Total synthesis of stereospecific sphingosine and ceramide

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Abstract A small-scale synthesis of the four sphingosine stereoisomers (D-*erythro*, L-*erythro*, D-*threo*, and L-*threo*) and lignoceroyl D- and L-*erythro*-sphingosines, which is suitable for synthesis of tritium-labeled compounds, is described. Ethyl DL-*erythro*-2-acetamino-3-hydroxy-4*t*-octadecenoate was esterified with L(+)-acetylmandeloyl chloride and the two diastereomers obtained were separated from each other by thin-layer or column chromatography. Each diastereomer was subjected to ethanolysis to obtain ethyl D- or L-*erythro*-2-amino-3-hydroxy-4*t*-octadecenoate which was then reduced with $LiAlH_4$ or $NaBH_4$ to yield D- or L-*erythro*-sphingosine. D-*erythro*-[1-^{3}H]Sphingosine with high specific activity was prepared by using $LiAl^3H_4$ in the last step. D- and L-*threo*-sphingosines were synthesized from ethyl DL-*threo*-2-acetamino-3-hydroxy-4*t*-octadecenoate by using a similar procedure.

Ceramide (lignoceroyl sphingosine) was prepared either by acylating sphingosine or by the following new method. Ethyl DL-*erythro*-2-amino-3-hydroxy-4*t*-octadecenoate was converted to the *N*-lignoceroyl derivative and esterified with L(+)-acetylmandeloyl chloride. The two diastereomers obtained were separated and each isomer was treated with a catalytic amount of sodium ethoxide. One of the products, ethyl D-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoate, was reduced with $NaBH_4$ to yield ceramide. *N*-palmitoyl DL-*erythro*-sphingosine was also prepared using an identical procedure. *N*-lignoceroyl D-*erythro*-[1-^{3}H]sphingosine was prepared by NaB^3H_4 reduction of the corresponding amide ester. A doubly labeled ceramide, [1-^{14}C]lignoceroyl [1-^{3}H]sphingosine, containing high specific activity, was prepared by mixing the above *N*-lignoceroyl D-*erythro*-[1-^{3}H]sphingosine and *N*-[1-^{14}C]lignoceroyl D-*erythro*-sphingosine. The conversion of the doubly labeled ceramide to 3-keto derivative is also described.

Supplementary key words Resolution of ethyl D- and L-*erythro*-2-acetamino-3-[L(+)-acetylmandeloyloxy]-4*t*-octadecenoate · resolution of D- and L-*erythro*-2-lignoceroyl amino-3-[L(+)-acetylmandeloyloxy]-4*t*-octadecenoate · [1-^{3}H]sphingosine · *N*-acyl [1-^{3}H]sphingosine · doubly labeled ceramide · $NaBH_4$ reduction of ethyl 2-acylamino-3-hydroxy-4*t*-octadecenoate · doubly labeled 3-ketoceramide

Ceramide (*N*-acyl-D-*erythro*-sphingosine) is a key intermediate in the biosynthesis and degradation of sphingolipids (1, 2). It is synthesized in vitro from sphingosine and fatty acyl CoA (3–5) or a free fatty acid (6) and hydrolyzed to free fatty acid and sphingosine (6, 7). Sphingosine is synthesized from palmitoyl CoA and serine. These compounds are first condensed to 3-ketodihydrosphingosine; this is followed by dehydrogenation at the 4,5-position and reduction of the 3-keto group (1, 8). Recently, Morell and Radin (4) reported that rat brain microsomes catalyze the condensation of 3-ketodihydrosphingosine with acyl CoA to form *N*-acyl 3-ketodihydrosphingosine. This observation suggests a new pathway of ceramide synthesis in which the dehydrogenation and reduction of 3-ketodihydrosphingosine occur after it is converted to the *N*-acyl derivative. To test this hypothesis, the synthesis of a doubly labeled 3-ketoceramide, as well as of a doubly labeled ceramide, becomes necessary.

Three methods have been established to prepare ^{3}H-labeled sphingosine and its derivative. The simplest method of labeling is the addition of tritium to the double bond of sphingosine by catalytic hydrogenation (9). The product of this procedure, however, is not sphingosine but dihydrosphingosine

Abbreviations: TLC, thin-layer chromatography; ORD, optical rotatory dispersion. Trivial names used: sphingosine is 4-*trans*-sphingenine (1,3-dihydroxy-2-amino-4-*trans*-octadecene). The absolute configurations of D-*erythro*-sphingosine, L-*erythro*-sphingosine, D-*threo*-sphingosine, and L-*threo*-sphingosine are [2*S*,3*R*], [2*R*,3*S*], [2*R*,3*R*], and [2*S*,3*S*], respectively. Ceramide is *N*-acyl sphingosine. 3-Ketosphingosine and 3-ketoceramide are 2-amino-3-oxo-4*t*-octadecenol-1 and its *N*-acyl derivative, respectively.

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which may behave differently than sphingosine during metabolic studies. In addition, [4,5-^{3}H]dihydrosphingosine and its derivatives, such as ceramide, will lose one-half of the tritium during conversion of sphingolipids containing sphingosine. Moreover, sphingolipids containing [4,5-^{3}H]dihydrosphingosine may be much less effectively converted to those containing sphingosine in vivo because of an isotope effect on the dehydrogenation step.

The second method involves reduction of the 3-keto derivative of a sphingolipid with NaB^3H_4 (10). The problem with this method is the formation of *threo* and *erythro* stereoisomers. In addition, the tritium at the 3-position of sphingosine may not be stable in some metabolic reactions because of the allylic nature of the 3-hydroxy group.

The final method is based on the total synthesis of sphingosine developed by Shapiro, Segal, and Flowers (11) and applied by Stoffel and Henning (12) to the synthesis of DL-*erythro*-[1-^{3}H]sphingosine. The tritium in this compound should be stable both chemically and metabolically. However a problem with the original procedure is that the sphingosine obtained has a racemic form and its resolution to enantiomers is required. Natural sphingolipids contain D-*erythro*-sphingosine, and the presence of the L-enantiomer, which is not a natural compound, in the substrate will confuse the results of metabolic studies. Such resolution of D- and L-*erythro*-dihydrosphingosine was performed in the past by converting the racemic base to a salt either with glutamic acid (11) or with mandelic acid (13). However, such preparation requires a relatively large-scale operation.

