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Synthesis of Lysogangliosides†

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ABSTRACT: The synthesis of gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a} solely lacking the fatty acid moiety, and thus called lysogangliosides in analogy to lysophospholipids, is described. Since a selective elimination of the fatty acid residue has not been achieved as yet, the gangliosides were first subjected to alkaline hydrolysis. By this procedure the fatty acyl as well as the acetyl groups of the sialic acid residue(s) were completely removed. The acetamido group of the *N*-acetylgalactosamine moiety of the gangliosides G_{M2}, G_{M1}, and G_{D1a} was very little ($\simeq$10%) hydrolyzed. In a two-phase system composed of water and ether, the selective protection of the sphingoid amino group was accomplished with a hydrophobic protective group (9-fluorenylmethoxycarbonyl). Lysogangliosides were obtained after re-N-acetylation of the sialooligosaccharide amino group(s) followed by removal of the protecting group. The overall yield was about 30%. The structures of the lysogangliosides were confirmed by chemical analysis as well as negative ion FAB mass spectrometry and ^{1}H NMR spectroscopy. By simple re-N-acylation of lysogangliosides with any labeled fatty acid, labeled gangliosides are now obtainable that are identical with their parent gangliosides except for their labeled fatty acid residue. This has been demonstrated by the synthesis of G_{M1} with a [1-^{13}C]palmitic acid moiety in its ceramide portion. If desired, double-labeled gangliosides may be obtained by use of labeled acetic anhydride in the synthesis of the lysogangliosides.

Gangliosides are characteristic components of mammalian plasma membranes in which they are located asymetrically with their sialooligosaccharide moiety facing the extracellular matrix. Though the ganglioside pattern observed on the cell surface seems to be differentiation- and cell-specific and is altered in a characteristic way by viral cell transformation, little is known about the physiological function of gangliosides and their influence on membrane properties [for a review, see Hakomori, (1981)]. Previously, individual gangliosides have been implicated as receptors for bacterial toxins (e.g., G_{M1}[1] for cholera toxin) and viruses (e.g., gangliosides G_{D1a}, G_{T1b}, and G_{Q1b} for binding of Sendai virus) [for reviews, see Fishman & Brady (1976) and Markwell et al. (1981)]. In addition, gangliosides are assumed to form clusters around certain membrane proteins and thus may regulate receptor function and influence the dynamic state of membrane lipids (Sharom & Grant, 1978).

To study the role of gangliosides in these crucial cellular phenomena, ganglioside derivatives carrying special probes in their ceramide portion that render them suitable for biophysical and biochemical analysis are needed. A prerequisite for these studies is the insertion of the appropriate ganglioside derivatives into plasma membranes. This is possible at least for nitroxide-labeled ganglioside analogues and for ganglioside G_{M3}, as has been shown previously (Schwarzmann et al., 1981, 1983; Schwarzmann & Sandhoff, 1983).

Thus, gangliosides with a nitroxide group at various positions in the *N*-acyl chain are suitable for the investigation of protein–ganglioside interactions, as well as for the study of the dynamic state of gangliosides in the lipid environment of the cell membrane. Likewise, gangliosides bearing a photoactivatable azido group in their hydrophobic moiety may be used to study their nearest neighbors. Further examples of the application of labeled gangliosides are their use in studying ganglioside metabolism as well as the lateral diffusion of gangliosides when radio- and fluorescent-labeled ganglioside derivatives are used, respectively.

The best way to prepare the above-mentioned ganglioside derivatives is the replacement of their fatty acid residue by an appropriately labeled fatty acid moiety. Thus, lyso-

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[1] Abbreviations: G_{M3}, II^{3}Neu5Ac-LacCer; G_{M2}, II^{3}Neu5Ac-GgOse$_3$Cer; G_{M1}, II^{3}Neu5Ac-GgOse$_4$Cer; G_{D1a}, IV^{3}Neu5Ac,-II^{3}Neu5Ac-GgOse$_4$Cer; lyso-G_{M3}, II^{3}Neu5Ac-LacSph; lyso-G_{M2}, II^{3}Neu5Ac-GgOse$_3$Sph; lyso-G_{M1}, II^{3}Neu5Ac-GgOse$_4$Sph; lyso-G_{D1a}, IV^{3}Neu5Ac,II^{3}Neu5Ac-GgOse$_4$Sph; Fmoc, 9-fluorenylmethoxycarbonyl; Cbz, benzyloxycarbonyl; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; Me_2SO-d_6, dimethyl-d_6 sulfoxide; D_2O, deuterium oxide; Me_4Si, tetramethylsilane; TLC, thin-layer chromatography.

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gangliosides, i.e., fatty acyl free gangliosides, have been prepared with their oligosaccharide and sphingoid moiety unaltered.

Experimental Procedures

Materials

Gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a} were available in this laboratory. Ganglioside G_{M3} was from postmortem human spleen. Ganglioside G_{M2} was prepared from the brain of a Tay-Sachs patient. Gangliosides G_{M1} and G_{D1a} were derived from either human or bovine brain. 9-Fluorenylmethyl chloroformate (Fmoc-Cl) and benzyl chloroformate (Cbz-Cl) were from Fluka, Buchs, Switzerland. Visking dialysis tubing type 20/32 was from Serva, Heidelberg, FRG. Precoated thin-layer Kieselgel 60 and Kieselgel 60_{F254} plates (0.25-mm layer thickness) and silica gel Lichroprep Si 60 were obtained from E. Merck AG, Darmstadt, FRG. All other chemicals were of the highest purity available.

Methods

Analytical Assay. Glycolipid-bound Neu5Ac was measured by the method of Svennerholm (1957) as modified by Mietinen & Takki-Luukkainen (1959) with α_1-acid glycoprotein (12.6% Neu5Ac) as reference.

Synthesis of Lysogangliosides. (Step a) Deacylation of Gangliosides. The solution of the appropriate ganglioside (20 μmol) in 1 M methanolic potassium hydroxide (10 mL) was stirred under argon at 102 °C in a screw-capped vial for various periods of time (18–27 h). The course of the deacylation of the gangliosides was followed by TLC in chloroform/methanol/2 M ammonia (60/40/9 by volume) and the reaction was stopped by neutralization with acetic acid when the slow-migrating and ninhydrin-positive component corresponding to the deacylated gangliosides exceeded 80% as determined densitometrically. Following evaporation of methanol, the residue was dissolved in water (3 mL) and freed of most of the salt by brief dialysis (3 h) and then freeze-dried.

