papers and notes on methodology

Conversion of *erythro*-D-sphinganine to its [1-$^2H_1$] and [1-$^3H_1$] derivatives

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Abstract A convenient chemical synthesis of *erythro*-D-[1-$^2H_1$]sphinganine and *erythro*-D-[1-$^3H_1$]sphinganine is described. The approach utilizes a stereospecific starting material (natural sphinganine prepared from bovine brain sphingomyelin) and applies a sequence of selective protection of functional groups yielding 2-acetamido-3-O-benzoyloctadecan-1-ol. Oxidation of the primary alcohol to an aldehyde followed by NaB^2H_4 or NaB^3H_4 reduction and hydrolysis of the protective groups yields *erythro*-D-[1-$^2H_1$]sphinganine or *erythro*-D-[1-$^3H_1$]sphinganine. The synthetic intermediates and isotopically labeled sphinganines are characterized by infrared analysis, ^{1}H-nuclear magnetic resonance, optical rotation, and gas–liquid radiochromatographic and mass spectral fragmentation analyses. The [1-$^2H_1$] and [1-$^3H_1$] derivatives were obtained with overall yields (and isotope enrichments) of 11% (min. 84 mol% $^2H_1$) and 8% (60 mCi/mmol), respectively.—**Crossman, M. W., and C. B. Hirschberg.** Conversion of *erythro*-D-sphinganine to its [1-$^2H_1$] and [1-$^3H_1$] derivatives. *J. Lipid Res.* 1984. **25:** 729–737.

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The understanding of sphingolipid metabolism has been greatly aided by the use of specifically radiolabeled precursors. One of the most desirable, yet difficult to synthesize, is the [1-^{3}H]sphingolipid long chain base. The elegant total synthesis of sphinganine by Shaprio, Segal, and Flowers (1) was applied by Stoffel and Sticht (2) for the production of racemic *erythro*-[1-$^3H_2$]sphinganine. Shoyama et al. (3) modified the original synthesis by Shapiro et al. (1) by esterifying the racemic pre-labeled intermediates with a L(+)-acetylmandeloyl moiety. This modification enables the resolution of enantiomers; however, the burden of total synthesis still remains. This study describes the conversion of *erythro*-D-sphinganine to its [1-$^2H_1$] and [1-$^3H_1$] derivatives without the requirement of total synthesis.

EXPERIMENTAL PROCEDURES

Materials and methods

Trityl chloride was purchased from Fisher Chemical Co., St. Louis, MO. Pyridinium dichromate was purchased from Aldrich Chemical Co., Milwaukee, WI. 4-Dimethylaminopyridine, benzoic anhydride, sodium borodeuteride [98 atom % ^{2}H], *erythro*-DL-sphinganine, and bovine brain sphingomyelin were purchased from Sigma Chemical Co., St. Louis, MO. Sodium borotritide (100 mCi/mmol) was purchased from New England Nuclear, Boston, MA. Silica gel (100–200 mesh, Sigma) or Unisil (100–200 mesh, Clarkson Chemical Co., Williamsport, PA) was activated at 120°C for 30 min before use with column chromatography. All solvents were reagent grade and were made anhydrous by standard procedures. All solvent ratios are expressed (v/v).

Melting points (uncorrected) were recorded on a Fisher-Johns melting point apparatus as read. IR spectra were recorded on a Perkin-Elmer Model 21 double beam spectrometer using 1% KBr discs. ^{1}H-NMR spectra were determined in $CDCl_3$ solutions of synthetic intermediates using a Jeol FT-100 MHz spectrometer operating at 100 MHz with tetramethylsilane as internal standard. Peaks are reported as ppm (δ) downfield from the tetramethylsilane (Me_4Si) signal.

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMS, trimethylsilyl; PMA, phosphomolybdic acid; PDC, pyridinium dichromate; DMAP, 4-dimethylaminopyridine; GLC–MS, gas–liquid chromatography–mass spectrometry; Dnp-SH_2, N-dinitrophenylsphinganine; Dnp-, dinitrophenyl; LCB, long chain base; RRT, relative retention time; SS, solvent system; sphinganine, *erythro*-D-2-aminooctadecan-1,3-diol; IR, infrared; NMR, nuclear magnetic resonance.

Gas–liquid chromatographic (GLC) analyses were performed on a Hewlett-Packard 5711A chromatograph with silanized all-glass columns (6 ft × 1/8 in i.d.) containing 3% SE-30 (on Gas Chrom Q 60/80 mesh) at column temperatures specified in the text. Injection port and detector were operated at 250°C. Gas–liquid radiochromatography was performed as described by Crossman and Hirschberg (4). Briefly, fractions of the column effluent were collected with Pasteur pipettes for 1-min intervals. Radioactivity was eluted from the pipettes with Aquasol® (New England Nuclear) and counted in a Searle Analytic 6893 liquid scintillation spectrometer.

Gas–liquid chromatography–mass spectrometry (GLC–MS) was performed on an LKB-9000 spectrometer coupled to a Logos data system model DS-2000WE. The acceleration voltage was 2.5 kV and the ionizing potential was 70 eV at 120 μA of emission. GLC–MS was also performed on an HP 5985 Quadrapole spectrometer (Michigan State University) with an ionizing potential of 70 eV at 300 μA of emission.

