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EFFICIENT SYNTHESIS OF DEUTERIUM- AND TRITIUM-LABELED D-*ERYTHRO*-SPHINGOSINE

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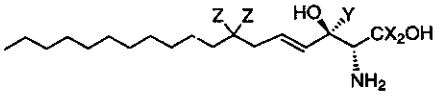
Summary

Deuterium- and tritium-labeled D-*erythro*-sphingosine (**1b** and **1c**) were prepared by an efficient approach based on a known stereoselective total synthesis. Tritium was introduced at C-1 by [^{3}H]$NaBH_4$ reduction in the final synthetic step to give **1c** of high radiochemical purity. 1,1,3-Trideuterio-D-*erythro*-sphingosine (**1b**) was prepared similarly and showed >99.5% enantiomeric purity and a high level of deuterium incorporation.

Key words: sphingosine, tritium labeling, NMR, MTPA derivative

Introduction

Sphingosine (**1a**) and other long-chain bases are the defining elements of sphingolipids, a complex and diverse class of lipids of current biomedical interest (1, 2). Among the reported preparations of deuterium- and tritium-labeled derivatives of sphingosine (Figure 1) and related sphingolipids, strategies for introducing the isotopic label have generally centered around hydride reductions of a carbonyl group at C-1 (3–6) or C-3 (7–12). Gaver and Sweeley (7) developed conditions for selective oxidation of *N*-acetyl sphingosine to the 3-keto analog, which was reduced



1a	X=H	Y=H	Z=H	**1d**	X=H	Y=D	Z=H
1b	X=D	Y=D	Z=H	**1e**	X=H	Y=T	Z=H
1c	X=T	Y=H	Z=H	**1f**	X=H	Y=H	Z=T

Figure 1. D-*erythro*-sphingosine (**1a**) and isotopically labeled derivatives.

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by $NaBD_4$ to a mixture of deuterium-labeled *threo*- and *erythro*-sphingosine (**1d**) accompanied by *threo*- and *erythro*-dihydrosphingosine arising from 1,4-addition. With various improvements, including milder oxidation conditions (13) and chromatographic separation of *erythro* and *threo* isomers, this methodology was used to prepare cerebrosides (8), gangliosides (9), and other sphingolipids (11) labeled with tritium at C-3. Alternative routes to labeled sphingosines include $[^3H]NaBH_4$ reduction of a 1-carboxylic ester intermediate in a total synthesis to furnish **1c** as either a DL mixture (6) or the D-*erythro*-isomer (5), similar reduction of a 1-aldehyde prepared from **1a** using labile trimethylsilyl protecting groups (4), reduction of 3-keto intermediates in stereospecific total syntheses of **1d** (12) and **1e** (10), and placement of tritium at C-7 in a total synthesis of DL-*erythro*-**1f** (14) (modifiable with current synthetic methodology (15) to produce exclusively the D isomer). Labeled dihydrosphingosine and other saturated sphingolipids have been prepared by total synthesis (12, 14), labeled $NaBH_4$ reduction of a suitably protected 1-aldehyde derivative (3), and catalytic addition of tritium gas (16) or catalytic D_2O exchange (17) with unsaturated sphingolipids.

These existing approaches to isotopically labeled sphingosine have various drawbacks. Methods beginning with natural D-*erythro*-sphingosine (**1a**) involve protection or oxidation reactions that suffer from limited selectivity. Other routes entail lengthy total syntheses. Reductions of 3-keto intermediates produce mixtures of *erythro* and *threo* isomers. Furthermore, most existing methods involve considerable synthetic or chromatographic manipulation after introduction of the label to remove protecting or masking groups and to separate stereoisomers. Interestingly, only one labeling scheme (10) utilizes any of the numerous stereoselective total syntheses reported for D-*erythro*-sphingosine. One of the more efficient total syntheses is noteworthy because the final step is a $NaBH_4$ reduction of a 1-carboxylic ester (**8a**) to sphingosine (15). This ideal opportunity for introduction of tritium is the basis of our synthesis of tritium-labeled sphingosine **1c** (Figure 2), which we needed for biological studies (18).

Results and Discussion

The unlabeled sphingosine ethyl ester derivative **8a** was prepared as described previously (15) in five steps in 45% overall yield from tetradecanal. A key step in this synthesis is the aldol-type reaction between aldehyde **5a** and imine-ester **6**. This stereoselective reaction creates two chiral centers to produce predominantly a single isomer **7a**. Although in principle the attached chiral auxiliary might also act as a resolving agent to permit chromatographic separation of **7a** from minor stereoisomeric byproducts, gradual hydrolysis of **7a** on silica gel precluded thorough purification. Nevertheless, hydrolysis of **7a** in HCl-tetrahydrofuran gave a single (19) diastereomeric product **8a** as judged by the 500-MHz ^1H NMR (20) spectrum. High enantiomeric purity might be expected because of the high stereoselectivity normally observed at the α carbon in similar additions of glycine enolate equivalents to aldehydes (21). This issue was not conclusively addressed previously (15) because evidence for the enantiomeric purity of **1a** was based only on specific rotation, which for D-*erythro*-sphingosine is very low (–1 for **1a** and –15 for its triacetate derivative (22)). Our analysis of the enantiomeric purity of the deuterium-labeled analog **1b** (see below) indicated a >200:1 ratio of D- to L-*erythro* isomers, a result indicating that **7a** is indeed formed with very high stereoselectivity at the α carbon. This finding adds to the already high appeal of the present total synthesis (15) as an efficient method for preparing both labeled and unlabeled D-*erythro*-sphingosine.

