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Synthesis of Double-labeled Lactosylceramide

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The total stereo-controlled synthesis of lactosylceramide and introduction of two kinds of isotopes, ^{3}H and ^{14}C, into synthetic lactosylceramide are described.

Lactosylceramide is a key intermediate in the biosynthesis of most complex glycosphingolipids. Gangliosides, ABO blood-type glycolipids and globo-type glycosphingolipids are derived from lactosylceramide by a series of glycosyl transferase reactions involving transfer of glycosyl residues from sugar nucleotides to the oligosaccharide chains of the glycolipids. Lactosylceramide, like other glycosphingolipids, can be purified from the plasma membranes of various tissues but the process is laborious and yields are low. Therefore, synthetic approaches for the preparation of this glycosphingolipid are still attractive.

Although numerous ceramides, containing long-chain bases such as 4-sphingenine, sphinganine (including all stereo-isomers), and 4-hydroxysphinganine have been synthesized, primarily by Shapiro and co-workers, syntheses of glycosphingolipids containing natural ceramides are limited. The first total synthesis, in low yield, of simple lactosylceramide was achieved by Shapiro *et al.* [1, 2].

To study the biosynthesis and function of G_{M3} ganglioside, the total synthesis of lactosylceramide containing radioisotopes has been undertaken. Our synthetic strategy for lactosylceramide featured two different approaches. The first was a modification of the total stereospecific method described by Koike *et al.* [3]. In this procedure the stereochemistry of the natural D-isomer of 4-sphingenine, *trans*-(2S,3R)-1,3-dihydroxy-2-amino-4-octadecene (or its *cis*-isomer) was retained during synthesis from D-glucose. The second approach involved the half-synthesis of lactosylceramide using native ceramide extracted from bovine brain. For double-labeled lactosylceramide, ^{14}C was introduced into the fatty acid moiety of ceramide and ^{3}H into the galactosyl residue of lactose.

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Materials and Methods

Commercial chemicals were of the highest grade available and were stored in a desiccator after complete drying. Common solvents were re-distilled before use and special anhydrous organic solvents were prepared by published procedures. Water for routine use was freshly re-distilled in a glass apparatus. Silica gel G for column chromatography (230-400 mesh) and galactose oxidase were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and silica gel precoated thin-layer plates (HPTLC, Kieselgel 60, 250 μm thick, 10 × 10 cm) were purchased from Merck, Darmstadt (W. Germany). Iatrobeads (6RS 8060) for column chromatography was purchased from Iatron Lab. (Tokyo, Japan). [1-^{14}C]Palmitic acid (56 mCi/mmol) and NaB^3H_4 (282 mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL, USA) and from New England Nuclear (Boston, MA, USA), respectively.

Melting points were taken on a Reichert micro hot-stage apparatus and uncorrected. ^{1}H-NMR (250 MHz) and natural abundance ^{1}H noise-decoupled ^{13}C FT NMR (62.5 MHz) were recorded on a Bruker WM-250 spectrometer using 5 mm spinning tubes at 27°C in DMSO-$^2H_6$. The chemical shifts are referred to internal tetramethylsilane (TMS) or to the solvent peak (2.49 ppm with respect to internal TMS). Optical rotations were measured with a Carl Zeiss LEP A1 Optischer Schwerpunkt (Germany). FAB-MS spectra were taken with a Varian MAT CH5 double-focusing mass spectrometer and a JEOL HX-110 mass spectrometer with triethanolamine as matrix. For determination of specific activity of labeled compounds, a Packard Tri-Carb 460C scintillation counter, equipped with the luminescence option and an ACD-18 Automatic Computing Densitometer (Gelman Instrument Company, Ann Arbor, MI, USA) were used to measure radioactivity and the quantities, respectively. Autoradiograms of labeled compounds on TLC were analysed on a Varian Aerograph LB 2723 radio-scanner.