This communication describes in detail a new synthesis of D-*erythro*-sphingosine, its stereoisomers, their *N*-acyl derivatives, and 3-ketoceramide. This synthesis, a modification of the original synthesis of sphingosine by Shapiro et al. (11), which involves resolution of intermediate amide esters by conversion to L(+)-acetylmandeloyl derivatives, is especially suitable for the synthesis of optically active [1-^{3}H]sphingosines and their *N*-acyl derivatives (ceramides) with high specific activity. In vivo metabolism of the doubly labeled ceramides, prepared by combining the above [^{3}H]ceramide with [1-^{14}C]acyl sphingosine, and of their 3-keto derivatives in rat liver (14) and brain (15, 16) have been published.

RESULTS

Synthesis of stereoisomers of sphingosine

The *erythro*- and *threo*-isomers of the intermediate, ethyl 2-acetamino-3-hydroxy-4*t*-octadecenoate were separated from each other by column chromatography. The key modifications for the sphingosine synthesis include the resolution of the DL-*erythro*-2-acetamino-3-hydroxy-4*t*-octadecenoate (I) by conversion to its L-(+)-acetylmandelates (II) as shown in **Chart 1.**

The resulting diastereomers are easily separated using either column chromatography or TLC. The structure of the D-*erythro* isomer was confirmed by infrared spectroscopy (**Fig. 1*A***), ultraviolet spectroscopy (not shown; weak absorption at 260 nm because of the isolated benzene ring), and mass spectrometry (**Fig. 2**). Identical spectra were obtained with the L-*erythro* isomer (not shown). Mass spectrometric fragmentation expected from the compound II is shown in **Chart 2**. All expected fragments were obtained in the mass spectrum (Fig. 2). In addition, *m/e* 99 was obtained by the removal of the ethoxy group from the *m/e* 144, and *m/e* 107 was produced by the loss of ketene form *m/e* 149.

Ethyl D- and L-*erythro*-2-amino-3-hydroxy-4*t*-octadecenoate (III) were obtained in good yields by ethanolysis of II without racemization. The optical purity was confirmed by *N*-acetylating the purified product (*erythro*-III) followed by esterification with L(+)-acetylmandeloyl chloride. TLC of the product showed a single spot of the corresponding L(+)-acetylmandelate without diastereomer. Absolute configurations of these isomers were confirmed from ORD spectra (**Fig. 3**). Similarly, ethyl D- and L-*threo*-2-amino-3-hydroxy-4*t*-octadecenoates (D- and L-*threo*-III) and ethyl D- and L-*erythro*-2-amino-3-hydroxy-octadecanoates (dihydro III), which are precursors of D- and L-*erythro*-dihydrosphingosines, were prepared. Their ORD spectra are also in Fig. 3. The ORD spectrum of triacetyl derivative of purified natural D-*erythro*-sphingosine obtained from cerebroside was used as the reference.

The last step of sphingosine synthesis was performed by either $LiAlH_4$ reduction as described by Shapiro et al. (11) or more conveniently by $NaBH_4$ reduction of III. D-*erythro*-[1-^{3}H]Sphingosine was prepared by using $LiAl^3H_4$.

Synthesis of radioactive ceramide

Radioactive ceramides were prepared either by *N*-acylation of D-*erythro*-[1-^{3}H]sphingosine with the *N*-hydroxysuccinimide ester of a nonradioactive fatty acid (17) or by *N*-acylation of nonradioactive D-*erythro*-sphingosine with a [1-^{14}C]-labeled free fatty acid by oxidation–reduction coupling (18). Both procedures satisfactorily provided the desired ceramide; however, the preparation of ceramide containing [1-^{3}H]sphingosine needed some improvement. The amount of [1-^{3}H]sphingosine with a high specific activity was so

