papers and notes on methodology

Preparation of GM1 ganglioside molecular species having homogeneous fatty acid and long chain base moieties

Sandro Sonnino, Günther Kirschner,* Riccardo Ghidoni, Domenico Acquotti, and Guido Tettamanti

Study Center for the Functional Biochemistry of Brain Lipids, Department of Biological Chemistry, The Medical School, University of Milan, Milano, and Department of Chemistry, FIDIA Research Laboratories,* Abano Terme, Italy

Abstract A new procedure is described for preparing the molecular species of GM1 ganglioside that carry a single fatty acid (myristic (C14:0), stearic (C18:0), arachidic (C20:0) or lignoceric (C24:0) acid) and a single long chain base (C18 or C20 sphingosine, C18 or C20 sphinganine, each of them in natural 3D(+)*erythro* or unnatural 3L(−)*threo* form). The procedure consisted of the following steps: *a*) alkaline hydrolysis of GM1 ganglioside in the presence of tetramethylammonium hydroxide, which produces de-N-acylation of the ceramide and de-N-acetylation of the sialic acid residue; *b*) specific re-N-acylation at the long chain base amino group with a new fatty acid (myristic, stearic, arachidic, or lignoceric) in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride; and *c*) final re-N-acetylation at the level of the sialic acid residue. GM1 ganglioside molecular species, completely homogeneous in the ceramide portion, were prepared by reversed phase high performance liquid chromatography. The GM1 ganglioside molecular species were analyzed for saccharide, fatty acid, and long chain base composition by chemical and spectrometric analyses. Using a combination of the two procedures, 32 different molecular species of GM1 ganglioside, over 99% homogeneous, have been prepared. — **Sonnino, S., G. Kirschner, R. Ghidoni, D. Acquotti, and G. Tettamanti.** Preparation of GM1 ganglioside molecular species having homogeneous fatty acid and long chain base moieties. *J. Lipid Res.* 1985. **26:** 248–257.

Supplementary key words GM1 ganglioside • HPLC separation • ganglioside molecular species • de-acylation and re-acylation of ganglioside

Gangliosides, glycosphingolipids containing one or more residues of sialic acid, are normal components of the plasma membranes of mammalian cells and are particularly abundant in neuronal membranes (1–3). They are constituted of a hydrophilic portion, the oligosaccharide chain, which is oriented towards the extracellular medium, and of a hydrophobic moiety, the ceramide, which is inserted in the lipid layer of the membrane. The individual gangliosides that are prepared by conventional techniques are characterized by the presence of a homogeneous oligosaccharide chain. However, each of them has a ceramide portion that is heterogeneous in both the fatty acid and long chain base composition.

The hydrophilic portion is assumed to be involved in recognition phenomena, mediated by specific interactions between an external ligand and the oligosaccharide chain (4, 5). The hydrophobic portion, which contributes to the structural organization of the membrane, might participate in the process of transduction of external signals through the membrane (5). In fact, the length and possible unsaturation of both alkyl chains, present in the

Abbreviations: The paper follows the ganglioside nomenclature of Svennerholm (22) and the IUPAC-IUB recommendations (23). GM1, II^{3}NeuAc-GgOse$_4$Cer, Galβ1→3GalNAcβ1→4(NeuAcα2→3)Galβ1→4Glcβ1→1'Cer; LCB, long chain bases; C18 sphingosine, 2D-amino-octadec-4-ene-1,3-D-diol; C20 sphingosine, 2D-amino-eicos-4-ene-1,3-D-diol; C18 sphinganine, 2D-amino-octadecan-1,3-D-diol; C20 sphinganine, 2D-amino-eicosan-1,3-D-diol; Neu, neuraminic acid; NeuAc, N-acetylneuraminic acid; DDQ, 2,3-dichloro-5,6-dicyano-benzoquinone; 3-keto-ganglioside, oxidized ganglioside at the C-3 of the sphingosines; TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TMA-OH, tetramethylammonium hydroxide; DEC, 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide-hydrochloride; Ac_2O, acetic anhydride; MetOH, methanol; ButOH, butan-1-ol.

ganglioside molecule, can influence membrane fluidity and permeability by means of the hydrophobic interactions with the other lipid components of the membrane. Moreover, the ceramide portion can interact and possibly modify the activity of membrane-associated proteins (6–9).

The availability of ganglioside molecular species that are homogeneous in both the oligosaccharide and lipid moieties is expected to greatly facilitate studies aimed at understanding the role played by the ceramide portion in the above-mentioned phenomena. Further improvement in these studies would derive from the use of gangliosides with a fatty acid with a paramagnetic, photoreactive, or fluorescent group. Recently, an HPLC method was reported (10) capable of preparing individual molecular species of GM1 and GD1a gangliosides containing a single long chain base and a fatty acid constituted primarily of stearic acid. The preponderance of stearic acid was dependent on the natural abundance of this fatty acid in the original gangliosides, which were prepared from bovine brain.

In the present investigation a procedure has been devised that enabled us to chemically substitute the fatty acid moiety present in gangliosides with a new fatty acid. The procedure was applied to ganglioside GM1 and consisted of the following steps: *a*) removal of the fatty acyl group from ceramide and of the acetyl group from sialic acid by alkaline hydrolysis of ganglioside; *b*) re-N-acylation of the long chain base with a new fatty acid (myristic, stearic, arachidic, or lignoceric); *c*) final re-N-acetylation of the sialic acid residue. Combination of this procedure with the one previously reported based on reversed phase HPLC, led to the preparation of 32 different molecular species of ganglioside GM1, each of them with a single fatty acid and long chain base moiety.

