Synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate

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Abstract The syntheses of D-*erythro*-sphingosine-1-phosphate and DL-*erythro*-sphinganine-1-phosphate are described starting from the commercially available D-*erythro*-sphingosine and DL-*erythro*-sphinganine. The phosphate group is introduced via phosphoramidite chemistry using bis(2-cyanoethyl)-N,N-diisopropylamino-phosphoramidite as the monophosphorylating reagent. The procedure generates the phosphorylated sphingoid bases in three steps and 32–39% overall yields.—**Boumendjel, A., and S. P. F. Miller.** Synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. *J. Lipid Res.* 1994. **35:** 2305–2311.

Supplementary key words sphinganine-1-phosphate • phosphoramidite • phosphorylation

Sphingolipids constitute an important group of compounds that occur in high abundance in cellular membranes (1). These compounds include a large number of complex lipids including ceramides, sphingomyelins, cerebrosides, and gangliosides. The long chain bases, sphingosine (D-*erythro*-2-amino-1,3-dihydroxy-*trans*-4-octadecene) and sphinganine (DL-*erythro*-2-amino-1,3-dihydroxy-octadecane)[2] are the major components of most mammalian sphingolipids (2) (**Scheme 1**).

Interest in sphingosine and related long-chain sphingoid bases increased when sphingosine was found to be a potent inhibitor of protein kinase C both in vitro and in vivo (3, 4). The route for catabolic degradation of sphingosine and sphinganine involves an initial ATP-dependent phosphorylation at the primary hydroxyl group to yield sphingosine-1-phosphate or sphinganine-1-phosphate. This phosphorylation step is catalyzed by a specific sphingosine-kinase reported to be located in the cytoplasm (5–10). Sphingosine-1-phosphate is then cleaved by the action of a pyridoxal phosphate-dependent lyase (aldolase) to yield ethanolamine-O-phosphate (corresponding to carbons 1 and 2) and 2-hexadecenal (the remainder of the molecule) (11–15) (**Fig. 1**).

In addition to its role as a catabolic intermediate, recent reports suggest further roles for S-1-P. Sphingosine-1-phosphate is a potent mitogen in Swiss 3T3 fibroblasts (16–18), and causes rapid translocation of calcium from intracellular stores (19). Thus, S-1-P may be a component of the intracellular second messenger system that is involved in calcium release and the regulation of cell growth induced by sphingosine (18, 19). Recently S-1-P was found to inhibit motility of melanoma cells at a very low concentration (10 nM) at which sphingosine has no inhibitory effect (20, 21). These findings suggest possible roles for S-1-P in cell metastasis and inflammatory processes.

A convenient method is needed for the preparation of S-1-P in sufficient quantities to allow studies of its physiological role(s). Synthetic methods suitable for producing labeled S-1-P would expedite studies on the enzyme S-1-P lyase, which remains poorly understood (15).

The synthetic attempts of Weiss (22) failed to produce sphingosine-1-phosphate, due to the catalytic hydrogenolytic removal of phosphate protecting groups in the presence of the allylic double bond, however the synthesis of sphinganine-1-phosphate was achieved. Van Veldhoven, Foglesong, and Bell (23) reported an enzymatic preparation of sphingosine-1-phosphate in which sphingosylphosphorylcholine was treated with phospholipase D isolated from *Streptomyces chromofuscus* and milligram quantities were isolated. However, S-1-P was isolated as a mixture of the L-*threo* and D-*erythro* diastereoisomers. The occurrence of the unnatural L-*threo* S-1-P is due presumably to the starting material that was used as mixture of two diastereoisomers.[3]

Abbreviations: ATP, adenosine triphosphate; Boc, *tert*-butyloxycarbonyl; $(Boc)_2O$, di-*tert*-butyl dicarbonate; CH_2Cl_2, dichloromethane; CH_3CN, acetonitrile; DEPT, distortionless enhancement by polarization transfer; DHS-1-P, dihydrosphingosine-1-phosphate or sphinganine-1-phosphate; DIPEA, diisopropylethylamine; EI-MS, electron impact mass spectrometry; DMSO, dimethyl sulfoxide; EtOH, ethanol; FAB-MS, fast atom bombardment mass spectrometry; HRMS, high resolution mass spectrometry; MeOH, methanol; NMR, nuclear magnetic resonance; noba, *m*-nitrobenzyl alcohol; RT, room temperature; S-1-P, sphingosine-1-phosphate; S-1-P lyase; sphingosine-1-phosphate lyase; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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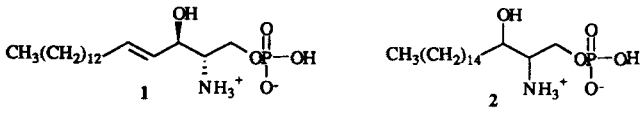
[2]Throughout this manuscript, the term sphinganine is used to describe dihydrosphingosine or DL-*erythro*-2-amino-1,3-octadecanediol.