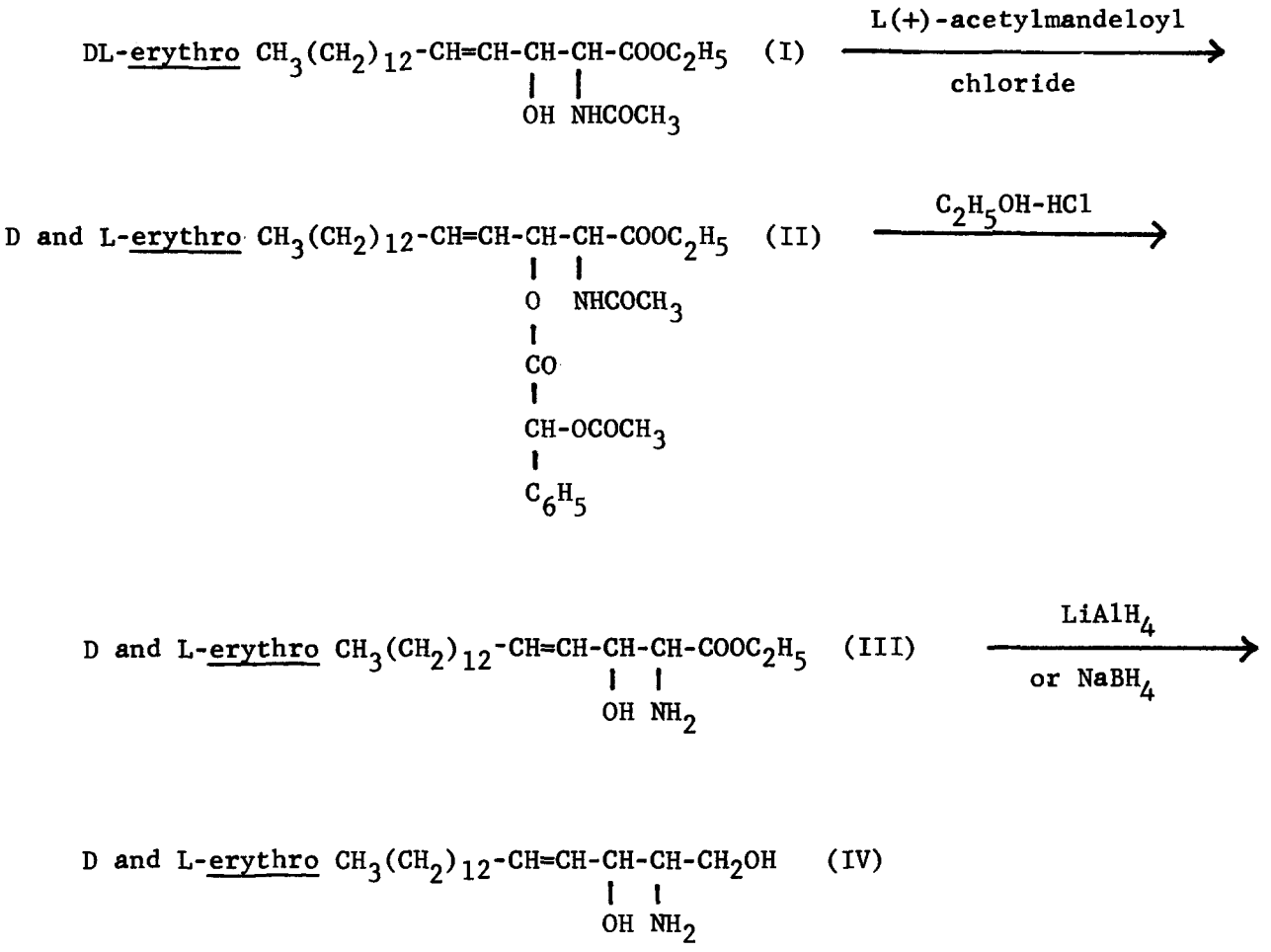

Chart 1. Synthesis of stereoisomers of sphingosine.

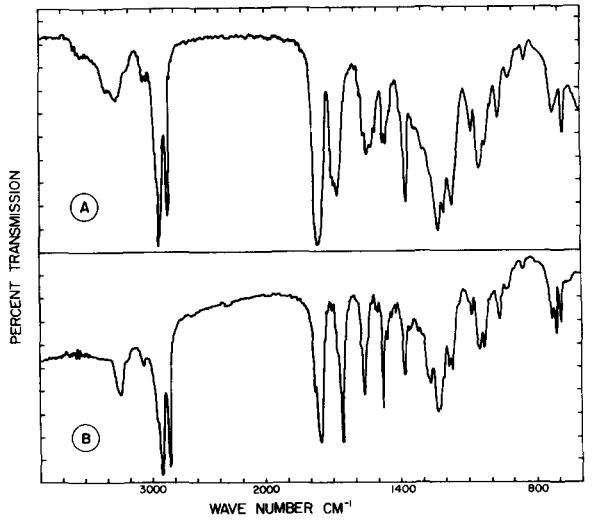

Fig. 1A. Infrared spectrum of ethyl D-*erythro*-2-acetylamino-3-[L-(+)-mandeloyloxy]-4*t*-octadecenoate (D-*erythro*II). The spectrum was taken from a neat film. **B.** Infrared spectrum of L-(+)-acetylmandelate of ethyl D-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoate (D-VI). The spectrum was measured from KBr pellet (1 mg sample in 150 mg KBr).

small that recrystallization was not possible. Thin-layer chromatography and column chromatography resulted in considerable loss of radioactive sphingosine. Another problem was the need for a two-step synthesis involving the radioactive material, namely, the reduction of ethyl 2-amino-3-hydroxy-4*t*-octadecenoate (III) by $LiAl^3H_4$ and the acylation of the [1-^{3}H]sphingosine obtained.

Our discovery that ethyl 2-amino-3-hydroxy-4*t*-oc-

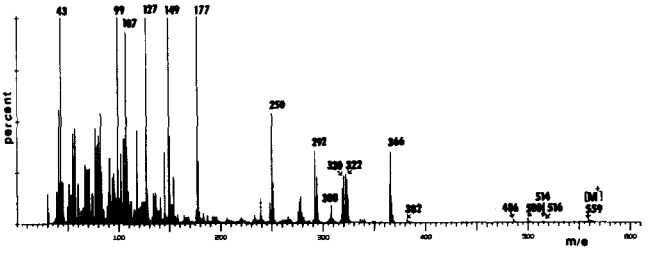

Fig. 2. Low resolution mass spectrum of ethyl D-*erythro*-2-acetamino-3-[L-(+)-mandeloyloxy]-4*t*-octadecenoate (D-*erythro*-III).

tadecenoates (III) could be converted to sphingosine by NaB^3H_4 in place of $LiAl^3H_4$ led us to a new convenient synthesis of ceramide as illustrated in **Chart 3.** Ethyl DL-*erythro*-2-amino-3-hydroxy-4*t*-octadecenoate (III) was converted to the *N*-lignoceroyl derivative (V) by using the oxidation–reduction coupling (18). The racemic compound was then converted to its 3-[L-(+)-acetylmandeloyl] derivatives (VI) and the diastereomers were separated by column chromatography. The structures of D- and L-*erythro*-VI were confirmed by infrared (Fig. 1*B* and UV spectra (not shown; weak adsorption at 260 nm) and elementary analysis. These diastereomers were subjected to a mild alkaline ethanolysis that yielded ethyl *N*-lignoceroyl D-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoate and its L-enantiomer (VII). These two enantiomers were then reduced by $NaBH_4$ to give *N*-lignoceroyl D-*erythro*-sphingosine and its L-enantiomer (VIII), respectively.

If the last step was performed using $LiAlH_4$ instead of $NaBH_4$, the amide group would also be reduced to a secondary amine (19). The configurations of ethyl D- and L-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoates and their $NaBH_4$ reduction products (ceramides) were confirmed by ORD (**Fig. 4**). When the last reduction was done with NaB^3H_4, lignoceroyl [1-^{3}H]sphingosine was obtained. The possibility that isomerization occurred during these reactions was ruled out by examining the product by TLC on a borate-impregnated silica gel G plate with chloroform–methanol 9:1 as the solvent; this showed a single spot corresponding to the *erythro*-sphingosine ceramide. If isomerization had occurred, the *threo*-isomer would have been detected under these conditions.