MATERIALS AND METHODS

Commercial chemicals were of analytical grade or the highest grade available. Common solvents were redistilled before use and water for routine use was freshly redistilled in a glass apparatus. Methanol and acetic anhydride were dehydrated before use by refluxing over metallic magnesium and sodium, respectively. HPLC-grade reagents were used for HPLC. Silica gel 100 for column chromatography (0.063–0.2 mm, 70–230 mesh, ASTM) and high performance silica gel precoated thin-layer plates (HPTLC Kieselgel 60, 10 × 10 cm) were purchased from Merck GmbH (Darmstadt, FRG). Tetramethylammonium hydroxide pentahydrate and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride were from Aldrich (Beerse, Belgium). N-acetylneuraminic acid (NeuAc), stearic acid, myristic acid, arachidic acid, lignoceric acid, and behenic acid methyl ester were from Sigma Chemical Co. (St. Louis, MO). Reagents for GLC were from C. Erba (Milano, Italy) and Merck (Darmstadt, FRG). Dialysis tubing was from A. Thomas (Philadelphia, PA). GM1 ganglioside was extracted from calf brain according to Tettamanti et al. (11), purified and structurally characterized as described by Ghidoni et al. (12). Its lipid moiety had the following composition (as mole %): long chain bases: *erythro* C18 sphinganine, 2.5%; *erythro* C18 sphingosine, 50.0%; *erythro* C20 sphinganine, 1.8%, *erythro* C20 sphingosine, 45.7%; fatty acids: stearic acid, 92%; palmitic acid, 3%; oleic acid, 0.5%; arachidic acid, 4.5%. The final purity of ganglioside GM1, with reference as conventionally used, to the oligosaccharide portion, was over 99%. Standard neutral glycosphingolipids (tetrahexosylceramide, trihexosylceramide, lactosylceramide, and glycosylceramide) were prepared by partial acid hydrolysis of GM1 and purified by silica gel column chromatography as previously described (13).

De-acylation and re-acylation of GM1 ganglioside

The procedure used for removal of the fatty acid moieties present in natural GM1 and for substitution with a new fatty acid is schematically given in the flow sheet of **Fig. 1.** The various compounds obtained are named according to this flow sheet.

Preparation of compound II, Galβ1→3GalNAcβ1→4(Neuα2→3)Galβ1→4Glcβ1→1' long chain base, by alkaline hydrolysis of GM1. The optimal conditions of alkaline hydrolysis of GM1 ganglioside for production of compound II were determined in preliminary experiments. For this purpose 1-mg aliquots of GM1 ganglioside were allowed to react for different times (up to 96 hr) and temperatures (from 50° to 100°C), at various concentrations of tetramethylammonium or potassium hydroxide (from 0.25 to 2 M). The optimal conditions were found to be as follows: GM1 ganglioside was dissolved in butan-1-ol (11.1 mg/ml) and 288 ml of this solution was mixed at 100°C with 32 ml of 10 M tetramethylammonium hydroxide in water. The reaction mixture was refluxed at 100°C under continuous stirring for 13 hr and then evaporated under vacuum at 50°C almost to dryness. The wet residue was diluted with a few ml of water, dialyzed at 4°C for 2 days against 4 liters of distilled water (changed three times a day), and lyophilized. Separation of the products (compound I, compound II, and remainder of ganglioside GM1) contained in the residue was accomplished by HPTLC using solvent system A (see below). The purification of the two main hydrolysis products (compound I and compound II) was performed by chromatography on silica gel 100 columns (200 × 4 cm) previously equilibrated and eluted with methanol–butan-1-ol–water 2:2:1 (by vol). The elution profile was monitored by HPTLC, using solvent system A (see below).

