Chemical synthesis of D-*ribo*-phytosphingosine-1-phosphate, a potential modulator of cellular processes

Shengrong Li, William K. Wilson, and George J. Schroepfer, Jr.[1]

Departments of Biochemistry and Cell Biology and of Chemistry, Rice University, 6100 Main St., Houston, TX 77005-1892

Abstract **D-*erythro*-Sphingosine-1-phosphate (2), an intermediate in sphingosine metabolism, shows a diversity of biological activities. Comparable roles might be anticipated for D-*ribo*-phytosphingosine-1-phosphate (1). We describe an efficient three-step chemical synthesis of 1 from D-*ribo*-phytosphingosine. Our approach is based on standard phosphoramidite methodology and on the finding of Boumendjel and Miller (*J. Lipid Res.* 1994. 35: 2305–2311) that sphingosine can be monophosphorylated at the 1-hydroxyl without protection of the 3-hydroxyl. However, we were unable to duplicate their reported synthesis of 2 without important modifications in reagents and reaction conditions. Under the reported conditions for preparing 2, we obtained a cyclic carbamate (14), which we have isolated and identified. The structures of 1 and the cyclic carbamate 14 were elucidated by a combination of mass spectrometry and 1D and 2D nuclear magnetic resonance spectroscopy.—**Li, S., W. K. Wilson, and G. J. Schroepfer, Jr. **Chemical synthesis of D-*ribo*-phytosphingosine-1-phosphate, a potential modulator of cellular processes. *J. Lipid. Res.* 1999.** 40: **117–125.**

Supplementary key words phytosphingosine-1-phosphate • sphingosine-1-phosphate • phosphorylation • NMR • mass spectrometry

Many years ago, we devoted a very considerable effort towards studies of the enzymatic formation of the 1-phosphate derivatives of sphingolipid bases (1, 2) and their enzymatic degradation (3–5). Since that time, interest in these matters has increased amazingly with the recognition of important biological actions and their demonstrated or implied involvement in a variety of critical cellular processes. Among many fascinating reports in the last 2 years alone have been the purification, to apparent homogeneity, of sphingosine kinase from rat kidney (6), reports on the levels of sphingosine-1-phosphate in various tissues (7) and in human plasma and serum (8), interesting studies of factors affecting sphingosine kinase activity (9–11), identification of the genes for sphingosine phosphate lyase (12) and dihydrosphingosine-1-phosphate phosphatase (13), an important report indicating high affinity binding of sphingosine-1-phosphate to the orphan receptor EDG-1 (14), and many other reports of interesting, diverse biological activities of sphingosine-1-phosphate. Earlier important actions of sphingosine-1-phosphate have been reviewed by Spiegel and Merrill (15). Whereas almost all investigations of the formation, metabolism, and actions of the phosphate derivatives of sphingoid long-chain bases (LCB) have concerned sphingosine, essentially nothing is known of the properties and actions of the 1-phosphate derivative of another major LCB of eukaryotes, i.e., phytosphingosine. Phytosphingosine is not only the major LCB in plants and yeasts (16) but is also present in several mammalian tissues (17–21). Moreover, its formation from labeled dihydrosphingosine in the rat has been reported (22).

We now report the first chemical synthesis and characterization of D-*ribo*-phytosphingosine-1-phosphate.[2] The enzymatic synthesis of this compound was reported in early studies of Stoffel, Assmann, and Binczek (23) and in recent studies of Nagiec et al. (24) and Lanterman and Saba (25). In each case, characterization of the product was limited. In the present manuscript we also present important improvements in a previously described chemical synthesis of sphingosine-1-phosphate (26, 27).

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods

Melting points (mp) were measured with a Thomas-Hoover apparatus in sealed and evacuated capillary tubes.

Abbreviations: Boc, *t*-butyloxycarbonyl; COSY, ^{1}H-^{1}H correlation spectroscopy; EI, electron impact; FAB, fast atom bombardment; GC, gas chromatography; HMBC, heteronuclear multiple bond correlation; HSQC, ^{1}H-^{13}C heteronuclear single quantum coherence; IR, infrared (spectrum); LCB, long-chain base; MPLC, medium pressure liquid chromatography; MS, mass spectrometry or mass spectrum; NBA, 3-nitrobenzyl alcohol; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, trimethylsilyl.

[1] To whom correspondence should be addressed.

[2] Chemical Abstracts nomenclature for selected compounds: **1**, 1,3,4-octadecanetriol, 2-amino-, 1-(dihydrogen phosphate), (2S, 3S, 4R); **2**, 4-octadecene-1,3-diol, 2-amino-, 1-(dihydrogen phosphate), [R-[R*, S–(E)]]; **4**, 4-octadecene-1,3-diol, 2-amino-, [R-[R*,S*-(E)]]; **8**, carbamic acid, [2-hydroxy-1-(hydroxymethyl)heptadecyl]-, 1,1-dimethylethyl ester, (R*,S*); **12**, 5,7-dioxa-2-aza-6-phosphanonanoic acid, 9-cyano-6-(2-cyanoethoxy)-3-(1-hydroxy-2-hexadecenyl)-, 1,1-dimethylethyl ester, 6-oxide, [R-[R*,S*-(E)]].

Optical rotations were measured on a JASCO DIP-4 digital polarimeter at room temperature (22°C) in pyridine solution. Infrared spectra (IR) were measured with KBr pellets on a Mattson Galaxy 6020 Fourier-transform infrared spectrometer. Nuclear magnetic resonance (NMR) spectra were measured on 3–50 mm $CDCl_3$ solutions (unless specified otherwise) with a Bruker AMX500 instrument (500 MHz for ^1H) and referenced as follows: internal tetramethylsilane (0.0 ppm, ^1H) and $CDCl_3$ (77.0 ppm, ^{13}C) for $CDCl_3$ and $CDCl_3$–CD_3OD mixtures; CD_3OD (3.30 ppm, ^1H and 50.0 ppm, ^{13}C) for CD_3OD and CD_3OD-CD_3COOD mixtures; internal[3] $P(OMe)_3$ (140.4 ppm, ^{31}P). $CDCl_3$–D_2O designates addition of a drop of D_2O to a $CDCl_3$ solution to improve spectral definition.[4] COSY(^1H–^1H correlation spectroscopy), HSQC (heteronuclear single quantum coherence), and heteronuclear multiple bond correlation (HMBC) spectra were acquired as described previously (28). Most ^1H NMR chemical shifts from $CDCl_3$ solutions are presented to ±0.001 ppm precision and are corrected for effects of strong coupling.[5] Coupling constants were measured from resolution enhanced spectra; an asterisk indicates splittings that may be due to chemical nonequivalence or spin-spin coupling.[6] Purities were estimated by integration of unapodized ^1H NMR spectra. Analytical thin-layer chromatography (TLC) was performed using aluminum-backed silica gel 60 F_{254} plates (EM Science, Gibbstown, NJ). TLC plates were charred by spraying with 5% ammonium molybdate in 10% sulfuric acid followed by heating for 5 min at 80°C. Flash chromatography and medium pressure liquid chromatography (MPLC) were done on glass columns dry-packed with silica gel (230–400 mesh; EM Science). Fraction volumes were 20 ml. Electron impact (EI) mass spectra (MS) were acquired at 70 eV by direct-inlet with a ZAB-HF reverse-geometry double-focusing instrument and are reported as *m*/*z* (relative intensity, suggested assignment); † indicates that the exact mass from high-resolution data was compatible (±3.0 mmu) with the suggested assignment. Fast atom bombardment (FAB) mass spectra were acquired using a 3-nitrobenzyl alcohol (NBA) or glycerol matrix by the Department of Chemistry of Rice University (Houston, TX) or the Washington University Resource for Biomedical and Bio-organic Mass Spectrometry (St. Louis, MO). Capillary gas chromatography (GC) was carried out on a Shimadzu GC-9A instrument using split injection (49:1 split ratio, flame ionization detection, and a 30 m × 0.25 mm i.d. DB-5 column (J&W Scientific; Folsom, CA) operated isothermally (200°C; nitrogen carrier gas at 26 cm/s linear velocity, unless specified otherwise).

d-*erythro*-Sphingosine (**4**) was isolated from cow brain as described previously (28, 29) and showed >98% purity by TLC and ^1H NMR. Crude d-*ribo*-phytosphingosine tetraacetate, isolated from cultures of *Hansenula ciferrii* (30), was a gift from H.E. Carter. 2-Cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (**5**), *N*,*N*-diisopropylethylamine, 3-hydroxypropionitrile, di-*t*-butyl dicarbonate, anhydrous acetonitrile, 1*H*-tetrazole (99+%, sublimed), and *t*-butyl peroxide were purchased from Aldrich Chemical Co. (Milwaukee, WI). Bis(2-cyanoethyl) *N*,*N*-diisopropylphosphoramidite (**6**) was prepared as described previously (26, 31) by reaction of **5** (0.50 g, 2.1 mmol), *N*,*N*-diisopropylethylamine (0.742 g, 3.0 mmol), and 3-hydroxypropionitrile (0.15 g, 2.1 mmol); flash chromatography[7] on silica gel (150 × 10 mm i.d. column; elution with ethyl acetate–hexane 1:3) gave **6** as a clear oil (0.48 g, 84% yield).[8] Trimethylsilyl (TMS) ethers were prepared by treatment of the sphingolipids with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide and pyridine for 1 h at 40°C, followed by evaporation to dryness under nitrogen.