Preparation of Ceramide

Two kinds of natural ceramide were isolated from bovine brain by chloroform-methanol extraction, Folch's washing procedure [4], mild saponification [5] and periodate oxidation, followed by hydrolysis under very mild acid conditions [6]. GC analysis confirmed that the upper ceramide (Ceramide I) on TLC consisted of non-hydroxy fatty acids, mainly stearic and tetracosanoic acids (13% and 48%, respectively) whereas the lower band (Ceramide II) contained primarily α-hydroxy-stearic and α-hydroxy-tetracosanoic acids (26% and 34%, respectively). These bovine brain ceramides contained mainly C18 sphingenine base (more than 90% by GC) (data not shown).

Chemical ceramides containing lignoceric, palmitic or [1-^{14}C]-labeled palmitic acid were synthesized according to Ogawa's procedure [3] after complete separation of Wittig olefinated cis- and trans-isomers of sphingenine by silica gel column chromatography.

Preparation of Lactosylceramide

Synthesis of 1,2-*trans*-linked glycosides [7, 8] is generally based upon the original work of Koenigs-Knorr [9] or upon the orthoester glycosidation method [10, 11]. In the former approach for the synthesis of 1,2-*trans*-linked di- and oligosaccharides, silver triflate has

been shown to be an efficient glycosidation promoter [12-14]. These conditions, particularly with nitromethane as a solvent, were not suitable for glycosidation of ceramide with sugar bromides because the ceramide did not dissolve in the nitromethane. Silver imidazolate-associated glycosidation [15] gave good yields of cerebrosides but with low stereospecificity at the anomeric position. For these reasons we chose to carry out the glycosidations for lactosylceramide synthesis with mercuric cyanide as a promoter, according to Pascher [16]. To a solution of the carefully dried ceramide (0.40 mmol) in dry benzene (45 ml), dry powdered mercuric cyanide (1.5 eq. mol), powdered Drierite (400 mg) and 4 Å molecular sieves (400 mg) were added and the mixture was heated under anhydrous conditions until about one third of the solvent had distilled off. To this solution, acetobromolactose (1.5 eq. mol) dissolved in dry nitromethane (15 ml) was added dropwise in three portions: one third at the beginning of the reaction and the rest in two equal portions after 2 and 4 h, respectively. The mixture was heated in an oil bath at 80°C under a nitrogen atmosphere for a total reaction time of 6 h. After cooling, the unreacted ceramide was precipitated, the solution was filtered and the precipitate was washed with benzene. The filtrate and washings were combined, washed with water and concentrated to give an oily residue. This residue was dissolved in dry methanol (20 ml) containing 0.2 N sodium methoxide (6 ml). After stirring for 1 h at room temperature, the mixture was neutralized with Bio-Rad AG 50W-X8 (H^+ form). The resin was filtered off and the filtrate was evaporated to a solid. This solid was chromatographed on Iatrobeads with chloroform/methanol/water, 100/30/2 by vol, to afford pure lactosylceramide (0.18-0.20 mmol; yield, 45-50%). Unreacted ceramide was recovered by extraction from the above precipitate with chloroform and from the first eluate of the Iatrobeads column (total, 0.1-0.12 mmol).

Synthesis of [^{14}C]-Labeled Ceramide and Lactosylceramide

Radioactive ceramides were prepared either by *N*-acylation of [1-^{3}H]sphingenine with *N*-hydroxysuccinimide ester of a non-radioactive fatty acid [17] or by *N*-acylation of non-radioactive 4-sphingenine with a [1-^{14}C]-labeled free fatty acid by oxidation-reduction coupling [18]. The synthetic approach afforded easy introduction of a [1-^{14}C]-labeled fatty acid into ceramide in high yield at the final stage (see below). Glycosidation of [^{14}C]-labeled ceramide with lactosyl bromide and mercuric cyanide, as described above, gave [^{14}C]-labeled lactosylceramide in 57% yield.