Thin-layer chromatography (TLC). Samples were separated on pre-coated silica gel 60 F-254 (5 × 20 cm, 0.2 mm; Brinkman). Preparative TLC was carried out on pre-coated silica gel 60 F-254 (20 × 20 cm, 0.5 mm; Brinkman). The following solvent systems were used for TLC; A, chloroform–methanol 8:2; B, benzene–acetone 4:1; C, benzene–acetone 1:1; D, hexane–diethyl ether 2:8; E, chloroform–n-propanol–acetic acid 95:5:1; F, chloroform–methanol–conc. NH_4OH 90:10:1; G, chloroform–methanol–2 N NH_4OH 40:10:1; H, methylene chloride–ethyl acetate 1:1; I, chloroform–methanol 25:1; J, chloroform–methanol–acetic acid 90:2:8; K, hexane–diethyl ether–acetic acid 7:3:1. The use of benzene should be restricted to well-ventilated fume hoods. Thin-layer radiochromatography was performed on a Packard Model 7201 radiochromatogram scanner. Compounds were visualized on TLC plates by brief exposure to iodine vapors, exposure to UV light, and/or spraying with phosphomolybdic acid (PMA) (10% in ethanol) and heating at 120°C for 5 min. A specific color reagent (5), 2% Purpald® (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole; Aldrich) made in 1 N NaOH, was used to visualize aldehydes. Trityl-containing compounds turn bright yellow after spraying the TLC plate with PMA reagent and heating the plate at 100°C for 5 min.

Optical rotation of the Dnp-derivatives of sphinganine (6, 7) in methanol (HPLC grade, Fisher Scientific Co.) were made at 589 nm in 1-cm cells with a Jasco Model 20 spectrometer operating in the ORD mode.

Preparation of *erythro*-D-sphinganine

Bovine brain sphingomyelin was converted to ceramide (N-acylsphingosine) using phospholipase C according to Karlsson (8). Ceramide was subjected to catalytic hydrogenation (6) followed by acidic methanolysis (1 N methanolic HCl (10 M H_2O), 80°C, 16 hr). Sphinganine was purified as described by Crossman and Hirschberg (4).

2-Acetamidooctadecan-1,3-diol (I)

To a solution of sphinganine (500 mg, prepared from bovine brain sphingomyelin, as described above) in dry methanol (25 ml), acetic anhydride (6 ml) was added and the solution was allowed to stand overnight at room temperature. Distilled water (6 ml) was added to the cooled reaction mixture (0–4°C); after 30 min, the methanol was removed by rotary evaporation and the residue was extracted five times with 25 ml of diethyl ether. The combined ether extracts were evaporated under reduced pressure to provide a white solid, which was crystallized from methanol; mp 126–126.5°C (lit. 122–124°C) (1); yield of I was 513 mg (99%); TLC, a single spot, R_f 0.43 (SS-A); GLC analysis (220°C) of the *bis*-TMS derivative showed one peak comigrating with the *erythro* isomer, RRT 2.00 (relative to sphinganine) (9); IR, γmax 3250 cm^{-1} (NH and OH), 2890 and 2830 cm^{-1} (CH_2 and CH_3), 1650 and 1552 cm^{-1} (amide bands I and II), 1080 cm^{-1} (2° OH), 1050 cm^{-1} (1° OH). The TMS derivative showed the following characteristic ions by GLC–MS (relative intensity) m/z 472 (7.4%, M–15), 384 (5.9%, $M-CH_2=\overset{+}{O}-Si(CH_3)_3$), 313 (22.4%, $CH_3(CH_2)_{14}CH=\overset{+}{O}-Si(CH_3)_3$), 247 (30.7%, $CH_3CO\overset{+}{N}H-[Si(CH_3)_3]-CHCH_2OSi(CH_3)_3$), 174 (7.7%, $CH_3CONHCHC\text{-}H_2O\text{-}Si(CH_3)_3$), 157 (100%, 247–trimethylsilanol).

1-O-Trityl-2-acetamidooctadecan-3-ol (II)

To a solution of I (306 mg) in dry pyridine (25 ml), trityl chloride (436 mg) was added and the reaction mixture was heated in a reflux apparatus at 100°C for 3 hr, under nitrogen. The reaction mixture was cooled to room temperature, then poured into ice-cold 1 N HCl (340 ml) and extracted three times with diethyl ether (500 ml). The combined ether extracts were washed with 3 × 250 ml of 2.5% $NaHCO_3$ followed by distilled water (250 ml) until the pH was neutral as measured by pH indicator paper. The solution was dried over anhydrous $MgSO_4$ and the solvent was evaporated under reduced pressure. The ether extract showed two UV absorbing spots, (R_f 0.73 and 0.29, SS-B) comigrating with authentic tritanol and the desired trityl ether (II), respectively; the residue was dissolved in benzene and purified on a silica gel column (40 g, 16 × 2.5 cm). Tritanol was eluted with 700 ml of benzene and II was eluted with 700 ml of benzene–acetone 8:1. Trityl ether (II) (252 mg, 70%) was crystallized from 80% methanol at −20°C; mp 70–72°C; TLC, R_f 0.29 (SS-B), R_f 0.60 (SS-C); IR: γmax 3050, 1495 and 1455 cm^{-1} (trityl); ^{1}H-NMR, (δppm): 0.88 (t, J = 6 Hz, 3H, C–18–CH_3), 1.25 (m, 28H,

$-(CH_2)_{14}-$), 2.04 (s, 3H, $NCOCH_3$), 2.91 (d, J = 9 Hz, 1H, C–2–H), δ_A 3.52 δ_B 3.28 (d of AB system, J_{AB} = 10 Hz, J_d = 3 Hz, 2H, C–1–H_A, H_B), 3.81–4.02 (m, 1H, C–3–H), 6.3 (d, J = 8 Hz, 1H, NH), 7.20–7.44 (m, 15H, phenyl). After mild acid hydrolysis of II (5 mg, 90% acetic acid, 100°C, 30 min), TLC (SS-B) of the reaction products showed two components, one with R_f 0.73 and the other migrating just off the origin. The faster moving component was UV-absorbing and comigrated with tritanol standard. In GLC, the TMS derivative of the slower moving component comigrated with authentic *erythro*-N-acetylsphinganine.