(Step b) Selective N-Acylation of Sphingoid and Re-N-acetylation of Sialooligosaccharide Moiety. The solution of the crude deacylated ganglioside of step a in 0.5 M sodium hydrogen carbonate (4 mL) was mixed with diethyl ether (4 mL) and cooled until the aqueous phase turned solid. After the addition of 27 μmol of Fmoc-Cl (7.0 mg) in *n*-hexane (0.7 mL), the mixture was stirred vigorously at 10 °C for 24 h. In the case of G_{M1}, the selective N-acylation of the sphingoid moiety was also performed appropriately with benzyl chloroformate. The reaction mixture was centrifuged at room temperature for 30 min at $5000g_{av}$ to yield two phases that were treated separately at room temperature with small portions of acetic anhydride (10 μL) under vigorous stirring until pH 5 was attained. If necessary, the gel-like upper phase was liquified by a little methanol before the addition of acetic anhydride. After 3 h, the etheral upper phase was dried under a stream of nitrogen, and the residue was dissolved in the aqueous lower phase. The reaction product (*N*-[(9-fluorenylmethoxy)carbonyl]lysoganglioside) was freed of most of the salt and acetic acid by brief dialysis (2.5 h) and freeze-dried. The *N*-[(9-fluorenylmethoxy)carbonyl]lysogangliosides were purified by chromatography on silica gel Lichroprep Si 60 with mixtures of chloroform, methanol, and water of increasing polarity. The fractions giving rise to fluorescent and resorcinol-positive spots on TLC, which contained the pure products, were pooled, and the latter was quantified by the Neu5Ac content.

(Step c) Deblocking of Sphingoid Amino Group. For the removal of the Fmoc group, the reaction products of step b were kept in liquid ammonia in a screw-capped vial for 2 h at room temperature. After evaporation of ammonia, the lysogangliosides were purified by chromatography on silica gel Lichroprep Si 60 with mixtures of chloroform, methanol, and 2 M ammonia of increasing polarity. The fractions containing the lysoganglioside were pooled, and the latter was quantified by the Neu5Ac content. In general, the overall yield was about 8 μmol (30%). The weight of the dry lysogangliosides corresponded to their Neu5Ac content within 5%, thus showing that the remaining salts left after dialysis had been removed by column chromatography. In the case of *N*-Cbz-lyso-G_{M1}, the protective group was cleaved by catalytic hydrogenation at 3 bar for 4 h at 20 °C following the addition of 10% palladium–charcoal (10 mg). The characterization of lysogangliosides was done by FAB mass spectrometry and ^{1}H NMR spectroscopy.

Preparation of N-Acetyllyso-G_{M1}. Aliquots of acetic anhydride (5 μL) were added under vigorous stirring at 20 °C to a solution of 3.9 μmol of lyso-G_{M1} (5 mg) in 1 M sodium hydrogen carbonate (2 mL) over a period of 1 h, until pH 5 was reached. After brief dialysis, the product was lyophilized. The *N*-acetyllyso-G_{M1} was composed of two components differing only in the nature of their sphingoid moiety (C_{18} and C_{20} sphingosine). These two components were separated by preparative TLC in chloroform/methanol/15 mM calcium chloride (60/40/9 by volume) and characterized by FAB mass spectra.

Preparation of Deuterated N-Acetyllysogangliosides. About 3 μmol each of gangliosides G_{M2}, G_{M1}, and G_{D1a} was deacylated as described above, freed of most of the salts by brief dialysis, and then freeze-dried. The lyophilizate was dissolved in 0.5 M sodium hydrogen carbonate (0.5 mL). Three aliquots of a total of 15 μL of $(CD_3CO)_2O$ were added at 20 °C under vigorous stirring over a period of 1 h. The resulting deuterated *N*-acetyllysogangliosides were briefly dialyzed and freeze-dried. Purification of these compounds was achieved by preparative thin-layer chromatography with chloroform/methanol/water (60/35/8 by volume).

Isolation of Sphingoid Bases. Solutions of 3 μmol each of *N*-acetyllyso-G_{M1} (3.8 mg) and G_{M1} (4.6 mg), in saturated sodium metaperiodate (0.2 mL) containing 1 μL of acetic acid, were kept at 20 °C for 72 h in the dark. The excess metaperiodate was destroyed by the addition of ethylene glycol (25 μL). Salts were removed by dialysis. Any aldehyde function was reduced by the addition of sodium borohydride (2.5 mg). After 3 h at 20 °C, salts were removed by dialysis for 3 h. The freeze-dried products were dissolved in a mixture of chloroform/methanol (2:1 by volume) (0.5 mL) and partially hydrolyzed at 20 °C for 25 h by the addition of 12 M hydrochloric acid (5 μL).

Following the addition of water (0.17 mL), the ceramides were partitioned according to Folch et al. (1957). The lower phase was dried in a stream of nitrogen, and the ceramides were hydrolyzed in 1 M methanolic potassium hydroxide (0.8 mL) at 90 °C for 24 h. Following the addition of chloroform (1.6 mL) and water (0.6 mL), the sphingoid bases were partitioned into the lower phase, which was washed once with solvent of the upper phase composition (1.5 mL) (Folch et al., 1957). The lower phase was dried in a stream of nitrogen and subjected to TLC in chloroform/methanol/2 M ammonia (40/10/1 by volume). Spots of sphingoid bases were visualized by ninhydrin.

Isolation of Monosialogangliotetraose. Monosialogangliotetraose (II^3Neu5AcGgOse$_4$) was prepared from G_{M1} and *N*-acetyllyso-G_{M1} by ozonolysis and alkaline fragmentation

(Wiegandt & Baschang, 1965) and subjected to TLC in 1-butanol/acetic acid/water (2/1/1 by volume). Spots of the ganglioside sugar were visualized with Ehrlich's reagent (Heacock & Mahon, 1965) and anisaldehyde (Stahl & Kaltenbach, 1960).

Desialylation of N-Acetyllyso-G_{M1} and G_{M1}. Sialic acid was released by hydrolysis of 1 μmol each of *N*-acetyllyso-G_{M1} (1.3 mg) and G_{M1} (1.5 mg) in 1 M formic acid (1.0 mL) at 80 °C for 2 h. After lyophilization, the residue was dissolved in methanol/water (4/1 by volume; 0.2 mL) and subjected to TLC in 1-butanol/acetic acid/water (2/1/1 by volume). Sialic acids were detected by Ehrlich's reagent.

Reacylation of Lyso-G_{M1}. The lysoganglioside (1 μmol) dissolved in chloroform/methanol/water (60/40/9 by volume; 1 mL) was reacylated in the presence of triethylamine (10 μL) by the addition of 5 μmol of *N*-succinimidyl octadecanoate or *N*-succinimidyl [1-^{13}C]hexadecanoate (2 mg) and subjected to TLC in chloroform/methanol/15 mM calcium chloride (60/40/9 by volume). The products of re-N-acylation were purified by chromatography on silica gel Lichroprep Si 60 with mixtures of chloroform, methanol, and water of increasing polarity.