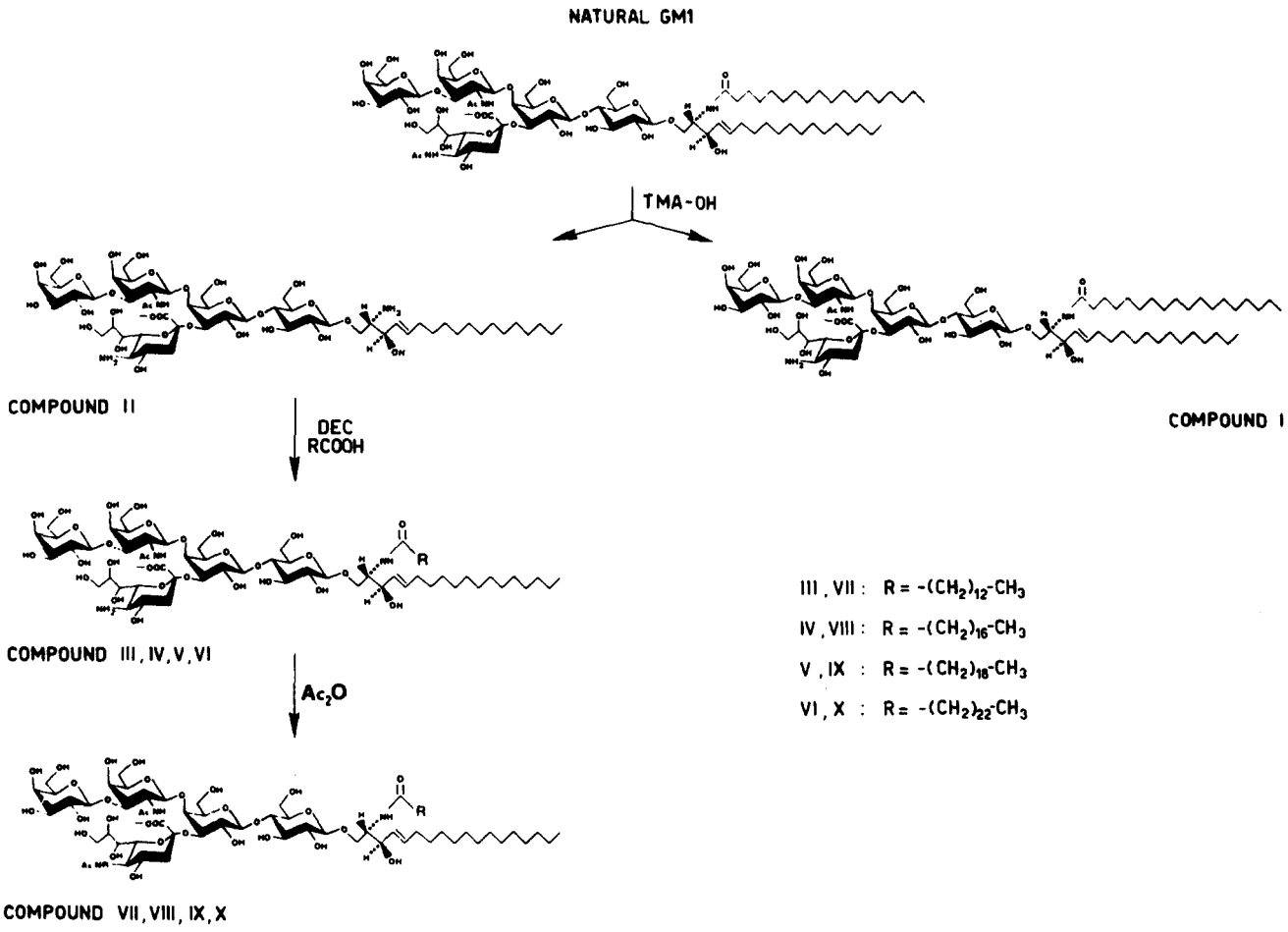

Fig. 1 Flow sheet of the procedural steps used for preparing GM1 ganglioside with homogeneous fatty acid moiety.

Preparation of compounds III–VI, Galβ1→3GalNAcβ1→4(Neuα2→3)Galβ1→4Glcβ1→1'N-acyl, long chain base, by acylation of compound II. The optimal conditions for specific acylation of the long chain base moiety of compound II at the level of free amino group were assessed in preliminary experiments. Myristic acid, stearic acid, arachidic acid, and lignoceric acid were employed. Each of these fatty acids was dissolved in various solvents (water, chloroform–methanol 1:1 (by vol), dimethylsulfoxide, dimethylformamide) in the presence of different amounts of DEC. Compound II was then added (in molar ratios, referred to fatty acid, varying from 0.1/1 to 1/1) and the mixtures were allowed to react for different times (up to 16 hr) and temperatures (from 20° to 80°C). The condition which resulted as optimal was the following: an aqueous solution (480 ml) containing fatty acid (myristic, stearic, arachidic, or lignoceric acid) (4.5 mM as final concentration) and DEC (5 mM as final concentration) was maintained at 50°C for 2 hr under continuous stirring. Then 250 mg of compound II was added and the mixture was allowed to react for 5 additional hours under the same conditions. At the end of the reaction the solution was chilled in an ice bath and 8 ml of 6 N NaOH was added in order to release nonspecific O-acyl ester groups possibly formed during the reaction. After standing at room temperature for 8 hr, the alkaline mixture was dialyzed at 4°C for 2 days against 3 liters of distilled water, changed three times a day, and lyophilized. Separation of the reaction products for analysis was carried out by HPTLC, using solvent system A (see below). The preparative separation and purification of the main products (compounds (III–VI) was performed on silica gel 100 (100 × 2 cm) previously equilibrated and eluted with chloroform–methanol–water 60:35:5 (by vol). The elution profile was monitored by HPTLC, using solvent system B (see below).

Preparation of compounds VII–X (GM1 ganglioside with homogeneous fatty acid moiety), by N-acetylation of compounds III–VI. One-hundred-fifty milligram of each of the compounds III–VI, which contained myristic, stearic, arachidic, and lignoceric acid, respectively, was dissolved in 200 ml of magnesium-dehydrated methanol. The solution was

mixed with 100 ml of sodium-dehydrated acetic anhydride and allowed to react overnight at room temperature. The mixture was then dried under vacuum at 40°C and the residue, dissolved in 10 ml of water, was lyophilized and stored at −20°C.

Preparation of GM1 ganglioside containing saturated long chain bases

Each of compounds VII–X was hydrogenated in the presence of PtO_2 as catalyst, at the level of the double bond of long chain base according to Sonnino et al. (10).

Preparation of GM1 ganglioside containing the *threo*-form of long chain bases

This was obtained by the previously described method (14) based on DDQ oxidation of gangliosides at the level of C-3 of unsaturated long chain bases, separation of the formed 3-keto derivatives, reduction with $NaBH_4$, and further purification by column chromatography.

HPLC preparation of the molecular species of GM1 ganglioside, differing in their long chain base moiety

Each of compounds VII–X, directly or after DDQ/$NaBH_4$ treatment or hydrogenation, was separated into the molecular species, which differ in length, presence of double bond in C-4 position, or configuration at C-3 of LCB, by reversed phase chromatography on Spherisorb-S5 OD S2 (10 × 250 mm), following the procedure described by Sonnino et al. (10).

Analytical procedures

Nuclear magnetic resonance spectroscopy. ^{1}H-NMR spectra (80 MHz) of 10^{-2} M solutions of natural GM1 ganglioside and of compounds I–X in ($^2H_6$)-dimethylsulfoxide were recorded at room temperature on a Varian CFT-20 pulse spectrometer operating in the Fourier transform mode. The pulse width was 12 μsec, the acquisition time was 4.095 sec, and the number of transients was 1000. Signals were assigned putting the central signal of dimethylsulfoxide at 2.52 ppm.

Compositional analyses. The carbohydrate, fatty acid, and long chain base composition of natural GM1 and of compounds I–X was determined by GLC and GLC–MS analyses as previously described (12). Fatty acid analysis was performed in the presence of behenic acid methyl ester as internal standard. Assessment of C-3 steric configuration of long chain base was accomplished following the directions given in a previous study (10).