EXPERIMENTAL PROCEDURES

Materials

The following chemicals were purchased from commercial sources: myristyl aldehyde from Aldrich Chemicals (Milwaukee, WI); NaB^3H_4 and $LiAl^3H_4$ from New England Nuclear (Boston, MA); D-*erythro*-sphingosine from Serdary Research Labs (London, Ontario, Canada); various nonradioactive fatty acids from Lachat Chemicals (Chicago Heights, IL) or Nu-Chek-Prep (Elysian, MN).

The sphinogosine was purified by column chromatography prior to use (20). The following chemicals were synthesized in this laboratory: [1-^{14}C]lignoceric acid (21); L(+)-acetylmandeloyl chloride (22); and *N*-hydroxysuccinimide lignocerate (23). Two kinds of silica gel, Unisil (100–200 mesh) and silica gel 60 extra pure (70–230 mesh), were obtained from Clarkson

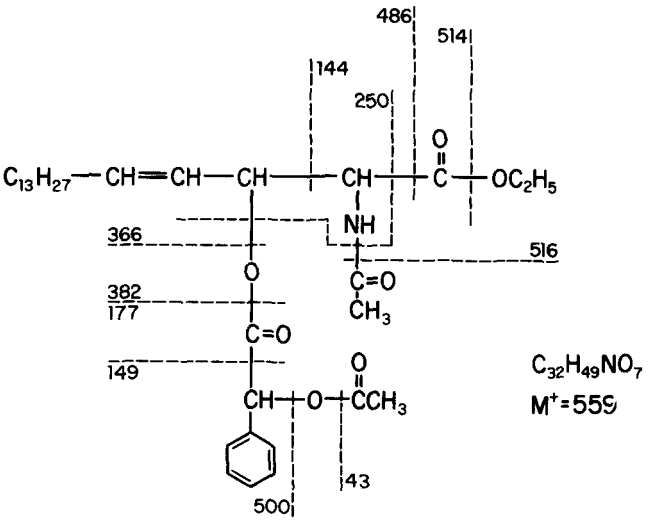

Chart 2. Expected mass spectrometric fragmentation of Compound II.

Chemicals (Williamsport, PA) and EM Labs (Elmsford, NY), respectively. Precoated thin-layer chromatographic plates were obtained from Analtech (Newark, DE) and Quantum Industries (Fairfield, NJ).

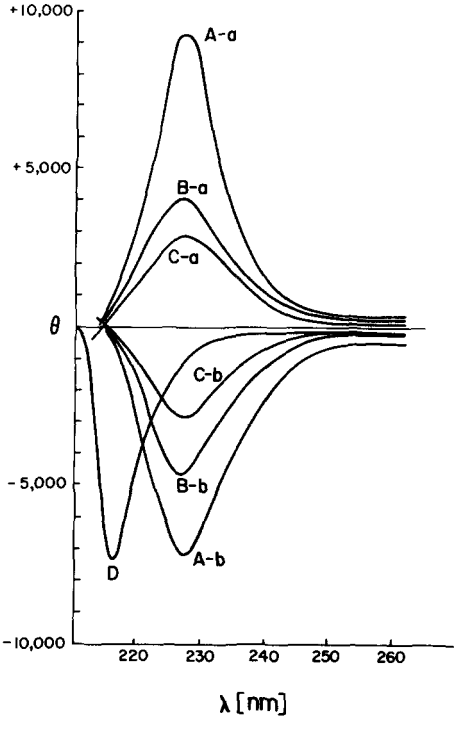

Fig. 3. ORD spectra of A-a, ethyl L-*threo*-2-amino-3-hydroxy-4*t*-octadecenoate (L-*threo*-III); A-b, D-*threo*-III; B-a, L-*erythro*-2-amino-3-hydroxy-octadecanoate (L-*erythro*-dihydro-III); B-b, D-*erythro*-dihydro-III; C-a, L-*erythro*-2-amino-3-hydroxy-4*t*-octadecenoate (L-*erythro*-III); C-b, D-*erythro*-III; and D, D-*erythro*-triacetyl sphingosine. Each sample was dissolved in 3 ml of hexane, except triacetyl sphingosine which was dissolved in 3 ml of methanol.