Purification of D-*ribo*-phytosphingosine tetraacetate

Crude d-*ribo*-phytosphingosine tetraacetate (2.0 g) was purified by MPLC (500 × 25 mm column; elution with ethyl acetate–hexane 1:9 (500 ml) and ethyl acetate–hexane 2:8). Fractions 50–65 gave the tetraacetate as a white solid (1.5 g, 75% recovery): mp, 45–46°C (lit. 49–50°C (30)); single component on TLC (R_f 0.30, chloroform–methanol 9:1) and on GC (t_R 17.3 min, 250°C, helium carrier gas at 1.1 kg/cm^2); EI–MS, 412† (3, M–CH_2OAc), 383 (3), 366 (9), 352† (11, M–$C_5H_9O_4$), 310† (11, M–$C_7H_{11}O_5$)), 305† (19, M–$C_9H_{10}O_3N$), 292† (14, M–$C_7H_{13}O_6$), 264 (6), 145 (41), 144† (67, $C_6H_{10}O_3N$), 84 (100); high resolution EI–MS, calcd. for $C_{26}H_{48}O_7N$ (M + H), 486.3431, found 486.3436; ^1H NMR, ≥99% purity, δ_H 6.005 (d, 9.4 Hz, NH), 5.103 (dd, 8.3, 3.1 Hz, H–3), 4.939 (dt, 9.9, 3.2, H–4), 4.475 (dddd, 9.4, 8.3, 4.9, 3.1 Hz, H–2), 4.289 (dd,

[3] Proton-coupled spectra were acquired before and after addition of $P(OMe)_3$. The $P(OMe)_3$ resonance was the anticipated 10-tet in $CDCl_3$ but a singlet in CD_3OD–CD_3COOD mixtures, evidently due to exchange with the solvent.

[4] Shaking $CDCl_3$ solutions with a drop of D_2O improved the resolution for some resonances, but others (such as H–2 and H–4 of **7** and H–2 of **8**) consisted of broad humps, even with strong resolution enhancement.

[5] NMR chemical shifts for the hydrophobic tail region of sphingolipid bases are quite reproducible (±0.001 ppm for ^1H, ±0.01 ppm for ^{13}C), but ^1H shieldings in the polar head region may vary by as much as 0.2 ppm, depending on concentration and moisture levels (28). For other solvents, precision is given to only ±0.01 ppm.

[6] Several ^1H and ^{13}C signals, notably those of the diastereotopic OCH_2CH_2CN groups of the phosphate intermediates **11**, **12**, and **13**, showed splittings that might arise from spin-spin couplings or chemical non-equivalence. In view of the possible presence of uncoupled phosphate contaminants in some samples, any significance of these splittings should be interpreted cautiously. Some unassigned splittings were also observed in the sphingolipid moiety.

[7] When crude **6** was subjected to slower (2 h) MPLC purification with a weaker solvent system, a mixture of **6**, 3-hydroxypropionitrile, and unidentified decomposition products were eluted. Later fractions contained only 3-hydroxypropionitrile, which was identified by its NMR signals (δ_H 3.90 (t, 6.2 Hz, 2H), 2.61 (t, 6.2 Hz, 2H)) and observed in at most trace amounts in other samples of **6**.

[8] Single component on TLC (R_f 0.50, ethyl acetate–hexane 1:1); EI–MS, 271† (22, M^+), 256† (64, M–CH_3), 228† (29, M–C_3H_7), 214† (40, M–C_4H_9), 201† (51, M–OCH_2CH_2CN), 171† (100, M–$N(C_3H_7)_2$), 54† (69, C_3H_4N); ^1H NMR, δ_H 3.903 (ddt, 10.4, 7.4, 6.3 Hz, 2H), 3.821 (ddt, 10.4, 8.0, 6.3 Hz, 2H), 3.628 (d of septet, 10.4, 6.8 Hz, 2H), 2.661 (tt, 6.3, 0.8 Hz, 4H), 1.198 (d, 6.8 Hz, 12H), in agreement with literature data (26, 31).

11.7, 4.9 Hz, H–1), 4.006 (dd, 11.6, 3.1 Hz, H-1), 2.080 (s, 3H), 2.048 (br s, 6H), 2.026 (s, 3H), 1.64 (m, 2H), 1.25 (m, 24H), 0.880 (t, 7 Hz, 3H); ^{13}C NMR, δ_C 171.10, 170.82, 170.05, 169.67, 72.93 (C–4), 71.97 (C–3), 62.81 (C–1), 47.58 (C–2), 31.89, 29.66, 29.65, 29.64, 29.62, 29.59, 29.55, 29.46, 29.32, 29.26, 28.14, 25.47, 23.26, 22.65, 21.01, 20.74, 20.71, 14.08.

D-*ribo*-Phytosphingosine (3)

To a solution of the tetraacetate of **3** (1.5 g) in methanol (100 ml) was added sodium hydroxide (4.0 g). The resulting solution was refluxed under nitrogen overnight. TLC analysis showed the disappearance of the starting material and formation of a polar component (R_f 0.50, chloroform–methanol–ammonia 100:25:2.5). After evaporation of methanol, the residue was extracted with chloroform–methanol (9:1, 100 ml). The organic extract was washed with water (30 ml) and brine (20 ml) and dried over anhydrous sodium sulfate. Evaporation gave a white solid that was subjected to MPLC (500 × 25 mm column; elution with chloroform–methanol–ammonia 100:15:1). Evaporation of fractions 20–45 gave **3** as a white solid (0.9 g, 92% yield): mp, 98–100°C (lit., 98–100°C (32), 98–101°C (33), 98–108°C (34)); $[\alpha]_D^{22}$ + 8.6 (c 1, pyridine) (lit. $[\alpha]_D^{20}$ +8.9 (c 0.6, pyridine) (32), $[\alpha]_D^{20}$ +8.7 (c 0.8, pyridine) (33)); single component on TLC (R_f 0.50, chloroform–methanol–ammonia 100:25:2.5) and GC (as the tetra–TMS ether; t_R, 35.0 min); FAB–MS (NBA), 340 (25, M + Na), 318 (100, M + H), 300 (32, M + H − H_2O), 282 (26, M + H − 2H_2O); ^{1}H NMR (CD_3OD), ~97% purity, δ_H 3.85 (dd, 11.3, 4.0 Hz, H–1), 3.67 (dd, 11.3, 7.9 Hz, H–1), 3.47 (m, H–4), 3.46 (m, H–3), 3.28 (dt, 7.9, 3.9 Hz, H–2), ~1.77 (m, 1H), 1.55 (m, 1H), 1.28 (m, 24H), 0.89 (t, 7 Hz, 3H); ^{13}C NMR (CD_3OD), δ_C 75.65, 74.76, 61.92 (C–1), 57.16 (C–2), 36.16, 34.07, 31.88, 31.79 (5C), 31.77, 31.76, 31.47, 27.40, 24.73, 15.45.