[3]Sphingosylphosphorylcholine is prepared by hydrolysis of sphingomyelin, and a sample obtained from Sigma Chemical Co. (catalog #S 4257) was determined by ^{13}C NMR spectroscopy to be a 6:4 mixture of D(+)-*erythro* and L(−)-*threo*-sphingosylphosphorylcholine, respectively.



Scheme 1.

The first total synthesis of S-1-P was reported in 1992 by Ruan et al. (21). The secondary hydroxyl group of a sphingosine derivative was protected as pivaloyl ester. Selective deprotection of the primary hydroxy group, followed by its phosphorylation with phosphorylchloride and deprotection of the allylic hydroxy group and the amino group resulted in S-1-P (overall yield of 11% for 10 steps). Kratzer and Schmidt (24) recently reported a chemical preparation of sphingosine-1-phosphate that involved phosphorylation of 3-O-t-*butyl*-dimethylsilyl protected D-*erythro*-azidosphingosine. The sphingosine precursor used in this synthesis was prepared in 10 steps and 18% yield starting from D-glucose (25). This synthesis involved 18 steps and affords a modest yield of S-1-P (9% overall yield).

In this manuscript we report a short (3 steps), simple, and practical (32–39% overall yield) synthesis of D-*erythro*-sphingosine-1-phosphate and DL-*erythro*-sphinganine-1-phosphate. This procedure uses commercially available D-*erythro*-sphingosine or DL-*erythro*-sphinganine as starting materials and uses the monofunctional phosphorylating reagent bis(2-cyanoethoxy)-N,N-diisopropylamino-phosphoramidite (26).

MATERIALS AND METHODS

D-*erythro*-Sphingosine (cat #S 6136) and DL-*erythro*-dihydrosphingosine (cat #D 6908) were purchased from Sigma Chemical Co. (St. Louis, MO). Purity of D-*erythro*-sphingosine and DL-*erythro*-sphinganine as checked by TLC, ^{1}H NMR, and ^{13}C NMR was higher than 97%. All reagents and silica gel 230–400 mesh, 60 Å (used for flash chromatography), were obtained from Aldrich Chemical Company (Milwaukee, WI). HPLC grade acetonitrile was purchased from Government Marketing Services, Inc., (Sterling, VA). Thin-layer chromatography (TLC) analysis was performed on silica gel G (Analtech, Newark, DE) or on Macherey Nagel silica gel plates (Brinkman Instruments Inc., Westbury, NY). Lipid spots were detected by iodine, while spraying with ninhydrin and molybdenum reagent was used to reveal the presence of amino and phosphate groups, respectively (27). Nuclear magnetic resonance (NMR) spectra were measured on a Varian Gemini spectrometer (300 MHz for ^{1}H, 75 MHz for ^{13}C, and 121.4 MHz for ^{31}P). Chemical shifts (δ) are indicated in ppm relative to internal tetramethylsilane TMS (^{1}H, 0 ppm), $CDCl_3$ (^{13}C, 77 ppm) or H_3PO_4 (^{31}P, 0 ppm) as internal standards; couplings constants are in Hertz. Elemental analyses were obtained from Galbraith Laboratories (Knoxville, TN). Electron impact mass spectra (70 eV) was acquired with a Jeol JMS SX 102 mass spectrometer. Positive ion fast atom bombardment mass spectra (FAB-MS) were acquired with a Jeol SX102 mass spectrometer (10 KV, Xe beam) using *m*-nitrobenzyl alcohol (noba), or glycerol as matrix.

RESULTS

Bis(2-cyanoethyl)-N,N-diisopropylamino-phosphoramidite, (compound) 4, Fig. 2.