1-O-Trityl-2-acetamido-3-O-benzoyloctadecane (III)

Benzoic anhydride (346 mg) and DMAP (68 mg) were added to a solution of II (300 mg) in dry pyridine (15 ml) and placed in a 37°C water bath for 4 hr. The reaction mixture was cooled to room temperature, transferred to a separatory funnel containing diethyl ether (200 ml), and partitioned successively with 100 ml each of distilled water, 2 N HCl, saturated $NaHCO_3$, and distilled water until the pH was neutral (pH indicator paper). The ether phase was dried over anhydrous $MgSO_4$ and the solvent was evaporated under reduced pressure. The benzoylation reaction and recovery of III was quantitative. Samples for spectral analysis were purified by preparative TLC (SS-D, R_f 0.34). IR, γmax 1725, 1265 and 1110 cm^{-1} (benzoate). TLC (SS-B, R_f 0.58) exhibited one UV-absorbing spot which turned bright yellow after spraying the plate with PMA reagent. R_f values for trityl ether (II) and tritanol in this same solvent system were 0.29 and 0.73, respectively. ^{1}H-NMR (δppm): 0.88 (t, J = 6 Hz, 3H, C–18–CH_3), 1.25 (m, 28H, $-(CH_2)_{14}-$), 2.04 (s, 3H, $NCOCH_3$), δ_A 3.48 δ_B 3.24 (d of AB system, J_{AB} = 10 Hz, J_d = 4 Hz, 2H, C–1–H_A,H_B), 4.30–4.59 (m, 1H, C–2–H), 5.44 (q, J = 7 Hz, 1H, C–3–H), 5.82 (d, J = 10 Hz, 1H, NH), 7.19–7.72 (m, 18H, trityl plus meta and para benzoate protons), 7.96–8.13 (d, J = 8 Hz, 2H, ortho benzoate protons). Two mg (III) was treated with 1.0 ml of 0.1 N methanolic NaOH for 30 min at room temperature followed by addition of 1.0 ml of 1 N HCl and Folch partitioning (10). TLC (SS-B) of the reaction products (lower Folch phase) showed one component (UV-absorbing, trityl group-containing) comigrating with II (R_f 0.29) and another component (UV-absorbing only) comigrating with benzoic acid (R_f 0.22).

2-Acetamido-3-O-benzoyloctadecan-1-ol (IV)

The clear oil (III, 167 mg) was dissolved in 90% acetic acid (20 ml), refluxed at 100°C for 30 min. The reaction mixture was then cooled to room temperature and poured into a separatory funnel containing diethyl ether (100 ml) and washed successively with three portions of 50 ml each of distilled water, 2.5% $NaHCO_3$, and distilled water until the pH was neutral (pH indicator paper). The ether solution was dried overnight with anhydrous $MgSO_4$. After the solvent was removed under reduced pressure, the oil was redissolved in chloroform and purified by silica gel chromatography (40 g, 16 × 2.5 cm). Tritanol was eluted with a liter of chloroform and the primary alcohol (IV) was recovered with a liter of 1% methanol in chloroform. The detritylation reaction and purification of IV was quantitative. The alcohol (IV) crystallized from 80% methanol at −20°C; mp 73–74°C. TLC showed a single, UV-absorbing spot (SS-E, R_f 0.25; SS-F, R_f 0.66). IR γmax 1074 cm^{-1} (1° OH). ^{1}H-NMR (δppm): 0.88 (t, J = 6 Hz, 3H, C–18–CH_3), 1.25 (m, 28H, $-(CH_2)_{14}-$), 2.05 (s, 3H, $NCOCH_3$), 3.64 (d, J = 3 Hz, 2H, C–1–H_2), 4.01–4.27 (m, 1H, C–2–H), 5.08 (d of t, J_t = 6.5 Hz, J_d = 5 Hz, 1H, C–3–H), 6.31 (d, J = 9 Hz, 1H, NH), 7.35–7.73 (m, 3H, meta and para benzoate protons), 8.06 (d of d, J = 8.5 Hz, J = 2 Hz, 2H, ortho benzoate protons). The TMS derivative of IV showed the following prominent ions on GLC–MS (temperature program, 210°C (5 min)–230°C (hold) @ 1°C/min): m/z 504 (1.2%, M–15), 429 (0.4%, M–trimethylsilanol), 416 (0.8%, M–$CH_2{=}\dot{O}$–Si–$(CH_3)_3$), 382 (6.2%, M–15–benzoic acid), 355 (7.9%, M–ketene–benzoic acid), 307 (1.9%, M–(benzoic acid + trimethylsilanol)), 294 (28.7%, M–(benzoic acid + 103)), 174 (64.8%, $[CH_3CONH{-}CHCH_2OSi(CH_3)_3]^+$) 105 (100%, benzoyl ion). Two mg (IV) was subjected to mild alkaline hydrolysis in 0.1 N methanolic NaOH for 30 min at room temperature. The reaction products were determined to be benzoic acid and *erythro*-N-acetylsphinganine.