Synthesis of [^{3}H]-Labeled Lactosylceramide and [^{14}C]- and [^{3}H]-Double-labeled Lactosylceramide

As an alternative, ^{3}H was introduced into the galactose residue of lactose as follows: galactose oxidase oxidizes the primary hydroxyl group of the galactosyl residue to an aldehyde, which then can be reduced back to the original alcohol form by tritiated sodium borohydride to give [6-^{3}H]galactosyl-β(1-4)-glucosylceramide. Radin and coworkers first used this procedure for specific labeling of galactocerebroside [19] and lactosylceramide [20]. Later, Y. Suzuki and K. Suzuki reported that this method was useful for labeling not only the terminal galactose but also terminal *N*-acetylgalactosamine residues of glycosphingolipids [21].

Recently, chemical oxidation of the primary hydroxyl groups of sugar moieties with CrO_3-graphite followed by reduction with [^{3}H]-labeled sodium borohydride has been

Table 1. Analytical data of ceramides.

Ceramide	Fatty acid	Mp (°C)	$[\alpha]_D^{23a}$	MS[b] $[M+Na]^+$	TLC[c] (R_f)
I (natural)	C18:0	84-87	-1.5°	588	0.34
	C24:0			672	
II (natural)	C18:0(α-OH)	85-88	+2.4°	604	0.17
	C24:0(α-OH)			688	
III (synthetic, E-isomer)	C24:0	90-92	-1.7°	672	0.34
IV (" ")	C16:0	81-84	-1.9°	560	0.33
V (synthetic, Z-isomer)	C24:0	83-85	-1.8°	672	0.34

[a] c = 1.0 in chloroform.
[b] Measured with a Varian MAT CH5 double-focusing mass spectrometer.
[c] Solvent, chloroform/methanol, 95/5 by vol; spraying reagent, cupric acetate-H_3PO_4.

reported [22]. For the introduction of ^3H into the sugar moiety of lactosylceramide, we adopted the galactose oxidase-NaB^3H_4 treatment using synthetic lactosylceramide and [^{14}C]-labeled lactosylceramide. Lactosylceramide was oxidized by the procedure described by Suzuki and Suzuki [21]. Lactosylceramide (3 mg) was dissolved in a mixture of 0.1 M potassium phosphate buffer, pH 7.0 (1 ml), and freshly distilled tetrahydrofuran (1 ml). To this solution of lactosylceramide, the galactose oxidase solution (0.2 ml, 136 unit/ml in 0.1 M potassium phosphate buffer) was added and the tube was gently shaken at room temperature for 4 h. Additional galactose oxidase (0.2 ml) was then added, and shaking was continued overnight. Tetrahydrofuran was evaporated from the mixture under a stream of nitrogen, and 5 vol of chloroform/methanol, 2/1 by vol, was added to the remaining aqueous suspension. The lower phase was washed three times with water and dried to give the reaction product, which was dissolved in tetrahydrofuran (2 ml). Tritiated sodium borohydride (0.1 ml, 10.6 mCi/ml in 0.1 N NaOH) was added to the above tetrahydrofuran solution and the tube was shaken at room temperature overnight. Additional solid, unlabeled sodium borohydride (4 mg) was then added, and the shaking was continued for 4 h. The excess sodium borohydride was destroyed by the addition of 10 N acetic acid (0.2 ml) in a fume hood. After evaporation of tetrahydrofuran under a stream of nitrogen, 5 vol of chloroform/methanol, 2/1 by vol, was added. The lower phase was evaporated and the crude product was purified by silica gel column chromatography using a mixture of chloroform/methanol, 6/1 by vol, as the eluting solvent. The fractions containing labeled lactosylceramide were identified by TLC and liquid scintillation spectrometry; they were combined and solvents removed to give [^3H]-labeled lactosylceramide.

Results and Discussion

Analytical data of native ceramides and chemical synthetic ceramides are shown in Table 1. Fatty acid composition and long-chain base composition of native ceramides were determined in the usual manner (acid-catalyzed methanolysis and hydrolysis, respectively, followed by GC analysis).