To a solution of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (compound **3**) (3.0 g, 12.67 mmol) and diisopropylethylamine (2.32 g, 18 mmol) in dichloromethane (10 ml) was added 3-hydroxypropionitrile (0.82 g, 11.65 mmol). The solution was stirred at room temperature for 30 min, diluted with dichloromethane (40 ml), washed with a saturated solution of sodium bicarbonate (30 ml), brine (2 × 20 ml), water (20 ml), dried over anhydrous sodium sulfate, and evaporated to dryness. The resulting residue (4.24 g) was purified by flash chromatography (20 cm × 4 cm, eluted with hexane–ethyl acetate 4:1, 600 ml; fractions 25 ml in volume were collected). Evaporation of fractions 4–17 furnished compound **4** as an oil (2.4 g, 69% yield). Single component on TLC in two

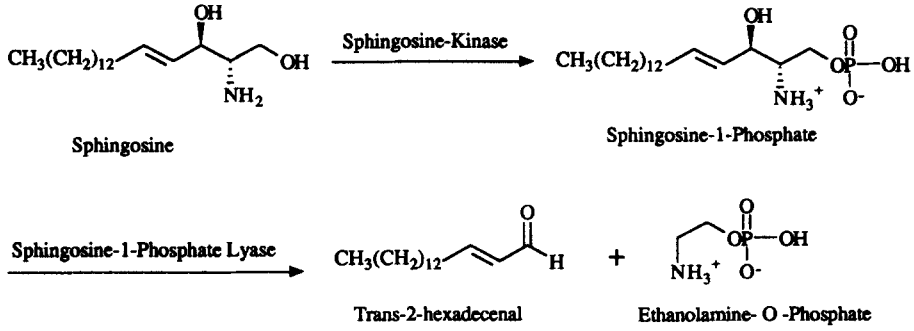


Fig. 1. The degradative pathway of sphingosine.

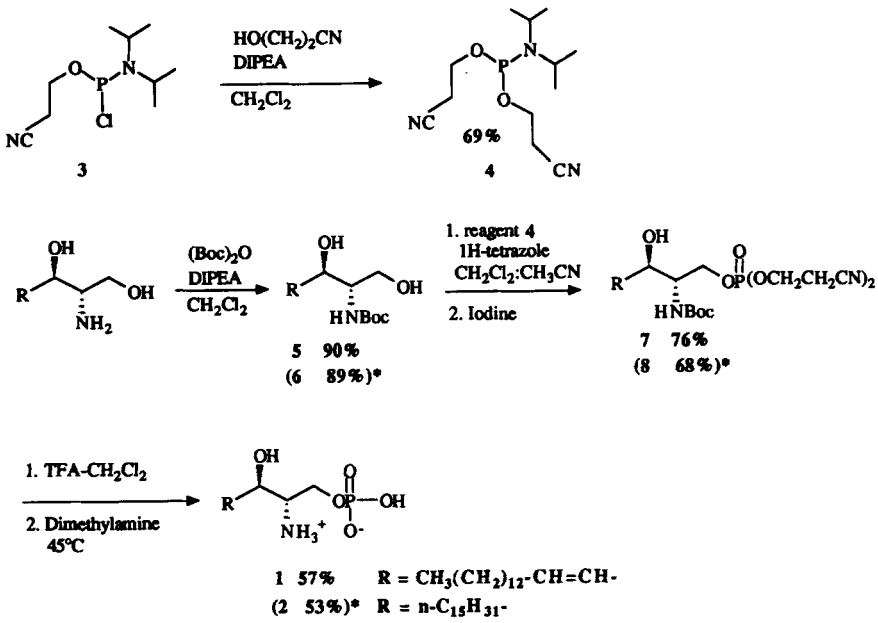


Fig. 2. Synthesis of sphingosine-1-phosphate from sphingosine. Data shown in parentheses correspond to the sphinganine series.

solvent systems (hexane–ethyl acetate 1:1, R_f 0.52; chloroform–methanol 95:5, R_f 0.71); >95% purity by ^{1}H NMR.

^{1}H NMR ($CDCl_3$) δ 3.92–3.73 (4H, m); 3.64–3.53 (2H, m); 2.66 (4H, t, J = 6.5 Hz); 1.20 (12H, d, J = 6.3 Hz). EI-MS *m/z* 270 (M^+, 100); 270 (64); 200 (36); 129 (43).

N-*tert*-butyloxycarbonyl-D-*erythro*-sphingosine, compound 5

To a stirred solution of D-*erythro*-sphingosine (100 mg, 0.33 mmol) in dichloromethane (4 ml) was added di-*tert*-butyl dicarbonate (144 mg, 0.66 mmol). The solution was cooled to 0°C and diisopropylethylamine (85 mg, 0.66 mmol) was added dropwise. The solution was stirred at room temperature for 5 h, diluted with dichloromethane (30 ml), washed with water (15 ml) and brine (20 ml). Drying over anhydrous sodium sulfate and evaporation provided a crude material (172 mg) that was purified by column chromatography (15 cm × 2 cm, using hexane–ethyl acetate 1:1, 300 ml as the eluant solvent: 10 ml-fraction volume). Evaporation of fractions 9–18 gave compound **5** (118 mg, 90% yield) showing a single component on TLC in two solvent systems (hexane–ethyl acetate 1:1, R_f 0.21; chloroform–methanol 9:1, R_f 0.53); >96% purity by ^{1}H NMR.