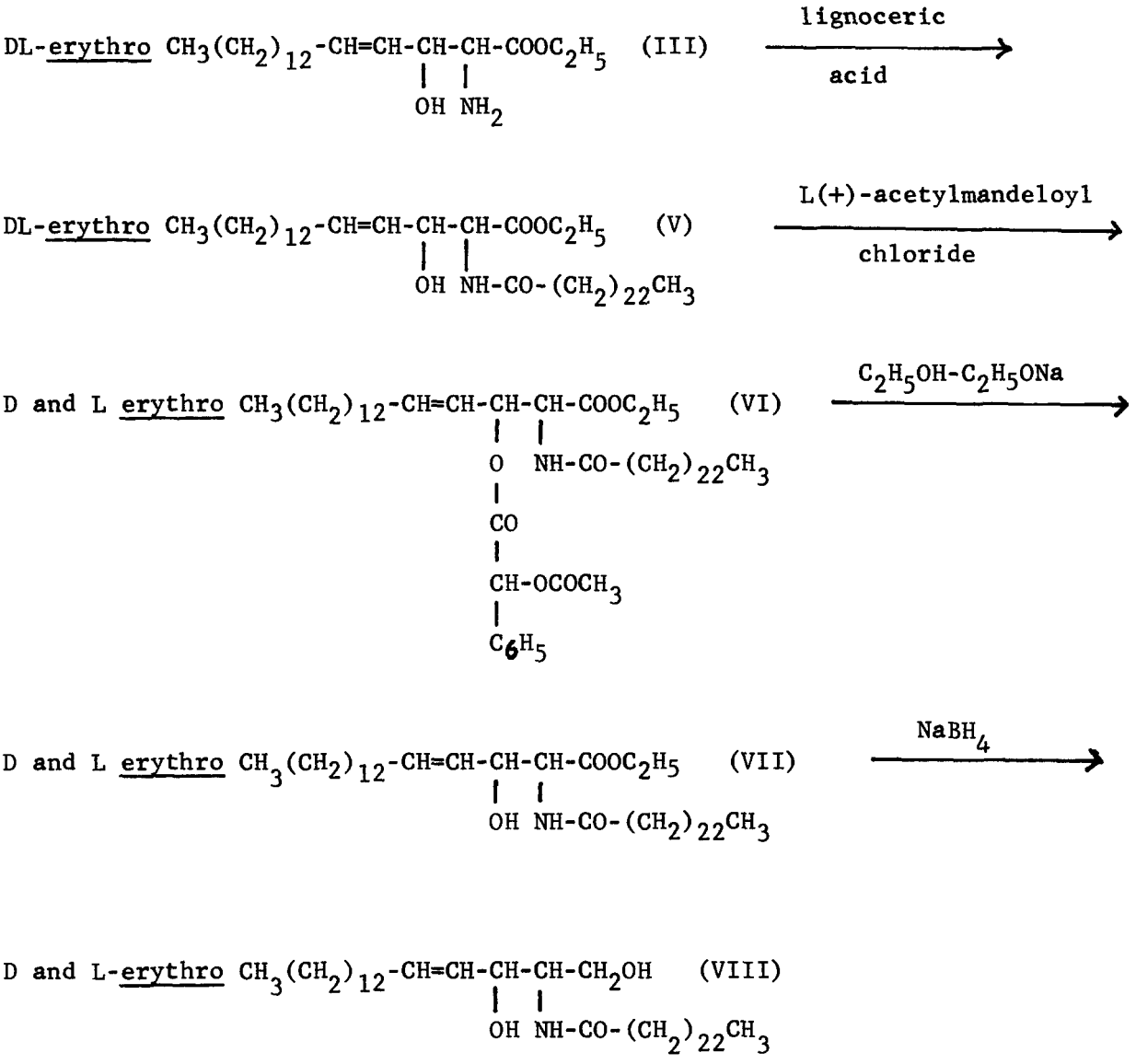

Chart 3. Synthesis of stereoisomers of ceramide.

Synthesis of sphingosines

Ethyl DL-*erythro-2-acetamino-3-hydroxy-4t-octadecenoate (I)*. This compound was synthesized in five steps starting from myristyl aldehyde according to Shapiro et al. (11). Yields and physical constants of this compound as well as intermediates of each step were in good agreement with those given by the authors. In addition, UV, IR, NMR, and mass spectra of the two intermediates and the final product all agreed with the assigned structures.

Ethyl D- *and* L-*erythro-2-acetamino-3-*[L(+)*-acetylmandeloyloxy*]*-4t-octadecenoates (II)*. DL-*erythro*-I (100 mg) was dissolved in a solution of 166.5 mg of L(+)-acetylmandeloyl chloride in 2.35 ml of dry benzene. The mixture was mixed with 0.7 ml of dry pyridine and allowed to stand for 1 hr at room temperature. The reaction mixture was diluted with 5 ml of benzene, washed three times with 2-ml portions of water and evaporated to dryness. The residue weighed 179 mg. Examination of the product by TLC (silica gel G with benzene–ether 1:2 as the solvent) revealed two spots, one with an R_f value of 0.56 and the other with an R_f value of 0.45. The starting material gave a spot with an R_f value of 0.19 in this system.

The product was fractionated by column chromatography on 40 g of silica gel 60 extra pure. The entire residue was dissolved in a small volume of benzene–ether 30:1 and applied to the column. The column was rinsed with 600 ml of the same solvent. The column was then eluted with 600 ml of benzene–ether 20:1, 100 ml of benzene ether 15:1, and 200 ml of

benzene–ether 10:1. The fraction eluted with benzene–ether 20:1 contained only the material (compound A) that produced the top spot in the TLC system. This weighed 69 mg and had an mp of 36–37°C. The fraction eluted with benzene–ether 10:1 contained only the material (Compound B) that gave the lower R_f value. The yield of this compound was 82 mg and its mp was 29–30°C.

Ethyl D-*erythro-2-amino-3-hydroxy-4t-octadecenoate (*D-*erythro-III).* The compound B (75 mg) was refluxed for 3 hr with 1 ml of aqueous ethanolic-HCl (8.55 ml of concentrated HCl diluted to 100 ml with absolute ethanol). This mixture was evaporated under a stream of nitrogen until a small amount remained. The residue was mixed with 1 ml of 3% NH_4OH at 0°C and then extracted five times with 3-ml portions of ice-cold ether. The pooled ether extracts were washed three times with 2-ml portions of water and evaporated to dryness. The residue, which weighed 55.9 mg, was recrystallized from hexane and 27.7 mg of colorless needles with an mp of 48–50°C was obtained. The material (28.7 mg) recovered from the mother liquor of the recrystallization was purified by column chromatography on 8.7 g of silica gel 60 extra pure. The column was eluted with chloroform–methanol 25:1; 1-ml fractions were collected. Each fraction was examined by TLC on silica gel G (chloroform–methanol 85:15. Fractions 34–45, containing D-*erythro*-III free from contaminants, were combined. Evaporation of the solvent yielded a residue that weighed 13.3 mg and had an mp of 51–52°C.

Ethyl L-*erythro-2-amino-3-hydroxy-4t-octadecenoate (*L-*erythro-III).* This compound was obtained from compound A of II using the procedure described for the preparation of the D-enantiomer. Compound A, 63 mg, yielded 30.2 mg of pure L-enantiomer, which had an mp of 52–53°C.

Ethyl D-*threo-2-amino-3-hydroxy-4t-octadecenoate (*D-*threo-III).* Ethyl DL-*threo*-2-acetamino-3-hydroxy-4*t*-octadecenoate (DL-*threo*-I) was obtained from the mother liquor of the recrystallization of DL-*erythro*-I described above and was purified by repeated silica gel 60 column chromatography. The column was eluted with a gradient of benzene–ether 10:1 to benzene–ether 1:2. Under these conditions the *threo*-isomer, which was an oil at room temperature, eluted slightly ahead of the *erythro*-isomer. The *threo*-II was prepared as described above for the *erythro*-isomer, and the product was separated into two diastereomers as described previously. The D-*threo*-II (colorless oil, 38.6 mg) was ethanolyzed to yield D-*threo*-III (29.3 mg) as described previously. After recrystallization this material melted at 61–62°C.