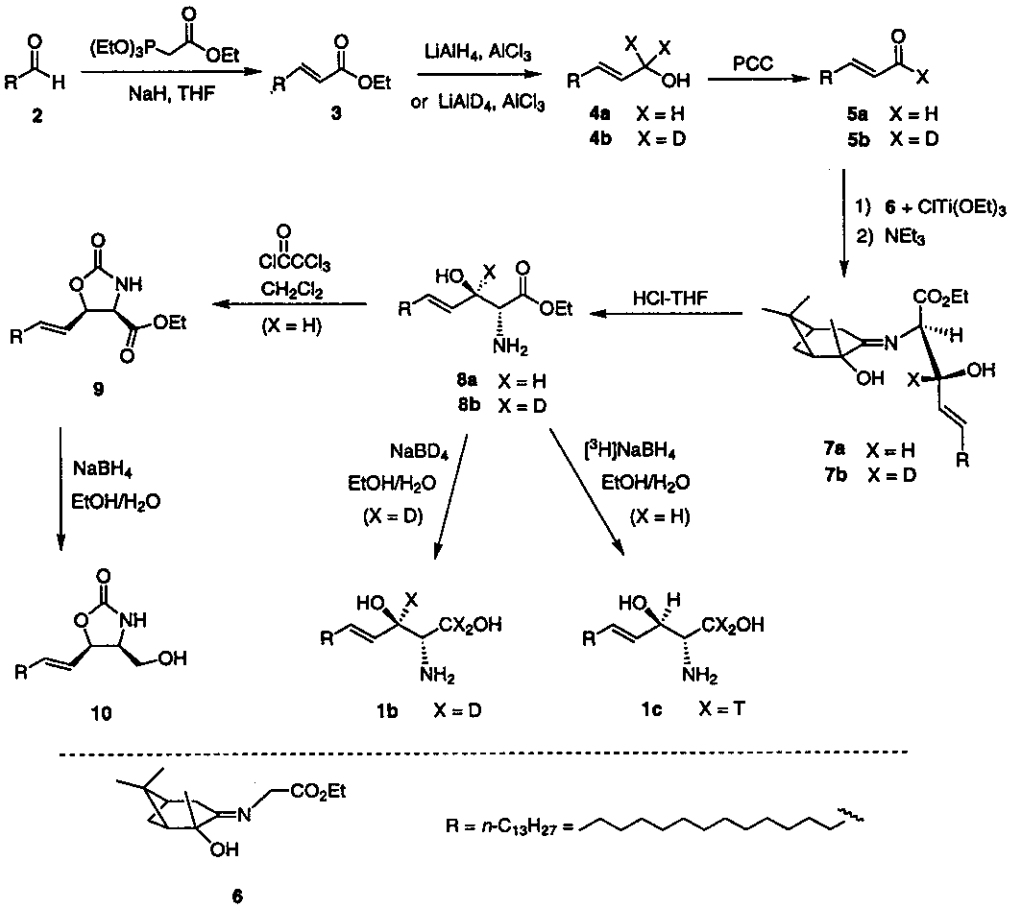

Figure 2. Synthesis of sphingosine labeled with deuterium (**1b**) or tritium (**1c**) based on a total synthesis described by Solladié-Cavallo and Koessler (15).

Ethyl ester **8a** was the starting material for our one-step synthesis of tritium-labeled sphingosine. Reduction of **8a** with [^{3}H]NaBH$_4$ gave [1-^{3}H]sphingosine (**1c**), which was purified by medium pressure liquid chromatography (MPLC) and characterized by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC). MPLC purification of the crude synthetic product gave **1c** of ~85% purity by radio-TLC and radio-HPLC. Further purification on reversed phase HPLC furnished **1c**, which was shown to be a single component on radio-TLC and radio-HPLC (Figure 3). This brief synthesis of **1c** from **8a** is simple and efficient. Furthermore, manipulation of radiolabeled material is minimized, an important consideration in view of the apparent lability of labeled sphingosine.

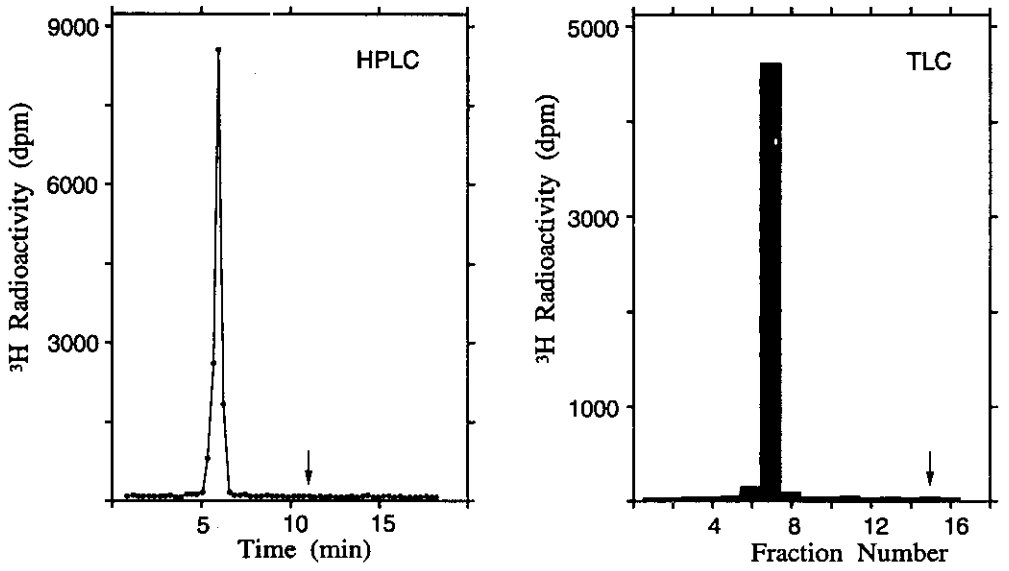

Figure 3. Radio-HPLC and radio-TLC analyses of [1-^{3}H]sphingosine (**1c**). HPLC conditions: 5-μm Alltima C_{18} column (250 × 4.6 mm i.d.); elution with methanol-buffer A 9:1 at 1 ml/min; 0.3-min fraction intervals. TLC conditions: 5 × 20 cm plate; developed with chloroform-methanol-water 65:30:5. The arrows indicate the location of nonpolar byproducts that were removed by chromatographic purification.

Stability studies showed the formation of nonpolar material when a solution of **1c** was evaporated to dryness and stored at –20°C for two weeks or when the solution was evaporated to dryness at elevated temperatures. No decomposition was observed in a buffered solution during 60 days storage at –20°C or from extraction into chloroform and evaporation to dryness (without storage). These findings are compatible with past observations (23) in our laboratory on the instability of tritiated dihydrosphingosine samples.