^{1}H NMR ($CDCl_3$) δ 5.81 (1H, dt, J = 15.6 Hz, J = 8.4 Hz); 5.55 (1H, dd, J = 15.6 Hz, J = 6.7 Hz); 4.33 (1H, m); 3.95 (1H, dt, J = 11.4 Hz, J = 4.0 Hz); 3.75–3.55 (2H, m); 3.60 (3H, m); 2.08 (2H, m); 1.50 (9H, s); 1.29 (22H, m); 0.90 (3H, t, J = 7 Hz). ^{13}C NMR ($CDCl_3$) δ 157.72 (N-$\underline{C}O_2C(CH_3)_3$); 135.00 (C-5); 129.81 (C-4); 80.59 (N-$CO_2\underline{C}(CH_3)_3$); 75.32 (C-3); 63.34 (C-1); 56.17 (C-2); 32.60 (C-6); 32.29 (C-16); 30.61–29.53 (C-7 - C-15 + N-$CO_2C(\underline{CH_3})_3$); 23.37 (C-17); 14.35 (C-18). FAB-MS *m/z* 400 (M+1, 25); 344 (18); 300 (92); 57 (100). HRMS (FAB-noba), calcd. for $C_{23}H_{46}NO_4$, (M+1), 400.3426. found, 400.3424. anal, calc. for $C_{23}H_{45}NO_4$, C: 69.13; H: 11.35; N: 3.50. found C: 68.93; H: 11.54; N: 3.53.

N-*tert*-butyloxycarbonyl-DL-*erythro*-dihydrosphingosine, compound 6

To a stirred solution of DL-*erythro*-sphinganine (150 mg, 0.5 mmol) in dichloromethane (6 ml) was added di-*tert*-butyl dicarbonate (218 mg, 1.0 mmol). The solution was cooled to 0°C and diisopropylethylamine (129 mg, 1.0 mmol) was added dropwise. The solution was stirred at room temperature for 5 h, diluted with dichloromethane (50 ml), washed with water (20 ml) and brine (20 ml). Drying over anhydrous sodium sulfate and evaporation provided a crude material (172 mg) that was purified by column chromatography (15 cm × 2 cm, using hexane–ethyl acetate 1:1, 350 ml as the eluant solvent: 10 ml-fraction volume). Evaporation of fractions 9–18 gave compound **6** (178 mg, 89% yield) showing a single component on TLC in two solvent systems (hexane–ethyl acetate 1:1, R_f 0.24; chloroform–methanol 9:1, R_f 0.53); >97% purity by ^{1}H NMR.

^{1}H NMR ($CDCl_3$) δ 4.35–4.05 (4H, m); 1.65 (2H, m); 1.50 (9H, s); 1.30 (26H, m); 0.93 (3H, t, J = 7 Hz). ^{13}C NMR ($CDCl_3$) δ 157.21 (N-$\underline{C}O_2C(CH_3)_3$); 80.10 (N-$CO_2\underline{C}(CH_3)_3$; 74.58 (C-3); 63.10 (C-1); 55.32 (C-2);

34.83 (C-4); 31.82 (C-16); 30.05-29.81 (C-5 - C-15); 29.10 ($N-CO_2C(\underline{C}H_3)_3$); 23.37 (C-17); 14.24 (C-18). FAB-MS *m/z* 402 (M+1, 15); 346 (98); 302 (100); 284 (64); 270 (28); 57 (83). HRMS (FAB-noba), calcd. for $C_{23}H_{48}NO_4$ (M+1), 402.3582. found 402.3579. anal, calcd. for $C_{23}H_{47}NO_4$. C: 68.78; H: 11.79; N: 3.48. found C: 68.50; H: 11.81; N: 3.40.