N-*t*-Butyloxycarbonyl-D-*ribo*-phytosphingosine (7) and *N*-*t*-butyloxycarbonyl-D-*erythro*-sphingosine (8)

Di-*t*-butyl dicarbonate (260 mg, 1.2 mmol) was added to a stirred solution of **3** (170 mg, 0.54 mmol) in dichloromethane (15 ml). The solution was cooled to 0°C and *N*,*N*-diisoproylethylamine (94 mg, 0.73 mmol) was added dropwise. The solution was stirred at room temperature for 24 h, diluted with chloroform (30 ml), washed with water (15 ml) and brine (20 ml), and dried over anhydrous sodium sulfate. Evaporation provided a crude product that was purified by MPLC (500 × 10 mm column, elution with methanol–chloroform 1:99 (500 ml) and methanol–chloroform 2:98). Evaporation of fractions 55–70 gave **7** (180 mg, 80% yield) as a white solid: single component on TLC (R_f 0.55, chloroform–methanol 9:1); FAB–MS (NBA), 440 (100, M + Na), 418 (9, M + H), 362 (34), 318 (67), 57 (32); high resolution FAB–MS (NBA), calcd. for $C_{23}H_{48}O_5N$ (M + H), 418.3532, found 418.3532; IR, ν_{max} 3275, 2953, 2920, 2851, 1672, 1547, 1469, 1364, 1254, 1175, 1057 cm^{-1}; ^{1}H NMR ($CDCl_3$–D_2O), >98% purity, δ_H 5.34 (d, 7.9 Hz, NH), 3.88 (dd, 11.2, 2.8 Hz, H–1), 3.84 (m, H–2), 3.74 (dd, 11.2, 5.5 Hz, H–1), 3.67 (m, H–4), 3.62 (dd, 6.1, 3.7 Hz, H–3), 1.71 (m, H–5), 1.50 (m, H–5), 1.45 (s, 9H), 1.26 (m, 24H), 0.88 (t, 7 Hz, 3H); ^{13}C NMR ($CDCl_3$–D_2O), δ_C 156.44 (Boc), 80.10 (Boc), 75.86 (C–3), 72.78 (C–4), 61.73 (C–1), 52.81 (C–2), 31.91, 29.69-29.64 (9C), 29.34, 28.35 (Boc), 25.93, 22.67, 14.09.

Similar treatment of **4** (106 mg) at room temperature for 5 h gave **8** as a white solid (120 mg, 85% yield): mp, 68–69°C; single component on TLC (R_f 0.53, chloroform–methanol 9:1); FAB–MS (NBA), 400 (7, M + H), 326 (81), 308 (16), 296 (18), 282 (34), 57 (100); ^{1}H NMR, >98% purity, δ_H 5.783 (dtd, 15.4, 6.8, 1.3, H–5), 5.533 (ddt, 15.4, 6.5, 1.4 Hz, H–4), 5.28 (d, 7.9 Hz, NH), 4.326 (ddd, 6.2, 4.2, 1.6 Hz, H–3), 3.940 (dd, 11.4, 3.7 Hz, H–1), 3.715 (dd, 11.4 Hz, 3.6, H–1), 3.606 (m, H–2), 2.06 (m, 2H), 1.455 (s, 9H), 1.37 (m, 2H), 1.26 (s, 20H), 0.881 (t, 7 Hz, 3H); ^{13}C NMR, δ_C 156.23 (Boc), 134.10 (C–5), 128.87 (C–4), 79.75 (Boc), 74.67 (C–3), 62.57 (C–1), 55.40 (C–2), 32.27, 31.89, 29.66, 29.65 (2C), 29.62, 29.58, 29.46, 29.32, 29.19, 29.09, 28.34 (Boc), 22.65, 14.08.

(*N*-*t*-Butyloxycarbonyl-D-*ribo*-phytosphingosine)-1-yl bis(2-cyanoethyl) phosphate (11) and (*N*-*t*-butyloxycarbonyl-D-*erythro*-sphingosine)-1-yl bis((2-cyanoethyl) phosphate (12)

A solution of **7** (100 mg, 0.24 mmol) in dichloromethane (4 ml) was dried over molecular sieves 3A overnight and added to a solution of **6** (64 mg, 0.24 mmol) in dry acetonitrile (4 ml) at 0°C, followed by dropwise addition of a solution of 1*H*-tetrazole (33 mg, 0.48 mmol) in dichloromethane–acetonitrile 1:1 (4 ml). TLC analysis of the reaction after 1 h indicated ~80% conversion to material of higher mobility (R_f 0.75, chloroform–methanol 9:1). To the solution was added dropwise *t*-butyl peroxide (70 μl, 5.0–6.0 M solution in decane) followed by stirring at room temperature for another 45 min. The reaction mixture was diluted with dichloromethane (30 ml), washed with water (20 ml) and brine (20 ml), dried over sodium sulfate and evaporated to dryness. The oily residue was subjected to MPLC (1000 × 10 mm column, elution with methanol–chloroform 1:99 (500 ml) and methanol–chloroform 2:98). Evaporation of fractions 37–47 gave **7** (15 mg). Fractions 54–60 gave **11** as a white solid (102 mg, 71% yield): single component on TLC (R_f 0.55, chloroform–methanol 9:1); FAB–MS (NBA), 626 (64, M + Na), 604 (4, M + H), 504 (100), 300 (16), 227 (46), 57 (17); IR, ν_{max} 3351, 2918, 2851, 2255, 1680, 1656, 1535, 1468, 1290, 1252, 1171, 1057, 1045, 1009 cm^{-1}; ^{1}H NMR, ~97% purity, δ_H 5.20 (d, 8.9 Hz, NH), 4.477 (ddd, 10.7, 8.7, 5.1 Hz, H–1), 4.326 (dt, 8.2, 6.1 Hz, 4H, OC$\underline{H}_2$CH_2CN), 4.32 (ddd, ~10.8, 7.4, 3.3 Hz, H–1), 3.964 (m, H–2), 3.68 (m, H–4), 3.66 (dd, 6.4, 4.6 Hz, H–3), 2.803 (tdd, 6.1, 1.3*, 1.0* Hz, 4H, C$\underline{H}_2$CN), 1.52 (m, H–5), 1.451 (s, 9H), 1.25 (m, 24H), 0.880 (t, 6.9 Hz, 3H); ^{13}C NMR, δ_C 155.79 (Boc), 116.56 (d, 4* Hz, CN), 80.10 (Boc), 73.92 (C–3), 72.62 (C–4), 68.51 (d, 6 Hz, C–1), 62.54 (t, 5* Hz, O$\underline{C}$H$_2$CH$_2$CN), 51.93 (d, 6 Hz, C–2), 31.88, 29.67 (~6C), 29.66, 29.64, 29.62, 29.33, 28.32 (Boc), 25.96, 22.65, 19.67 (d, 7.1* Hz, $\underline{C}$H$_2$CN), 14.08; ^{31}P NMR, δ_P −2.3 (br quintet or septet, ~8 Hz).

Similar treatment of **8** (100 mg, 0.25 mmol) gave **12** as a

white solid (110 mg, 75% yield):[9] single component on TLC (R_f 0.43, chloroform–methanol 9:1); FAB–MS (NBA), 608 (1, M + Na), 586 (7, M + H), 512 (63), 486 (44), 308 (43), 264 (71), 246 (28), 57 (100); high resolution FAB–MS (NBA), calcd. for $C_{29}H_{53}O_7N_3P$ (M + H), 586.3621, found 586.3604; IR, ν_{max} 3350, 2953, 2918, 2851, 2255, 1682, 1534, 1468, 1283, 1177, 1078, 1040, 1005, 961 cm^{-1}; ^1H NMR, ~95% purity,[10] δ_H 5.776 (dtd, 15.4, 6.8, 1.1 Hz, H–5), 5.496 (ddt, 15.4, 7.2, 1.5 Hz, H–4), 4.97 (d, 8.4 Hz, NH), 4.36 (m, H–1), 4.314 (dt, 8.2, 6.1 Hz, 4H, $OC\underline{H}_2CH_2CN$), 4.241 (ddd, 10.5, 6.9, 3.5 Hz, H–1), 4.154 (br t, ~6.8 Hz, H–3), 3.83 (m, H–2), 2.796 (td, 6.2, 1.0* Hz, 4H, $C\underline{H}_2CN$), 2.04 (m, 2H), 1.442 (s, 9H), 1.37 (m, 2H), 1.26 (m, 20H), 0.881 (t, 7 Hz, 3H); ^{13}C NMR, δ_C 155.58 (Boc), 135.36 (C–5), 128.30 (C–4), 116.39 (d, 4* Hz, $\underline{C}N$), 79.7 (d, 3* Hz, Boc), 72.31 (d, 2 Hz, C–3), 67.63 (d, 5.8 Hz, C–1), 62.40 (dd, 5.7*, 3.1* Hz, $O\underline{C}H_2CH_2CN$), 54.70 (dd, 5*, 3* Hz, C–2), 32.27, 31.88, 29.65 (3C), 29.61, 29.57, 29.47, 29.31, 29.23, 29.03, 28.31, 22.65, 19.66, (d, 7.1 Hz, $\underline{C}H_2CN$), 14.08; ^{31}P NMR, δ_P −2.7, −3.9 (20:1 ratio) (lit. 1.23 (26)).