2-Acetamido-3-O-benzoyloctadecan-1-al (V) and 2-acetamido-(1-$^2H_1$)octadecan-1,3-diol (VI-$^2H_1$)

Pyridinium dichromate (175 mg) was added to a solution of IV (148 mg) in dry methylene chloride (15 ml) and the mixture was vigorously stirred under nitrogen at room temperature for 8 hr. The reaction mixture was cooled on ice and diethyl ether (50 ml) was added to precipitate the PDC. The reaction mixture was poured over a small column of anhydrous $MgSO_4$ (2 g) to remove the PDC precipitate and the solvent was evaporated under reduced pressure. The oily products were redissolved in diethyl ether and washed with ice-cold 1 N HCl followed by cold distilled water. Examination of the reaction products by TLC (SS-E) showed a UV-absorbing, Purpald®-reactive component at R_f 0.48. Spectral characterization of the aldehyde (V) was not achieved due to chemical instability; therefore the aldehyde (V) was immediately purified by preparative TLC (SS-E, R_f 0.48) and rapidly prepared for reduction to the primary alcohol (VI-$^2H_1$ or VI-$^3H_1$). For this, the oily product was redissolved in ice-cold absolute ethanol and reduced with solid NaB^2H_4 (30 mg). After 3 hr (25°C), a few drops of 1 N HCl were

added to destroy excess NaB^2H_4 and the solvent was removed under reduced pressure. The reaction products were redissolved in diethyl ether, washed three times with distilled water, and the solvent was evaporated under reduced pressure. TLC (SS-E) indicated one UV-absorbing compound that comigrated with benzoic acid standard (R_f 0.58). PMA spray reagent disclosed the presence of a non-UV-absorbing compound near the origin. This reaction product, VI-$^2H_1$, comigrating with authentic *erythro*-N-acetylsphinganine, was purified by preparative TLC (SS-A, R_f 0.43) and eluted from the absorbent with chloroform–methanol 2:1. The TMS derivative of VI-$^2H_1$ showed a single peak comigrating with authentic *erythro*-N-acetylsphinganine and no evidence of *threo* isomer formation, as determined by GLC (9). The overall yield from reactions IV–VI was 12 mg (11%).

Two mg (VI-$^2H_1$) was subjected to acidic methanolysis (1 ml, 1 N methanolic HCl (10 M H_2O), 16 hr, 80°C). The free long chain base (VII-$^2H_1$) was isolated as previously described (4). TLC (SS-G, R_f 0.33) showed one component comigrating with authentic sphinganine, after spraying the plate with either ninhydrin or PMA reagent. GLC (215°C) showed a single peak comigrating with the TMS derivative of authentic sphinganine. VII-$^2H_1$, following N-acetylation and derivatization with TMS, exhibited one peak (GLC, 220°C) comigrating with authentic *erythro*-N-acetylsphinganine.

Dinitrophenylation of sphinganine (VII-$^1H_2$ and -$^2H_1$)

The Dnp-derivative of sphinganine (VII-$^1H_2$ and -$^2H_1$) was prepared quantitatively according to Karlsson, Samuelsson, and Steen (6). TLC (SS-H, R_f 0.44) showed a single UV-absorbing, yellow spot. TLC (silica gel G impregnated with borate (6); SS, hexane–chloroform–methanol 10:10:3) showed a single UV-absorbing, yellow spot comigrating with authentic *erythro*-Dnp-sphinganine (R_f 0.61). No *threo*-Dnp-sphinganine (R_f 0.50) was observed under UV light or after spraying the plate with PMA reagent. The TMS derivative of Dnp-(VII-$^1H_2$) showed a single component, RRT 3.4 (relative to cholesterol) as determined by GLC (3% OV-1, 280°C).

1,3-O-Acetyl-2-acetamido-(1-$^2H_1$)octadecane

Authentic *erythro*-N-acetylsphinganine (I, 7 mg) and *erythro*-N-acetyl-(1-$^2H_1$)sphinganine (VI-$^2H_1$, 7 mg) were acetylated at room temperature overnight (1 ml of dry pyridine and 0.5 ml of acetic anhydride). After the solutions were cooled to 4°C, the reaction was stopped by addition of 10 ml of distilled water. The triacetates were extracted from the reaction mixtures with three 10-ml portions of diethyl ether. The combined ether extracts were washed with three 30-ml portions each of 1 N HCl, 2.5% $NaHCO_3$, and distilled water. The solvent was removed under reduced pressure and the white solid was redissolved in methylene chloride. TLC analysis indicated one component after spraying the plate with PMA reagent (SS-I, R_f 0.33).

2-Acetamido-(1-$^3H_1$)octadecan-1,3-diol (VI-$^3H_1$)

The primary alcohol (IV, 30 mg) was treated with PDC (36 mg) in CH_2Cl_2 (8 ml). After 4 hr, the reaction mixture was transferred into a separatory funnel containing 10 ml of diethyl ether and 10 ml of 1 N HCl and distilled water. The aldehyde (V) was isolated by TLC as described earlier and reduced with NaB^3H_4 (ca. 1 mCi). After 30 min (4°C), nonradioactive $NaBH_4$ (5 mg) was added and the ethanolic solution was allowed to stand for 1 hr at 25°C. The reaction was stopped and the products were isolated as described earlier for VI-$^2H_1$. The yield of VI-$^3H_1$ was 80 μCi (60 mCi/mmol).

RESULTS

Identification of *erythro*-(1-$^2H_1$)sphinganine (VII-$^2H_1$)

Identification of this structure was obtained from several chromatographic and spectral analyses of VII-$^2H_1$ and its N-acetyl, triacetyl, and Dnp derivatives. The N-acetyl derivative comigrated with authentic *erythro*-N-acetylsphinganine on two separate TLC systems and as the TMS derivative on GLC.

O-acetylation of VI-$^2H_1$ yielded a triacetyl derivative that comigrated on TLC with authentic standard. The triacetyl derivative of VI-$^2H_1$, along with a protium reference species of triacetylsphinganine, were subjected to ^{1}H-NMR and the results of this analysis are shown in **Table 1.** The spectra of the two samples are essentially identical, except within the C–1–$H_{A,B}$ multiplet region, where a decrease in intensity and disappearance of some

TABLE 1. ^{1}H-NMR chemical shifts (ppm from internal Me_4Si[a]) and coupling constants (Hz) of protium and VI-$^2H_1$ triacetylsphinganines

	Protium Reference	VI-$^2H_1$
CH_3	0.88 t[b] (6)	0.88 t (6)
$-(CH_2)_{14}-$	1.25 m	1.25 m
C–3–H	5.03 q (6)	4.91 q (6)
C–2–H	4.31–4.52 m	4.29–4.52 m
C–1–$H_{A,B}$	3.97–4.23 m	4.02–4.25 m
NH	5.92 d (8)	5.87 d (9)
$NCOCH_3$	2.00 s	2.00 s
$OCOCH_3$	2.07 d (1)	2.07 d (1)

Authentic *erythro*-N-acetylsphinganine (7 mg) and Vi-$^2H_1$ (7 mg) were O-acetylated with 1 ml of dry pyridine and 0.5 ml of acetic anhydride at 25°C overnight. The triacetates were isolated as described in the text and, following preparative TLC (SS-I), 5 mg of each compound was redissolved in 0.5 ml of $CDCl_3$ for analysis.