N-*tert*-butyloxycarbonyl-D-*erythro*-sphingosine-1-[0,0'-di-(2-cyanoethyl)-phosphate], compound 7

To a solution of compound **4** (68 mg, 0.25 mmol) in acetonitrile (4 ml) was added compound **5** (100 mg, 0.25 mmol) in solution in dichloromethane (4 ml) followed by dropwise addition of [^{1}H]tetrazole (35 mg, 0.5 mmol) in solution in dichloromethane-acetonitrile 1:1 (4 ml). TLC analysis of the reaction after 1 h revealed the absence of the starting material and the formation of a higher R_f product (hexane-ethyl acetate 1:1; R_f 0.61). To the solution was added dropwise a solution of iodine (0.4 M solution in pyridine-dichloromethane-water 3:1:1) until added iodine was no longer decolorized (yellow color persisted, 2.5 ml was added), the solution was stirred for 15 min and diluted with dichloromethane (30 ml), washed with an aqueous solution of sodium thiosulfate (5 M, 2 × 20 ml), water (20 ml), and brine (10 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated to provide crude material (208 mg) that was purified by flash chromatography (10 cm × 2 cm, using chloroform-methanol 95:5, 200 ml as the eluting solvent; 10 ml-fraction volume). The contents of fractions 9-15 were evaporated to give **7** (111 mg, 76% yield). There was a single component on TLC in two solvent systems (hexane-ethyl acetate 3:2; R_f 0.12; chloroform-methanol 9:1; R_f 0.34). Purity as checked by ^{1}H NMR was higher than 98%.

^{1}H NMR ($CDCl_3$) δ 5.81 (1H, dt, J = 15.6 Hz, J = 8.2 Hz); 5.5 (1H, dd, J = 15.4 Hz, J = 8.1 Hz); 5.02 (1H, br s); 4.40-4.12 (7H, m); 3.83 (1H, m); 2.80 (4H, t, J = 6.1 Hz); 2.05 (2H, m); 1.48 (9H, s); 1.42-1.20 (22H, m); 0.89 (3H, t, J = 6.6 Hz). ^{13}C NMR ($CDCl_3$) δ 156.13 ($N-\underline{C}O_2C(CH_3)_3$); 136.01 (C-5); 128.83 (C-4); 117.05 ($OCH_2CH_2\underline{C}N$); 116.98 ($OCH_2CH_2\underline{C}N$); 80.40 ($N-CO_2\underline{C}(CH_3)_3$); 72.89 (C-3); 68.16 (d, J = 5.8 Hz, ($O\underline{C}H_2CH_2CN$); 62.93 (d, J = 5.1 Hz, C-1); 55.23 (d, J = 5.4 Hz, C-2); 32.79 (C-6); 32.41 (C-16); 30.25-28.92 (C-7 - C-15); 28.84 ($N-CO_2C(\underline{C}H_3)_3$); 23.17 (C-17); 20.20 (d, J = 6.8 Hz, $OCH_2\underline{C}H_2CN$); 14.60 (C-18). ^{31}P NMR ($CDCl_3$) δ 1.23. FAB-MS *m/z* 586 (M+1, 10); 512 (100); 486 (84); 264 (95), 57 (70). HRMS (FAB-noba), calcd. for $C_{29}H_{53}N_3O_7P$ (M+1), 586.3623. found 586.3618. anal, calcd. for $C_{29}H_{52}N_3O_7P$. C: 59.46; H: 8.94; N: 7.17. found C: 59.45; 8.91; N: 7.14.

N-*tert*-butyloxycarbonyl-DL-*erythro*-dihydrosphingosine-1-[0,0'-di-(2-cyanoethyl)-phosphate], compound 8

To a solution of compound **4** (86 mg, 0.32 mmol) in acetonitrile (5 ml) was added compound **6** (128 mg, 0.32 mmol) in solution in dichloromethane (5 ml) followed by dropwise addition of [^{1}H]tetrazole (45 mg, 0.64 mmol) in solution in dichloromethane-acetonitrile 1:1 (6 ml). TLC analysis of the reaction after 1 h revealed the absence of the starting material and the formation of a higher R_f product (hexane-ethyl acetate 1:1; R_f 0.63). To the solution was added dropwise a solution of iodine (0.4 M solution in pyridine-dichloromethane-water 3:1:1) until added iodine was no longer decolorized (yellow color persisted, 3 ml was added), the solution was stirred for 15 min and diluted with dichloromethane (50 ml), washed with an aqueous solution of sodium thiosulfate (5 M, 2 × 25 ml), water (30 ml), and brine (25 ml). The organic layer was dried over anhydrous sodium sulfate and evaporated to provide crude material (273 mg) that was purified by flash chromatography (17 cm × 2 cm, using chloroform-methanol 95:5, 250 ml as the eluting solvent; 10 ml-fraction volume). Evaporation of fractions 7-18 gave compound **8** (149 mg, 68% yield). There was a single component on TLC in two different solvent systems (hexane-ethyl acetate 3:2; R_f 0.11; chloroform-methanol 9:1; R_f 0.32). Purity as checked by ^{1}H NMR was higher than 97%.