D-*ribo*-Phytosphingosine-1-phosphate (1) and D-*erythro*-sphingosine-1-phosphate (2)

Trifluoroacetic acid (1 ml) was added to a stirred solution of **11** (60 mg, 0.10 mmol) in dichloromethane (1 ml), followed by continued stirring at room temperature for 1 h. The solution was evaporated under a gentle nitrogen stream and further dried in vacuo. Traces of trifluoroacetic acid were removed by dissolving the product in methanol followed by nitrogen evaporation. The crude product was dissolved in dimethylamine (40% in ethanol, 10 ml). The solution was stirred at 45°C for 48 h and evaporated to dryness. The residue was dissolved quickly in hot acetic acid (0.5 ml). The product was immediately precipitated by addition of water (0.5 ml) followed by vortexing and centrifugation at 4°C for 5 min. The precipitate was washed with water (2 × 0.5 ml), acetone (2 × 1 ml), and diethyl ether (2 ml) and dried in vacuo for 4 h to furnish **1** as a white solid (30 mg, 76% yield): single component on TLC (R_f 0.28, chloroform–methanol–water–acetic acid 30:30:2:5); FAB–MS (glycerol), 525[11] (8), 398 (100, M + H); high resolution FAB–MS (NBA), calcd. for $C_{18}H_{41}O_6NP$ (M + H), 398.2671, found 398.2670; IR, ν_{max} 3331, 2920, 2850, 1645, 1541, 1458, 1190, 1067, 930, 824, 721 cm^{-1}; ^1H NMR (CD_3OD–CD_3COOD, 1:1), ~95% purity,[10] δ_H 4.28 (ddd, 12.0, 7.9, 3.6 Hz, H–1), 4.23 (ddd, 12.0, 9.2, 7.6 Hz, H–1), 3.75 (ddd, 7.6, 5.0, 3.6 Hz, H–2), 3.67 (dd, 8.8, 5.1 Hz, H–3), 3.61 (td, 8.5, 2.7 Hz, H–4), 1.76 (m, H–5), 1.49 (m, H–5), 1.24 (m, 24 H), 0.83 (t, 7 Hz, 3H); ^{31}P NMR (CD_3OD-CD_3COOD, 1:1), δ_P 0.2 (dd, ~9.0, 7.9 Hz).

Similar treatment of **12** (40 mg, 0.07 mmol) gave **2** as a white solid (13 mg, 50% yield): single component on TLC (R_f 0.32, chloroform–methanol–water–acetic acid 30:30:2:5); ~98% purity by ^1H NMR; FAB-MS (glycerol), 507[11] (16), 380 (32, M + H), 241 (40), 149 (100); high resolution FAB–MS (NBA), calcd. for $C_{18}H_{39}O_5NP$, 380.2566, found 380.2541 (M + H); IR, ν_{max} 3435, 2920, 2851, 1632, 1547, 1465, 1250, 1184, 1069, 1036, 930 cm^{-1}; ^1H NMR (CD_3OD–CD_3COOD, 1:1), ~98% purity,[10] δ_H 5.85 (dtd, 15.4, 6.8, 1.1 Hz, H–5), 5.48 (ddt, 15.5, 7.0, 1.3 Hz, 1H), 4.38 (ddd, 6.9, 5.0, 1.1 Hz, H–3), 4.17 (ddd, 12.1, 8.6, 3.3 Hz, H–1), 4.10 (12.2, 9.7, 8.4 Hz, H–1), 3.57 (ddd, 8.4, 5.0, 3.4 Hz, H–2), 2.03 (m, 2H), 1.35 (m, 2H), 1.23 (m, 20H), 0.83 (t, 7 Hz, 3H); ^{31}P NMR (CD_3OD–CD_3COOD, 1:1), δ_P 0.1 (t, 8.6 Hz) (lit. δ_P 8.11 (26), δ_P (CD_3COOD), 2.42 (35, 36)). Deprotection with dimethylamine in ethanol was monitored by ^1H NMR after 6 h (50% completion), 18 h (95% completion), and 48 h (complete reaction).

Cyclic carbamate derivative of (D-*erythro*-sphingosine-1-yl) bis(2-cyanoethyl) phosphate (13)

A solution of **8** (103 mg, 0.26 mmol) in dichloromethane (4 ml) was dried over type 3A molecular sieves overnight and added to a solution of **6** (72 mg, 0.26 mmol) in dry acetonitrile (4 ml) at 0°C, followed by dropwise addition of a solution of 1*H*-tetrazole (37 mg, 0.52 mmol) in dichloromethane–acetonitrile 1:1 (4 ml). TLC analyses of the reaction after 1 h and 1.5 h indicated ~80% conversion to material of higher mobility (R_f 0.85, chloroform–methanol 9:1). To the solution was added dropwise a solution of iodine (0.4 M solution in pyridine–dichloromethane–water, 3:1:1) until the iodine was no longer decolorized (about 2.5 ml), followed by stirring at room temperature for another 10 min. TLC analysis revealed the formation of polar material (R_f 0.35, chloroform–methanol 9:1). The reaction mixture was diluted with dichloromethane (30 ml), washed with aqueous sodium thiosulfate (5 M, 2 × 20 ml), water (20 ml), and brine (20 ml), dried over anhydrous sodium sulfate and evaporated to dryness. The residue was subjected to MPLC (1000 × 10 mm column; elution with methanol–chloroform 2:98 (1000 ml) and methanol–chloroform

[9] TLC analysis of the reaction mixture immediately prior to addition of *t*-butyl peroxide showed the formation of **10** (R_f 0.55, chloroform–methanol 9:1, 70%) and an unidentified byproduct of higher mobility (R_f 0.90, chloroform–methanol 9:1, 5–10%). In similar reactions performed without drying the solution of **8** over molecular sieves, TLC indicated low conversion (30%) to **10**. TLC analysis of similar reactions carried out at room temperature, with or without drying of **8**, indicated major contamination of **10** with material of high mobility (R_f 0.90, chloroform–methanol 9:1). A faint upper spot (R_f 0.9, chloroform–methanol 9:1) was also observed in crude **9**.

[10] Integration of ^1H NMR spectra was not a highly reliable method for estimating purities because the nature of the impurities (and their formula weights) was usually not known. Extraneous signals observed for **1**, **2**, and **14** (Fig. 2) may represent solvent or other non-sphingolipid impurities. Only one ^{31}P NMR signal was observed for **1** and **2**, but hydrolysis to the unphosphorylated base (39) would not be detected. Extraneous NMR signals at δ_H ~4.3 and 2.82 (td, 6.0, 1.1 Hz) and δ_P–3.9 representing ~5 mole % of **12** were attributed to uncoupled material derived from **6**. Corresponding ^1H or ^{31}P NMR signals were not observed in samples of **11**.

[11] An unidentified FAB ion at *m*/*z* 507 was observed in mass spectra of **2** obtained from both glycerol and NBA matrices. The corresponding ion at *m*/*z* 525 (among other less abundant high-mass ions) was observed in FAB spectra of **1** (glycerol and NBA matrices).