We also prepared [1,1,3-^{2}H]sphingosine (**1b**) for future use as an internal standard for quantitating sphingosine levels in samples of biological origin by isotope-dilution mass spectrometry. Two deuterium atoms were introduced at the incipient C-3 position by reduction of ester **3** with $LiAlD_4$-$AlCl_3$. This reagent, like the diisobutylaluminum hydride used previously to prepare unlabeled material (15), reacted almost exclusively by 1,2-addition to give **4b**. Pyridinium chlorochromate (PCC) oxidation to **5b** resulted in loss of one deuterium, but two additional deuterium atoms were added at C-1 in the final synthetic step by reduction of **8b** with $NaBD_4$. The final product **1b** showed 5% d_1, 9% d_2, 86% d_3 by fast atom bombardment mass spectrometry (FAB-MS). The ^{2}H NMR spectrum contained no signals other than the anticipated resonances for deuterium at C-1 and C-3, and the ^{13}C NMR spectrum showed almost no intensity for signals of C-1

and C-3. The ^{1}H NMR spectrum indicated ~99% deuterium incorporation at C-3 and lower incorporation at C-1. Other ^{1}H and ^{13}C NMR signals were compatible with those reported (22) for D-*erythro*-sphingosine (**1a**). The stereoisomeric purity of **1b** was evaluated by analysis of the ^{1}H and ^{19}F NMR spectra of its (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetate (MTPA) derivative (Figure 4). Stereoisomeric impurities were identified based on comparisons with NMR spectra of MTPA derivatives of **1a**, its L enantiomer, its *threo* epimer, and a DL-*threo* mixture. As illustrated in Figure 4, **1b** contained 3% L-*threo*, ~1% D-*threo*, and <0.5% of the L-*erythro* enantiomer (24, 25).

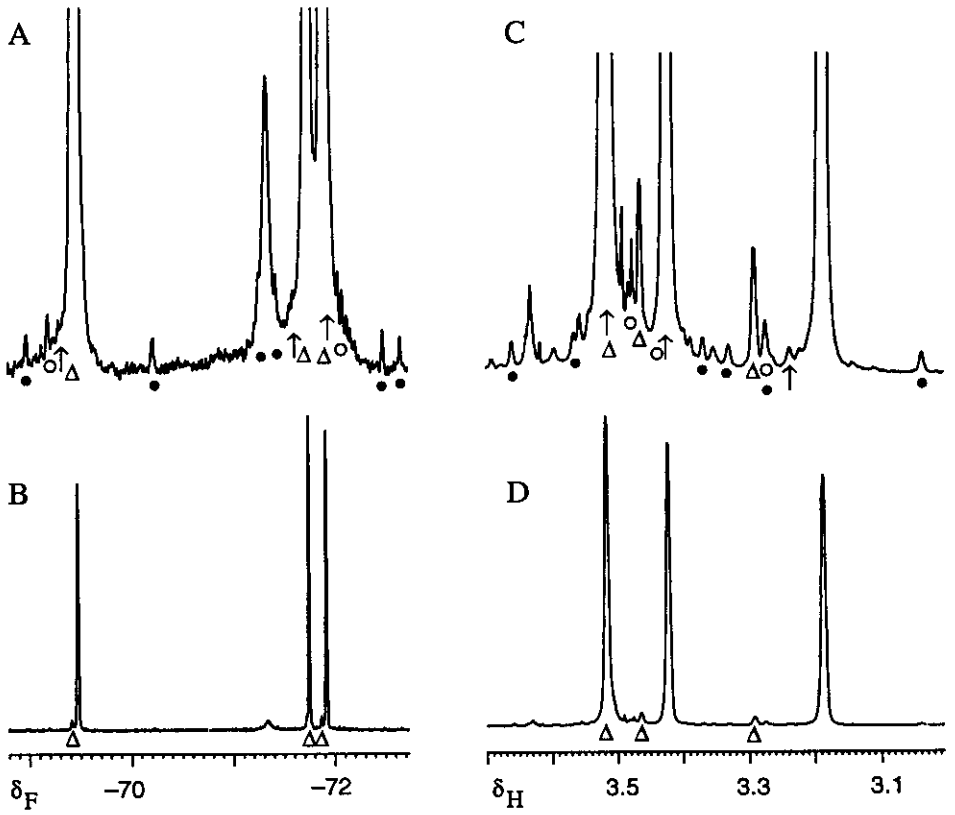

Figure 4. Analysis of the stereoisomeric purity of deuterium-labeled sphingosine (**1b**) from ^{1}H and ^{19}F NMR spectra of its (*S*)-MTPA derivative. The 235-MHz ^{19}F NMR spectrum is shown (A) with 2-Hz line broadening and vertical expansion and (B) with mild Gaussian apodization. The 500-MHz ^{1}H NMR spectrum with 0.5-Hz line broadening is shown (C) with and (D) without vertical expansion. Signals corresponding to stereoisomeric contaminants are marked by a triangle (Δ, L-*threo*), an open circle (o, D-*threo*), or an arrow (↑, L-*erythro*). ^{13}C satellite signals are marked by a closed circle (•). Large minor signals represent unidentified non-sphingolipid MTPA byproducts.

Ester **8a** also provided access to the 2,3-cyclic carbamate derivative **10** of sphingosine. As shown in Figure 2, treatment of **8a** with diphosgene gave the carbamate-ester intermediate **9**, reduction of which afforded **10**. Replacement of $NaBH_4$ by deuterium- or tritium-labeled borohydride as described above for the preparation of **8b** and **8c** should provide a more efficient method for introducing an isotopic label into carbamate **10** than modification of the previously reported synthesis of **10** from 2-amino-2-deoxy-D-glucose (26).