^{1}H NMR ($CDCl_3$) δ 5.21 (1H, br s); 4.42-4.10 (6H, m); 3.62-3.50 (2H, m); 2.92-2.75 (4H, t, J = 6.3 Hz); 1.49 (9H, s); 1.35-1.20 (26H, m); 0.90 (3H, t, J = 7.1 Hz). ^{13}C NMR ($CDCl_3$) δ 159.82 ($\underline{C}O_2C(CH_3)_3$); 116.92 ($-OCH_2CH_2\underline{C}N$); 116.84 ($-OCH_2CH_2\underline{C}N$); 82.65 ($N-CO_2\underline{C}(CH_3)_3$); 71.93 (C-3); 65.10 (d, J = 6.2 Hz, $-O\underline{C}H_2CH_2CN$); 63.15 (d, J = 4.8 Hz, C-1); 56.75 (d, J = 5.2 Hz, C-2); 33.68 (C-4); 32.28 (C-16); 30.00-29.72 (C-5 - C-15); 28.52 ($CO_2C(\underline{C}H_3)_3$); 23.03 (C-17); 20.10 (d, J = 4.6 Hz, $OCH_2\underline{C}H_2CN$); 14.45 (C-18). ^{31}P NMR ($CDCl_3$) δ 1.24. FAB-MS *m/z* 688 (M+1, 17); 488 (100); 99 (93). HRMS (FAB-noba), calcd. for $C_{29}H_{55}N_3O_7P$ (M+1), 588.3780. found 588.3775. anal, calcd. for $C_{29}H_{54}N_3O_7P$. C: 59.26; H: 9.43; N: 7.15. found C:59.25; H: 9.24; N: 7.12.

D-*erythro*-sphingosine-1-phosphate, compound 1

To a stirred solution of compound **7** (82 mg, 0.14 mmol) in dichloromethane (2 ml) was added trifluoroacetic acid (2 ml). After 1 h of stirring at room temperature, the solution was evaporated under reduced pressure and the residue was dissolved in methanol. The methanol was evaporated, and the residue was again dissolved in methanol and recovered by evaporation. The crude product was then dissolved in dimethylamine (40% in ethanol, 10 ml) and the solution was stirred at 45°C for 6 h. The solution was evaporated to dryness and the residue was

dissolved in boiled acetic acid (1.5 ml). The product was precipitated by adding water (1.5 ml) to the solution. After vortexing and centrifugation at 4°C for 10 min, the precipitate was washed with water (2 × 1 ml), acetone (2 × 2 ml), and diethyl ether (2 × 2 ml) consecutively, then the precipitate was dried under vacuum for 4 h to provide compound **1** (30 mg, 57%). The purity of sphingosine-1-phosphate was confirmed by analysis of the product in three different solvent systems. (n-butanol–acetic acid–water 3:1:1, R_f 0.47; chloroform–methanol–water 60:35:8, R_f 0.22; chloroform–methanol–water–acetic acid 30:30:2:5, R_f 0.33). In each system, there was only a single component on TLC that was positive with ninhydrin and molybdenum spray.

In the ^{1}H NMR spectra of compounds **1** and **2** (CD_3OD, CD_3CO_2D, DMSO-d_6) there is considerable broadening of resonances, presumably due to their self-association.

^{1}H NMR (CD_3OD+CD_3COOD, 2:1) δ 5.84 (1H, m); 5.63 (1H, br s); 4.45–4.00 (4H, m); 2.10 (2H, m); 1.42–1.20 (22H, m); 0.89 (3H, br s). ^{31}P NMR (CD_3COOD + H_3PO_4 9:1) δ 2.39. FAB-MS *m/z* 380 (M+1, 23); 249 (285, 100); 249 (46). HRMS (FAB-noba), calcd. for $C_{18}H_{39}NO_5P$ (M+1), 380.2567. found. 380.2564. anal, calcd. for $C_{18}H_{38}NO_5P$. C: 56.97; H: 10.09; N: 3.68; P: 8.16. found. C: 56.96; H: 10.12; N: 3.68; P: 8.11.