4:96). Evaporation of fractions 17–21 gave **8** (15 mg). Fractions 52–55 gave **13** (60 mg, 46% yield) as a clear oil: single component on TLC (chloroform–methanol 9:1, R_f 0.35); FAB–MS (NBA), 534 (31, M + Na), 512 (85, M + H), 308 (70, M–OP(O)($OCH_2CH_2CN)_2$), 264 (95), 55 (100); high resolution FAB–MS (NBA), calcd. for $C_{25}H_{42}O_6N_3PNa$, 534.2709, found 534.2734; IR, ν_{max} 3430, 2924, 2855, 2259, 1788, 1466, 1402, 1275, 1227, 1082, 1036, 1009, 974 cm^{-1}; ^1H NMR, >96% purity, δ_H 5.68 (br s, NH), 5.898 (dtd, 15.4, 6.8, 0.9 Hz, H–5), 5.550 (ddt, 15.3, 7.9, 1.5, H–4), 4.720 (ddd, 7.9, 5.6, 0.9 Hz, H–3), 4.333 and 4.329 (dt, 8.0, 5.9 Hz and dtd, 8.2, 5.9, 1.3 Hz, 4H, $OC\underline{H}_2CH_2CN$), 4.271 (ddd, 11.1, 8.1, 3.1 Hz, H–1), 4.121 (ddd, 11.1, 7.9, 5.2 Hz, H–1), 3.797 (tdt, 5.3, 3.1, 1.1 Hz, H–2), 2.814 (tdd, 5.9, 2.6*, 1.1* Hz, 4H, 2 × $OCH_2C\underline{H}_2CN$), 2.08 (m, 2H), 1.39 (m, 2H), 1.26 (m, 20 H), 0.881 (t, 7 Hz, 3H); ^{13}C NMR, δ_C 158.84, 138.14 (C–5), 125.18 (C–4), 116.71 (d, 11.2* Hz, CN), 78.82 (C–3), 68.02 (d, 5.6 Hz, C–1), 62.76 (t, 5.5* Hz, $O\underline{C}H_2CH_2CN$), 57.88 (d, 6.9 Hz, C–2), 32.05 (C–6), 31.85, 29.62 (2C), 29.61, 29.58, 29.52, 29.39, 29.29, 29.13, 28.57 (C–7), 22.62, 19.66 (dd, 7.4, 1.8* Hz, $\underline{C}H_2CN$), 14.06.

Cyclic carbamate derivative of D-*erythro*-sphingosine-1-phosphate (14)

A solution of **13** (35 mg) in dimethylamine (40% in ethanol, 10 ml) was stirred at 45°C for 48 h. The solution was evaporated to dryness and the residue was dissolved quickly in hot acetic acid (0.5 ml). The product was immediately precipitated by addition of water (1.0 ml) followed by vortexing and centrifugation at 4°C for 10 min. The precipitate was washed with water (2 × 0.5 ml), acetone (2 × 1 ml), and diethyl ether (2 × 1 ml) and dried in vacuo for 4 h to yield **14** as a white solid (10 mg, 36% yield): single component on TLC (R_f 0.76, chloroform–methanol–water–acetic acid 30:30:2:5); FAB–MS (glycerol + NaCl), 450 (28), 428 (100, M + Na), 406 (M + H); FAB (glycerol + AcOH), 406 (100); IR, ν_{max} 3400, 2920, 2851, 1726, 1466, 1404, 1227, 1113, 1053, 1020, 964 cm^{-1}; ^1H NMR (CD_3OD–CD_3COOD, 1:1), ~95% purity,[10] δ_H 5.88 (dt, 15.4, 6.8 Hz, H–5), 5.578 (ddt, 15.4, 7.6 Hz, 1.4 Hz, H–4), 4.82 (br dd, 7.3, 5.8 Hz, H–3), 3.98 (br, H–1), 3.93 (br, H–1), 3.77 (br, H–2), 2.06 (m, 2H), 1.37 (m, 2H), 1.24 (m, 20 H), 0.84 (t, 7 Hz, 3H).

DISCUSSION

Most chemical syntheses of sphingosine-1-phosphate (**2**) have been quite lengthy owing to the perception that regioselective monophosphorylation at the 1-hydroxyl requires protection of the 3-hydroxy group. In early work by Weiss (37), *N*-protected sphingosine underwent 1,3-diphosphorylation with diphenylphosphoryl chloride, although monophosphorylation at the 1-hydroxyl was observed with dihydrosphingosine. Catalytic hydrogenolysis of the dihydro intermediate gave dihydrosphingosine-1-phosphate, but several attempts to prepare sphingosine-1-phosphate were unsuccessful (37). In a total synthesis of dl-sphingomyelin, Shapiro, Flowers, and Spector-Shefer (38) monophosphorylated a 2,3-diprotected sphingosine intermediate, but this methodology was not used to prepare **2**. Enzymatic methods were also used to convert sphingosine, phytosphingosine, and other sphingolipid bases to their 1-phosphate derivatives on a microscale (23, 39). As synthetic routes to 1-phosphate derivatives, all these approaches had serious deficiencies.

Interest in nucleotide synthesis led to the development of better methods for phosphorylation of alcohols. An efficient synthetic scheme, entailing the condensation of alcohols with monochlorophosphite esters followed by oxidation with aqueous iodine to phosphate esters (40), was later improved by the introduction of the base-labile 2-cyanoethyl esters for deprotection under mild conditions (41) and replacement of phosphite esters by dialkylamino phosphoramidite esters (31, 42, and references therein). Phosphoramidites can be activated for coupling with alcohols under mild conditions, and fine control over the entire phosphorylation scheme can be maintained by choosing from a variety of dialkylamino groups, ester groups, and oxidizing reagents (31). Also, phosphoramidites can be manipulated to produce either phosphates or phospho-diesters (31), such as sphingomyelin. The phosphoramidites are relatively stable, and some can even withstand rapid chromatographic purification (31). Moreover, coupled phosphate ester intermediates can be purified on silica gel prior to deprotection. This purification step is especially important in syntheses of sphingosine-1-phosphate and its analogs, which are quite difficult to purify owing to their very limited solubilities in most solvent systems (39).

The phosphoramidite methodology, widely used in nucleotide and carbohydrate work, has been applied in several syntheses of sphingolipid phosphates. Sphingomyelin was prepared by sequential coupling of a monochlorophosphoramidite reagent with a ceramide (protected at the 3-hydroxyl) and choline tosylate (43). Subsequent syntheses of sphingomyelin (35) and d-*erythro*-sphingosine-1-phosphate (**2**) (35, 36, 44) by phosphoramidite coupling also involved long-chain bases protected at the 3-hydroxyl. In 1994, Boumendjel and Miller (26) reported an efficient three-step synthesis of **2** from d-*erythro*-sphingosine. Under standard phosphoramidite coupling conditions, the *N*-Boc derivative of sphingosine and its saturated analog underwent monophosphorylation at the 1-hydroxyl without any protection of the 3-hydroxyl. Considering the availability of gram-quantities of sphingosine by isolation from cow brain (28, 45) and the difficulty of protecting and deprotecting the labile 3-hydroxy group, this synthesis represented a major advance over enzymatic preparations (23, 39) of **2** and lengthy approaches based on total synthesis (35, 36, 44). The successful monophosphorylation of *N*-Boc-sphingosine (**8**) suggested possible application to the synthesis of d-*ribo*-phytosphingosine-1-phosphate (**1**).

Our chemical synthesis of (**1**) started with d-*ribo*-phytosphingosine (**3**), which was obtained in high purity by saponification of the tetraacetate derivative. This work

was guided by a parallel synthesis of d-*erythro*-sphingosine-1-phosphate (**2**) from d-*erythro*-sphingosine (**4**) according to the method of Boumendjel and Miller (26). These syntheses are outlined in **Fig. 1**. Protection of **3** and **4** as their *N*-Boc derivatives **7** and **8** proceeded smoothly in high yield. Owing perhaps to poor solubility in dichloromethane, **3** reacted more slowly than **4**. The next step consisted of regioselective coupling of the *N*-Boc derivatives **7** and **8** to phosphoramidite **6**, a rather labile reagent that can be prepared from the commercially available phosphoramidite **5** and purified by flash chromatography (26) or short-column chromatography (31) on silica gel. We observed significant hydrolysis of **6** when the chromatographic purification exceeded 30 min.

