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SYNTHESIS AND CHARACTERIZATION OF lyso-GM_3 ($II^3Neu5Ac$ LACTOSYL SPHINGOSINE), de-*N*-ACETYL-GM_3 (II^3NeuNH_2 LACTOSYL Cer), AND RELATED COMPOUNDS*

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ABSTRACT

Various GM_3 derivatives which are present in A431 cells have different effects on the activity of the EGF receptor kinase. In order to systematically study these effects, the following GM_3 derivatives have been synthesized: de-*N*-acetyl-GM_3 (D_1), de-*N*-acetyl-lyso-GM_3 (D_2), lyso-GM_3 (D_3), de-*N*-acetyl-GM_3 with *N*-acetylsphingosine (D_4), and GM_3 with *N*-acetylsphingosine (D_5). A crucial step for the preparation of D_1 is the use of mild alkaline conditions of hydrolysis under which the *N*-acetyl group of sialic acid is preferentially hydrolyzed. For the preparation of D_3, conditions which allowed preferential *N*-acetylation of the amino group of the neuraminic acid moiety were devised, *i.e.*, D_2 was incorporated in a dipalmitoylphosphatidylcholine (dpPC) liposome in which the sphingosine moiety was protected and the amino group of neuraminic acid was *N*-acetylated with acetate and a water-soluble catalyst, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (DEC). When an aqueous micellar solution of D_2 was treated with acetate and DEC, *N*-acetylation occurred mainly at the amino group of sphingosine in D_2, yielding D_4. In contrast, when an aqueous solution of D_2 was treated with acetic anhydride and sodium hydrogencarbonate, *N*-acetylation occurred at the amino groups of both neuraminosyl and sphingosyl residues, yielding D_5. The structures of these derivatives were verified by ^{1}H-n.m.r. spectroscopy and mass spectrometry.

INTRODUCTION

During investigations[1–3] into the effect of GM_3 on the activity of EGF-dependent receptor kinase activity, it was found that some derivatives of GM_3 had effects that were qualitatively or quantitatively different from that of GM_3 on the activity of the kinase. Therefore, the synthesis and characterization of various GM_3

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derivatives and analogs are of interest for the study of the functional role of membrane gangliosides.

The lyso form of GM_3 was prepared[4] by treatment of GM_3 with a refluxing solution of M KOH in aqueous 90% 1-butanol for 2.5 h. The product was claimed to have a strong hemolytic activity (twice that of lysolecithin) and was called lysosphingolipid or lysohematoside. The compound, however, had free amino groups at both neuraminic acid* and sphingosine. More recently, various gangliosides (GM_3, GM_2, GM_1, and GD_{1a}) have been derivatized[6] into their lyso forms, in which the amino groups of sialic acid and hexosamines were *N*-acetylated and only the amino group of the sphingosine was unsubstituted. For this derivatization, the amino group of the sphingosine was first blocked by a hydrophobic protective group (9-fluorenylmethoxycarbonyl), followed by acetylation of the amino groups of sialic acid and hexosamines, and subsequent removal of the protective group by liquid ammonia. The procedure involved several steps, and the yield was poor (~30%).

We now describe synthesis procedures which allow preferential hydrolysis of the *N*-acetyl group of sialic acid in GM_3, and preferential re-*N*-acetylation of the free amino group of sialic acid with protection of the sphingosine amino group by hydrophobic interaction in the presence of dpPC liposome. Thus, various derivatives of GM_3 (D_1–D_5) have been prepared, and their structures verified by ^{1}H-n.m.r. and mass spectrometry.

EXPERIMENTAL

GM_3 ganglioside. — GM_3 was extracted from dog erythrocytes and purified by chromatography[7] on DEAE Sephadex followed by h.p.l.c. on a column of Iatrobeads 6RS8010 in a 2-propanol–hexane–water system[8]. 1-(3-Dimethylamino-propyl)-3-ethylcarbodiimide (DEC) was obtained from Aldrich, and synthetic dpPC from Sigma.

*Preparation of de-*N*-acetyl-GM_3 (D_1) and de-*N*-acetyl-lyso-GM_3 (D_2).* — A solution of GM_3 (6 μmol) in M KOH in aqueous 90% 1-butanol (3 mL) was heated for 2 h at 117°. Under these conditions, originally described by Taketomi and Yamakawa[9], >95% of the GM_3 was converted into D_2. On hydrolysis with 0.1M KOH in aqueous 90% 1-butanol for 4 h at 80°, the *N*-acetyl group of GM_3 sialic acid was preferentially hydrolysed to give D_1 as the main product (>70%). The hydrolysate in aqueous 1-butanol was neutralized with 12M HCl, and concentrated to dryness, and a solution of the residue in water (6 mL) was passed through a column of C_{18} silica (Bond Elut, Analytichem International). After washing with water to eliminate salts, the lipids were eluted with methanol. The derivatives were finally purified by h.p.l.c.