[a] Tetramethylsilane.

[b] t, Triple; m, multiplet; q, quartet; s, singlet.

signals (relative to the protium species of equal concentration) was noted. This is presumably the result of the incorporation of deuterium at C–1.

The Dnp derivative of VII-$^2H_1$ behaved chromatographically (TLC, GLC) as the standard Dnp-sphinganine. The IR spectrum of this derivative was obtained from a chloroform solution and was essentially the same as reported by Karlsson et al. (6).

Localization of deuterium within the sphinganine moiety

The localization of the isotope within the sphinganine moiety was determined by GLC–MS analysis. The relative intensities and the observed $\frac{[a+1]^+}{a^+}$ values, representing the major fragmentation and rearrangement ions of a protium reference of *erythro*-N-acetylsphinganine (11, 12) and VI-$^2H_1$, are presented in **Table 2.** The mole percentage of monodeuterium-labeled species was calculated for several characteristic ions by subtracting the observed isotopic abundance in the protium reference from the observed isotopic abundance in VI-$^2H_1$, as outlined by Biemann (13). These values are presented in Table 2.

TABLE 2. Comparative GLC–MS analysis of protium and C–1 monodeuterio species of *erythro*-N-acetylsphinganine[a]

m/z	Protium		VI-$^2H_1$		mol %$^2H_1$[b]
	percent relative isotopic abundance				
472	7.4	(0.45)[c]	1.1	(11.0)[c]	69
473	3.3		12.1		
428	0.6	(0.50)	0.2	(4.50)	
429	0.3		0.9		
384	5.9	(0.29)	9.0	(0.26)	
385	1.7		2.4		
313	22.4	(0.33)	32.4	(0.32)	
314	7.3		10.3		
247	30.7	(0.26)	4.2	(8.50)	
248	9.0		35.7		83
217	16.9	(0.20)	6.2	(2.11)	
218	3.4		13.1		
174	7.7	(0.38)	1.6	(5.25)	72
175	2.9		8.4		
157	100.0	(0.17)	15.2	(6.58)	84
158	17.3		100.0		
129	22.4	(0.23)	24.5	(0.27)	
130	5.2		6.6		
103	24.5	(0.12)	16.2	(0.41)	
104	2.9		6.7		
85	67.0	(0.06)	10.2	(4.25)	79
86	4.5		43.4		

[a] TMS derivative.
[b] The mole percentage of monodeuterium-labeled species obtained after correcting for the natural isotope abundance.
[c] The value in parentheses represents the observed ratio of $\frac{[a+1]^+}{a^+}$.

A shift is observed in the M–15 ion from m/z 472 to m/z 473 indicating incorporation of a single deuterium atom in our product. Fragment ions (473, 248, 175, 158, 104, 86) containing the C–1 terminal carbon show high isotope enrichment. The absence of an isotope shift from m/z 384 to m/z 385 is an important criterion for deuterium assignment in the sphinganine fragmentation pattern (11). This ion results from a cleavage between C–2 and C–1 with charge retention in the larger fragment. One would have expected to observe a shift from m/z 384 to m/z 385 if deuterium substitution had occurred at any position other than the C–1 terminal carbon. The absence of a m/z 384 to 385 shift and the isotope enrichment for fragments containing C–1, strongly suggest that deuterium was incorporated at C–1, as predicted from the reaction scheme presented in **Fig. 1.**

Maintenance of stereochemical configuration

The stereochemical configuration of naturally occurring sphinganine is of the D-*erythro* type. It was our aim to label sphinganine selectively at C–1 without appreciably altering the existing stereochemistry. Isomerization about C–3, resulting in *threo* isomer formation, was not observed in VI (all species), or at any time during the synthesis, as determined by GLC (9). The configuration of VII (all species) was confirmed to be *erythro* following N-acetylation, TMS derivatization, and analysis by GLC and/or GLC–radiochromatography. The optical rotations of *erythro*-Dnp-VII-$^1H_2$ and Dnp-SH_2 (obtained as described earlier from bovine brain sphingomyelin) were measured in order to ascertain whether our procedure resulted in racemization about C–2.

The specific rotations $[\alpha]^{23}_{589}$ for Dnp-VII-$^1H_2$ and Dnp-SH_2 (natural) were $+57 \pm 5°$ and $+54 \pm 5°$, respectively (lit. $[\alpha]^{23}_{D} + 60 \pm 6°$ (6)). The positive rotation and chromatographic mobilities on GLC and TLC of our synthetic products are supportive evidence for a D-*erythro* configuration.