Experimental

Materials and Methods. Tetradecanal (trimer), triethyl phosphonoacetate, pyridinium chlorochromate, (+)-(1*R*,2*R*,5*R*)-2-hydroxy-3-pinanone, titanium tetraethoxide, glycine ethyl ester hydrochloride, sodium borodeuteride (98% D), and (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (98% ee) were purchased from Aldrich Chemical Co. (Milwaukee, WI), diphosgene from Lancaster Synthesis (Windham, NH), lithium aluminum deuteride (99% D) from Cambridge Isotope Laboratories (Andover, MA), and sodium borotritide from ICN Pharmaceuticals (Costa Mesa, CA). Iminoglycinate **6** was prepared in 90% yield as described previously (27) by condensation of (1*R*,2*R*,5*R*)-2-hydroxy-3-pinanone and ethyl glycinate in benzene with a catalytic amount of boron trifluoride etherate. Titanium monochloride triethoxide was made in 80% yield as described previously (28) by reaction of titanium tetraethoxide with acetyl chloride under nitrogen. Trimethylsilyl ethers were prepared by treating long-chain bases with a 1:1 mixture of bis(trimethylsilyl)trifluoroacetamide and pyridine for 2 h at 40°C, followed by evaporation to dryness under nitrogen. Buffer A (for purification of **1c**) was 25 mM KH_2PO_4-H_3PO_4 in water (pH 2.5).

^1H, ^2H, and ^{13}C NMR spectra were acquired at 25°C on a Bruker AMX500 instrument (500 MHz for ^1H) and referenced to tetramethylsilane (^1H) or $CDCl_3$ (77.0 ppm for ^{13}C; 7.26 ppm for ^2H). Spectra were measured on $CDCl_3$ (~5 mM for ^1H and ~50 mM for ^{13}C) or $CHCl_3$ solutions (5-50 mM for ^2H). Assignments of most ^1H and ^{13}C NMR signals can be made directly by chemical shift comparisons with NMR data presented previously (22). Purities were estimated from ^1H NMR spectra by comparing the intensity of extraneous signals with those of ^{13}C satellites. ^{19}F NMR spectra were acquired at 235 MHz at ~22°C on a Bruker AC250 spectrometer and referenced to the downfield line of internal $CFCl_3$. FAB-MS were acquired using a 3-nitrobenzyl alcohol matrix with added NaCl and KCl on a ZAB-HF reverse-geometry double-focusing instrument. Electron impact (EI) mass spectra were acquired with the ZAB-HF instrument at 70 eV after gas chromatography (GC) or direct-inlet sample introduction 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. GC-MS was carried out on a Hewlett-Packard 5890A instrument with a 60 m × 0.25 mm i.d. DB-5ms column (J&W Scientific; Folsom, CA) held at 220°C for 3 min and increased to 250°C at 1°C/min. Infrared (IR) spectra were measured with KBr pellets on a Mattson Galaxy 6020 Fourier-transform spectrometer. Melting points (mp) were measured with a Thomas-Hoover apparatus in sealed, evacuated capillary tubes. MPLC was done on glass columns dry packed with silica gel (230-400 mesh; EM Science); fraction volumes were 20 ml. HPLC was performed at 1 ml/min on a 5-μm C_{18} Alltima column (250 × 4.6 mm; Alltech, Deerfield, IL) with

UV detection at 210 nm. Analytical 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 5 min at 80°C. Radio-TLC analyses were carried out on 5 x 20 cm glass TLC plates (0.25-mm layer of silica gel G; Analtech, Newark, DE) followed by scraping 1-cm sections directly into 7-ml vials for scintillation counting. Radioactivity was measured on a Packard model 4640 liquid scintillation analyzer using ScintiVerse from Fisher Scientific (Fair Lawn, NJ) as scintillation fluid.

(2*E*)-Ethyl 2-hexadecenoate (3). Reaction of NaH (0.8 g, 33 mmol) in anhydrous tetrahydrofuran (20 ml) with triethyl phosphonoacetate (5.3 ml, 27 mmol) followed by addition of crude de-trimerized (29) tetradecanal (5.65 g, 27 mmol) in dichloromethane (30 ml) as described previously (15) gave **3** as a colorless oil (6.9 g, 91% yield, 99% purity): single component on TLC (R_f 0.24, ether-hexane 5:95); EI-MS, 282 (6, M^+), 237 (46, M–EtO), 194 (27), 155 (20), 152 (18), 141 (17), 127 (33), 115 (26), 101 (100), 88 (60), 55 (68); IR, 2924, 2854, 1724, 1655, 1465, 1390, 1367, 1309, 1265, 1178, 1045, 979 cm^{-1}; ^1H NMR, δ 6.96 (dt, 15.6, 6.9 Hz, H-3), 5.81 (dt, 15.6, 1.6 Hz, H-2), 4.18 (q, 7.1 Hz, OEt), 2.19 (m, H-4), 1.45 (m, H-5), 1.29 (t, 7.1 Hz, OEt), 1.26 (m, 20H), 0.88 (br t, 7 Hz, H-16).

(2*E*)-[1,1-^{2}H] 2-Hexadecen-1-ol (4b). To a solution of $AlCl_3$ (0.89 g, 6.7 mmol) in anhydrous ether (20 ml) was added $LiAlD_4$ (0.84 g, 20 mmol) under nitrogen at 0°C. The resulting slurry was stirred for 15 min, and a solution of **3** (2.82 g, 10 mmol) in dry ether (10 ml) was added dropwise over 15 min. After continued stirring at 0°C for another 40 min, saturated ammonium chloride (1 ml) was added. Inorganic precipitates were removed by filtration and washed thoroughly with ether. Evaporation of the combined filtrates gave a white solid that was subjected to MPLC (500 x 25 mm column; elution with ethyl acetate-hexane 8:92). Evaporation of fractions 31-49 gave **4b** as a white solid (2.2 g, 94% yield, 98% purity): mp, 38-39°C (lit. (15) for **4a**, 36-37°C); single component by TLC (R_f 0.28, diethyl ether-hexane 1:1); high-resolution EI-MS, calcd. for $C_{16}H_{30}D_2O$, 242.2579, found 242.2594; EI-MS, 242† (1, M^+), 224† (6, M–H_2O), 194† (2, $C_{14}H_{26}$), 166† (3, $C_{12}H_{22}$), 139† (5, $C_{10}H_{15}D_2$), 125† (11, $C_9H_{13}D_2$), 111† (26, C_8H_{15}), 97† (64, C_7H_{13}), 83† (81, C_6H_{11}), 69† (76, C_5H_9), 59† (100, $C_3H_3D_2O$), 57† (71, C_4H_9), 55† (73, C_4H_7); IR, 3315, 2872, 2849, 2177, 2085, 1471, 1462, 1176, 1087, 968 cm^{-1}; ^1H NMR, δ 5.699 (dt, 15.4, 6.4 Hz, H-3), 5.624 (br d, 15.4 Hz, H-2), 4.08 (m, 0.1 H), 2.04 (m, H-4), 1.37 (m, H-5), 1.26 (m, 20H), 0.88 (br t, 7 Hz, H-16); ^2H NMR, δ 4.07 (s).