Deacetylation and Re-N-acetylation of N-Acetyllyso-G_{M1}. About 0.75 μmol of either C_{18} or C_{20} sphingoid containing *N*-acetyllyso-G_{M1} (1 mg) was deacylated as described above. Following dilution of the hydrolysate with an equal volume of water (1 mL), aliquots to a total of 20 μL of acetic anhydride were added under vigorous stirring over a period of 1 h at 20 °C. Salts were removed by brief dialysis, and the freeze-dried products were examined by TLC in chloroform/methanol/15 mM calcium chloride (60/40/9 by volume).

Mass Spectrometry. FAB mass spectrometry (Barber et al., 1981a,b) was carried out with a VG analytical ZAB 1F, reverse geometry, mass spectrometer fitted with a FAB source and an Ion-Tech atom gun. Samples were dissolved in methanol, and 0.5–1.0 μL of the solution containing 1–10 μg of the sample and 0.1% sodium acetate was added to 2–4 μL of 1-mercapto-2,3-propanediol on the stainless steel target. The target was bombarded with argon or xenon atoms having 8–9-keV energy. Spectra were obtained with a Hall probe controlled linear mass scan of up to 500-s duration for a full scan from 3000 to 12 mass units. The spectra were recorded on UV-sensitive chart paper and calibrated manually by counting.

Proton NMR Spectroscopy. For removal of exchangeable protons, each sample (about 3 mg) was repeatedly freeze-dried from D_2O. The lyophilized sample was dissolved in 0.6 mL of freshly prepared Me_2SO-d_6–D_2O (98:2 by volume) containing a Me_4Si reference (4 mM). The NMR spectra were obtained at 25 °C with a Bruker WM 400 spectrometer (16K data points) in 5-mm sample tubes at 400 MHz (^{1}H). Chemical shifts were referenced to internal Me_4Si.

Results

Lysogangliosides (lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a}) have been synthesized following the route outlined in Figure 1. Deacylation of the gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a} was performed in 1 M methanolic KOH at 102 °C. The course of the reaction was followed by measuring the increase of the deacylated products and the decrease of the parent gangliosides, respectively, as revealed by TLC (data not shown). Generally, a reaction time of 20 h was necessary to totally deacylate ganglioside G_{M3}, whereas the formation of deacylated gangliosides G_{M2}, G_{M1}, and G_{D1a} and the concomitant disappearance of their parent gangliosides required longer

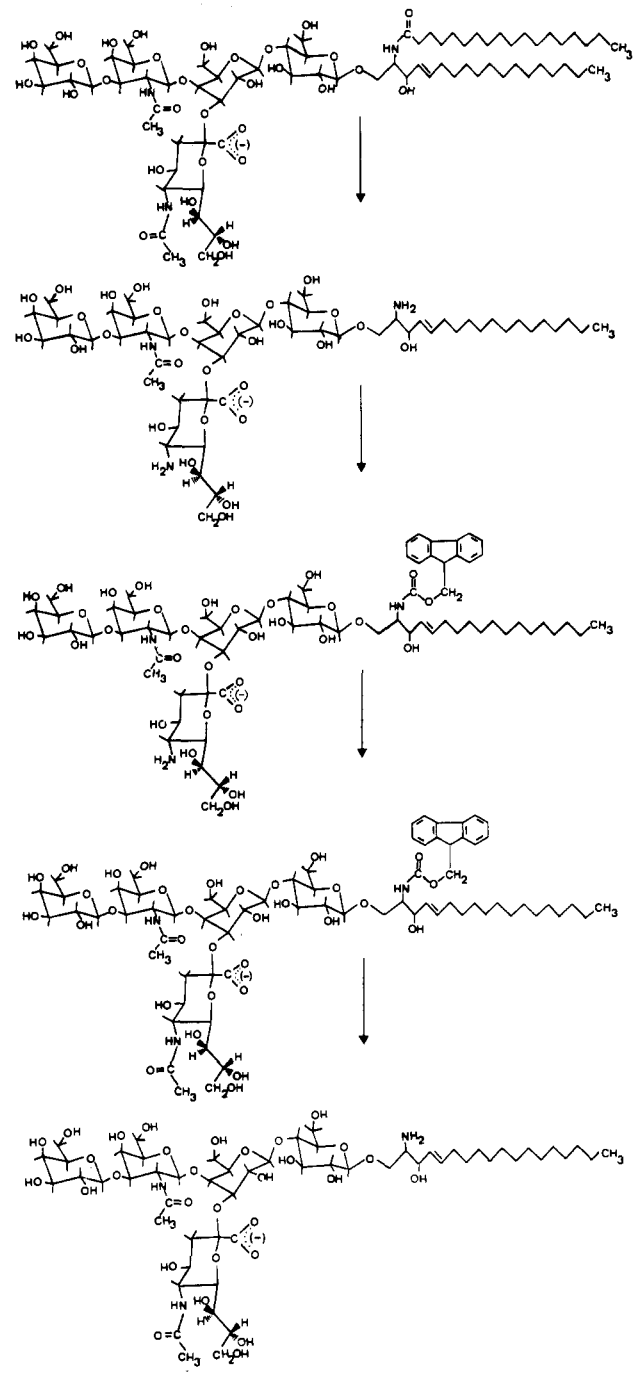

FIGURE 1: Reaction route to lyso-G_{M1}.

reaction times (up to 25 h). This very probably reflects the different solubilities of the gangliosides in methanol. The degree of deacylation of the gangliosides has been investigated as illustrated for G_{M1}. By use of [1-^{14}C]acetic anhydride, the deacylated G_{M1} was selectively N-acetylated and the resulting *N*-acetyllyso-G_{M1} was analyzed. If all three amide bonds had been cleaved during alkaline hydrolysis of G_{M1} and three *N*-[1-^{14}C]acetyl groups were subsequently introduced into the deacylated G_{M1}, then one would expect that two-thirds of the radioactivity would reside in the monosialooligosaccharide and one-third in the sphingoid moiety. However, the results of Table I show that the ^{14}C-labeled *N*-acetyllyso-G_{M1} had a specific radioactivity of 12.1 Ci/mol, and when this compound was subjected to ozonolysis and alkaline fragmentation, a monosialogangliotetraose with about half the specific radioactivity (6.3 Ci/mol) was obtained. In addition, *N*-acetylneuraminic acid released from the ^{14}C-labeled *N*-acetyllyso-

Table I: Comparison of Specific Radioactivities of Ganglioside G_{M1} Derivatives

N-[1-^{14}C]acetylated products	sp radioactivity (Ci/mol)
N-acetyllyso-G_{M1}	12.1
monosialogangliotetraose	6.3 (6.1)[a]
N-acetylneuraminic acid	6.1 (5.9)[a]

[a] Specific radioactivity of monosialogangliotetraose and *N*-acetylneuraminic acid that was released from ^{14}C-labeled *N*-Fmoc-lyso-G_{M1}.