Partial acid hydrolysis. A sample of natural GM1 ganglioside and compounds I–X (1.5 mg) was dissolved in 1 ml of 0.5 N HCl and heated in a screw-capped tube at 100°C for 45 min (12). The mixture was dialyzed at 5°C for 3 days against 500 ml of distilled water (changed three times a day) and evaporated to dryness. Separation of the individual neutral glycosphingolipids, contained in the residue, was accomplished by HPTLC, using solvent system C (see below). The preparation of each individual glycolipid was performed on silica gel 100 columns (40 × 0.5 cm), equilibrated and eluted with chloroform–methanol–water 110:40:6 (by vol) (12). The elution profile was monitored by HPTLC using the solvent system C.

Thin-layer chromatography. TLC of the various compounds was performed on HPTLC plates under the following conditions: temperature, 18–20°C; solvent system A: chloroform–methanol–2.5 M NH_4OH 60:40:9 (by vol); solvent system B: chloroform–methanol–0.2 % aqueous $CaCl_2$ 50:42:11 (by vol); solvent system C: chloroform–methanol–water, 110:40:6 (by vol); development time: 1 hr; detection of the spots by treatment with a *p*-dimethylaminobenzaldehyde spray reagent followed by heating at 120°C for 10 min (15) for acidic glycosphingolipids, and by treatment with an anisaldehyde spray reagent, followed by heating at 120°C for 15 min (13) for neutral glycosphingolipids. The percentage of products separated by HPTLC was determined by densitometric scanning of the plate, with a Camag densitometer equipped with a 3390 A Hewlett-Packard integrator (15).

Colorimetric methods. GM1 ganglioside and compounds I–X were assayed as bound sialic acid by the resorcinol method (16, 17), pure NeuAc being used as the reference standard.

RESULTS

Preparation of compound II

The pattern of the compounds formed under the different conditions for alkaline hydrolysis of ganglioside GM1 was monitored by HPTLC. The percentage of each of the compounds present on the plates was determined by densitometric scanning. Moreover, the possible degradation of sialic acid during hydrolysis was assessed by determination of total bound sialic acid. Densitometric and colorimetric responses of identical molar amounts of purified (see below) natural GM1 and compounds I–II (previously dried over P_2O_5) were found not to be dependent on the nature of the sialic acid residue present in the molecule.

As shown in **Fig. 2,** four main spots could be detected on HPTLC plate, having R_f values of 0.07, 0.15, 0.29, and 0.41, respectively. They corresponded to compound II, compound I, unreacted GM1, and to an unidentified, less polar product, respectively. As shown in **Table 1,** the amount of compound II obtained was dependent on temperature and time of reaction, and on the concentration of tetramethylammonium hydroxide. The conditions providing the highest yield of compound II in terms of final recovery were as follows: tetramethylammonium

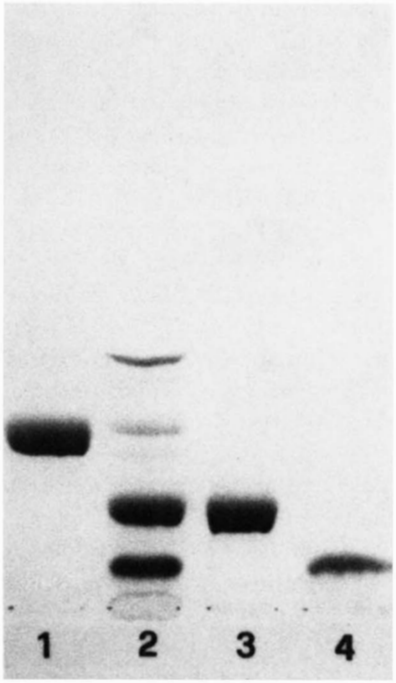

Fig. 2 TLC of the products formed by alkaline hydrolysis of GM1 ganglioside. 1, Starting GM1 ganglioside; 2, products obtained after alkaline hydrolysis of GM1 ganglioside; 3, purified compound I; 4, purified compound II.

hydroxide, 1 M; temperature, 100°C; reaction time, 13 hr. Under these conditions, the yield of compound II (as bound sialic acid) was 45%. The yield of compound II was much higher using TMA-OH than KOH. The compositional data of compound I and II, derived from natural GM1 by alkaline hydrolysis, are reported in **Table 2.** Compound II had the same composition of compound I, but lacked the fatty acid. The NMR spectra of natural GM1 ganglioside and of compounds I and II are reported in **Fig. 3.** In the NMR spectrum of natural GM1, the acetyl protons of N-acetyl-neuraminic acid and N-acetyl-galactosamine resonate at 1.89 and 1.75 ppm, respectively, and the methyl protons of the alkyl portion resonate at 0.87 ppm. This agrees with previously reported data (18). The NMR spectra of compounds I and II completely lacked the peaks at 1.89 ppm, suggesting that alkaline hydrolysis caused de-acetylation of bound NeuAc.

Peak intensity measurements (**Table 3**) showed that the ratio between methyl protons of the alkyl region and acetyl protons of N-acetyl-galactosamine, which was about 2 in the natural GM1 and in compound I, became 1 in compound II, indicating that this latter compound contained only one alkyl chain. Partial acid hydrolysis of compound I gave rise to four different neutral glycosphingolipids having the same thin-layer chromatographic behavior and the same saccharide and lipid composition as those of the corresponding products obtained from natural GM1 ganglioside processed in parallel. They corresponded to glucosylceramide, lactosylceramide, trihexosylceramide, and tetrahexosylceramide, respectively. Partial acid hydrolysis of compound II also gave rise to four different neutral glycosphingolipids that had lower migration on TLC than the corresponding ones obtained from natural GM1. They had the same saccharide and long chain base composition as the above-mentioned neutral glycosphingolipids but lacked fatty acid.