(2*E*)-[1-^{2}H]2-Hexadecenal (5b). Oxidation of **4b** (1.12 g, 4.7 mmol) with PCC (2.0 g, 9.4 mmol) as described (15) for **4a** gave **5b** as a white solid (0.75 g, 65% yield, 99% purity): mp, 42-43°C; single component by TLC (R_f 0.14, ether-hexane 5:95); high-resolution EI-MS, calcd. for $C_{16}H_{29}DO$, 239.2359, found 239.2360; EI-MS, 239† (4, M^+), 221† (3, M–H_2O), 194† (5, $C_{14}H_{26}$), 166† (2, $C_{12}H_{22}$), 150† (4, $C_{11}H_{18}$), 136† (10, $C_{10}H_{16}$), 122† (16, C_9H_{14}), 112† (17, C_8H_{16}), 96† (39, C_7H_{12}), 84† (62, C_6H_{12}, and C_5H_8O), 71† (100, C_5H_{11} and C_4H_5DO), 55† (62, C_4H_7); IR, 2916, 2872, 2847, 2094, 1678, 1653, 1469, 1155, 1016, 986, 947, 719 cm^{-1}; ^1H NMR, δ 9.5 (absent,

< 0.01H), 6.85 (dt, 15.6, 6.8 Hz, H-3), 6.12 (d of quintet, 15.6, 1.3 Hz, H-2), 2.34 (m, H-4), 1.51 (m, H-5), 1.26 (m, 20H), 0.88 (br t, 7 Hz, H-16); ^{2}H NMR, δ 9.55 (s).

[1*R*-[1α,2β,3(2*R,3*R**,4*E*),5α]] [3-^{2}H]Ethyl 3-hydroxy-2-[(2-hydroxy-2,6,6-trimethylbicyclo[3.1.1]hept-3-ylidene)amino]-4-octadecenoate (7b).** To a solution of iminoglycinate **6** (0.81 g) in dichloromethane (3 ml) were added a solution of titanium chloride triethoxide (19) (0.70 g) in dichloromethane (5 ml) dropwise at 0°C followed by the addition of a solution of **5b** (0.70 g) in dichloromethane (5 ml) and anhydrous triethylamine (0.90 ml). The reaction mixture was stirred at 0°C for 4 h. The product was extracted with ethyl acetate (3 × 100 ml). The extracts were washed with water and brine, dried over anhydrous sodium sulfate, and evaporated to a yellow oil that was subjected to MPLC (500 × 25 mm column prewashed with triethylamine (50 ml) and ethyl acetate-hexane 2:8 (200 ml); elution with ethyl acetate-hexane 2:8). Evaporation of fractions 35-58 gave **7b** as a light yellow oil (1.1 g, 70% yield): single component on TLC (R_f 0.40, ethyl acetate-hexane 4:6); FAB-MS, 531 (13, M+K), 515 (35, M+Na), 493 (67, M+H), 325 (15), 276 (10), 252 (14), 236 (19), 235 (16), 162 (38), 55 (100).

(2*R*,3*R*)-[3-^{2}H]Ethyl 2-amino-3-hydroxy-4-octadecenoate (8b). Hydrolysis of **7b** (1.0 g) in 1.2 N HCl (15 ml) and tetrahydrofuran (4 ml) as described (15) for **7a** gave **8b** as a white solid (0.40 g, 80% yield, 98% purity (19)): mp, 51-53°C; single component on TLC (R_f 0.20, ethyl acetate-methanol-aqueous NH_3, 98:1:0.5); high-resolution EI-MS, calcd. for $C_{17}H_{33}DNO$ (M–CO_2Et), 269.2731, found 269.2712; EI-MS, 269† (10, M–CO_2Et), 251† (7, M–H_2O–CO_2Et), 143† (2, $C_7H_{13}NO_2$), 117† (11, $C_5H_9DNO_2$), 103† (100, $C_4H_5NO_2$), 74† (40, $C_2H_4NO_2$); FAB-MS, 381 (6, M+K), 365 (19, M+Na), 343 (24, M+H), 325 (100, M–H_2O+H), 251 (21); IR, 3364, 3192, 2916, 2850, 2108, 1722, 1585, 1471, 1246, 1209, 1035, 974 cm^{-1}; ^{1}H NMR, δ 5.743 (dt, 15.4, 6.8 Hz, H-5), 5.344 (dt, 15.4, 1.5 Hz, H-4), 4.34 (m, 0.02H, H-3), 4.21 (dq, 10.8, 7.1 Hz, OEt), 4.18 (dq, 10.8, 7.1 Hz, OEt), 3.62 (s, H-2), 2.02 (m, H-6), 1.34 (m, H-7), 1.282 (t, 7.1 Hz, OEt), 1.25 (m, 20H), 0.881 (br t, 7 Hz, H-18); ^{2}H NMR, δ 4.33 (s).