G_{M1} by mild acid hydrolysis showed a specific radioactivity of 6.1 Ci/mol. Thus, during alkaline hydrolysis of G_{M1} only two of the three amide bonds had been cleaved, and the acetamido group of the *N*-acetylgalactosamine moiety was left intact.

To confirm this unexpected observation, the *N*-acetylgalactosamine-containing gangliosides G_{M2}, G_{M1}, and G_{D1a} were deacylated and re-N-acetylated with deuterated acetic anhydride $(CD_3CO)_2O$. The resulting deuterated *N*-acetyllysogangliosides were subjected to FAB mass spectrometry. The characteristic pseudo molecular ions (M − 1) were found at m/z 1164 for *N*-acetyllyso-G_{M2}, at m/z 1326 and 1354 for *N*-acetyllyso-G_{M1}, and at m/z 1620 and 1648 for *N*-acetyllyso-G_{D1a}, reflecting the C_{18} and C_{20} sphingosine content of the latter two ganglioside derivatives. All the observed pseudo molecular ions were found to be higher by 6 and 9 mass units than those calculated for the nondeuterated mono- and disialoganglioside derivatives, respectively. This clearly demonstrates that all but one of the *N*-acetyl groups of the sialooligosaccharide moieties were replaced by an *N*-[^{3}H]acetyl group. If all *N*-acetyl groups had been removed during deacylation the pseudo molecular ions should have been higher by 9 and 12 mass units for the mono- and disialoganglioside derivatives, respectively. In addition, the *N*-acetylneuraminic acid residue(s) was (were) easily split, giving rise to a fragment ion at m/z 311 and to a daughter ion at m/z 293 due to loss of water from the former. Both ions were higher by 3 mass units than that calculated for the nondeuterated ions; no ions at m/z 308 and 290 were found. These results confirm the radiochemical analysis. However, in the case of *N*-acetyllyso-G_{M2} a pseudo molecular ion at m/z 1167 was also found. Although the intensity of this ion was far less than that at m/z 1164, it indicates that during alkaline hydrolysis a few percent of G_{M2} had been completely deacylated. On the other hand, only traces of completely deacylated gangliosides G_{M1} and G_{D1a} were revealed by FAB mass analysis.

The ^{1}H NMR spectra of the deuterated *N*-acetyllysogangliosides totally lacked the characteristic signal from the methyl protons of the *N*-acetyl group of sialic acid at 1.88 ppm (see below). On the other hand, the signal for the methyl protons of the acetamido group of *N*-acetylgalactosamine was found at 1.76 ppm, again confirming the radiochemical analysis.

Protection of Sphingoid Amino Group of Deacylated Gangliosides and Re-N-acetylation of Oligosaccharide Moiety. A selective N-acylation of the sphingoid amino group of the deacylated gangliosides G_{M3}, G_{M2}, G_{M1}, and G_{D1a} was achieved with the hydrophobic reagent 9-fluorenylmethyl chloroformate and, additionally, in the case of G_{M1} with benzyl chloroformate in a two-phase solvent system consisting of diethyl ether and water of equal volumes at low temperatures. Re-N-acetylation of the sialooligosaccharide residues was performed with acetic anhydride following separation of the aqueous and organic phases (see Experimental Procedures). After purification *N*-Fmoc-lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a} and *N*-Cbz-lyso-G_{M1} were obtained in about 50% yield. The specificity of the protection of the sphingoid amino group, i.e., that none of the carbohydrate amino groups were acylated by 9-fluorenylmethyl chloroformate, was demonstrated by the fact that monosialogangliotetraose released from ^{14}C-labeled *N*-Fmoc-lyso-G_{M1} showed almost the same specific radioactivity as the monosialogangliotetraose derived from ^{14}C-labeled *N*-acetyllyso-G_{M1} (cf. Table I). In addition, Neu5Ac released from the labeled Fmoc derivative, as well as from the labeled *N*-acetyllyso-G_{M1}, demonstrated comparable specific radioactivities.

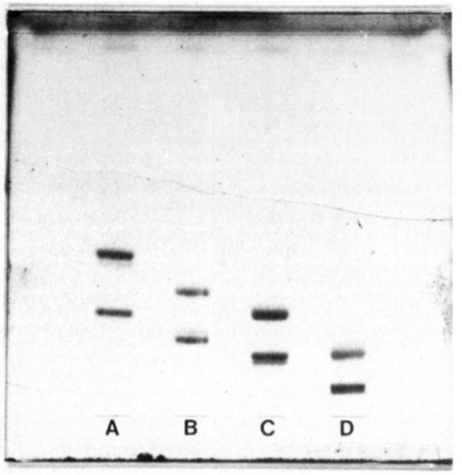

FIGURE 2: Thin-layer chromatogram of lysogangliosides and their parent gangliosides. TLC plates were developed in chloroform/methanol/15 mM calcium chloride (60/35/8 by volume). Spots were visualized according to Stahl & Kaltenbach (1961). (Lane A) G_{M3} and lyso-G_{M3}; (lane B) G_{M2} and lyso-G_{M2}; (lane C) G_{M1} and lyso-G_{M1}; (lane D) G_{D1a} and lyso-G_{D1a}. The upper bands represent the parent gangliosides.

Deblocking of Sphingoid Amino Group of N-Protected Lysogangliosides. Removal of 9-fluorenylmethoxycarbonyl from the amino-protected lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a} was achieved in liquid ammonia (Bodanszky et al., 1979) to yield quantitatively the lysogangliosides. Lyso-G_{M1} was also obtained by hydrogenolysis of *N*-Cbz-lyso-G_{M1}. Figure 2 shows the different chromatographic properties of the lysogangliosides as compared to their parent gangliosides. When ninhydrin was used to detect the products on thin-layer plates, only the lysogangliosides became visible.

Characterization of Lysogangliosides. Figure 2 reveals double bands for lyso-G_{M2}, -G_{M1}, and -G_{D1a} but simply one band for lyso-G_{M3}, thus suggesting that the former may be composed of at least two different sphingoid bases. In this respect, lyso-G_{M3} is expected to be composed of mainly one single type of sphingoid.