DL-*erythro*-sphinganine-1-phosphate, compound 2

To a stirred solution of compound **8** (106 mg, 0.18 mmol) in dichloromethane (4 ml) was added trifluoroacetic acid (4 ml). The solution was stirred for 1 h at room temperature, the solution was evaporated under reduced pressure, and the residue was dissolved in methanol (4 ml). The methanol was evaporated, and the residue was again dissolved in methanol and recovered by evaporation. The residue was then dissolved in dimethylamine (40% in ethanol, 12 ml) and the solution was stirred at 45°C for 6 h. The solution was evaporated to dryness and the residue was dissolved in boiled acetic acid (1.5 ml). The product was precipitated by adding water (1.5 ml) to the solution. After vortexing and centrifugation at 4°C for 10 min, the precipitate was washed with water (2 × 1 ml), acetone (2 × 2 ml), and diethyl ether (2 × 2 ml) consecutively, then the precipitate was dried under vacuum for 4 h to provide compound **2** (36 mg, 53%). The purity of sphinganine-1-phosphate was confirmed by analysis of the product in three different solvent systems that showed a single component. (n-butanol–acetic acid–water 3:1:1, R_f 0.45; chloroform–methanol–water 60:35:8, R_f 0.21; and chloroform–methanol–water–acetic acid 30:30:2:5, R_f 0.33).

^{1}H NMR ($CD_3OD+CD_3CO_2D$ 2:1) δ 4.40–3.95 (4H, m); 1.54 (2H, m); 1.41–1.25 (26H, m); 0.90 (3H, br s). ^{31}P NMR ($CD_3COOD+H_3PO_4$ 9:1) δ 2.37. FAB-MS *m/z* 382 (M+1, 16); (365, 18); 287 (100). HRMS (FAB-noba), calcd. for $C_{18}H_{41}NO_5P$ (M+1), 382.2724. found. 382.2720. anal, calcd. for $C_{18}H_{40}NO_5P$. C: 56.67; H: 10.57; N: 3.67; P: 8.12. found. C: 56.64; H: 10.61; N: 3.66; P: 8.10.

DISCUSSION

The synthetic route used to obtain S-1-P (compound **1**) and DHS-1-P (compounds **2**) is outlined in Fig. 2. The monophosphorylating reagent (compound **4**) was easily prepared from the commercially available bifunctional reagent chloro-2-cyanoethyl-N,N-diisopropylamino-phosphoramidite (compound **3**) which was previously used for the phosphorylation of carbohydrates (26, 28, 29) and DNA-fragments (26). Treatment of the bifunctional reagent **3** with 0.92 equivalent of 3-hydroxypropionitrile in the presence of diisopropylethylamine at room temperature for 30 min resulted in complete conversion to compound **4** (as judged by TLC). Compound **4** was purified by flash chromatography and could be stored at −20°C for at least 4 months without detectable decomposition. The synthesis of S-1-P begins with the protection of the amino group of sphingosine as the *tert*-butyloxycarbonyl (Boc) derivative **5**. Reaction of compound **5** with one equivalent of phosphoramidite (compound **4**) in the presence of [^{1}H]tetrazole (2 eq) resulted in the formation of the corresponding phosphite which was not isolated. Oxidation in situ by dropwise addition of a solution of iodine (0.4 M in pyridine–dichloromethane–water 3:1:1) gave the phosphate **7** in 76% yield after purification by flash chromatography. The structure of compound **7** was unambiguously confirmed by FAB-mass spectrometry and NMR spectroscopy. In the positive ion FAB-mass spectrum, a peak at 586 (M+1) confirmed that only one phosphate group was incorporated per molecule. No ions were observed at *m/z* = 766, which would correspond to the diphosphorylated derivative. Monophosphorylation was confirmed by the presence of a triplet in the ^{1}H NMR at 2.80 ppm (J = 6.1 Hz) that integrated for four protons and was attributed to (2 × $OCH_2\underline{C}H_2CN$). The regiochemistry of phosphorylation was determined by ^{13}C NMR, which confirmed that the phosphate group was on the primary hydroxyl group. **Figure 3** shows the proton decoupled ^{13}C NMR spectra of N-Boc sphingosine-1-phosphate triester **7**. All assignments of the resonances were made by comparison with the data reported for naturally occurring sphingosine, ceramide, and glycosphingolipids (30–33) and were confirmed by the degree of protonation obtained by means of DEPT experiment. Scale expansions of the C-1, C-2, C-3, C-4, C-5 and $O\underline{C}H_2CH_2CN$ regions are shown as insets in Fig. 3. The resonances for C-1 and C-2 at 62.93 and 55.23 ppm appear as doublets due to the J_2 and J_3 coupling with the phosphorus ($J_{C1\text{-}P}$ = 5.1 Hz, $J_{C2\text{-}P}$ = 5.4 Hz). Neither