On the basis of all the above results, compound I was identified as GM1 de-N-acetylated at the level of sialic acid [Galβ1→3GalNAcβ1→4(Neuα2→3)Galβ1→4Glcβ1→1′Cer] and compound II as GM1 de-N-acetylated at the level of sialic acid and de-N-acylated at the level of the ceramide portion [Galβ1→3GalNAcβ1→4(Neuα2→3)Galβ1→4Glcβ1→1′LCB], respectively.

Preparation of compounds III-VI

Preliminary experiments, carried out as described in the Methods section, and based on HPTLC monitoring

TABLE 1. Formation of compound II by tetramethylammonium hydroxide hydrolysis of natural GM1: effect of different parameters on the reaction and on the relative yield of the different products

	Time (hr)																			
	1	2	4	7	13	13	13	13	13	13	16	16	16	24	24	24	32	32	64	96
Temperature (°C)	100	100	100	100	50	75	100	100	100	100	50	75	100	50	75	100	50	75	50	50
TMA-OH concentration (M)	1	1	1	1	1	1	0.25	0.5	1	2	1	1	1	1	1	1	1	1	1	1
Recovery (%)[a]	100	100	100	100	100	100	100	100	100	60	100	100	90	100	95	60	100	80	100	80
Percent distribution[b]																				
Unknown compound		2	3	4		2	1	3	5	5	1	3	5	2	5	5	1	5	2	2
Unreacted GM1	74	52	33	15	90	43	61	29	5	3	88	35	5	83	30		78	27	55	54
Compound I	26	42	52	54	10	45	36	54	45	40	11	47	45	15	43	50	20	43	40	41
Compound II		4	12	27		10	2	14	45	50		15	45		22	45	1	25	3	3

[a]Recovery refers to total lipid-bound sialic acid.
[b]Compound II, compound I, unreacted GM1, and unknown compound were densitometrically assayed as bound sialic acid after HPTLC. Each compound is expressed as percentage of total recovered sialic acid on the plate.

TABLE 2. Chemical composition of natural GM1 and of compounds I-X[a]

	Glc	Gal	GalNAc	Neu	NeuAc	Long Chain Base	Fatty Acid
Natural GM1	1.00	1.89	0.93		0.98	0.92	1.03
Compound I	1.00	1.91	0.97	1.01		0.95	0.91
Compound II	1.00	2.03	0.99	1.08		1.01	
Compound III	1.00	2.01	1.09	0.97		0.91	0.99
Compound IV	1.00	2.00	0.91	0.99		0.91	0.99
Compound V	1.00	1.81	0.94	1.00		0.93	1.00
Compound VI	1.00	1.97	1.05	1.02		1.05	0.97
Compound VII	1.00	2.06	1.03		0.96	0.97	0.99
Compound VIII	1.00	2.11	1.10		1.03	0.89	1.05
Compound IX	1.00	1.87	1.09		1.02	1.10	0.91
Compound X	1.00	1.94	0.89		1.00	1.02	1.11

[a]The different components are given as relative molar amounts, referred to glucose as 1.00.

of the reaction products and assessment of total recovery of bound sialic acid, gave indications regarding the optimal conditions. These were: molar stoichiometry among compound II, fatty acid (myristic, stearic, arachidic, and lignoceric), and DEC, 1:8:9; temperature of the reaction, 50°C; reaction time, 2 hr with fatty acid and DEC, plus 5 hr in the presence of compound II. As shown in **Fig. 4** (lane 2), different products were present at the end of the reaction. Of them, the most abundant one had a TLC mobility higher than compound II but lower than natural GM1, indicating an intermediate polarity between these two substances. Several minor reaction products, which were fast moving on TLC, were not characterized and were disregarded. They might correspond to ganglioside derivatives acylated at the level of neuraminic acid, or deacylated, or to products derived from ganglioside condensation.

Under optimal reaction conditions, the yield of the compounds primarily formed (compounds III-VI), determined by HPTLC densitometric quantification, was 47% for compound III (containing myristic acid), 50% for compound IV (containing stearic acid), 49% for compound V (containing arachidic acid), and 45% for compound VI (containing lignoceric acid).

Compounds III-VI contained the same saccharide residues (Table 2) as natural GM1, with the only exception that neuraminic acid substituted N-acetyl-neuraminic acid. In all these compounds the fatty acid was present in unitary molar ratio with long chain base and glucose.

NMR spectra (Fig. 3, Table 3) of compounds III, IV, V, and VI were similar to that of compound II in that no peak at 1.89 ppm (acetyl protons of N-acetyl-neuraminic acid residue) was present. Instead, the intensity ratio between methyl protons of alkyl region and acetyl protons of N-acetyl-galactosamine had a value of 2, identical to that present in the starting natural GM1. This evidence, certifying the presence of two alkyl chains, confirms the insertion of a fatty acid residue in the molecule.