On the other hand, it had to be considered that during deacylation of the gangliosides byproducts besides the intact deacylated gangliosides could arise, thus leading to double bands of lyso-G_{M2}, -G_{M1}, and -G_{D1a}. The question whether one of these double bands was due to structural alteration of one or more of the ganglioside components during the alkaline hydrolysis (or re-N-acetylation) or was due to different sphingoid moieties has been answered by the following studies. The two products referred to as lyso-G_{M1} (cf. Figure 2) could easily be separated following conversion to their *N*-acetyl derivatives. Deacetylation and re-N-acetylation of each of the separated products yielded the identical starting compound with no detectable formation of the other product. Thus, we assume that different sphingoid moieties rather than structural alterations of any of their components caused lyso-G_{M2}, -G_{M1}, and -G_{D1a} to separate into two bands on thin-layer chromatograms. Indeed, these lysogangliosides were mainly composed of C_{18} and C_{20} sphingosines as shown by negative ion FAB

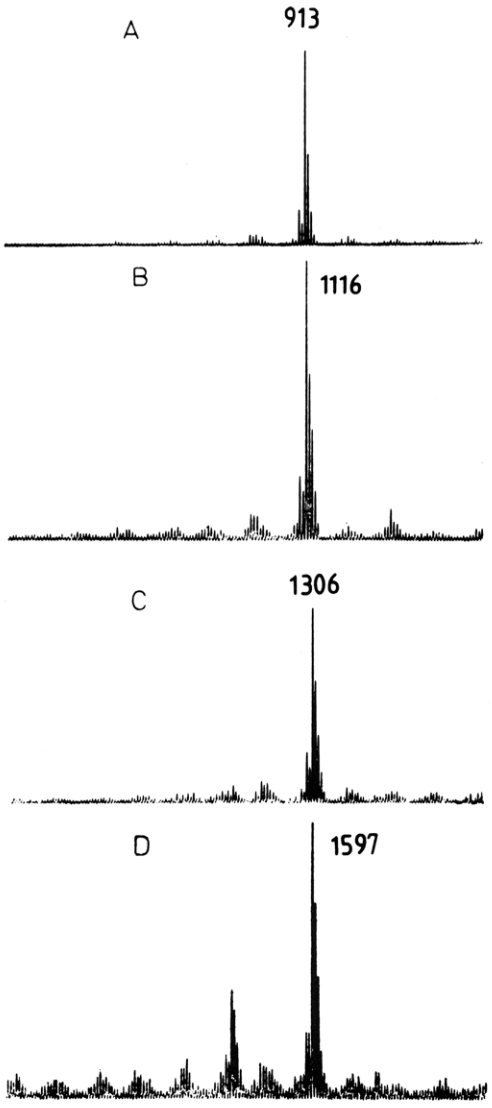

FIGURE 3: Mass spectra (part of the higher mass unit region) of lysogangliosides showing the pseudo molecular ions (M − 1) of lyso-G_{M3} (A), lyso-G_{M2} (B), lyso-G_{M1} (C), and lyso-G_{D1a} (D).

mass spectrometry (see below). By this method, lyso-G_{M3} was found to contain almost exclusively C_{18} sphingosine.

The structural integrity of lyso-G_{M1} was also demonstrated by its conversion to the *N*-octadecanoyl derivative, which on TLC was indistinguishable from native G_{M1}. Furthermore, *N*-acyllyso-G_{M1} when subjected to ozonolysis and alkaline fragmentation yielded monosialogangliotetraose with identical chromatographic properties with those of the sialooligosaccharide released from native G_{M1}. In addition, mild acid hydrolysis of both *N*-acetyllyso-G_{M1} and native G_{M1} liberated identical sialic acids. To investigate the sphingoid moiety of *N*-acetyllyso-G_{M1}, it was released from the latter by a procedure (Klenk & Huang, 1969) that does not cause any alteration of sphingoid bases. As revealed by TLC, the pattern of the sphingoid bases thus obtained was the same as for the parent G_{M1} with no indication of *threo*-sphingoid formation. Hence, we may conclude that during the synthesis of lysogangliosides no changes in their sphingoid moieties had occurred.

The structures of lysogangliosides were also analyzed by negative ion FAB mass spectrometry. The high relative intensity of pseudo molecular ions (M − 1) at m/z 913, 1116, 1306, and 1597 for lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a}, respectively, as shown in Figure 3 confirms their composition

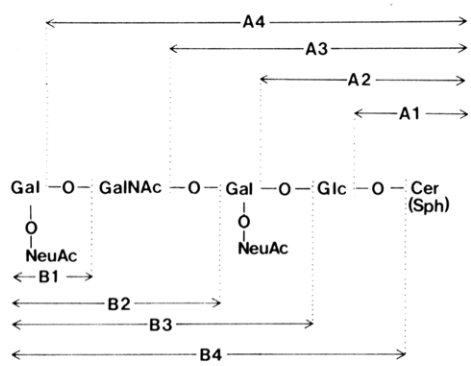

FIGURE 4: Fragmentation of gangliosides (lysogangliosides) by fast atom bombardment.

of a sialooligosaccharide and sphingoid moiety. The negative ion at m/z 1306 indicates that the purified fraction of lyso-G_{M1} contains almost exclusively C_{20} sphingosine. In contrast, in lyso-G_{M3} and lyso-G_{M2} mostly C_{18} sphingosine is found. Lyso-G_{D1a} contains both C_{18} and C_{20} sphingosine as major sphingoid bases.

During fast atom bombardment the glycosidic bonds are easily split forming negative ions according to the fragmentation scheme illustrated in Figure 4. Fragments A always contain the sphingoid or ceramide moiety of lysogangliosides and gangliosides, respectively, whereas fragments B contain the terminal sugars. Furthermore, the sialic acid residue is preferentially split, giving rise to a negative ion at m/z 308 and a daughter ion by loss of water from the former at m/z 290. Both these ions and the pseudo molecular ion (M − 1) are rather intense as shown in the mass spectrum of lyso-G_{M1} (Figure 5). In addition, fragments A and B are found in the spectrum at the expected mass per charge values and are highly indicative of the structure of lyso-G_{M1}. Those fragments B that contain sialic acid residues give rise to an additional fragment smaller by 2 mass units due to dehydrogenation of the former.

The negative ions that are characteristic of the structures of the lysogangliosides are listed in Table II. For comparison, the corresponding negative ions of the parent gangliosides have been included. The structures of *N*-Fmoc-lyso-G_{D1a} and -G_{M1}, as well as G_{M1} bearing a [1-^{13}C]palmitic acid in its ceramide moiety, were also analyzed by FAB mass spectrometry, and their characteristic negative ions are also found in Table II. As indicated in Table II, all the expected pseudo molecular ions (M − 1) are found in the spectra of the appropriate compounds and are of high relative intensity.