Identification of *erythro*-(1-$^3H_1$)sphinganine (VII-$^3H_1$): 3-O-benzoyl- and 1-O-benzoyl- isomers of N-acetyl-(1-$^3H_1$)sphinganine (VI-$^3H_1$)

Fig. 2 shows a TLC-radiochromatographic scan of radiolabeled products resulting from the NaB^3H_4 reduction of aldehyde (V). Component A, (ca. 9% of total radioactivity), comigrated with authentic N-acetylsphinganine and was not characterized further. Component B (R_f 0.24) comigrated with 2-acetamido-3-O-benzoyloctadecan-1-ol. Component C (R_f 0.32) is tentatively assigned as the 1-O-benzoyl isomer of N-acetylsphinganine; this compound may result from benzoyl migration following reductive

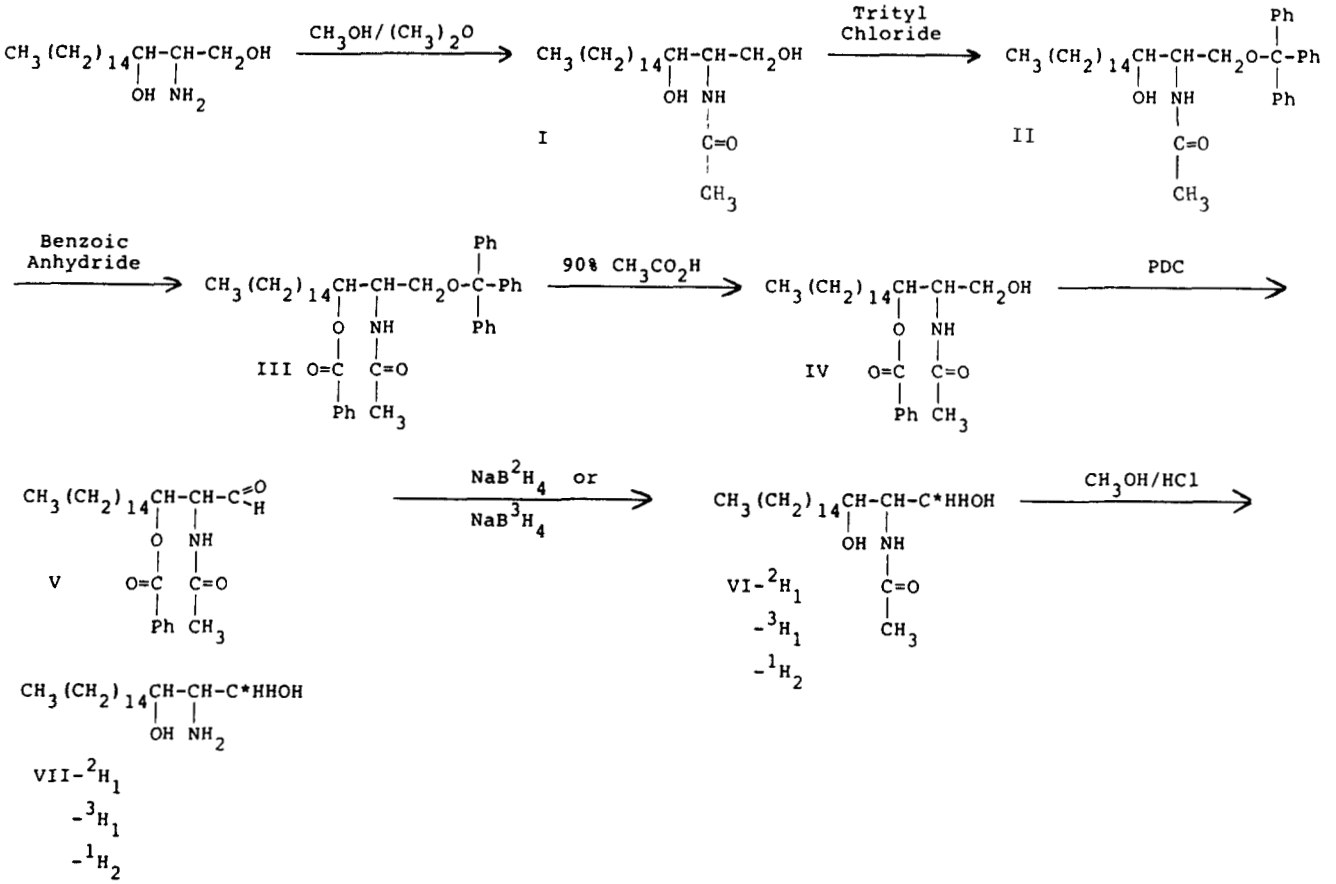

Fig. 1. Synthesis of [1-$^2H_1$] and [1-$^3H_1$]sphinganine from naturally occurring sphinganine. I, 2-acetamidooctadecan-1,3-diol; II, 1-O-trityl-2-acetamidooctadecan-3-ol; III, 1-O-trityl-2-acetamido-3-O-benzoyloctadecane; IV, 2-acetamido-3-O-benzoyloctadecan-1-ol; V, 2-acetamido-3-O-benzoyloctadecan-1-al; VI-$^2H_1$, 2-acetamido-[1-$^2H_1$]octadecan-1,3-diol; VI-$^3H_1$, 2-acetamido-[1-$^3H_1$]octadecan-1,3-diol; VII-$^2H_1$, [1-$^2H_1$]sphinganine; VII-$^3H_1$, [1-$^3H_1$]sphinganine. (VI- and VII-$^1H_2$ obtained by non-isotopic reduction of V with $NaBH_4$ followed by hydrolysis as indicated.)