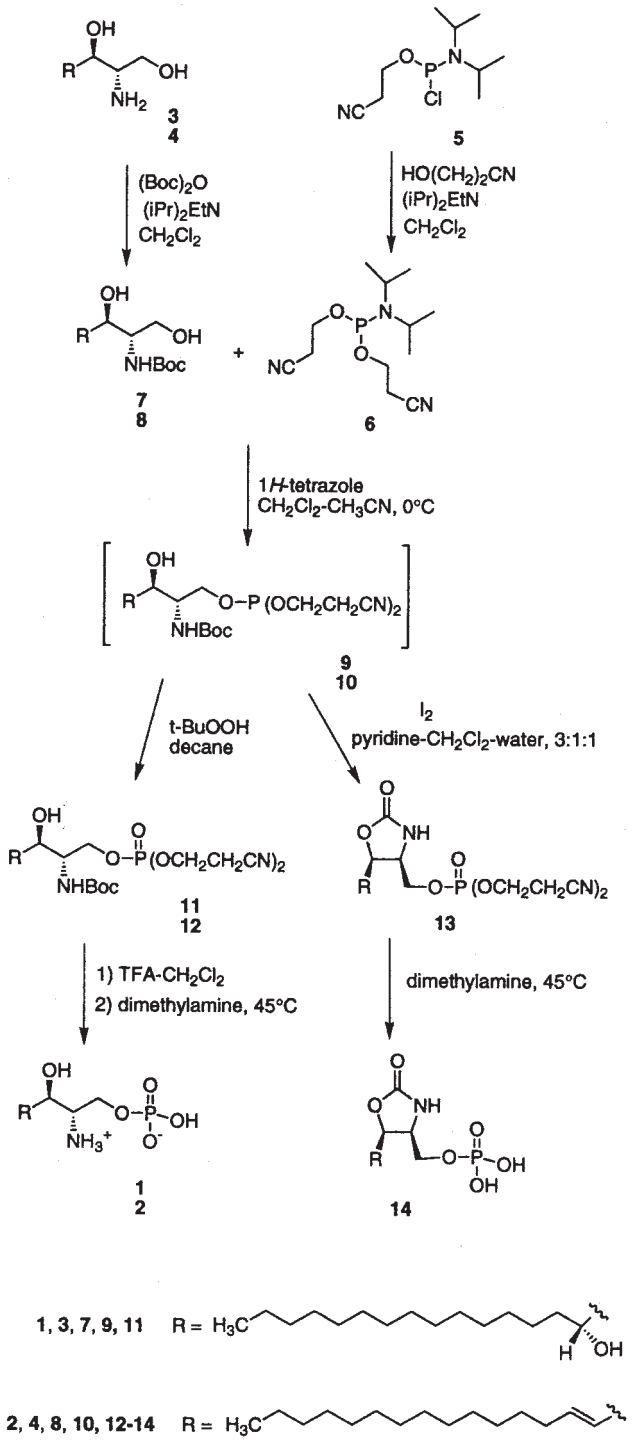


Fig. 1. Synthesis of d-*ribo*-phytosphingosine-1-phosphate (**1**) and d-*erythro*-sphingosine-1-phosphate (**2**).

We encountered several difficulties in carrying out the coupling reaction according to the procedure of Boumendjel and Miller (26). Our initial reaction attempts using standard anhydrous conditions and drying of the *N*-Boc sphingosine **8** in vacuo for 48 h resulted in inefficient conversion to the coupled phosphite intermediate **10** as judged by TLC. Drying the reaction solution containing **8** overnight over molecular sieves gave much better results, presumably due to removal of moisture tenaciously bound to **8**. We initially carried out the coupling reaction at room temperature, as no reaction temperature was specified (26). Under these conditions, TLC showed a 2:3 mixture of the desired **10** and a nonpolar byproduct. We discovered that the coupling reaction also proceeded at 0°C. The lower reaction temperature markedly reduced the amount of nonpolar material, which may represent 1,3-diphosphorylation of **8**.

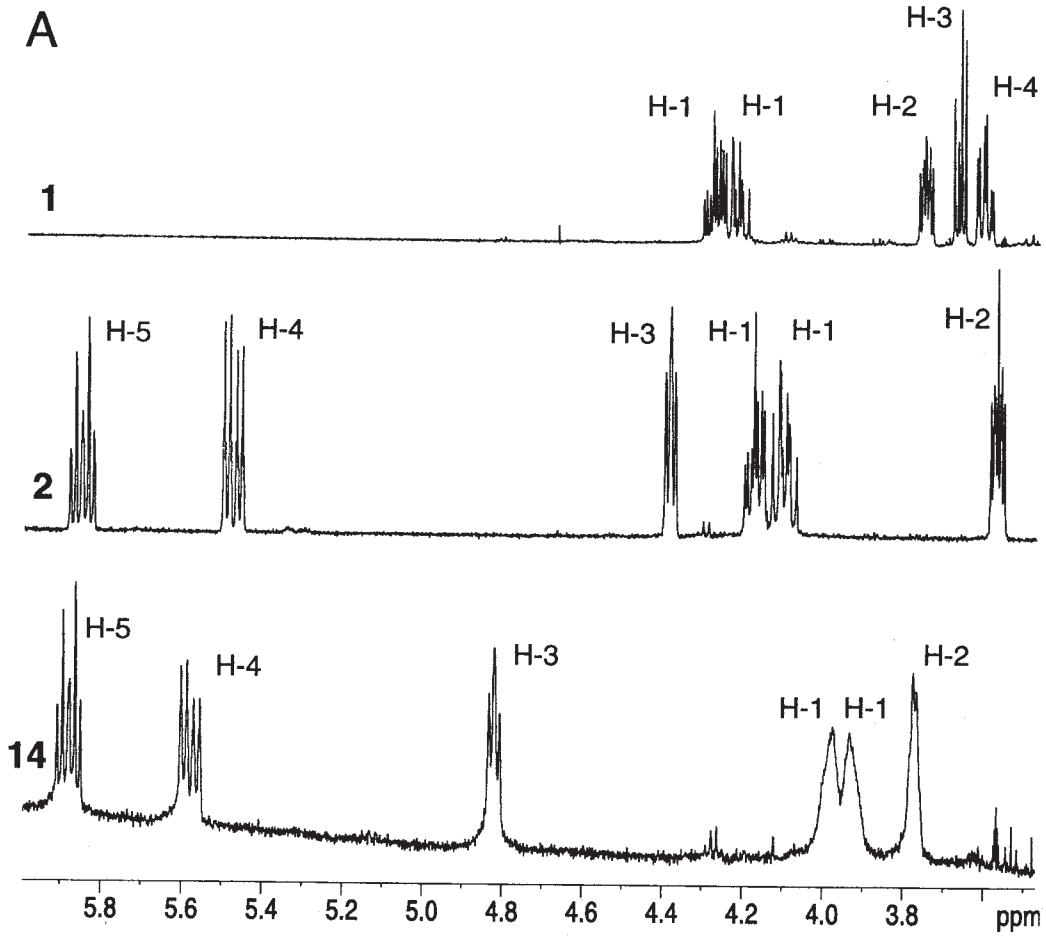
Further difficulties were encountered in reproducing the reported oxidation and deprotection conditions. Boumendjel and Miller (26) described the in situ oxidation of phosphite **10** to phosphate **12** by treatment with iodine in pyridine–dichloromethane–water 3:1:1. In several attempts to duplicate this reaction, we consistently obtained the cyclic carbamate **13** but detected no formation of the desired phosphate **12**. Based on studies of the other reagents known to oxidize similar phosphites to phosphates (31), we replaced iodine by *t*-butyl peroxide, which furnished **11** and **12** in good yields without any formation of cyclic carbamate. The *N*-Boc protecting group was easily removed with trifluoroacetic acid, but complete deprotection to d-*erythro*-sphingosine-1-phosphate (**2**) with warm dimethylamine required 48 h rather than the 6 h specified by Boumendjel and Miller (26). Because the deprotection is difficult to follow by TLC (owing to poor charring of the free bases with molybdic acid) and NMR (due to elaborate sample preparation requirements), we carried out all phosphate ester hydrolyses for 48 h. Comparable reaction times (36–72 h) were allotted by others for similar deprotections (36). As shown by NMR (see below), the acidic deprotection conditions did not cause detectable epimerization of **2** to its *threo* isomer.

