H, s), 1.48 (9H, s), 1.44 (3H, s), 1.37 (6H, t, *J* = 7.0 Hz): ^{19}F NMR (376 MHz, $CDCl_3$) δ −49.9 ∼ −51.6 (2F, m), ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.53 (t, J_{PF} = 99.8 Hz); IR (neat) 3357, 2983, 2936, 1701, 1391, 1259, 1166, 1062 cm^{-1}; MS (EI) *m*/*z* 494 ($M^+ + 1$). HRMS (EI) calcd for $C_{21}H_{31}NO_7F_2P$ (M^+–Me): 478.1806. Found: 478.1812.

***tert* - Butyl (4*S*,5*S*) - 4 - [2 - (diethoxyphosphoryl) - 2,2 - difluoroethyl]-2,2-dimethyl-5-phenyl-1,3-oxazolidine-3-carboxylate (16).** This compound was obtained in 97% yield as an oil from **15** in an analogous manner for that of the preparation of **9**. $[\alpha]_D^{25}$ −28.3 (*c* 1.1, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.47–7.26 (5H, m), 5.20–5.12 (1H, m), 4.66–4.55 (1H, m), 4.33–4.18 (4H, m), 2.71–2.48 (2H, m), 1.59 (3H, s), 1.53 (3H, s), 1.48 (9H, s), 1.39 (3H, t, *J* = 7.0 Hz), 1.38 (3H, t, *J* = 7.0 Hz); ^{19}F NMR (376 MHz, $CDCl_3$) δ −47.4 ∼ −51.2 (2F, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.79 (t, J_{PF} = 106.2 Hz); IR (neat) 2982, 1703, 1377, 1259, 1168, 1023 cm^{-1}; MS (EI) 478 ($M^+ + 1$). Anal. calcd for $C_{22}H_{34}NO_6F_2P$: C, 55.34; H, 7.18; N, 2.93. Found: C, 54.91; H, 7.20; N, 2.63.

Diethyl (3*S*,4*S*)-1,1-difluoro-4-hydroxy-3-(palmitoylamino)-4-phenylbutylphosphonate (17). This compound was obtained in 60% yield as an oil from **16** in an analogous manner to that for the preparation of **10**. $[\alpha]_D^{25}$ +12.6 (*c* 1.4, $CHCl_3$); ^1H NMR (400 MHz, $CDCl_3$) δ 7.40–7.18 (5H, m), 6.00 (1H, d, *J* = 7.6 Hz), 4.90 (1H, d, *J* = 4.2 Hz), 4.34–4.13 (5H, m), 2.64–2.43 (2H, m), 2.04 (2H, dd, *J* = 6.7, 8.1 Hz), 1.50–1.41 (2H, m), 1.38 (3H, t, *J* = 7.0 Hz), 1.36 (3H, t, *J* = 6.9 Hz), 1.32–1.08 (24H, m), 0.88 (3H, t, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.0, 141.2, 128.1, 127.4, 125.9, 120.4 (dt, t, J_{CP} = 214.3, J_{CF} = 261.1 Hz), 74.6, 64.9 (d, J_{CP} = 6.8 Hz), 50.6, 36.5, 34.7 (dt, J_{CP} = 15.2 Hz, J_{CF} = 20.1 Hz), 31.8, 29.6 (2 carbons), 29.4, 29.3, 29.0, 25.4, 22.6, 16.3 (d, J_{CP} = 5.1 Hz), 14.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −45.5 ∼ −47.5 (2F, m); ^{31}P NMR (162 MHz, $CDCl_3$) δ 6.87 (t, J_{PF} = 107.4 Hz); IR (neat) 3308, 2924, 2853, 1650, 1550, 1261, 1027 cm^{-1}; MS (EI) 576 ($M^+ + 1$), 558 (M^+-OH). Anal. calcd for $C_{30}H_{52}NO_5F_2P$: C, 62.59; H, 9.10; N, 2.43. Found: C, 62.63; H, 8.90; N, 2.38.