Ethyl L-*threo-2-amino-3-hydroxy-4t-octadecenoate (*L-*threo-III).* L-*threo*-II (colorless oil, 38.3 mg) was hydrolyzed to yield ethyl L-*threo*-III (25.2 mg), which had an mp of 60–61°C after recrystallization.

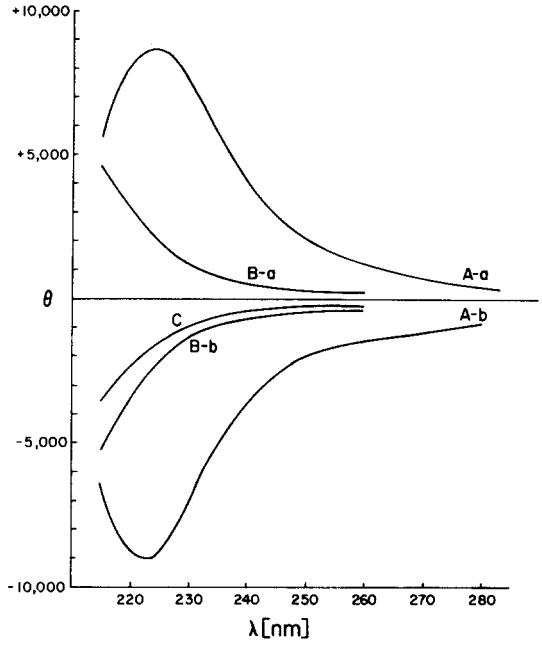

Fig. 4. ORD spectra of A-a, ethyl L-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoate (L-VI); A-b, D-VI; B-a, L-*N*-lignoceroyl sphingosine (L-VII), B-b, D-VII; and C, ceramide containing nonhydroxy fatty acids prepared from calf brain cerebrosides. The samples (6–8 mg) were dissolved in hexane–ethanol 8:2.

Ethyl D-*erythro-2-amino-3-hydroxyoctadecanoate (*D-*erythro-dihydro-III).* This compound was prepared from ethyl DL-*erythro*-2-acetamino-3-hydroxyoctadecanoate which was obtained as the by-product of the DL-*erythro*-II preparation (1). The racemic compound was converted to L-(+)-acetylmandelate (DL-dihydro-II) and the two diastereomers were separated from each other by silica gel 60 column chromatography as described above for the separation of *erythro*-II. L-Dihydro-II was eluted from the column slightly ahead of the D-isomer. The D-*erythro*-dihydro-II (mp 42–44°C, 25.7 mg) thus obtained was treated with ethanolysis as described above to yield D-*erythro*-dihydro-III (19.3 mg) which had an mp of 53–55°C.

Ethyl L-*erythro-2-amino-3-hydroxy-octadecanoate (*L-*erythro-dihydro-III).* This compound was synthesized by the ethanolysis of L-*erythro*-dihydro-II (25.3 mg). The purified product (16.7 mg) had an mp of 54–56°C.

D-*erythro-sphingosine (*D-*erythro-IV) synthesis by* $LiAlH_4$ *reduction.* D-*erythro*-III (8.2 mg) was converted to D-*erythro*-IV by the procedure of Shapiro et al. (11). The structure of D-*erythro*-IV was confirmed by converting it to its triacetyl derivative. IV was dissolved in 0.2 ml of acetic anhydride and pyridine (1:1) and left standing at room temperature for 8.5 hr. Two ml of iced water

was added to this mixture and precipitates were collected by centrifugation. The precipitates were washed twice with water and lyophilized. The residual white powder weighed 9.2 mg. TLC examination (silica gel G, chloroform–methanol 25:1) showed a single spot corresponding to the spot of authentic D-*erythro*-sphingosine triacetate.

The crude product was purified by preparative TLC on silica gel G using chloroform–methanol 25:1. The band of triacetyl sphingosine was detected by I_2 vapor and eluted with chloroform–methanol 85:15. Removal of the solvent from the eluant left a colorless crystalline material which weighed 6.85 mg and had an mp of 97–101°C. The triacetate prepared from natural D-*erythro*-sphingosine had an identical melting point. A mixture of the synthetic and natural triacetate melted at 96–100.5°C.

Synthesis of DL-*erythro-sphingosine (*DL-*erythro-IV) by* $NaBH_4$ *reduction*. A mixture of 20 mg each of DL-*erythro*-III and $NaBH_4$ was suspended in 0.5 ml of methanol and gently refluxed for 20 min. After the addition of 2 ml of chloroform, the solution was washed twice with 1-ml portions of water and evaporated to dryness. The product was purified by column chromatography. A column containing 8 g of silica gel 60 extra pure was eluted with chloroform–methanol–concentrated NH_4OH 90:9:1 and the effluent was monitored by TLC. The fractions containing DL-sphingosine were combined and evaporated to dryness. The residue was further purified by recrystallization from ethyl acetate. The crystalline white powder weighed 6 mg and had an mp of 65–68°C. TLC (silica gel G plate, chloroform–methanol–2N NH_4OH 40:10:1) gave one spot identical to that of the authentic sample of sphingosine. This compound was again identified by converting it to its triacetate. The triacetate had an mp of 95–95.5°C and an R_f value and an infrared spectrum identical to those of the authentic sample.

Synthesis of D-*erythro*-[*1-*^{3}*H*]*sphingosine*. D-*erythro*-III (4.88 mg) was mixed with 1.5 mg of $LiAl^3H_4$ (132.7 mCi/mmol) and 0.3 ml of tetrahydrofuran–ether 1:1; the mixture was sonicated for 5 min and refluxed for 2 hr. Two ml of ether was added and refluxing was continued for an additional 30 min. This product was treated as described above, and 4.19 mg of the crude reaction product containing 215×10^6 cpm was obtained. TLC–radioscanning indicated that most of the radioactivity was contained in D-*erythro*-sphingosine.