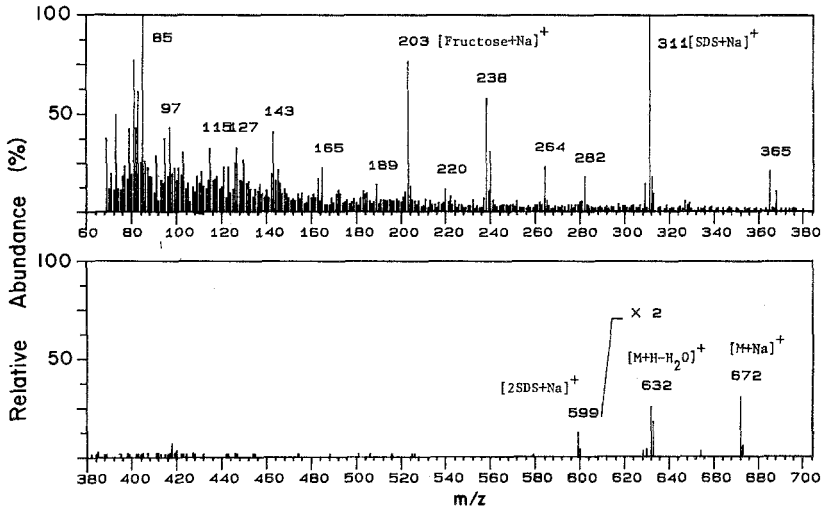


Figure 1. FAB-MS spectrum of synthetic ceramide III.

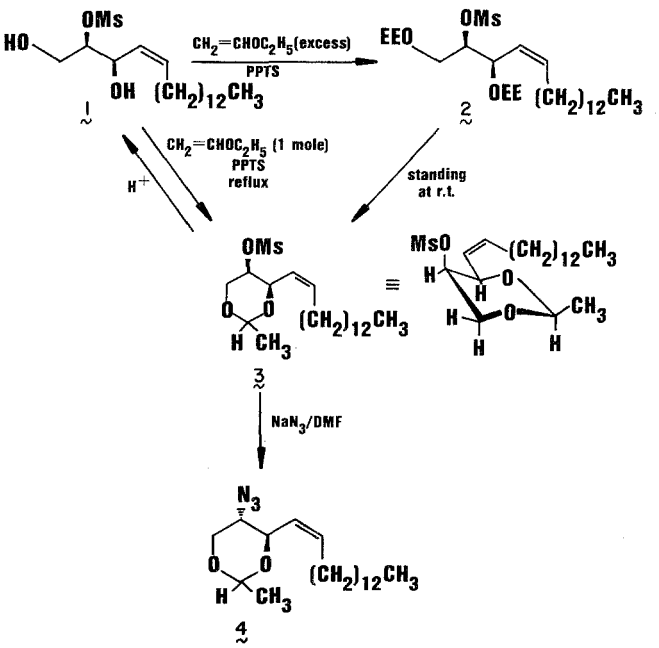


Figure 2. Desirable side-reaction of -OEE derivatization. Abbreviations: Ms, methanesulfonyl; PPTS, pyridinium *p*-toluenesulfonate; EE, ethoxyethyl; DMF, dimethylformamide.