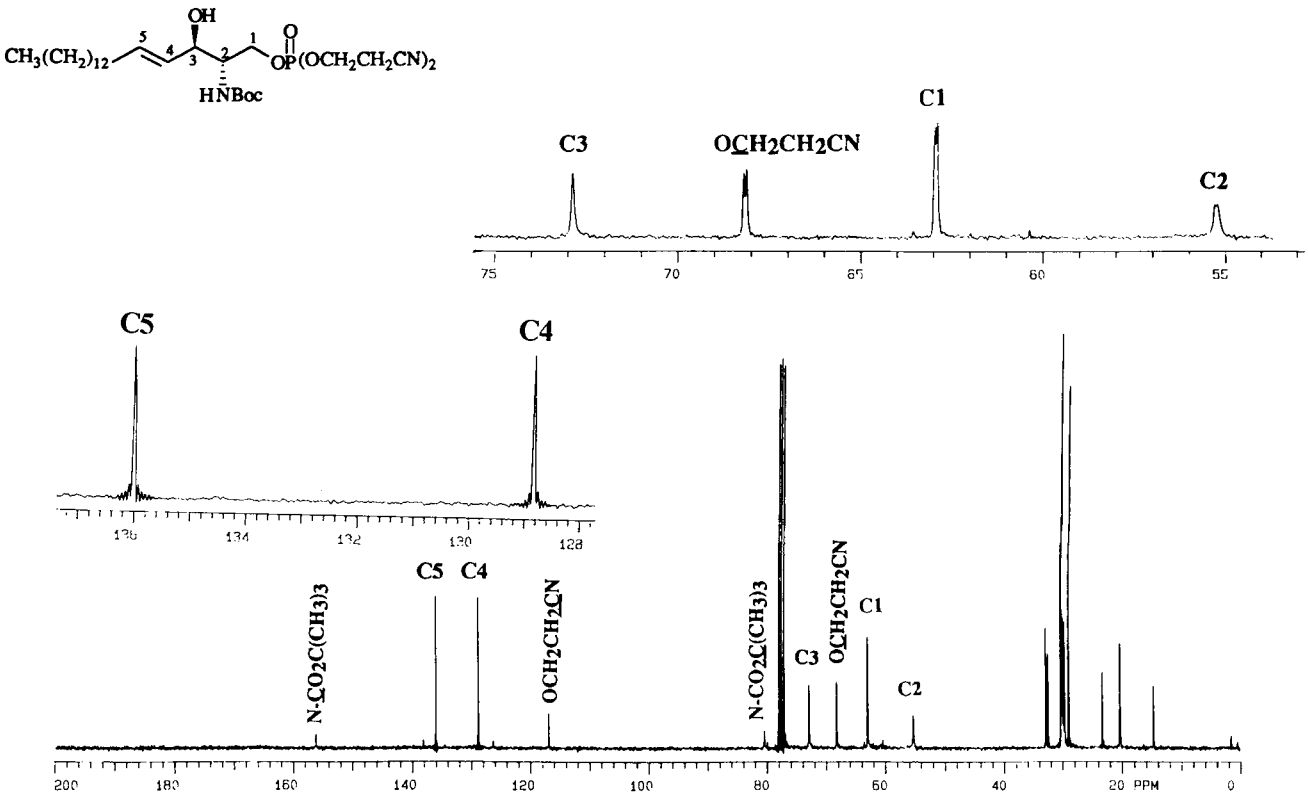


Fig. 3. ^{13}C-NMR proton-decoupled spectrum of sphingosine phosphate triester **7** at 75 MHz.

C-3 nor the C-4 resonances show any coupling to phosphorus (singlets at 72.89 and 128.83), thus confirming that the protected phosphate group of **7** was on the primary hydroxyl.

Conversion of compound **7** to S-1-P (compound **1**) was achieved using the deprotection sequence shown in Fig. 2. Treatment of phosphate triester **7** with trifluoroacetic acid (TFA) in dichloromethane resulted in complete removal of the Boc group. The free amine was not isolated prior to phosphate deprotection with a solution of dimethylamine (40% in ethanol). The purification of S-1-P by chromatography was precluded by the lack of solubility in most organic solvents. Instead, purification was achieved by dissolution of crude compound **1** in boiled acetic acid and precipitation by addition of water. After successive washings with water, acetone and diethylether, compound **1** was obtained in 57% yield. This corresponds to an overall yield of 39% from sphingosine. The synthesis of DHS-1-P (compound **2**) proceeded in an analogous fashion to that of S-1-P, and resulted in an overall yield of 32% from sphinganine (Fig. 2).

This phosphorylation method was successfully used to prepare 2-vinyl-dihydrosphingosine-1-phosphate, the most potent inhibitor of S-1-P lyase described to date (34, 35). We are currently using this procedure to phosphorylate other analogs of sphingosine, in order to better understand the biological role(s) of S-1-P and S-1-P lyase.

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