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

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Abstract: d-erythro-Sphingosine-1-phosphate (2), an intermediate in sphingosine metabolism, shows a diversity of biological activities. Comparable roles might be anticipated to mediate in sphingosine metabolism, shows a diversity of 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. We now report the first chemical synthesis and characterization of d-ribo-phytosphingosine-1-phosphate. 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-1H correlation spectroscopy; EI, electron impact; FAB, fast atom bombardment; GC, gas chromatography; HMBEC, heteronuclear multiple bond correlation; HSQC, 1H-13C 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.

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1Chemical 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, (2hydroxy-1-hydroxymethyl)heptadecyl-1,1-dimethyl ester, (R-S*); 12, 5,7-dioxa-2-aza-6-phosphonanoic acid, 9-cyano-6-(2-cyanoethoxy)-3-{1-hydroxy-2-hexadecenyl}, 1,1-dimethyl 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 CDCl₃ solutions (unless specified otherwise) with a Bruker AMX 500 instrument (500 MHz for ¹H) and referenced as follows: internal tetramethylsilane (0.0 ppm, ¹H) and CDCl₃ (77.0 ppm, ¹³C) for CDCl₃ and CDCl₃-CD₂OD mixtures; CD₂OD (3.30 ppm, ¹H and 50.0 ppm, ¹³C) for CD₂OD and CD₂OD-CD₂COOD mixtures; internal ³P(O)(OMe)₂ (140.4 ppm, ³¹P). CDCl₃-D₂O designates addition of a drop of D₂O to a CDCl₃ solution to improve spectral definition. COSY (H–H 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 CDCl₃ 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 F₂₅₄₄ 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 as a 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 x 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-ribophytosphingosine tetracetate, isolated from cultures of Hansenula ciferri (30), was a gift from H.E. Carter. 2-Cyanoethyl N,N-diisopropylchlorophosphoramidite (5), N,N-diisopropylethylamine, 3-hydroxypropionitrile, di-i-butyl dicarbonate, anhydrous acetonitrile, ¹H-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 on silica gel (150 x 10 mm i.d. column; elution with ethyl acetate-hexane 1:3) gave 6 as a clear oil (0.48 g, 84% yield). Triphenylsilylethyltrimethylsilyl (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-ribophytosphingosine tetracetate**

Crude d-ribophytosphingosine tetracetate (2.0 g) was purified by MPLC (500 x 25 mm column; elution with ethyl acetate-hexane 1:9 (500 ml) and ethyl acetate-hexane 2:8). Fractions 50-65 gave the tetracetate as a white solid (1.5 g, 75% recovery): mp, 45-46°C (lit. 49-50°C (30)); single component on TLC (Rₐ 0.30, chloroform-methanol 9:1) and on GC (tR 17.3 min, 250°C, helium carrier gas at 1.1 kg/cm²): EI-MS, 412† (3, M–CH₂OAc), 383 (3), 366 (9), 353 (11, M–C₄H₉O₂), 310† (11, M–C₅H₉O₂), 305† (19, M–C₆H₁₀O₂N), 292† (14, M–C₅H₁₀O₂), 264 (6), 145 (41), 144 (67, C₇H₁₀O₆N), 84 (100); high resolution EI-MS, calcd. for C₂₄H₄₈O₇N (M †), 486.3436, found 486.3436; ¹H NMR, (largely aqueous) 6H 0.05 (d, 9.4 Hz, NH), 5.10 (dd, 8.3, 3.1 Hz, H–3), 4.93 (dt, 9.9, 3.2 Hz, H–4), 4.47 (dd, 9.4, 8.3, 4.9, 3.1 Hz, H–2), 4.28 (dd, 9.9, 3.2 Hz, H–4). ¹H NMR, 3.90 (t, 6.2 Hz, 2H), 2.61 (t, 6.2 Hz, 2H)) and observed 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 (δH 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.

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 (δH 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.

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**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 (Rf 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)); [α]D 22 +8.6 (c 1, pyridine) (lit. [α]D 22 +8.9 (c 0.6, pyridine) (32), [α]D 22 +8.7 (c 0.8, pyridine) (33)); single component on TLC (Rf 0.50, chloroform–methanol–ammonia 100:25.5:2.5) and GC (as the tetra-TMS ether; tR, 35.0 min); FAB–MS (NBA), 340 (25, M + Na), 318 (100, M + H), 300 (32, M + H–H2O), 282 (26, M + H – 2H2O); 1H NMR (CD3OD), 3.85 (dd, 11.3, 4.0 Hz, H–1), 3.60 (dd, 11.3, 7.9 Hz, H–1), 3.47 (m, 4–H), 3.46 (m, 4–H), 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); 13C NMR (CD3OD), δc 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-ribo-phytosphingosine (7) and N-t-butyloxycarbonyl-erythro-phytosphingosine (8)**