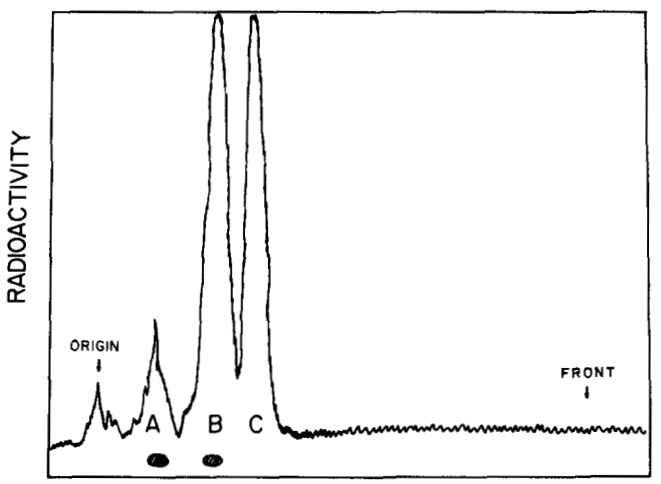

Fig. 2. TLC-radioscan of products following NaB^3H_4 reduction of 2-acetamido-3-O-benzoyloctadecan-1-al (V). Authentic N-acetylsphinganine (A) and 2-acetamido-3-O-benzoyloctadecan-1-ol (B) were co-chromatographed with the reduction products in SS-E and visualized by spraying the plate with PMA reagent.

labeling of the 2-acetamido-3-O-benzoyloctadecan-1-al (V). The position of the isotope and the isomeric relationship of compounds B and C (Fig. 2) were determined by the following procedures. Compounds B and C, putatively the 3-O- and 1-O-benzoyl isomers of N-acetyl-(1-$^3H_1$)sphinganine, respectively, were separated by TLC (SS-E) localized by TLC–radiochromatographic scanning and eluted separately from the silica gel plate with methanol. Compounds B and C contained 1.0×10^6 and 0.8×10^6 cpm, respectively; this accounts for ca. 91% of the radioactivity applied to the TLC plate.

Mild alkali-lability of the 3-O- and putative 1-O-benzoyl isomers resulting in conversion to *erythro*-N-acetyl-(1-$^3H_1$)sphinganine (VI-$^3H_1$)

Compounds B and C were subjected to mild alkaline hydrolysis (1 ml, 0.1 N methanolic NaOH, 25°C, 30 min), after which the reaction was stopped by the addition of 2 ml of chloroform and 1 ml of 1 N HCl. The samples

were mixed with a vortex mixer and centrifuged to partition the phases. The upper layer of each sample contained less than 0.1% of tritium and was discarded. The lower layer was reduced in volume by placing the samples in a 37°C bath and under a stream of nitrogen. TLC–radiochromatographic scanning of the hydrolysis product(s) indicated all the radioactivity from compounds B and C, comigrating with authentic N-acetylsphinganine (SS-F, R_f 0.14; SS-J, R_f 0.23). To further characterize the identity of the radiolabeled products released by alkaline hydrolysis, the samples were dried in vacuo, converted to the TMS derivatives, and analyzed by GLC–radiochromatography. **Fig. 3** shows the results from these analyses. The mass profile represents an exogenously added mixture of TMS derivatives of *threo*- and *erythro*-N-acetylsphinganines, in order of elution, respectively. After mild alkaline hydrolysis, greater than 97% of the radioactivity is shown to comigrate with *erythro*-N-acetylsphinganine (Fig. 3A, B).

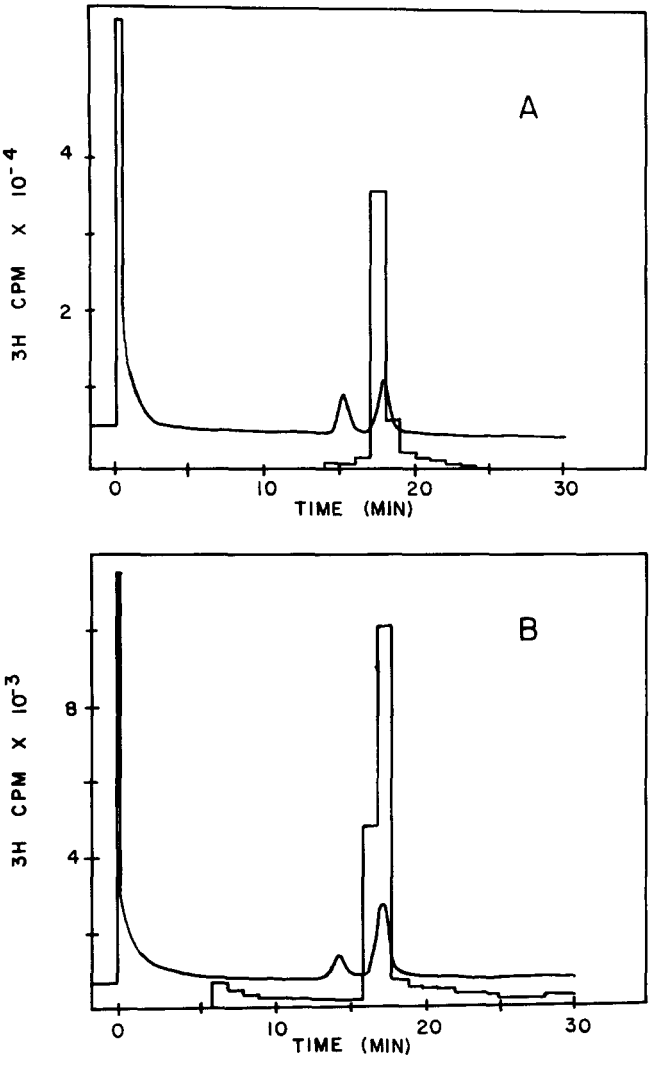

Fig. 3. GLC-radiochromatographic analysis of N-acetyl-[1-$^3H_1$]-sphinganine obtained from 2-acetamido-3-O-benzoyl-[1-$^3H_1$]-octadecan-1-ol (A) and putative 1-O-benzoyl-2-acetamido-[1-$^3H_1$]-octadecan-3-ol (B) after mild alkaline hydrolysis and TMS derivatization. Mass profile represents exogenous addition of *threo* and *erythro* isomers (in order of elution).