This product was purified on a chromatography column containing 0.83 g of silica gel extra pure 60. The column was eluted with chloroform–methanol–2N NH_4OH 40:10:1 and 0.5-ml fractions were collected. Each fraction was monitored by TLC–radioscanning. Fractions 6–10 contained only radioactive sphingosine and were combined. Evaporation of the solvent from the pooled fractions yielded D-*erythro*-[1-^{3}H] sphingosine which weighed 0.50 mg and contained 41.45×10^6 cpm. Specific activity was 49.6 mCi/mmol.

Synthesis of radioactive ceramide and 3-ketoceramide from sphingosine

N-Lignoceroyl D-*erythro*-[*1-*^{3}*H*]*sphingosine*. A mixture of 0.5 mg of [1-^{3}H]sphingosine, containing 41.45×10^6 cpm, and 2.0 mg of *N*-hydroxysuccinimide lignocerate in 0.5 ml of freshly redistilled tetrahydrofuran was allowed to stand for 36 hr at room temperature. After the solvent was evaporated the product was purified by preparative TLC on a silica gel G plate with benzene–acetone 3:1 as the solvent. The band was detected by spraying with methanol–water 1:1, eluted with chloroform–methanol 2:1, and washed (24). Removal of the solvent from the lower layer yielded *N*-lignoceroyl D-*erythro*-[1-^{3}H]sphingosine which contained 16.36×10^6 cpm and weighed 0.45 mg. TLC–radioscannings of the product chromatographed in two other solvent systems (chloroform–methanol–acetic acid 90:2:8 and chloroform–methanol–concentrated NH_4OH 90:10:1) showed only a single radioactive peak that corresponded to authentic *N*-lignoceroyl D-*erythro*-sphingosine in each case.

*N-Lignoceroyl 3-keto-*D-[*1-*^{3}*H*]*sphingosine*. The above *N*-lignoceroyl D-*erythro*-[1-^{3}H]sphingosine, containing 10.9×10^6 cpm, was reacted with 0.13 ml of 3% dichlorodicyanobenzoquinone in dioxane (25). The reaction product was purified by preparative TLC using a silica gel GF plate and chloroform–methanol 25:1. The 3-ketoceramide band was detected using ultraviolet absorption and was eluted with chloroform–methanol 10:1. The eluted material weighed 0.135 mg and contained 8.85×10^6 cpm.

N-[*1-*^{14}*C*]*Lignoceroyl* D-*erythro-sphingosine*. [1-^{14}C]Lignoceric acid (0.43 mg containing 78.5×10^6 cpm) was reacted with 1 mg of D-*erythro*-sphingosine, 0.62 mg of triphenyl phosphine, and 0.53 mg of 2,2′-dipyridyl disulfide in 0.035 ml of methylene chloride as described previously (18). TLC–radioscanning of the product revealed two radioactive peaks; one corresponded to lignoceric acid and the other to ceramide. The ceramide was purified by preparative TLC (silica gel GF, chloroform–methanol–concentrated NH_4OH 90:10:1) and further purified by TLC on a sodium borate-impregnated silica gel G plate with chloroform–methanol 10:1 as the solvent. The purified material contained 13.84×10^6 cpm. The specific activity was 56.3 mCi/mmol.

N-[*1-*^{14}*C*]*Lignoceroyl-3-keto-*D-*sphingosine*. *N*-[1-^{14}C]Lignoceroyl D-*erythro*-sphingosine was converted to the

3-keto derivative and purified as described for the synthesis of *N*-lignoceroyl 3-keto-D-[1-^{3}H]sphingosine.

Synthesis of ceramide by $NaBH_4$ reduction of ethyl 2-acylamino-3-hydroxy-4*t*-octadecenoate

N-Palmitoyl DL-*erythro sphingosine.* DL-*erythro*-III (92 mg) was stirred for 24 hr at room temperature with triphenylphosphine (133 mg), 2,2′-dipyridyl disulfide (94 mg), and palmitic acid (95 mg) in 4 ml of dioxane. The solvent was removed by evaporation and the residue was twice recrystallized from methanol. The fine white needles of ethyl DL-*erythro*-2-palmitoylamino-3-hydroxy-4*t*-octadecenoate weighed 95 mg and had an mp of 69–69.5°C. The infrared spectrum as a KBr pellet showed absorptions (in cm^{-1}) at 3200–3600 (OH group), 3330 (NH), 1750 (-$COOC_2H_5$) and 1655, 1545 (-CO-NH-).

Elemental analysis $C_{36}H_{69}NO_4$, calcd. N, 2.41; found N, 2.39.

The above ethyl DL-*erythro*-2-palmitoylamino-3-hydroxy-4*t*-octadecenoate (20 mg) was suspended in 1.2 ml of methanol, and 27 mg of $NaBH_4$ was gradually added to the solution at room temperature. The mixture was then refluxed gently for 15 min. The reaction mixture was mixed with 3 ml each of chloroform and water and a small volume of 1 N acetic acid to make the aqueous phase slightly acidic. The lower layer was washed with water and evaporated to dryness. The residue was recrystallized from methanol and 11 mg of white powder which melted at 93–94°C was obtained. The infrared spectrum of this material was almost identical to that of the authentic *N*-palmitoyl-D-*erythro*-sphingosine (mp 97–100°C).

Elemental analysis $C_{34}H_{67}NO_3$, calcd. N, 2.61; found N, 2.57.

N-Lignoceroyl DL-*erythro-sphingosine (VIII).* Ethyl DL-*erythro*-2-lignoceroyl amino-3-hydroxy-4*t*-octadecenoate (V) was prepared from 160 mg of III by reacting with a mixture of 161 mg of lignoceric acid, 235 mg of triphenylphosine, and 160 mg of 2,2′-dipyridyl disulfide in 5 ml of dioxane. This product was recrystallized twice from methanol. The yield was 280 mg and had an mp of 74–75°C. The infrared spectrum was similar to that of ethyl DL-*erythro*-2-palmitoylamino-3-hydroxy-4*t*-octadecenoate described above.