(3*S*,4*S*)-1,1-Difluoro-4-hydroxy-3-(palmitoylamino)-4-phenylbutylphosphonic acid (18). This compound was obtained as crystals (mp 85–86 °C) from **17** in an analogous manner to that for preparation of **3**. $[\alpha]_D^{20}$ −8.90 (*c* 1.1, MeOH); ^1H NMR (400 MHz, CD_3OD) δ 7.40–7.19 (5H, m), 4.85 (1H, d, *J* = 3.7 Hz, C*H*OH), 4.68–4.58 (1H, m, C*H*NHCO), 2.43–2.23 (2H, m), 2.17 (2H, t, *J* = 7.6 Hz, $COCH_2$), 1.52–1.38 (2H, m), 1.37–1.12 (24H, m), 0.89 (3H, t, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 176.7, 142.8, 129.1, 128.5, 127.5, 121.7 (dt, J_{CP} = 211.1 Hz, J_{CF} = 258.0 Hz), 75.1, 51.2, 36.3, 35.6 (dt, J_{CP} = 15.3 Hz, J_{CF} = 20.1 Hz), 33.0, 30.7, 30.4, 30.1, 27.0, 23.7, 14.5; ^{19}F NMR (376 MHz, CD_3OD) −50.1 ∼ −50.8 (2F, m), ^{31}P NMR (162 MHz, CD_3OD) δ 4.28 (t, J_{PF} = 104.1 Hz). This compound was unstable for evaluation of the biological activity; the palmitoyl group completely migrated to the neighboring hydroxyl to give *O*-palmitoyl derivative **20** on standing at room temperature within 24 h. This fact was estimated on the basis of the ^1H NMR (400 MHz, CD_3OD) analysis; the signals of the methylene protons α to the carbonyl as well as the methin proton α to OH were gradually shifted to the downfield δ 2.47 (2H, dt, *J* = 3.3, 7.4 Hz) and δ 5.90 (2H, d, *J* = 8.0 Hz), respectively.

Assay and inhibition of SMases. Microsomal or *Bacillus cereus* N-SMase activity was measured as described previously.[19] Briefly, *N*-palmitoyl SM (20 nmol, Sigma) in 50 μL of 20 mM Tris–HCl buffer (pH 7.4)/50 mM $MgCl_2$/0.1% Tween 20 were mixed with 200 μL of enzyme source, where indicated, contained SMase inhibitor. Bovine brain microsomes as an enzyme source were prepared as follows. The brain tissues were homogenized with 5 vol. 20 mM Tris–HCl buffer (pH 7.4)/0.15 M NaCl/0.25 M sucrose. After a centrifugation at 8000*g* for 20 min at 4 °C, the supernatant was further centrifuged at 105,000*g* for 60 min at 4 °C. The pellet (microsomal fraction) was suspended in 9 vol. 20 mM Tris–HCl buffer (pH 7.4) and used at a protein concentration of 500 μg/mL. *Bacillus cereus* SMase was the product of Sigma. Enzyme reaction was carried out at 37 °C for 45 min (microsomal N-SMase) or for 10 min (*Bacillus cereus* N-SMase) and terminated by adding 4 mL of chloroform/methanol (2:1, v/v). After termination, 1 mL of water was added, and the mixture was vortexed and centrifuged. The lower layer was collected, and the chloroform was allowed to evaporate. The amounts of ceramide in the residues were determined

fluorometrically on HPLC as described by Previati et al.[21] For the assay of A-SMase, lysosome-rich fraction was obtained by centrifugation at 8000*g* for 20 min at 4 °C of the above brain homogenate and used as the enzyme source. Following assay procedures were the same as those described in microsomal N-SMase assay, except that 250 mM sodium acetate buffer (pH 5.6) was used for the suspension of lysosomes and enzyme reaction.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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