For all compounds listed in Table II with the exception of lyso-G_{M1}, -G_{M3}, and G_{M3}, two pseudo molecular ions differing by 28 mass units are found reflecting their C_{18} and C_{20} sphingosine content. Consequently, all their other sphingosine-containing fragments appear as two peaks. In the case of lyso-G_{M1}, the C_{18} sphingosine containing component had been chromatographically removed prior to analysis.

Lyso-G_{M3} contained exclusively C_{18} sphingosine as shown by its pseudo molecular ion with m/z 913, whereas the ceramide portion of G_{M3} that was isolated from human spleen was mainly composed of C_{24}:0, C_{24}:1, C_{22}:0, and C_{22}:1 fatty acids besides C_{18} sphingosine as indicated by four pseudo molecular ions with m/z 1263, 1261, 1235, and 1233, respectively.

Fragments B of comparable relative intensity were found in the FAB mass spectra of lysogangliosides and their parent gangliosides, thus indicating that during the preparation of lysogangliosides no perceptible alteration of their sialooligosaccharide residue had taken place. Fragment B_4 and its

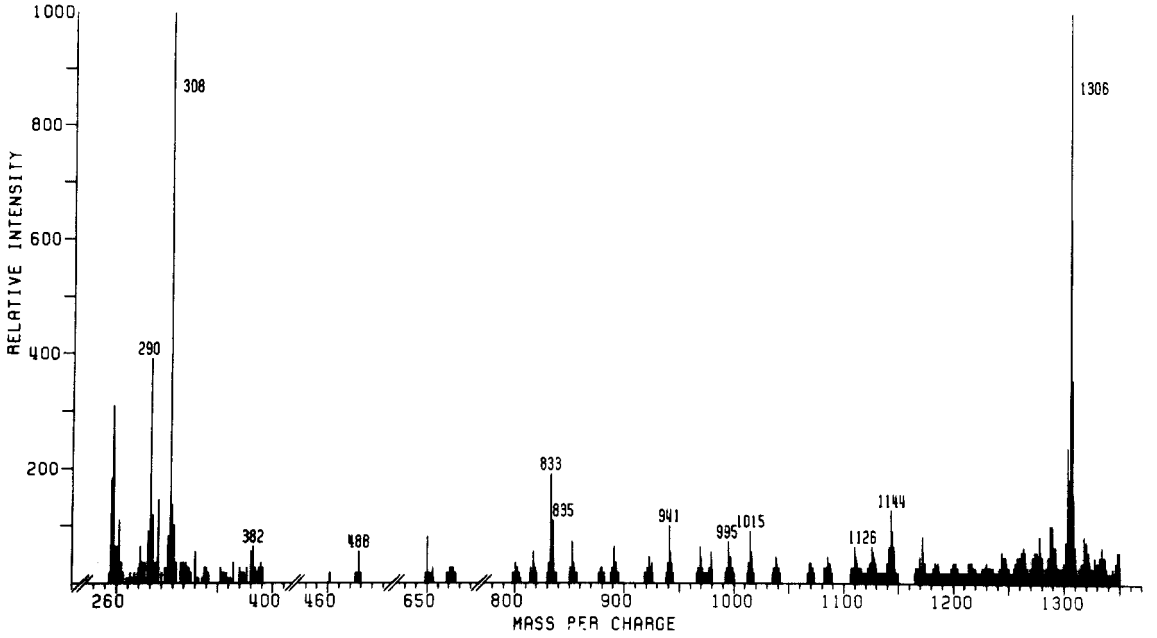

FIGURE 5: FAB mas spectrum of the purified fraction of lyso-G_{M1} containing C_{20} sphingosine.

Table II: Characteristic Negative Ions of Gangliosides and Ganglioside Derivatives in FAB Mass Spectra[a]

glycolipids	M-1	M-1-NeuAc	A1	A2	A3	A4	B1	B2	B3	B4
G_{D1a}	1835 (s)	1544 (s)	564 (m)	726 (m)	1179 (w)	1382 (w)	470 (w)	673 (w)	1126 (w)	1288 (nd)
	1863 (s)	1572 (s)	592 (m)	754 (m)	1207 (w)	1410 (w)	468 (w)	671 (w)	1124 (w)	1286 (nd)
lyso-G_{D1a}	1569 (m)	1278 (m)	298 (nd)	460 (nd)	913 (w)	1116 (w)	470 (m)	673 (m)	1126 (w)	1288 (m)
	1597 (s)	1306 (s)	326 (nd)	488 (nd)	941 (m)	1144 (m)	468 (m)	671 (m)	1124 (w)	1286 (w)
N-Fmoc-lyso-G_{D1a}	1791 (m)	1500 (m)	520 (nd)	682 (nd)	1135 (w)	1338 (w)	470 (m)	673 (nd)	1126 (nd)	1288 (nd)
	1819 (s)	1528 (s)	548 (nd)	710 (nd)	1163 (m)	1366 (m)	468 (m)	671 (nd)	1124 (nd)	1286 (nd)
G_{M1}	1544 (s)	1253 (nd)	564 (s)	726 (m)	1179 (w)	1382 (nd)	179	382 (nd)	835 (m)	997 (w)
	1572 (s)	1281 (nd)	592 (s)	754 (m)	1207 (w)	1410 (nd)			833 (m)	995 (w)
lyso-G_{M1}	1278 (nd)	987 (nd)	298 (nd)	460 (nd)	913 (nd)	1116 (nd)	179	382 (w)	835 (w)	997 (w)
	1306 (s)	1015 (w)	326 (nd)	488 (w)	941 (w)	1144 (w)			833 (m)	995 (w)
N-Fmoc-lyso-G_{M1}	1500 (s)	1209 (nd)	520 (nd)	682 (nd)	1135 (w)	1338 (w)	179	382 (nd)	835 (m)	997 (w)
	1528 (s)	1237 (nd)	548 (w)	710 (nd)	1163 (m)	1366 (s)			833 (m)	995 (w)
G_{M2}	1382 (s)	1091 (m)	564 (m)	726 (m)	1179 (w)			220	673 (m)	835 (w)
	1410 (m)	1119 (w)	592 (w)	754 (w)	1207 (w)				671 (m)	833 (m)
lyso-G_{M2}	1116 (s)	825 (m)	298 (nd)	460 (w)	913 (w)			220	673 (m)	835 (w)
	1144 (w)	853 (w)	326 (nd)	488 (nd)	941 (w)				671 (m)	833 (w)
G_{M3}	1263 (s)	972 (w)	648 (w)	810 (w)					470 (m)	632 (w)
	1261 (s)	970 (w)	646 (w)	808 (w)					468 (m)	630 (w)
	1235 (m)	944 (w)	620 (nd)	782 (w)						
	1233 (w)	942 (nd)	618 (nd)	780 (nd)						
lyso-G_{M3}	913 (s)	622 (m)	298 (nd)	460 (w)					470 (m)	632 (m)
	941 (nd)	650 (nd)	326 (nd)	488 (nd)					468 (m)	630 (m)
$^{13}C_{16}$-G_{M1}[b]	1517 (s)	1226 (w)	537 (w)	699 (w)	1152 (nd)	1355 (nd)	179	382 (w)	835 (s)	997 (w)
	1545 (s)	1254 (w)	565 (s)	727 (m)	1180 (nd)	1383 (nd)			833 (s)	995 (w)