*The term "neuraminic acid" is used according to the original definition[5], *i.e.*, de-*N*-acyl sialic acid is defined as "neuraminic acid", while *N*-acetyl, *N*-glycolyl, and *O*-acyl derivatives of neuraminic acid are collectively called "sialic acid".

*Synthesis of lyso-GM_3 (D_3), de-*N*-acetyl-GM_3 with* N*-acetylsphingosine (D_1), and GM_3 with* N*-acetylsphingosine (D_5).* — The neuraminic acid residue of D_2 was preferentially *N*-acetylated by protecting the amino group of the sphingosine by placing the D_2 in a liposome of dpPC. D_2 (1 μmol) was dried together with dpPC (10 μmol). A solution of DEC (20 mg) in water (1 mL) was added, and the lipids were resuspended by sonication with the needle probe of a sonicator (Braun-Sonic 1510) using 30 watts of power usually for 5 min. The suspension was cooled to 4° and the *N*-acetylation reaction was initiated by adding 0.1M acetate buffer (0.2 mL, pH 5.2). After incubation for 24 h at 4°, the reaction was stopped by the addition of ethanolamine (20 μmol) followed by chloroform–methanol (5 mL, 2:1). The lower phase was washed with the same volume of chloroform–methanol–water (3:47:48), and the combined upper phases were dried, resuspended in water, desalted by passage through a C_{18} silica column, and purified by h.p.l.c. to give D_3 (70–80%).

D_4 was synthesized from D_2 when an aqueous micellar solution of D_2 was subjected to catalytic *N*-acetylation, *i.e.*, a solution of D_2 (1 μmol) in 20mM acetate buffer (pH 5.2, 1 mL), followed by the addition of DEC (20 mg). The mixture was stored for 24 h at room temperature, and the reaction was then stopped by the addition of ethanolamine (20 μmol). The mixture was desalted using a C_{18} silica column, and the products were purified by h.p.l.c. to give D_4 as the main product, with D_5 as a by-product. In order to convert D_2 completely into D_5, D_2 (1 μmol) was resuspended in 0.5M $NaHCO_3$ (1 mL) and acetic anhydride (0.05 mL) was added. After storage for 1 h at room temperature, the mixture was desalted as described above. Since the acetylation was quantitative, no further purification was needed.

Characterization of GM_3 derivatives. — The derivatives D_1–D_5 were characterized by n.m.r. spectroscopy and negative ion f.a.b.-mass spectrometry. Solutions of each compound (400 μg) in $(CD_3)_2SO$–D_2O (98:2, 0.3 mL) were stored for 5 min in order to allow deuterium exchange of hydroxyl and amino protons. Each solution was then lyophilized and a solution of the residue in $(CD_3)_2SO$–D_2O (98:2, 0.5 mL) was used immediately for n.m.r. spectroscopy. ^{1}H-N.m.r. spectra were obtained at 35° with a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer using quadrature detection, a spectral width of 5000 Hz over 16k data points, and a relaxation delay of 2 s. The number of transients collected varied from 200 to 500. Chemical shifts are referenced to the terminal methyl resonance(s), the shift of the resonance of which was assumed to be 0.85 p.p.m.

Negative ion f.a.b.-mass spectrometry was performed using a JEOL HX-110 mass spectrometer/DA-5000 data system. Solutions of samples in methanol were transferred to a triethanolamine or glycerol matrix on the f.a.b. target and bombarded with a xenon beam. The acceleration voltage was 10 kV and the resolution was 3000. Data were acquired in the accumulation mode from 100–1500 a.m.u. with a scan slope of 1 min/decade. Each spectrum represents the accumulation of three scans. Sodium iodide in glycerol was used as the mass calibration standard.