D-*Erythro*-[1,1,3-^{2}H]sphingosine (1b). To a solution of **8b** (100 mg, 0.3 mmol) in ethanol-water (3:1, 3 ml) at 0°C was added in one portion $NaBD_4$ (45 mg, 11 mmol). The mixture was stirred at 0°C for 3 days followed by addition of water (20 ml) and extraction with chloroform (3 × 20 ml). The combined extracts were washed with water and brine, dried over anhydrous sodium sulfate, and evaporated to a white solid that was subjected to MPLC (1000 × 10 mm, elution with chloroform-methanol-water 100:20:4). Evaporation of fractions 12-28 gave **1b** as a white solid (55 mg, 65% yield): mp, 81-84°C; single component by TLC (R_f 0.47, chloroform-methanol-water 65:30:5); high-resolution EI-MS, calcd. for $C_{17}H_{33}DNO$ (M–CHD_2O), 269.2702, found 269.2703; EI-MS, 303 (0.2, M+H), 302 (0.1, M$^+$), 269† (6, M–CHD_2O), 251† (3, $C_{17}H_{27}D_3N$), 62† (100, $C_2H_4D_2NO$); GC-EI-MS of the trimethylsilyl ether (>99% purity, t_R 16.0 min), 446 (0.1, M$^+$), 431 (3, M–CH_3), 341 (13), 312 (7), 134 (100), 73 (44); FAB-MS, 341 (5, M+K), 325 (20, M+Na), 303 (49, M+H), 285 (100, M–H_2O+H), 267 (5), 253 (11), 251 (11); IR, 3369, 2918, 2849, 2173, 2108, 1583, 1467, 1134, 1095, 1060, 970 cm^{-1}; ^{1}H NMR, δ 5.767 (dt, 15.4, 6.8 Hz, H-5), 5.475 (br d, 15.4 Hz, H-4), 4.06 (absent, H-3), 3.66 (m, 0.2H, H-1), 2.88 (br s, H-2), 2.06 (m, H-6), 1.38 (m, H-7),

1.26 (m, 20H), 0.881 (br t, 7 Hz, H-18); ^{13}C NMR, δ 134.76, 128.60, 56.03, 32.37, 31.92, 29.70 (3C), 29.66 (2C), 29.51, 29.36, 29.30, 29.18, 22.68, 14.11; ^{2}H NMR, δ 4.13 (D-3), 3.69 (D-1).

The tris-(*S*)-MTPA derivative was prepared by adding (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (30 μl) to a solution of **1b** (2 mg) in pyridine (100 μl) followed by shaking for 5 min to mix the reagents. The mixture was left overnight at room temperature, quenched with water (2 ml), and extracted with dichloromethane (3 × 1.5 ml). The combined extracts were washed with water (1 ml) and brine (1 ml), dried over anhydrous sodium sulfate, and evaporated to a reside that was filtered through silica gel (30 × 5 mm column; elution with ethyl acetate-hexane 3:7 (5 ml)). The eluate was evaporated to dryness in a stream of nitrogen and further dried in vacuo to a colorless oil: single component by TLC (R_f 0.52, hexane-ethyl acetate 85:15), identical to that of the MTPA derivative of authentic **1a**; ^{1}H NMR, δ 6.97 (d, 9.2 Hz), 5.778 (dt, 15.4, 6.8 Hz, H-5), 5.224 (dt, 15.4, 1.5 Hz, H-4), 4.515 (d, 9.2 Hz, H-2), 3.516 (q, 1.2 Hz, OCH_3), 3.422 (q, 1.2 Hz, OCH_3), and 3.187 (q, 1.4 Hz, OCH_3), 1.90 (m, H-6), 1.26 (m, 20H), 0.880 (br t, 7 Hz, H-18); ^{19}F NMR, –69.47 (br s), –71.74 (br s), –71.91 (br s). Minor NMR signals were observed at δ_H 3.464 (3%), 3.290 (3%), 3.275 (1%) and δ_F –69.41 (3%), –71.86 (3%), –69.19 (1%), –72.04 (1%). Integration of methoxy signals in ^{1}H NMR and trifluoromethyl signals in ^{19}F NMR spectra (Figure 4) indicated a 96 : 3 : 1 : 0.3 mixture of **1b**, its L-*threo*, D-*threo*, and L-*erythro* isomers (24).

(2*R*,3*R*,4*E*)-Ethyl 2-amino-3-hydroxy-4-octadecenoate (8a). Ester **8a** was prepared as described previously (15) and obtained as a white solid (45% overall yield from **2**, 98% purity (19)): mp, 52-53°C; single component on TLC (R_f 0.20, ethyl acetate-methanol-aqueous NH_3 98:1:0.5); high-resolution EI-MS, calcd. for $C_{17}H_{34}NO$ (M–CO_2Et), 268.2640, found 268.2650; EI-MS, 341† (0.1, M^+), 268† (9, M–CO_2Et), 250† (5, M–H_2O–CO_2Et), 103† (100, $C_4H_9NO_2$), 74† (41, $C_3H_6O_2$); FAB-MS, 342 (32, M+H), 324 (100, M–H_2O+H), 250 (16); IR, 3364, 3331, 3198, 2918, 2850, 1724, 1471, 1207, 1037, 970 cm^{-1}; ^{1}H NMR, δ 5.743 (dtd, 15.3, 6.8, 1.2 Hz, H-5), 5.346 (ddt, 15.3, 6.6, 1.5 Hz, H-4), 4.338 (ddq, 6.6, 5.0, 1.0 Hz, H-3), 4.21 (dq, 10.8, 7.1 Hz, OEt), 4.18 (dq, 10.8, 7.1 Hz, OEt), 3.602 (d, 5.1 Hz, H-2), 2.02 (m, H-6), 1.34 (m, H-7), 1.28 (t, 7.1 Hz, OEt), 1.25 (m, 20H), 0.881 (br t, 7 Hz, H-18).

D-*Erythro*-[1,1-^{3}H]sphingosine (1c). To a solution of **8a** (10 mg, 0.03 mmol) in ethanol-water (3:1, 3 ml) at 0°C was added in one portion [^{3}H]$NaBH_4$ (nominally 2.5 mg, 25 mCi). The reaction was stirred at 0°C for 3 days, followed by addition of water (3 ml) and extraction with chloroform (3 × 3 ml). The combined extracts were washed with water (1 ml) and brine (1 ml), dried over anhydrous sodium sulfate, and evaporated to a white solid that was subjected to MPLC (500 × 10 mm, elution with chloroform-methanol-water 100:20:4). Evaporation of fractions 12-28 gave **1c** as a white solid (5.8 mg, 13 mCi) showing ~85% purity by radio-TLC and reverse phase radio-HPLC. Impure **1c** (0.45 μCi) in methanol (100 μl) was subjected to reversed phase HPLC purification (elution with methanol containing 10% aqueous buffer A at 1 ml/min; 0.3 ml-fraction volumes). Fractions 19-21 gave a solution of **1c** (0.38 μCi) showing (Figure 3) a single radioactive component

comigrating with authentic unlabeled D-*erythro*-sphingosine on radio-TLC (R_f 0.47, chloroform-methanol-water 65:30:5) and a single component coeluting with unlabeled D-*erythro*-sphingosine on radio-HPLC (t_R 6.1 min).