[a] The relative intensities of the negative ions are indicated as follows: s, strong; m, medium; w, weak; nd, not detectable. Fragments with $m/z \lesssim 220$ were not considered owing to the high background noise level of this region. For fragments M-1, M-1-NeuAc, A1 to A4, and B1 to B4, see text and Figure 4. [b] $^{13}C_{16}$-G_{M1}, ganglioside G_{M1} bearing a [1-^{13}C]palmitic acid in its ceramide residue.

daughter ion (see above) are not detected in the spectra of G_{D1a} and *N*-Fmoc-lyso-G_{D1a}. This is presumably due to the fact that either of the two sialic acid residues is easily lost during the fragmentation process. An exception seems to be fragment B_4 of lyso-G_{D1a}. However, the negative ion with m/z 1288 (and its daughter ion) of detectable relative intensity is very probably derived from the pseudo molecular ion by loss of a sialic acid residue and water.

In addition, lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a} were characterized by their ^{1}H NMR spectra. The spectra of lyso-G_{M2} and G_{M2} are compared in Figure 6. The most diagnostic proton chemical shifts of lyso-G_{M3}, -G_{M2}, -G_{M1}, and -G_{D1a} and those of their parent gangliosides are listed in Table III.

All signals of the alkyl chain methyl protons were near 0.85 ppm in the spectra of gangliosides as well as those of their lyso forms. Due to the lack of the fatty acid residue, the relative intensity of this signal in the spectra of lysogangliosides was about 50% of the relative intensity of the corresponding signal for intact gangliosides, thus confirming the structures of lysogangliosides. The signal of the *N*-acetyl methyl protons of the sialic acid residues was found in the spectra of gangliosides and lysogangliosides near 1.88 ppm with twice the relative intensity in the case of ganglioside G_{D1a} and lyso-G_{D1a}. The signal of the *N*-acetyl methyl protons of the *N*-acetylgalactosamine residue was found near 1.76 ppm with the same intensity as for the methyl protons at 1.88 ppm in the spectra

Table III: Chemical Shifts[a] (ppm) for Methyl, Anomeric, and Olefinic Protons of Gangliosides and Lysogangliosides

	G_{M3}	lyso-G_{M3}	G_{M2}	lyso-G_{M2}	G_{M1}	lyso-G_{M1}	G_{D1a}	lyso-G_{D1a}
methyl protons								
alkyl chain (s)	0.85	0.86	0.86	0.86	0.85	0.85	0.86	0.86
GalNAc			1.77	1.77	1.75	1.74	1.75	1.75
Neu5Ac	1.89	1.90	1.88	1.88	1.88	1.88	1.89	1.89
anomeric protons								
Glc	4.15	4.18	4.15	4.25	4.14	4.15	4.15	4.25
Gal (II)	4.19	4.25	4.28	4.28	4.27	4.27	4.26	4.25
Gal (IV)					4.20	4.20	4.24	4.25
GalNAc			4.78	4.77	4.85	4.84	4.78	4.77
olefinic protons								
C4 (sphingosine)	5.54	5.72	5.53	5.72	5.52	5.56	5.53	5.74
C5 (sphingosine)	5.34	5.45	5.34	5.45	5.33	5.42	5.34	5.45

[a] Obtained at 400 MHz at 25 °C in Me_2SO-d_6–D_2O (98/2 v/v); referenced to internal Me_4Si.

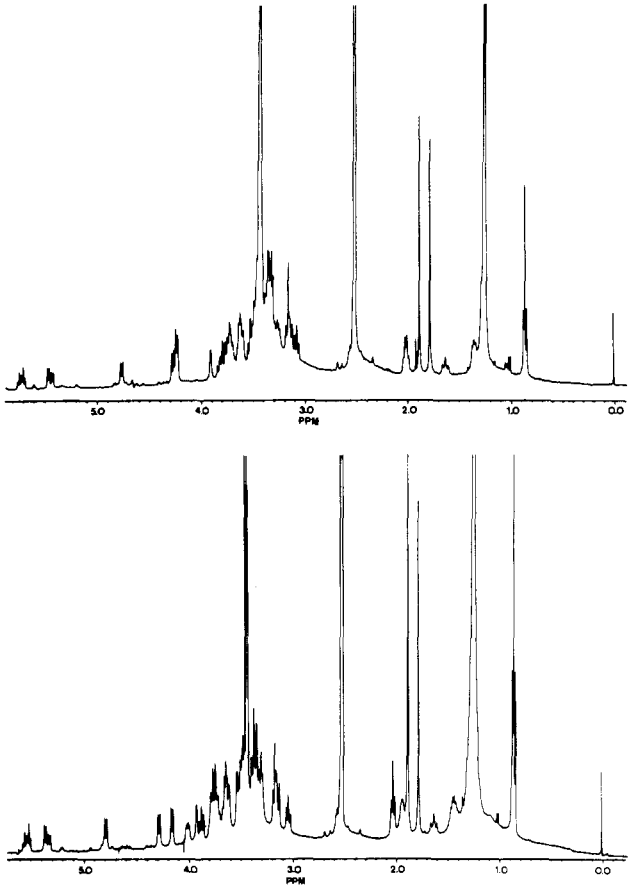

FIGURE 6: Proton NMR spectra of lyso-G_{M2} (upper panel) and G_{M2} (lower panel) obtained at 400 MHz and 25 °C; Me_4Si was the internal standard.

of monosialolysogangliosides and half the intensity of the methyl protons at 1.88 ppm in the spectrum of lyso-G_{D1a}. This clearly demonstrates that all amino groups in the sialooligosaccharide portion of the lysogangliosides are acetylated. The chemical shifts in the region of 4.1–4.9 ppm in the spectra of gangliosides and lysogangliosides were assigned to the anomeric protons according to the proton assignments proposed by Koerner et al. (1983). In case of lyso-G_{M2} and lyso-G_{D1a}, the anomeric proton resonance of the glucose moiety was observed about 0.1 ppm downfield from that in the corresponding intact gangliosides. This downfield shift might be due to steric perturbation caused by the free sphingoid amino group of these lysogangliosides. Nevertheless, the signals of the anomeric protons observed in the spectra of gangliosides, as well as in those of their lyso forms, showed the same coupling constants as published for the appropriate gangliosides G_{M3}, G_{M2}, and G_{M1} by Koerner et al. (1983). Thus, a change in anomeric linkages during the preparation of lysogangliosides can be excluded. A perceptible downfield shift of about 0.2 and 0.1 ppm was observed for the signal of the protons at carbons 4 and 5, respectively, of the sphingoid moiety in lysogangliosides (see Table III). This is probably due to different shielding of the neighboring free amino group as compared to the acylated amino group in the parent gangliosides.