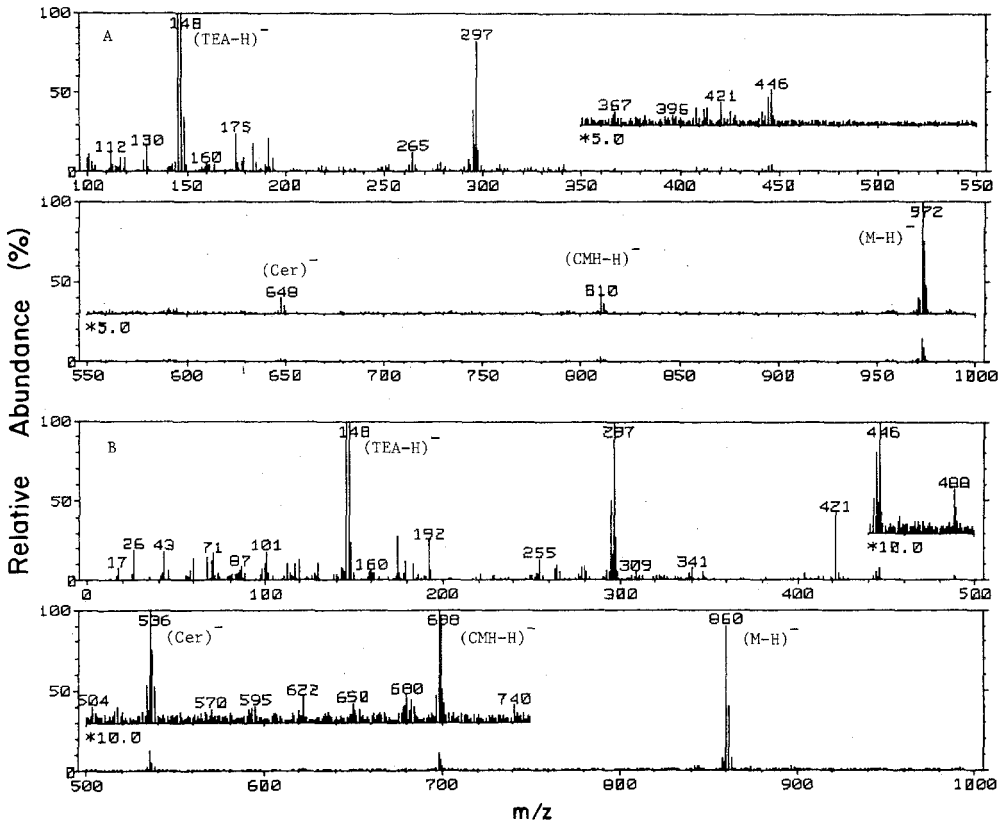
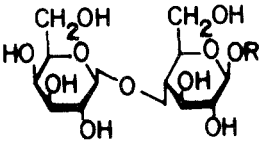


Figure 3. FAB-MS spectra of synthetic lactosylceramides, (A) derived from ceramide III, and (B) derived from ceramide IV.

Fig. 1 is the FAB-MS spectrum of synthetic ceramide (lignocerylsphingenine). All ceramides gave the strong $[M+Na]^+$ peaks in their FAB-MS spectra. As shown in Fig. 2, the diol intermediate **1** was easily cyclized to give the ethylidene derivative **3** in crystalline form (m.p. 47°C), which was identified by the NMR spectrum, showing three protons at δ 1.42 ppm which corresponds to a doublet signal of the methyl function. There was no ethoxy-generated signal in the same spectrum. Compound **3** was converted to the corresponding azide **4** in a shorter reaction time than that of the di-*O*-ethoxyether derivative **2**.

For the glycosidation of ceramide with lactosylbromide as the lactosyl donor, we used ceramide without protecting the secondary hydroxyl group(s) in the long-chain base because the reactivity of primary hydroxyl groups is much higher than that of secondary hydroxyl groups. After the glycosidation reaction in dry benzene-nitromethane, significant amounts of ceramide remained in the reaction mixture. Most of the unreacted ceramide was readily recovered as a solid from the cooling mixture. Prolonged reaction times were not more effective for complete glycosidation, as previously

Table 2. Analytical data of synthetic lactosylceramides.



R	Mp[a] (°C)	$[\alpha]_D^{23b}$	MS[c] $[M-H]^-$	TLC[d] (R_f)
Ceramide I	231-234	-5.5°	888	0.60
			972	
" II	230-235	-3.8°	904	0.56
			988	0.54
" III	230-233	-5.8°	972	0.60
" IV	227-230	-5.4°	860	0.56
" V	228-231	-4.8°	972	0.58

[a] Lactosylceramide becomes liquid at 150—160°C, but does not form a meniscus.
[b] c = 1.0 in pyridine.
[c] Measured with a JEOL HX-110 mass spectrometer.
[d] Solvent, chloroform/methanol/water, 60/35/8 by vol; spraying reagent, orcinol.