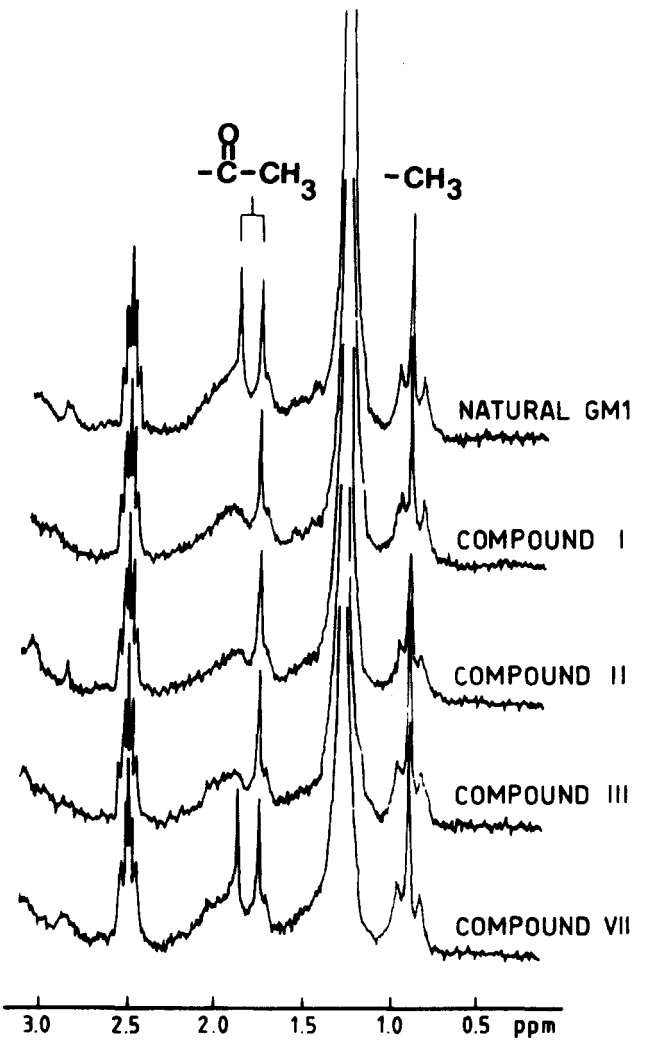

Fig. 3 NMR spectra of the various compounds obtained and purified in the course of the preparation of GM1 ganglioside with homogeneous fatty acid moiety. NMR spectra of compounds IV, V, and VI were identical to that of compound III and those of compounds VIII, IX, and X were identical to that of compound VII.

TABLE 3. H-NMR studies of natural GM1 and of compounds I–X, obtained by alkaline treatment of GM1 and further processing: relative intensities[a] of the alkyl and acetamido methyl protons

	GalNAc (1.75 ppm)	NeuAc (1.89 ppm)	Cer (0.87 ppm)
Natural GM1	1.0	0.9	1.9
Compound I	1.0		1.9
Compound II	1.0		1.0
Compound III	1.0		2.0
Compound IV	1.0		2.1
Compound V	1.0		2.1
Compound VI	1.0		2.0
Compound VII	1.0	1.0	1.9
Compound VIII	1.0	1.1	1.9
Compound IX	1.0	1.1	2.0
Compound X	1.0	1.0	1.8

[a]The relative peak intensities are referred to acetamido methyl protons of N-acetyl-galactosamine, stated as 1.0.

The four neutral glycosphingolipids, obtained from compounds III–VI by partial acid hydrolysis, had the same chemical composition as those derived from natural GM1 ganglioside and corresponded to glucosylceramide, lactosylceramide, trihexosylceramide, and tetrahexosylceramide. On the basis of overall analyses, compounds III–VI were the products of re-N-acylation of compound II at the level of long chain base, and had the common structure Galβ1→3GalNAcβ1→4(Neuα2→3)Galβ1→4Glcβ1→1′Cer, with a ceramide portion containing an individual fatty acid, myristic, stearic, arachidic, or lignoceric, respectively. To verify whether compounds III–VI were contaminated by the isomeric compounds in which the neuraminic acid NH_2 group was acylated instead of the LCB NH_2 group, the neutral glycosphingolipid patterns obtained by partial acid hydrolysis of compounds III–VI were spotted at progressively higher levels on an HPTLC plate (**Fig. 5**) and compared to that obtained by partial acid hydrolysis of compound II. By this experiment the presence of isomeric by-products due to undesired acylation on the neuraminic acid residue was excluded.

Preparation of compounds VII–X

Compounds III, IV, V, and VI, when submitted to re-N-acetylation, gave rise (Fig. 4) to compounds VII, VIII, IX, and X, respectively, having HPTLC behavior identical to that of natural GM1. Compounds VII, VIII, IX, and X contained glucose, galactose, N-acetyl-galactosamine, N-acetyl-neuraminic acid, long chain bases, and fatty acid in molar ratios, with reference to glucose content, identical to those of natural GM1 (Table 2).

The NMR spectra (Fig. 3, Table 3) and the pattern of neutral glycosphingolipids obtained by partial acid hydrolysis of compounds VII, VIII, IX, and X were indistinguishable from those provided by natural GM1.

Thus, it can be concluded that compounds VII, VIII, IX, and X have the same chemical structure as that of natural GM1, with the only difference that each of them had an individual and different fatty acid residue. Starting from about 3 g of natural GM1, the four different GM1 gangliosides prepared, homogeneous in the fatty acid moiety, were obtained in the following amounts: 145 mg for compound VII (GM1-myristic acid), 149 mg for compound VIII (GM1-stearic acid), 152 mg for compound IX (GM1-arachidic acid), and 157 mg for compound X (GM1-lignoceric acid).

Preparation of different molecular species of GM1 ganglioside, completely homogeneous in the ceramide portion

Compounds VII–X (140 mg each), submitted to DDQ oxidation, gave rise to the corresponding 3-keto derivatives, which were purified (yield: about 65 mg each) and reduced with $NaBH_4$. In each case a mixture was obtained of the diasteroisomer with LCB in the natural *erythro* form (65%) and of the diasteroisomer in the unnatural *threo* form (35%). A portion of the original compounds VII–X and of the DDQ/$NaBH_4$-treated compounds were fully hydrogenated in the presence of PtO_2 as catalyst at the level of the double bond of the LCB.