Discussion

Gangliosides, as well as other glycosphingolipids so far described, contain a ceramide residue, that is a double-tailed lipophilic moiety. Bilayer-forming phospholipids are also comprised of a double-tailed lipophilic residue though lysophospholipids with a single lipophilic chain are found in nature, too. In contrast, lysogangliosides have never as yet been found in biological material. The ganglioside derivatives described in this paper are solely lacking the fatty acid residue, this being the only difference with regard to their parent gangliosides. Hence, they are called lysogangliosides in analogy to lysophospholipids. The lysogangliosides of the present study are not to be confused with lyso-G_{M1} ganglioside and lysohematoside, which had been prepared previously by deacylation of G_{M1} (Tayot et al., 1981) and G_{M3} (Taketomi & Kawamura, 1970) and therefore are missing *N*-acetyl groups in their oligosaccharide moiety.

In the present study the first step in the route to lysogangliosides is the cleavage of amide bonds in the parent gangliosides since a selective removal of the fatty acid residue could not as yet be achieved in satisfying yields. Owing to the acid lability of the glycosidic linkages, splitting of the ganglioside amide bonds had to be performed under alkaline conditions. The hydrolysis seemed to be more effective in methanol than in water or higher alcohols. In water, the formation of micelles probably impedes the hydrolysis of the fatty acid amide bond. On the other hand, gangliosides are less soluble in higher alcohols.

An interesting feature of the gangliosides is the resistance of the acetamido group of their *N*-acetylgalactosaminyl residue to alkaline hydrolysis. We are inclined to explain this fact by shielding of this acetamido group by the negatively charged carboxyl group of the sialic acid residue. The pivotal step in the synthesis of lysogangliosides is the selective acylation of the sphingoid amino group. This is accomplished by use of a hydrophobic acylating agent in a two-phase system composed of water and a hydrophobic solvent, here ether. The acylating agent and the sphingoid residue are predominantly partitioned into the nonaqueous phase, thus enhancing the regioselectivity in the acylation reaction.

As protecting group, fluorenylmethoxycarbonyl proved to be favorable because its removal by liquid ammonia leaves the acid-sensitive glycosidic linkages untouched and has no effect

on the carbon–carbon double bond of the sphingosine moiety. On the other hand, hydrogenolytic cleavage of the benzyloxycarbonyl group converts any sphingosine into sphinganine residues.

The protection of the sphingoid amino group followed by re-N-acetylation of the sialooligosaccharide residue and removal of the protective group proceeded in high yields. However, the final yield of lysogangliosides was only about 30% due to loss of material during the initial step of alkaline deacylation followed by dialysis. Nevertheless, this is the only synthetic route to lysogangliosides published to date. In addition, it allows labeling of the *N*-acetylneuraminic acid residue(s) by use of either [^{3}H]-, [^{14}C]-, or [^{13}C]acetic anhydride in the re-N-acetylation step.

The investigation of the lysogangliosides by chemical analysis, as well as FAB mass spectrometry and ^{1}H NMR spectroscopy, showed that their sphingoid and carbohydrate portion are identical with those of their parent gangliosides. Previously, Schwarzmann et al. (1981, 1983) have shown that synthetic ganglioside analogues with a nitroxide group in their hydrophobic part are well suited to studying the insertion of gangliosides into the membrane of cultured cells. However, the synthetic analogues differ from true gangliosides in two respects: (a) the hydrophobic part is comprised of two long fatty acid chains rather than ceramide, and (b) the sialooligosaccharide part contains a sorbitylamine instead of a glucose moiety. Moreover, the synthesis of the ganglioside analogues requires free sialooligosaccharides. Consequently, a more simple approach to labeled gangliosides was looked for and, indeed, was found in the synthesis of lysogangliosides. N-Acylation of the latter with labeled fatty acid derivatives (e.g., *N*-succinimidyl ester) yields labeled gangliosides that are entirely comparable to native gangliosides. With nitroxide-labeled fatty acids, spin-labeled gangliosides are obtained, thus enabling the study of ganglioside cell membrane insertion, as well as ganglioside–protein interactions. These interactions may also be studied with gangliosides bearing a photoactivatable group (such as azido) in their ceramide residue. Of high interest is the determination of the cell membrane viscosity in the vicinity of gangliosides. This now seems possible by use of gangliosides that carry the membrane probe diphenylhexatriene (DPH) as part of their ceramide moiety. The appropriate labeled acid [4-*p*-phenylene-1-(1-phenylhexa-1,3,5-triene-6-yl)butanoic acid] is already available in this laboratory.

Thus far, lyso-G_{M3} has been N-acylated with 1-pyrenedodecanoic acid yielding fluorescent G_{M3}, which has been found to label membranes of cultured cells (fibroblasts and neurones; unpublished results). The fluorescent gangliosides may prove useful in the study of ganglioside distribution within cell membranes and in the investigation of ganglioside metabolism in cells.

We are inclined to think that various labeled gangliosides will be of particular importance in studying the functional role of gangliosides at the cell surface. The synthesis of labeled gangliosides seems also possible by selectively reacting the deacylated gangliosides with activated labeled fatty acids prior to re-N-acetylation of the oligosaccharide moiety. Indeed, when deacylated G_{M1} was treated with *N*-hydroxysuccinimidyl octadecanoate in the two-phase system followed by re-N-acetylation of the monosialoganglioteotraose moiety, G_{M1} was obtained in low yield. This procedure, at a first glance, seems to be simpler than the synthesis of labeled gangliosides via their lysogangliosides. However, the yield of labeled gangliosides is considerably higher when lysogangliosides are acylated. This is of particular importance if the labeled fatty acid either is difficult to obtain, is high in price, or is prone to react with acetic anhydride.

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