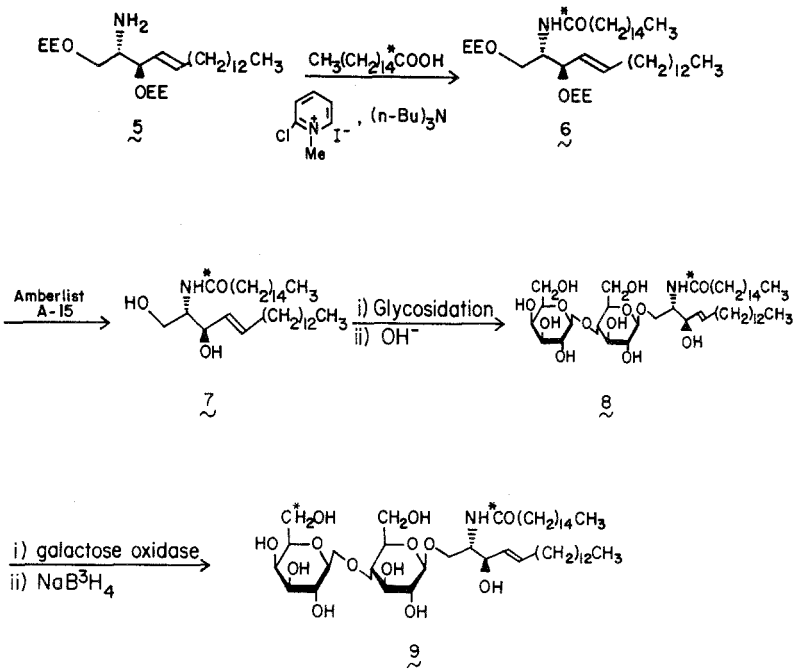


Figure 4. Synthetic scheme. Abbreviation: EE, ethoxyethyl.

Table 3. Specific activities of labeled compounds.

Compound	Specific Activity (cpm/nmol)
[^{14}C]-labeled ceramide	17890
[^{3}H]-labeled lactosylceramide	9750
[^{14}C]-labeled lactosylceramide	10200
[^{14}C]- and [^{3}H]-double-labeled lactosylceramide	6430 for [^{14}C] 13100 for [^{3}H]

mentioned by Pascher [15]. The yields after glycosidation followed by deacetylation with sodium methoxide were about 50% (net yields were more than 70% as calculated from the actual reacted ceramide). The proportions of α- and β-anomers for synthetic lactosylceramide were determined by the intensities of the anomeric proton signals (δ 4.20 ppm, J = 7.5 Hz for the β-anomer and δ 4.76 ppm, J = 3.5 Hz for the α-anomer) in the NMR spectrum before purification. The ratio of α-anomer to β-anomer was less than 0.10. Table 2 shows the analytical data of synthetic lactosylceramides. The Rf values on HPTLC agreed with that of the natural product from dog intestine. Identification of the synthetic lactosylceramides was confirmed by NMR, compared with the literature spectrum [23], and by FAB-MS (Fig. 3).

As shown in Fig. 4, the 4-sphingenine intermediate **5** was easily transformed to radioactive ceramide **6** using [1-^{14}C]-labeled free fatty acid by the Mukaiyama method [24] in high yield (80%). [^{14}C]-Labeled ceramide **7**, after removal of the protecting group with Amberlist A-15, was readily purified on a short silica gel column with chloroform/methanol, 15/1 by vol, as the eluting solvent. Glycosidation of **7** with acetobromolactose and deacetylation, as described above, gave [^{14}C]-labeled lactosylceramide. Galactose oxidase-NaB^{3}H$_4$-treatment of this [^{14}C]-labeled lactosylceramide, as previously described with the unlabeled lactosylceramide, gave [^{14}C]- and [^{3}H]-double-labeled lactosylceramide **9**. Specific activities of the labeled compounds are listed in Table 3.

Using the above synthetic non-labeled and labeled lactosylceramide, studies of sialyltransferases and other aspects of glycosphingolipid biochemistry are in progress.

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