Stability studies of 1c. After storage of purified **1c** (0.37 μCi in 0.9 ml of methanol-buffer A 9:1) at –20°C for 60 days, no detectable impurity was observed by radio-TLC. After addition of water (2 ml) to purified **1c** (0.23 μCi in buffer A, 0.6 ml), extraction with chloroform-methanol 9:1 (3 × 2 ml), and drying over anhydrous sodium sulfate, radio-TLC indicated no decomposition. These extracts (6 ml) were divided into three portions for stability studies. Evaporation of one portion (2 ml) to dryness at ca. 10°C in a stream of nitrogen followed by immediate redissolution in methanol (0.2 ml) and analysis by radio-TLC indicated a trace of nonpolar material (~2%, R_f 0.88, chloroform-methanol-water 65:30:5). A parallel experiment with storage in a –20°C freezer for two weeks showed additional nonpolar material (40%, R_f 0.70, chloroform-methanol-water 65:30:5). Evaporation of a third portion (2 ml) to dryness at ca. 40°C in a stream of nitrogen followed by immediate redissolution in methanol (0.2 ml) and analysis by radio-TLC showed some nonpolar material (20%, R_f 0.88, chloroform-methanol-water 65:30:5).

[4*R*-[4α,5α(*E*)]] Ethyl 2-oxo-5-(1-pentadecenyl)-4-oxazolidinecarboxylate (9). Diphosgene (40 mg) was added to a solution of **8a** (60 mg) in dichloromethane (10 ml) under nitrogen at room temperature. The resulting solution was stirred at room temperature for 1 h. After addition of dichloromethane (50 ml), the reaction mixture was washed with water (10 ml) and brine (10 ml) and dried with anhydrous sodium sulfate. Evaporation gave a yellow oil that was subjected to MPLC (500 × 10 mm column; elution with ethyl acetate-hexane 2:8). Evaporation of fractions 14-26 gave **9** as a white solid (60 mg, 92% yield, ~96% purity): mp 68-70°C; single component on TLC (R_f 0.23, ethyl acetate-hexane 3:7); high resolution EI-MS, calcd. for $C_{21}H_{37}NO_4$, 367.2723, found, 367.2716; EI-MS, 367† (2, M^+), 323† (6, M–CO_2), 306† (6, $C_{20}H_{34}O_2$), 294† (29, M–CO_2Et), 250† (100, M–CO_2–CO_2Et), 239† (10, $C_{16}H_{31}O$), 233† (9, $C_{17}H_{29}$), 200† (6, $C_9H_{14}NO_4$), 129† (37, $C_5H_7NO_3$); IR, 3254, 3169, 2914, 2849, 1764, 1749, 1728, 1471, 1371, 1217, 1124, 988 cm^{-1}; ^1H NMR (assigned with sphingosine numbering), δ 5.941 (dtd, 15.4, 6.8, 1.0 Hz, H-5), 5.393 (ddt, 15.4, 7.9, 1.5 Hz, H-4), 5.176 (ddd, 8.8, 7.9, 0.9, H-3), 4.455 (d, 8.8 Hz, H-2), 4.210 and 4.209 (q, 7.2 Hz, OEt), 2.05 (m, H-6), 1.36 (m, H-7), 1.26 (m, 20H), 0.881 (br t, 7 Hz, H-18); ^{13}C NMR, δ 168.75, 158.60, 139.08, 121.73, 78.53, 61.83, 58.50, 32.15, 31.89, 29.65, 29.64, 29.62 (2C), 29.52, 29.42, 29.32, 29.10, 28.60, 22.66, 14.16, 14.09.

[4*S*-[4α,5α(*E*)]]-4-(Hydroxymethyl)-5-(1-pentadecenyl)-2-oxazolidinone (10) (2,3-cyclic carbamate derivative of D-*erythro*-sphingosine). To a solution of **9** (50 mg) in ethanol-water (3:1, 3 ml) at 0°C was added in one portion $NaBH_4$ (25 mg, 6 mmol). The mixture was stirred at 0°C for 3 days and extracted with chloroform (3 × 20 ml). The combined extracts were washed with water and brine, dried over anhydrous sodium sulfate, and evaporated to a white solid that was subjected to MPLC (500 × 10 mm, elution with chloroform-methanol 96:4). Evaporation of fractions 12-17 gave **9** as a white solid (38 mg, 78% yield): mp, 101-102°C (lit. (25) 104-105°C); single component on TLC (R_f 0.35, ethyl acetate-hexane 1:1); high-resolution EI-MS, calcd. for $C_{19}H_{35}NO_3$, 325.2617, found 325.2601; EI-MS, 325† (2, M^+), 294† (26, M–CH_2OH), 250† (100, M–CO_2–CH_2OH), 239†

(41, $C_{16}H_{31}O$), 237† (22, $C_{16}H_{29}O$), 123† (14, C_9H_{15}), 109† (33, $C_7H_{11}N$), 95† (59, C_6H_9N), 87† (62, $C_3H_5NO_2$), 57† (77, C_4H_9); FAB-MS, 651 (20, 2M+H), 326 (77, M+H), 308 (45, M–H_2O+H), 296 (35), 282 (100), 250 (17); IR, 3284, 2920, 2850, 1703, 1467, 1410, 1232, 1088, 1024, 970 cm^{-1}; ^1H NMR (assigned with sphingosine numbering), δ 5.923 (dtd, 15.4, 6.8, 1.0 Hz, H-5), 5.65 (br s, NH), 5.558 (ddt, 15.4, 8.1, 1.5 Hz, H-4), 5.085 (td, 8.2, 1.0, H-3), 3.893 (dddd, 8.1, 7.1, 4.0, 0.7 Hz, H-2), 3.68 (br dd, 11.6, 4.0 Hz, H-1), 3.64 (br dd, 11.6, ~7 Hz, H-1), 2.09 (m, H-6), 1.39 (m, H-7), 1.26 (m, 20H), 0.881 (br t, 7 Hz, H-18); ^{13}C NMR, δ 159.95, 139.13, 121.92, 79.98, 61.91, 57.24, 32.24, 31.91, 29.68, 29.66 (2C), 29.64, 29.58, 29.43, 29.35, 29.15, 28.76, 22.68, 14.11.