Using our modified set of reagents and conditions, we obtained the desired sphingosine-1-phosphate (**2**) and phytosphingosine-1-phosphate (**1**). The overall yield of **1** from **3** was 43% and that of **2** from **4** was 32%. The sphingolipid phosphates **1** and **2** and their synthetic precursors **11** and **12** were characterized by NMR, MS, IR, and TLC. The spectroscopic data were critical in establishing the presence of a single monosubstituted phosphate group at C–1. High-resolution FAB–MS of **1** and **2** showed ions corresponding to M + H. The MS evidence was confirmed by integrations of the ^{1}H NMR spectra of **11** and **12** showing two cyanoethoxy groups and one C_{18} sphingolipid chain. ^{1}H–^{31}P couplings in the ^{1}H NMR spectra of **1**, **2**, **11**, and

12 indicated the presence a phosphate substituent only at C–1, and this conclusion was confirmed by ^{13}C–^{31}P couplings in the ^{13}C spectra of **11** and **12**. These findings were based on signal assignments established rigorously from HSQC and COSY spectra. The NMR spectra of **1**, **2**, **11**, and **12** showed good to excellent purities, and spectra of **2** and **12** were compatible with data reported previously (26, 44). Similarities between the NMR spectra of **11** and **12** and those of **1** and **2** further supported the structure of synthetic d-*ribo*-phytosphingosine-1-phosphate (**1**).

^1H NMR spectra of **1**, **2**, and the cyclic carbamate **14** are shown in **Fig. 2**, with slight resolution enhancement. The spectra of **1** and **2** show well-resolved multiplets representing the polar head region (H–1 to H–5). The relatively clean baselines indicate the absence of significant levels of other stereoisomers, such as the *threo* isomer of **2**, for which ^1H NMR data have been shown (44). With the aid of stronger Gaussian apodization, most resonances shown in Fig. 2 became first-order multiplets, analysis of which led to precise coupling constants and reliable signal assignments. This first detailed set of coupling constants for 1-phosphate derivatives (Fig. 2, panel B) should be useful for identification of samples of **1** and **2** and for studies of conformational analysis (43, 46) by comparison with similar data for sphingomyelin (43, 46) and long-chain bases (28).

We also isolated the cyclic carbamate **13** and converted it to the 1-phosphosphingosine derivative **14** (Fig. 1). The structures of **13** and **14** were elucidated from their NMR and mass spectra. The FAB–MS showed ions correspond-



B

	H1a-H1b	H1a-H2	H1b-H2	H1a-P	H1b-P	H2-H3	H3-H4	H4-H5
1	12.0	3.6	7.6	7.9	9.2	5.1	8.8	8.2, 2.7
2	12.1	8.4	3.3	7.9	8.6	5.0	9.2	15.4
14						~5.6	7.6	15.4

Fig. 2. Panel A: 500 MHz ^1H NMR spectra of d-*ribo*-phytosphingosine-1-phosphate (**1**), d-*erythro*-sphingosine-1-phosphate (**2**), and cyclic carbamate **14**, each ~3 mM in CD_3OD–CD_3COOD 1:1, apodized with slight resolution enhancement (LB −1, GB 0.2). Panel B: selected coupling constants in Hz derived from analysis of the above spectra (processed with stronger Gaussian apodization).

ing to M + H and M + Na. The ^{1}H NMR spectra of **13** and **14** showed olefinic signals closely resembling those of sphingosine, with H–5 showing a COSY correlation to an allylic H–6 pair at δ 2.1 (thus excluding any type of iodolactonization). Based on these assignments for H–4 and H–5, signals corresponding to C–3, C–2, and C–1 and their attached protons were established from HSQC and COSY spectra of **13**. ^{13}C–^{31}P couplings of 5.6 and 7.1 Hz for C–1 and C–2 indicated phosphorylation at C–1. Spectra of **13** showed the absence of ^{1}H and ^{13}C signals corresponding to the *t*-butyl group but the presence of a carbonyl signal at δ_C 159, which was correlated to H–2 and H–3 in the HMBC spectrum. These observations, indicating cyclization of the Boc carbonyl to the C–3 hydroxyl, were supported by the observation of downfield shifts of H–3 and C–3 typical of esterification and by the shift of the carbonyl IR absorption from 1682 cm^{-1} in **12** to 1755 cm^{-1} in **13**. Interestingly, the two diastereotopic cyanoethyl groups showed increased non-equivalence in the ^{1}H and ^{13}C NMR spectra of **13** relative to spectra of **11** and **12**. Although we have not studied the mechanism for the formation of **13**, the generation of pyridinium and triiodide ions during the reaction may be involved in byproduct formation. No cyclic carbamate byproduct was observed in phosphite oxidations with *t*-butyl peroxide, which produces only *t*-butanol.

In summary, we have demonstrated a brief, efficient chemical synthesis of d-*ribo*-phytosphingosine-1-phosphate (**1**) from d-*ribo*-phytosphingosine (**3**). Our approach is based on standard phosphoramidite methodology for preparing phosphate derivatives of alcohols (31) and on the finding that sphingosine can be monophosphorylated at the 1-hydroxyl without protection of the 3-hydroxyl (26). We were unable to duplicate the Boumendjel and Miller synthesis (26) of d-*erythro*-sphingosine-1-phosphate from d-*erythro*-sphingosine without important modifications in reagents and reaction conditions. These synthetic improvements should be valuable to researchers wishing to prepare 1-phosphate derivatives of sphingolipid bases. The availability of the 1-phosphate derivative of phytosphingosine should facilitate research on its potential activities in a variety of systems.

We gratefully acknowledge the support of the National Institutes of Health (HL-49122) and the Robert A. Welch Foundation (C-583).

Manuscript received 8 July 1998 and in revised form 18 September 1998.