Identification of the long chain base moiety contained within compounds B and C

The radiolabeled compound comigrating with N-acetylsphinganine on TLC (SS-A, R_f 0.42) was eluted from the silica gel with methanol and subjected to acidic methanolysis (1 N methanolic HCl (10 M H_2O), 80°C, 16 hr); the long chain base was isolated as previously described (4). TLC–radiochromatographic scanner (SS-G, R_f 0.33) showed a radioactive component comigrating with authentic sphinganine, as detected by spraying the plate with ninhydrin.

Localization of the tritium within the sphinganine moiety from compounds B and C

The radiolabeled sphinganine, from compounds B and C, was eluted from the silica gel with methanol and each sample was taken to dryness under a stream of nitrogen. Periodate oxidation of the putative [1-$^3H_1$]sphinganine derived from compounds B and C, followed by sodium borohydride reduction, was performed according to Sweeley and Moscatelli (14), as modified by Carter and Hirschberg (15). Greater than 95% of the tritium associated with sphinganine was released into water-soluble products (presumably [^{3}H]–CH_2O). The resulting hexadecanol (carbons 3 thru 18) was mixed with authentic hexadecanol and analyzed by TLC (SS-K). The hexadecanol was visualized on the plate by iodine vapors and the area corresponding to hexadecanol (R_f 0.44) was scraped and eluted with methanol from the silica gel into a scintillation vial. The solvent was removed under a stream of nitrogen. No radioactivity was detected with hexadecanol.

DISCUSSION

Hitherto, *erythro*-D-[1-^{2}H] and [1-^{3}H]sphinganine were synthesized only by total synthesis (2, 3). These procedures, based upon the synthesis by Shapiro et al. (1), result in four stereoisomers (D-*erythro*, D-*threo*, L-*erythro*, L-*threo*). Carter, Shapiro, and Harrison (16) and Carter and Shapiro (17) showed that the D-*erythro* configuration corresponds to the naturally occurring sphinganine base. Stoffel and Bister (18) compared the precursor potential of these four isomeric sphinganines in vivo and found only the D-*erythro* isomer to be utilized for the biosynthesis of complex sphingolipids such as sphingomyelin and cerebrosides. Thus, meaningful investigation of sphingolipid pathways require the availability of isotopically labeled, stereospecific precursors.

This report describes a convenient chemical synthesis of *erythro*-D-[1-$^2H_1$] and [1-$^3H_1$]sphinganine without the burden of total synthesis and purification of stereoisomers. Our dissatisfaction with the resolution of optical isomers from previous total syntheses (2, 3) led us to pursue an approach that avoids generation of isomers. Our rationale was to utilize a stereospecific starting material (LCB's, natural sources) and to maintain the original configuration throughout the synthesis.

All the reactions described except the tritylation and PDC oxidation are quantitative. Acetylation of the C–2 amino provides protection as well as the ability to detect sphinganine isomers by GLC via the N-acetyl TMS derivatives. The yield of the tritylation reaction was highly dependent upon the freshness of the trityl chloride and the dryness of the pyridine. The benzoylation reaction, as described by McCluer and Ullman (19), was employed due to its ease and avoidance of N-benzoylation by-products which occur with benzoyl chloride in pyridine at 60°C. Initially, we used an acetyl group to protect the C–3 hydroxyl. This selection was abandoned due to rapid acyl migration of the acetate from C–3 to C–1 hydroxyl following detritylation of III (Fig. 1). We have not observed benzoyl migration following detritylation. We have observed, however, benzoyl migration after PDC oxidation of the primary alcohol (IV) followed by NaB^3H_4 of the resulting aldehyde (V). A mixture of 1-O- and 3-O-benzoyl isomers of *erythro*-N-acetyl-[1-$^3H_1$]sphinganine has been detected, as suggested in Fig. 2. This migration was not observed for the synthesis of [1-$^2H_1$]sphinganine. In this case, the 1-O- and 3-O-benzoyl group was hydrolyzed from the sphinganine base (during NaB^2H_4 reduction) most likely due to the large molar excess of reductant present in the reaction mixture and the extended reaction time at 25°C. In contrast, the products resulting from the NaB^3H_4 reduction, retained the benzoyl group yielding 2-acetamido-3-O-benzoyl-[1-$^3H_1$]octadecan-1-ol and 1-O-benzoyl-2-acetamido-[1-$^3H_1$]octadecan-3-ol isomers (Fig. 2). Retention of the benzoyl group after NaB^3H_4 reduction is most likely explained by the shorter reaction time at 4°C used in the synthesis of [1-$^3H_1$]sphinganine (VII-$^3H_1$).

Previous procedures (excluding total syntheses) for the preparation of ^2H- and ^3H-labeled LCB's have been described (20, 21, 22). NaB^2H_4 (20) or NaB^3H_4 (21) reduction of a 3-keto LCB derivative produces a mixture of isotopically labeled *erythro* and *threo* isomers. Catalytic reduction (with tritium) of the double bond of sphingosine yields a [4,5-$^3H_2$]sphinganine (22). This labeled LCB is the easiest to produce, yet is least suitable for metabolic studies. Catabolism of this compound yields [2,3-$^3H_2$]palmitic acid, a substrate displaying a high potential of reutilization for LCB biosynthesis.

This synthesis has several advantages over previous methods of labeling long chain bases. They are: *1*) utilization of an easily acquired stereospecific precursor; *2*) maintenance of configuration throughout synthesis; *3*) the absence of generating biologically inactive stereoisomers; and *4*) specific localization of the isotope, thereby eliminating metabolic reutilization of the isotope following catabolism by the cell. [1-^3H]LCB catabolism produces a degradation product ([^3H]phosphoethanolamine) with negligible potential of reutilization for LCB biosynthesis (23, 24). The use of [1-^3H]sphinganine in investigations of the biosynthesis of glycosphingolipids and localization of their subcellular site(s) of synthesis will be reported elsewhere.

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