Elemental analysis $C_{44}H_{85}NO_4$, calcd. N, 2.03; found N, 2.10.

Resolution of ethyl D- *and* L-*erythro-2-lignoceroylamino-3-hydroxy-4t-octadecenoates (VI).* Ethyl DL-*erythro*-2-lignoceroylamino-3-hydroxy-4*t*-octadecenoate (108 mg) was dissolved in 0.5 ml of benzene containing 108 mg of L-(+)-acetylmandeloyl chloride and then cooled in ice. Dry pyridine (0.5 ml) was added dropwise while stirring, and then the mixture was brought to room temperature. The mixture, which turned brown and contained precipitates, was stirred at this temperature for 40 min and then diluted with chloroform. It was washed twice with 2-ml portions of water and evaporated to dryness. The residue was fractionated on a column containing 20 g of silica gel 60; benzene–acetone 99.5:0.5 was used as the eluting solvent. This chromatographic procedure yielded two distinct fractions.

The first fraction contained 51.6 mg of material (Compound C) which produced a single (R_f 0.47) spot on a thin-layer Q5F plate developed in benzene–acetone 98:2. The starting material gave an R_f value of 0.23 using this system. Recrystallization of the material from methanol yielded a white powder which weighed 35 mg and had an mp of 66–67°C. This compound was identified as L-*erythro*-VI by IR, and UV [$\lambda_{max}^{CH_3OH}$ nm (ϵ):260 (180)] spectra, and by ORD of the ethanolysis product (see below).

Elemental analysis $C_{54}H_{93}NO_7$, calcd. C, 74.74; H, 10.72; N, 1.61; found C, 74.26; H, 10.76; N, 1.62.

The second fraction (Compound D) which weighed 66 mg and produced a single spot at R_f 0.38 by the TLC system described above was recrystallized from methanol and yielded a white powder which weighed 52.3 mg and had an mp of 65–66°C. This compound was identified as D-erythro-VI by IR and UV spectra and ORD of the ethanolysis product (see below).

Elementary analysis $C_{54}H_{93}NO_7$, calcd. C, 74.74; H, 10.72; N, 1.61; found C, 74.39; H, 10.75; N, 1.63.

Synthesis of N-lignoceroyl L-*erythro-sphingosine (L-VIII) by $NaBH_4$ reduction.* Compound C (15 mg) was suspended in 1.5 ml of absolute ethanol by agitating in a sonic cleaner. To this mixture, 15 μl of 0.2 N sodium ethoxide in ethanol was added. The suspension became clear after stirring for 5 min at room temperature, and then a white precipitate appeared after stirring for another 5 min. The mixture was stirred for an additional 5 min and then diluted with 3 ml of water. The precipitates were filtered and recrystallized from methanol. The white powder weighed 10 mg and had an mp of 79.0–80.5°C. The infrared spectrum (KBr) of this compound was identical to that of DL-*erythro*-VII.

The L-*erythro*-VII (15.5 mg) was reduced with 19.5 mg of $NaBH_4$ in 0.9 ml of methanol as described for the DL-isomer preparation. Recrystallization of the reaction product from methanol yielded 9.0 mg of white powder which melted at 91–94°C. The infrared spectrum of this compound was identical to that of *N*-lignoceroyl DL-*erythro*-sphingosine.

Synthesis of N-lignoceroyl D-*erythro-sphingosine (D-VIII) by $NaBH_4$ reduction.* This enantiomer was ob-

tained from Compound D by the procedure described for the L-isomer. From 18.7 mg of this material and a proportional amount of sodium ethoxide, 13 mg of D-VII was obtained after recrystallization from methanol. This compound had an mp of 76–78°C and its infrared spectrum was identical to that of the corresponding L-enantiomer. The ethanolysis product obtained from another preparation (17.5 mg) was reacted with 25 mg of $NaBH_4$. The product was purified on a 3-g silica gel 60 column by eluting with benzene–acetone 5:1. The ceramide fractions were combined and recrystallized from methanol with a yield of 7 mg of white powder, mp 93–95°C. The infrared spectrum of this compound was identical to that of L-VIII.

Preparation of N-lignoceroyl D-*erythro*-[*1-*^{3}*H*]*sphingosine by* NaB^3H_4 *reduction.* D-VII (4.2 mg) was reacted with 1 mg of NaB^3H_4 (sp act 272 mCi/mmol) in 0.2 ml of methanol as described above. The product contained 49×10^6 cpm and produced a single radioactive spot in three different thin-layer chromatographic solvent systems (chloroform–methanol–acetic acid 90:2:8, chloroform–methanol–concentrated NH_4OH 90:9:1, and benzene–acetone 3:1, all on silica gel G plates). The product was purified on a 1-g silica gel 60 column. The column was eluted with 5 ml of chloroform, 10 ml of chloroform–methanol 98:2, and finally with chloroform–methanol–concentrated NH_4OH 90:9:1. The first fraction contained the starting material (3.8 mg); both the second and third fractions contained pure radioactive ceramide, which weighed 0.77 mg and contained 47×10^6 cpm. TLC–radioscanning of this material (silica gel G, benzene–acetone 3:1) produced a single radioactive peak with an R_f value corresponding to that of authentic ceramide.

Analytical procedures

Radioactivity was measured in a Packard liquid scintillation counter (TriCarb Model 3380) and a Beckman LS 230 liquid scintillation counter. Samples were dissolved in a cocktail containing 4 g of PPO and 0.6 g of dimethyl POPOP in one 1 of toluene–ethanol 95:5. Radioactivity on TLC plates was scanned by a Berthold TLC scanner (Varian Aerograph). Beckman IR-33, Acta III, and Cary 60 spectrophotometers were used to obtain IR, UV, and ORD spectra, respectively. Low resolution mass spectra were obtained with a Hitachi RMU-6L interfaced to a Perkin-Elmer 990 gas chromatograph with IBM 1800 Computer Data Aquisition. Elemental analysis was performed at the Central Analytical Laboratory, Kyushu University, Japan.

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