Di-tert-butyldicarbonate (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-disopropylethylamine (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 (Rf 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 C23H46O2N (M + H), 418.3532, found 418.3532; IR, νmax 3275, 2953, 2920, 2851, 1617, 1547, 1469, 1364, 1254, 1175, 1057 cm–1; 1H NMR (CDCl3-D2O), ~98% purity; δH 5.34 (d, 7.9 Hz, NH), 3.88 (dd, 11.2, 2.8 Hz, H–1), 3.84 (m, H–4), 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); 13C NMR (CDCl3-D2O), δc 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 8 (100 mg, 0.25 mmol) gave 12 as a...
white solid (110 mg, 75% yield): single component on TLC (Rf 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 (10); high resolution FAB-MS (NBA), calcld. for C<sub>29</sub>H<sub>55</sub>O<sub>7</sub>N<sub>2</sub>P (M + H), 586.3621, found 586.3604; IR, υ<sub>max</sub> 3331, 2920, 2850, 1645, 1541, 1458, 1190, 1067, 930, 824, 721 cm<sup>-1</sup>;<sup>1</sup>H NMR (CD<sub>3</sub>OD-CD<sub>3</sub>COOD, 1:1), δ<sub>H</sub> 4.28 (d, 12.0, 7.9, 3.6 Hz, H-1), 4.23 (3d, 12.0, 9.2, 7.6 Hz, H-1), 3.75 (dd, 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); 31p NMR (CD<sub>3</sub>OD-CD<sub>3</sub>COOD, 1:1), δ<sub>p</sub> 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 (Rf 0.32, chloroform-methanol-water-acetic acid 30:30:2:5); ~98% purity by δ<sub>p</sub> NMR; FAB-MS (glycerol), 507<sup>11</sup> (16), 380 (32, M + H), 241 (40), 149 (100); high resolution FAB-MS (NBA), calcld. for C<sub>18</sub>H<sub>39</sub>O<sub>5</sub>N<sub>2</sub>P, 380.2566, found 380.2541 (M + H); IR, υ<sub>max</sub> 3350, 2953, 2918, 2851, 1643, 1541, 1458, 1190, 1067, 930 cm<sup>-1</sup>; 1H NMR (CD<sub>3</sub>OD-CD<sub>3</sub>COOD, 1:1), δ<sub>H</sub> 8.11 (26), 8.0, 7.9, 7.8 Hz). Corresponding 1H or 31P NMR signals were not observed in samples of 11.

**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 1H-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<sub>f</sub> 0.85, chloroform-methanol 9:1). To the solution was added dropwise a solution of iodine (0.4 ml in pyridinedichloromethane–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<sub>f</sub> 0.35, chloroform–methanol 9:1). The reaction mixture was diluted with dichloromethane (30 ml), washed with aqueous sodium thiosulfate (5 ml, 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 9:1).

<sup>9</sup>TLC analysis of the reaction mixture immediately prior to addition of t-butyl peroxide showed the formation of 10 (R<sub>f</sub> 0.55, chloroform–methanol 9:1, 70%) and an unidentified byproduct of higher mobility (R<sub>f</sub> 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<sub>f</sub> 0.90, chloroform–methanol 9:1). A faint upper spot (R<sub>f</sub> 0.9, chloroform–methanol 9:1) was also observed in crude 8.

<sup>10</sup>Integration of δ<sub>H</sub> 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-spinning signals. Only one IP NMR signal was observed for 1 and 2, but hydrolysis to the unphosphorylated base (39) would not be detected. Extraneous NMR signals at δ<sub>p</sub> < −4.3 and 2.62 (td, 6.0, 1.1 Hz) and δ<sub>H</sub> < −3.9 representing ~5 mole % of 12 were attributed to uncoupled material derived from 6. Corresponding δ<sub>H</sub> or δ<sub>P</sub> NMR signals were not observed in samples of 11.
Fraction 52–55 gave a single component on TLC (chloroform–methanol 9:1, 2c), precipitate was washed with water (2c), quickly in hot acetic acid (0.5 ml). The product was immediately evaporated to dryness and the residue was dissolved in vacuo for 4 h to yield a single component on TLC (chloroform–methanol 9:1, 2c). Evaporation of fractions 17–21 gave a single component on TLC (chloroform–methanol–water–acetic acid 30:30:2:5); FAB–MS (glycerol Na), 406 (100); IR, $\nu_{\text{max}}$ 3400, 2950, 2850, 1750, 1470, 1420, 1240, 1070 cm$^{-1}$; $^1$H NMR, $\delta$ C 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 (dd, 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, H–2), 2.814 (tdd, 5.9, 2.6*, 1.1* Hz, 4H, 2C), 1.39 (m, 2H), 1.26 (m, 20 H), 0.881 (t, 7 Hz, 3H); $^{13}$C NMR, $\delta$ C 68.02 (d, 5.6 Hz, C–1), 62.76 (t, 5.5* Hz, OC), 58.47 (s, Nd), 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, $\text{CH}_3$CN), 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 x 0.5 ml), acetone (2 x 1 ml), and diethyl ether (2 x 1 ml) and dried in vacuo for 4 h to yield 14 as a white solid (10 mg, 36% yield): single component on TLC (Rf 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, $\nu_{\text{max}}$ 3400, 2920, 2851, 1726, 1466, 1404, 1240, 1153, 1053, 1020, 964 cm$^{-1}$; $^1$H NMR (CD$_3$OD–CD$_3$COOD, 1:1), ~95% purity; $^1$H NMR, 5.88 (dt, 15.4, 6.8 Hz, H–5), 5.578 (dtd, 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 dihydroxyphosphinosine. Catalytic hydrolysis of the dihydro intermediate gave dihydroxyphosphinosine-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 monochlorophosphoryl 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 protection under mild conditions (41) and replacement of phosphate 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 phosphodiesters (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.

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 phosphate 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 phosphate 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 tert-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 2 and 14, the acidic deprotection conditions did not cause detectable epimerization of 2 to its three isomers.

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 2 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 1H NMR spectra of 11 and 12 showing two cyanoethoxy groups and one C18 sphingolipid chain. 1H–31P couplings in the 1H NMR spectra of 1, 2, 11, and
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).

$^1$H 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 $^1$H 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-
ing to M + H and M + Na. The 1H 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 HSQ and COSY spectra of 13. 13C–31P 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 1H and 13C signals corresponding to the t-butyl group but the presence of a carbonyl signal at δC 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 1H and 13C 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 phosphate 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 (2). 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 Boumendel and Miller synthesis (26) of 3-ethyl-sphingosine-1-phosphate from d-ethyl-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.

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