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References and Notes

1. Spiegel S., Merrill A.H. Jr. – FASEB J. 10: 1388-1397 (1996).
2. Merrill A.H. Jr, Schmelz E.M., Dillehay D.L., Spiegel S., Shayman J.A., Schroeder J.J., Riley R.T., Voss K.A., and Wang E. – Toxicol. Appl. Pharmacol. 142: 208-225 (1997).
3. Crossman M.W. and Hirschberg C.B. – J. Lipid Res. 25: 729-737 (1984).
4. Toyokuni T., Nisar M., Dean B., and Hakomori S.-I. – J. Labelled Compd. Radiopharm. 29: 567-574 (1991).
5. Shoyama Y., Okabe H., Kishimoto Y., Costello C. – J. Lipid Res. 19: 250-259 (1978).
6. Stoffel W. and Henning R. – Z. physiol. Chem. 349: 1400-1404 (1968).
7. Gaver R.C. and Sweeley C.C. – J. Am. Chem. Soc. 88: 3643-3647 (1966).
8. Iwamori M., Moser H.W., and Kishimoto Y. – J. Lipid Res. 16: 332-336 (1975).
9. Ghidoni R., Sonnino S., Masserini M., Orlando P., Tettamanti G. – J. Lipid Res. 22: 1286-1295 (1981).
10. Birk R., Brenner-Weiß G., Giannis A., Sandhoff K., Schmidt R.R. – J. Labelled Compd. Radiopharm. 29: 289-298 (1991).
11. Iwamori M., Moser H.W., McCluer R.H., and Kishimoto Y. – Biochem. Biophys. Acta 380: 308-319 (1975).
12. Hoffman R.V. and Tao J. – J. Org. Chem. 63: 3979-3985 (1998). Deprotection to sphingosine **1d** and the corresponding dihydrosphingosine was not carried out for deuterium-labeled species.
13. Kishimoto Y. and Mitry M.T. – Arch. Biochem. Biophys. 161: 426-434 (1974).
14. Stoffel W. and Sticht G. – Z. physiol. Chem. 348: 1561-1569 (1967).
15. Solladié-Cavallo A. and Koessler J.L. – J. Org. Chem. 59: 3240-3242 (1994).

16. Di Cesare J.U. and Rapport M.M. – Chem. Phys. Lipids 13: 447-452 (1974).

17. Rigby A.C., Barber K.R., and Grant C.W.M. – Biochim. Biophys. Acta 1240: 75-82 (1995).

18. We required tritium-labeled sphingosine for measuring the uptake and subsequent metabolism of free sphingosine by cultured cells and for determining whether sphingosine can be converted to *N,N*-dimethyl derivatives. In experiments to measure the levels of free sphingosine in biological tissues, tritium-labeled sphingosine may also be useful as an added internal standard to monitor losses during processing.

19. As reported in earlier work (15), we also observed by ^{1}H NMR some isopropyl ester (ca. 8% for **7b**, **8a**, and **8b**) owing to ~15% isopropoxide material in $Ti(OEt)_4$ obtained from Aldrich.

20. Abbreviations: EI, electron impact; FAB, fast atom bombardment; GC, gas chromatography; IR, infrared (spectrum); mp, melting point; MPLC, medium pressure liquid chromatography; MTPA, α-methoxy-α-(trifluoromethyl)phenylacetate; MS, mass spectrometry or mass spectrum; NMR, nuclear magnetic resonance; PCC, pyridinium chlorochromate; TLC, thin-layer chromatography.

21. Blaskovich M.A., Evindar G., Rose N.G.W., Wilkinson S., Luo Y., and Lajoie G.A. – J. Org. Chem. 63: 3631-3646 (1998).

22. Kisic A., Tsuda M., Kulmacz R.J., Wilson W.K., and Schroepfer G.J. Jr. – J. Lipid Res. 36: 787-803 (1995).

23. Ostrow R.S. – Ph.D. Thesis, 1976; Dissertation Abstr. 1670-B (1977).

24. MTPA derivatives of **1a**, its L enantiomer, L-*threo* isomer, and a DL-*threo* mixture were prepared analogously to that of **1b** and analyzed by ^{1}H and ^{19}F NMR. A full account of methodology for determining the enantiomeric and diastereomeric purity of sphingosine has been described: Li S., Wilson W.K., and Schroepfer G.J., Jr. – J. Lipid Res. 40: in press (1999).

25. Low levels of stereoisomeric impurities in **1b** evidently arose from incomplete chromatographic purification of its precursor **8b**. More careful purification of **8a** produced material showing no ^{1}H NMR signals corresponding to the *threo* epimer.

26. Sugawara T. and Narisada M. – Carbohydr. Res. 194: 125-138 (1989).

27. Solladié-Cavallo A., Simon-Wermeister M.-C., and Schwarz J. – Organometallics 12: 3743-3747 (1993).

28. Holloway H. – Chem. Ind. 214-215 (1962).

29. The commercial tetradecanal was predominantly in the form of the trimer, which was converted to the monomer **1** by stirring overnight in a solution of dichloromethane containing *p*-toluenesulfonic acid (5 mg). Similar treatment in tetrahydrofuran and benzene resulted in <40% conversion to **1**. Conversion to the monomer was monitored by TLC (R_f 0.65 for trimer; R_f 0.20 for **1**; ether-hexane 5:95). The residual *p*-toluenesulfonic acid did not adversely affect the subsequent condensation reaction.