Each of compounds VII–X (40 mg each) was submitted to semi-preparative reversed phase HPLC, and the molecular species containing *erythro* C18 sphingosine and *erythro* C20 spingosine were thus prepared. The same compounds VII–X (40 mg each), after PtO_2-catalyzed hy-

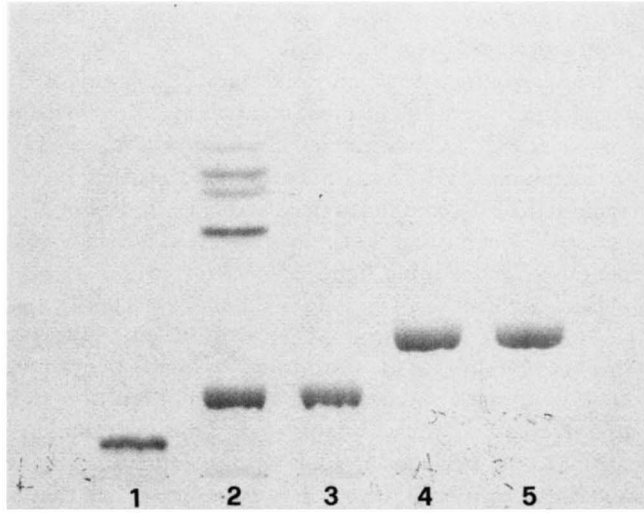

Fig. 4 TLC of the products obtained by re-N-acylation and re-N-acetylation of compound II. 1, Purified compound II; 2, same as 1 after re-N-acylation in the presence of DEC and myristic acid (a similar pattern was obtained when re-N-acylation was performed with stearic, arachidic, and lignoceric acids); 3, purified compound III (compounds IV, V, and VI had the same TLC behavior as compound III); 4, same as 3 after re-N-acetylation (compound VII) (compounds VIII, IX, and X had the same TLC behavior as compound VII); 5, standard natural GM1 ganglioside.

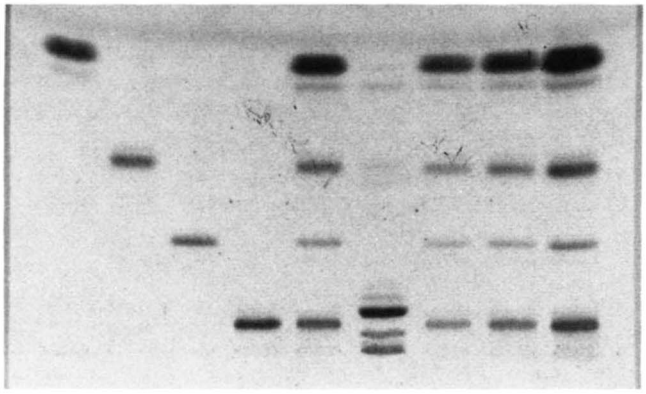

Fig. 5 TLC of the products obtained by partial acid hydrolysis of natural GM1 ganglioside, compound II, and compound III. 1, Standard glucosylceramide; 2, standard lactosylceramide; 3, standard trihexosylceramide; 4, standard tetrahexosylceramide; 5, partial acid hydrolysis of natural GM1 ganglioside; 6, partial acid hydrolysis of compound II; 7, 8, 9, partial acid hydrolysis of compound III spotted at progressively higher amounts.

drogenation and HPLC separation, provided the molecular species containing *erythro* C18 sphinganine and *erythro* C20 sphinganine. The corresponding molecular species containing *threo* C18 and C20 sphingosine and sphinganine were prepared by HPLC using DDQ/$NaBH_4$-treated compounds and DDQ/$NaBH_4$- and PtO_2/H_2-treated compounds (in all cases 20 mg each). Therefore, for each of compounds VII–X, 8 different molecular species were obtained and, by consequence, 32 different molecular species of GM1 with homogeneous and different ceramide portion were prepared. **Fig. 6** shows the chemical structures of all the newly synthesized molecular species of GM1.

DISCUSSION

This report describes a new procedure for the preparation, from natural GM1, of a number of molecular species having a homogeneous fatty acid and long chain base composition. The strategy consisted of two main steps:

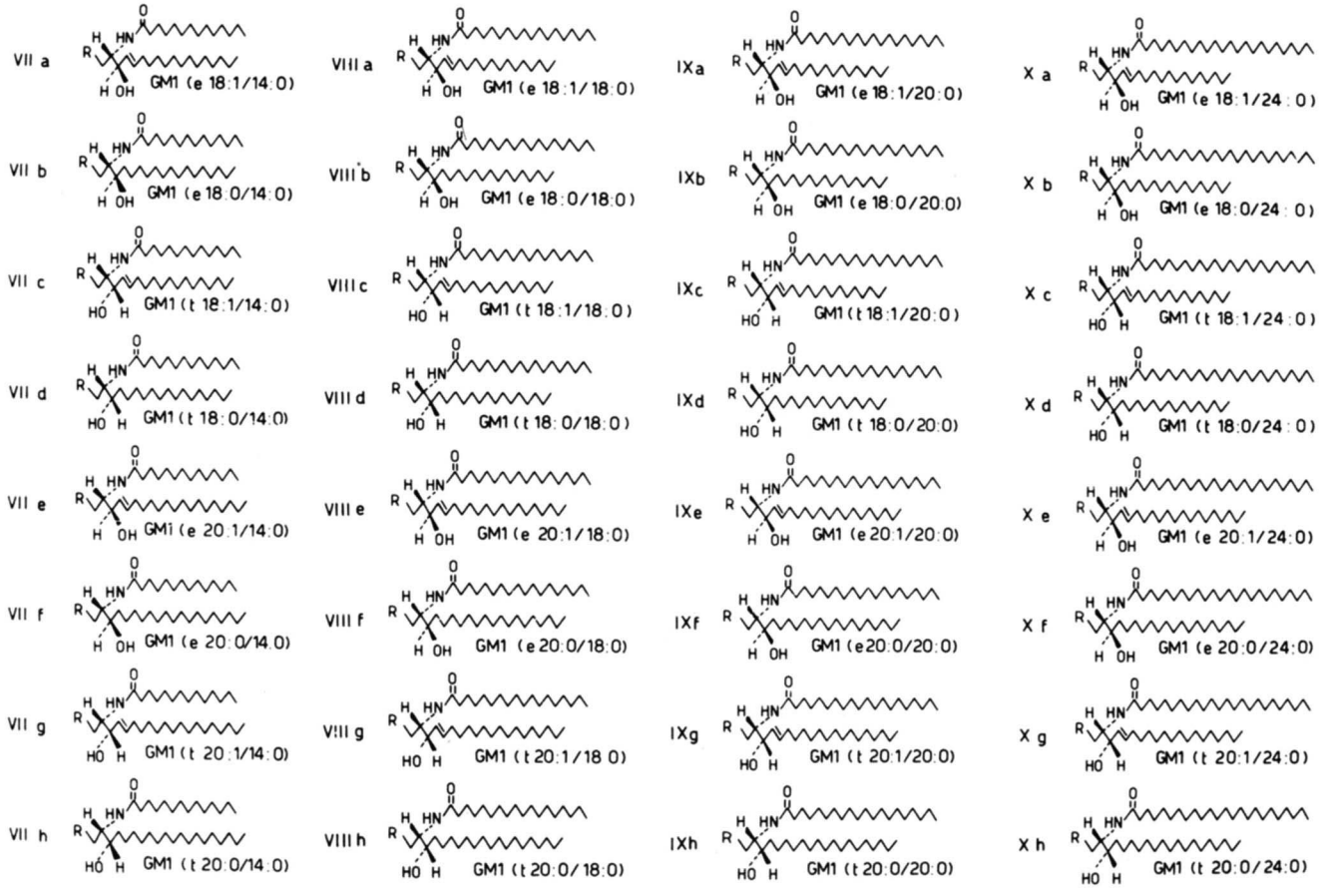

Fig. 6 Chemical structures of the 32 molecular species of GM1, homogeneous in the long chain base and fatty acid moiety. R, oligosaccharide chain of GM1 ganglioside. Each compound is numbered following the flow sheet of Fig. 1 and, in addition, a small letter has been included to distinguish each one on the basis of LCB content. A shorthand formula is also suggested, on the basis of the following logic: ganglioside designation, according to Svennerholm (22), is followed in parenthesis by LCB configuration (e, *erythro;* t, *threo*), length, and degree of unsaturation and, separated by a space-bar, fatty acid length and degree of unsaturation.

first, to remove the fatty acids present in natural GM1 and to introduce a new single fatty acid, myristic, stearic, arachidic, or lignoceric acid; and second, to fractionate, by reversed phase HPLC, each GM1 ganglioside, homogeneous in the fatty acid content, according to the species containing an individual long chain base.

The preparation of so called "lyso"-derivative of gangliosides was introduced by Taketomi and Kawamura (19). They started from GM3 ganglioside and prepared, by butanol–aqueous KOH hydrolysis, the "lyso"derivative Neuα2→3Galβ1→4Glcβ1→1'LCB with a final yield of 50%. Holmgren, Mansson, and Svennerholm (20) and later Tayot and Tardy (21) applied the same conditions to GM1 ganglioside, but did not mention the yield of the reaction. In these latter reports the alkaline conditions used gave rise to a product lacking not only fatty acyl group, but also the acetyl group N-acetyl-galactosamine and of N-acetyl-neuraminic acid. In our hands, alkaline hydrolysis of GM1 with butanol–aqueous KOH was followed by a low recovery of lipid-bound sialic acid. In the search for better conditions, we observed that substitution of KOH with tetramethylammonium hydroxide provided three main advantages: *a*) a marked increase in the solubility of starting natural GM1 in the reaction mixture; *b*) a drastic reduction in base strength, thus avoiding chemical degradation of sialic acid; and *c*) no de-N-acetylation of N-acetyl-galactosamine.

Alkaline treatment of natural GM1 by the present methodology provided two main products, a GM1 molecule de-N-acetylated at the level of sialic acid (compound I) and a GM1 de-N-acetylated at the level of sialic acid and de-N-acylated at the long chain base moiety (compound II). The yield of this latter compound was 45%, practically the same as that given by Taketomi and Kawamura (19) for "lyso"-GM3. A kinetic analysis of the reaction (see Table 1) allowed us to infer that compound I was produced first, and then converted into compound II.

The use of DEC as an activating agent for fatty acid carboxyl group greatly facilitated re-N-acylation of compound II at the level of the free amino group of long chain base. This preferential attack may be explained by considering that the reaction was carried out in aqueous solution. Under these conditions, activated fatty acid and compound II were likely present as micelles (mixed micelles); in fact they do not dialyze. Therefore, the activated fatty acid carboxyl group and the LCB free $-NH_2$ might be placed in a sterically vicinal position, favoring their condensation, with formation of an amide linkage. By employing different fatty acids, myristic, stearic, arachidic, and lignoceric, the reaction yield was always about 50%, indicating that the method could be conveniently extended, with possibly minor modifications, to other natural fatty acids and to synthetic fatty acids carrying paramagnetic, photoreactive, and fluorescent groups. Application of the procedure to these latter cases has already been successful. Furthermore, the method can be used for preparing gangliosides, isotopically labeled on the fatty acid moiety, by employing commercially available radioactive fatty acids.

Combination of the methodology here described with the reversed phase HPLC procedure (10) led to the fractionation of each of the gangliosides with a homogeneous fatty acid moiety in the molecular species containing an individual long chain base. Therefore, a full series of entirely homogeneous molecular species of GM1 gangliosides can be prepared. The results of pilot experiments indicate that the methodology described here can be applied to gangliosides other than GM1. The development and perfection of these methodological approaches are expected to facilitate investigations aimed at ascertaining the molecular aspects of the interaction and binding properties of gangliosides and their physicochemical behavior.

This work was supported by a grant from the Consiglio Nazionale delle Ricerche (CNR) No. 82.01089. We wish to thank Miss Nadia Toppi for her skillful technical assistance.

Manuscript received 16 July 1984.

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