REFERENCES

1. Hirschberg, C. B., A. Kisic, and G. J. Schroepfer, Jr. 1970. Enzymatic formation of dihydrosphingosine-1-phosphate. *J. Biol. Chem.* **245:** 3084–3090.
2. Louie, D. D., A. Kisic, and G. J. Schroepfer, Jr. 1976. Sphingolipid base metabolism. Purification and properties of sphinganine kinase from brain. *J. Biol. Chem.* **251:** 4557–4564.
3. Akino, T., T. Shimojo, Y. Miura, and G. J. Schroepfer, Jr. 1974. Stereospecificity of proton uptake in the enzymatic conversion of sphinganine-1-phosphate to ethanolamine-1-phosphate. *J. Am. Chem. Soc.* **96:** 939–940.
4. Shimojo, T., and G. J. Schroepfer, Jr. 1976. Sphingolipid base metabolism. Sphinganine-1-phosphate lyase: identification of ethanolamine-1-phosphate as product. *Biochim. Biophys. Acta* **431:** 433–446.
5. Shimojo, T., T. Akino, Y. Miura, and G. J. Schroepfer, Jr. 1976. Sphingolipid base metabolism. Stereospecific uptake of proton in the enzymatic conversion of sphinganine-1-phosphate to ethanolamine-1-phosphate. *J. Biol. Chem.* **251:** 4448–4457.
6. Olivera A., T. Kohama, Z. Tu, S. Milstien, and S. Spiegel. 1998. Purification and characterization of rat kidney sphingosine kinase. *J. Biol. Chem.* **273:** 12576–12583.
7. Yatomi, Y., R. J. Welch, and Y. Igarashi. 1997. Distribution of sphingosine-1-phosphate, a bioactive sphingolipid, in rat tissues. *FEBS Lett.* **404:** 173–174.
8. Yatomi, Y., Y. Igarashi, L. Yang, N. Hisano, R. Qi, N. Asazuma, K. Satoh, Y. Ozaki, and S. Kume. 1997. Sphingosine-1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem. (Tokyo).* **121:** 969–973.
9. Buehrer, B. M., E. S. Bardes, and R. M. Bell. 1996. Protein kinase C-dependent regulation of human erythroleukemia (HEL) cell sphingosine kinase activity. *Biochim. Biophys. Acta.* **1303:** 233–242.
10. Kleuser, B., O. Cuvillier, and S. Spiegel. 1998. 1α,25-Dihydroxyvitamin D_3 inhibits programmed cell death in HL-60 cells by activation of sphingosine kinase. *Cancer Res.* **58:** 1817–1824.
11. Rius, R. A., L. C. Edsall, and S. Spiegel. 1997. Activation of sphingosine kinase in pheochromocytoma PC12 neuronal cells in response to trophic factors. *FEBS Letts.* **417:** 173–176.
12. Zhou, J., and J. D. Saba. 1998. Identification of the first mammalian sphingosine phosphate lyase gene and its functional expression in yeast. *Biochem. Biophys. Res. Commun.* **242:** 502–507.
13. Mao, C., M. Wadleigh, G. M. Jenkins, Y. A. Hannun, and L. M. Obeid. 1997. Identification and characterization of *Saccharomyces cerevisiae* dihydrosphingosine-1-phosphate phosphatase. *J. Biol. Chem.* **272:** 28690–28694.
14. Lee, M-J., J. R. Van Brocklyn, S. Thangada, C. H. Liu, A. R. Hand, R. Menzeleev, S. Spiegel, and T. Hla. 1998. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science.* **279:** 1552–1555.
15. Spiegel, S., and A. H. Merrill, Jr. 1996. Sphingolipid metabolism and cell growth regulation. *FASEB J.* **10:** 1388–1397.
16. Karlsson, K-A. 1970. Sphingolipid long chain bases. *Lipids.* **5:** 878–891.
17. Karlsson, K-A., and E. Martensson. 1968. Studies on sphingosines. XIV. On the phytosphingosine content of major human kidney glycolipids. *Biochim. Biophys. Acta.* **152:** 230–233.
18. Carter, H. E., and C. B. Hirschberg. 1968. Phytosphingosines and branched sphingosines in kidney. *Biochemistry.* **7:** 2296–2300.
19. Breimer, M. E., G. C. Hansson, K. A. Karlsson, H. Leffler, W. Pimlott, and B. E. Samuelsson. 1979. Selected ion monitoring of glycosphingolipid mixtures. Identification of several blood group type glycolipids in the small intestine of an individual rabbit. *Biomed. Mass Spectrom.* **6:** 231–241.
20. Bouhours, D., and J-F. Bouhours. 1984. Phytosphingosine in intestinal glycolipids of the rat fetus. *Biochim. Biophys. Acta.* **794:** 169–171.
21. Mikami, M., K. Tukazaki, S. Nozawa, M. Iwamori, and Y. Nagai. 1992. Menstrual cycle-associated expression of 2-hydroxy fatty acyl phytosphingosine-containing GlcCer, LacCer and Gb_3Cer in human uterine endometrium. *Biochim. Biophys. Acta.* **1125:** 104–109.
22. Crossman, M. W., and C. B. Hirschberg. 1977. Biosynthesis of phytosphingosine in the rat. *J. Biol. Chem.* **252:** 5815–5819.
23. W. Stoffel, G. Assmann, and E. Binczek. 1970. Metabolism of sphingosine bases, XIII. Enzymatic synthesis of 1-phosphate esters of 4t-sphingenine (sphingosine), sphinganine (dihydrosphingosine), 4-hydroxysphinganine (phytosphingosine) and 3-dehydrosphinganine by erythrocytes. *Hoppe-Seyler's Z. Physiol. Chem.* **351:** 635–642.
24. Nagiec, M. M., M. Skrzypek, E. E. Nagiec, R. L. Lester, and R. C. Dickson. 1998. The LCB4 (*YOR171c*) and LCB5 (*YLR260w*) genes of *Saccharomyces* encode sphingoid long chain base kinases. *J. Biol. Chem.* **273:** 19437–19442.
25. Lanterman, M. M., and J. D. Saba. 1998. Characterization of sphingosine kinase (SK) activity in *Saccharomyces cerevisiae* and isolation of SK-deficient mutants. *Biochem. J.* **332:** 525–531.
26. Boumendjel, A., and S. P. F. Miller. 1994. Synthesis of sphingosine-1-phosphate and dihydrosphingosine-1-phosphate. *J. Lipid Res.* **35:** 2305–2311.

27. Boumendjel, A., and S. P. F. Miller. 1995. Method for preparation of sphingoid bases. U.S. Patent 5,430,169.
28. Kisic, A., M. Tsuda, R. J. Kulmacz, W. K. Wilson, and G. J. Schroepfer, Jr. 1995. Sphingolipid bases. A revisitation of the O-methyl derivatives of sphingosine. Isolation and characterization of diacetate derivatives, with revised ^{13}C nuclear magnetic resonance assignments for d-*erythro*-sphingosine. *J. Lipid Res.* **36:** 787–803.
29. Kulmacz, R. J., A. Kisic, and G. J. Schroepfer, Jr. 1979. Sphingolipid base metabolism. Chemical syntheses and properties of *N*-acetyl derivatives of 4R-, 4S-, 5R-, and 5S-hydroxysphinganine. *Chem. Phys. Lipids.* **23:** 291–319.
30. Stodola, F. H., and L. J. Wickerham. 1960. Formation of extracellular sphingolipids by microorganisms. II Structural studies on tetraacetylphytosphingosine from the yeast *Hansenula ciferrii*. *J. Biol. Chem.* **235:** 2584–2585.
31. Bannwarth, W., and A. Trzeciak. 1987. A simple and effective chemical phosphorylation procedure for biomolecules. *Helv. Chim. Acta.* **70:** 175–186.
32. Li, Y-L., X-H. Mao, and Y-L. Wu. 1995. Stereoselective syntheses of d-*ribo*- and l-*lyxo*-phytosphingosine. *J. Chem. Soc., Perkin Trans. 1:* 1559–1563.
33. Murakami, T., H. Minamikama, and M. Hato. 1994. Regio- and stereocontrolled synthesis of d-*erythro*-sphingosine and phytosphingosine from d-glucosamine. *Tetrahedron Lett.* **35:** 745–748.
34. Carter, H. E., W. D. Celmer, W. M. Lands, K. L. Mueller, and H. H. Tomizawa. 1954. Biochemistry of the sphingolipids. VIII. Occurrence of a long chain base in plant phosphatides. *J. Biol. Chem.* **206:** 613–623.
35. Kratzer, B., and R. R. Schmidt. 1995. Efficient synthesis of sphingosine-1-phosphate, ceramide-1-phosphate, lysosphingomyelin, and sphingomyelin. *Liebigs Ann. Chem.* 957–963.
36. Kratzer, B., and R. R. Schmidt. 1993. An efficient synthesis of sphingosine-1-phosphate. *Tetrahedron Lett.* **34:** 1761–1764.
37. Weiss , B. 1957. Synthesis of several phosphorylated derivates of dihydrosphingosine. *J. Am. Chem. Soc.* **79:** 5553–5557.
38. Shapiro, D., H. M. Flowers, and S. Spector-Shefer. 1959. Synthetic studies on sphingolipids. IV. The synthesis of sphingomyelin. *J. Am. Chem. Soc.* **81:** 4360–4364.
39. Van Veldhoven, P. P., R. J. Foglesong, and R. M. Bell. 1989. A facile enzymatic synthesis of sphingosine-1-phosphate and dihyrosphingosine-1-phosphate. *J. Lipid Res.* **30:** 611–616.
40. Letsinger, R. L., J. L. Finnan, G. A. Heavner, and W. B. Lunsford. 1975. Phosphite coupling procedure for generating internucleotide links. *J. Am. Chem. Soc.* **97:** 3278–3279.
41. Sinha, N. D., J. Biernat, and H. Köster. 1983. β-Cyanoethyl N,N-dialkylamino/N-morpholinomonochloro phosphoamidities, new phosphitylating agents facilitating ease of deprotection and work-up of synthesized oligonucleotides. *Tetrahedron Lett.* **24:** 5843–5846.
42. Westerduin, P., G. H. Veeneman, J. E. Marugg, G. A. van der Marel, and J. H. van Boom. 1986. An approach to the synthesis of α-l-fucopyranosyl phosphoric mono- and diesters via phosphite intermediates. *Tetrahedron Lett.* **27:** 1211–1214.
43. Bruzik, K. S. 1988. Synthesis and spectral properties of chemically and stereochemically homogeneous sphingomyelin and its analogues. *J. Chem. Soc., Perkin Trans. 1:* 423–431.
44. Ruan, F., Y. Sadahira, S. Hakomori, and Y. Igarashi. 1992. Chemical synthesis of d-*erythro*-sphingosine-1-phosphate and its inhibitory effect on cell motility. *Bioorg. Med. Chem. Lett.* **2:** 973–978.
45. Carter, H. E., W. J. Haines, W. E. Ledyard, and W. P. Norris. 1947. Biochemistry of the sphingolipides. Preparation of sphingolipides from beef brain and spinal cord. *J. Biol. Chem.* **169:** 77–82.
46. Bruzik, K. S. 1988. Conformation of the polar headgroup of sphingomyelin and its analogues. *Biochim. Biophys. Acta.* **939:** 315–326.