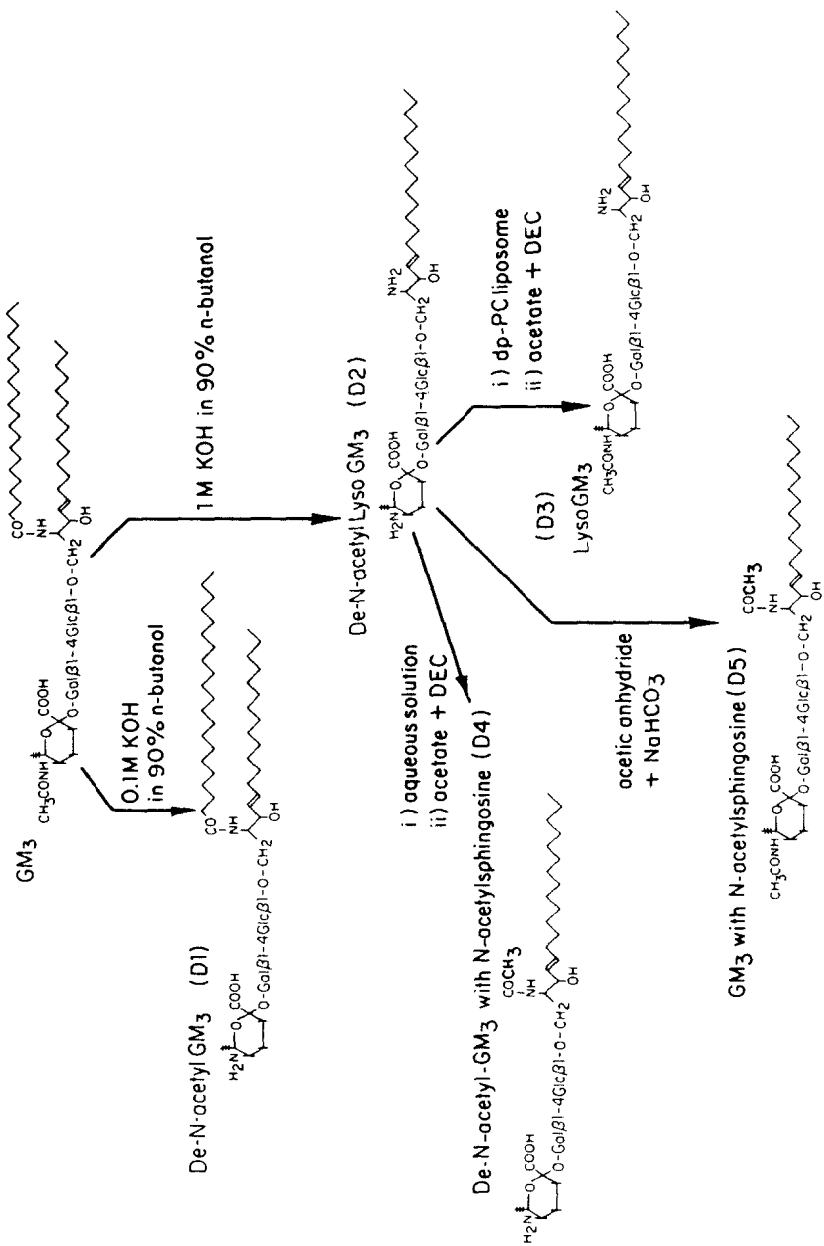


Fig. 1. Reaction scheme for synthesis of the derivatives of GM_3.

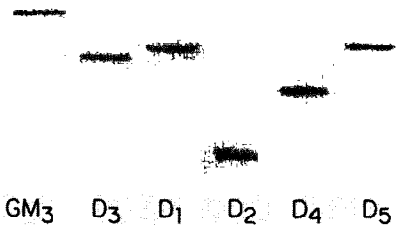


Fig. 2. Behavior of the derivatives (2 nmol of each) of GM_3 in h.p.t.l.c., using chloroform–methanol–aqueous 0.2% $CaCl_2$ (5:4:1) and detection with resorcinol.

RESULTS

*Preparation of de-*N*-acetyl-GM_3 (D_1) and de-*N*-acetyl-lyso-GM_3 (D_2) from GM_3.* — Partial hydrolysis of GM_3 in 0.1M KOH in aqueous 90% 1-butanol at 80° gave, as the major product, D_1, which has a free amino group at the sialic acid moiety of GM_3. Treatment of GM_3 with M KOH in aqueous 90% 1-butanol at 117° gave D_2, which was characterized by the absence of the *N*-acetyl group at the sialic acid moiety and the *N*-acyl group at the ceramide moiety of GM_3 (see Fig. 1). The behavior in h.p.t.l.c. and in h.p.l.c. is shown in Figs. 2 and 3, respectively.

Synthesis of lyso-GM_3 (D_3). — D_3 was synthesized by preferential *N*-acetylation of the neuraminic acid moiety of D_2, which was performed by incorporation of

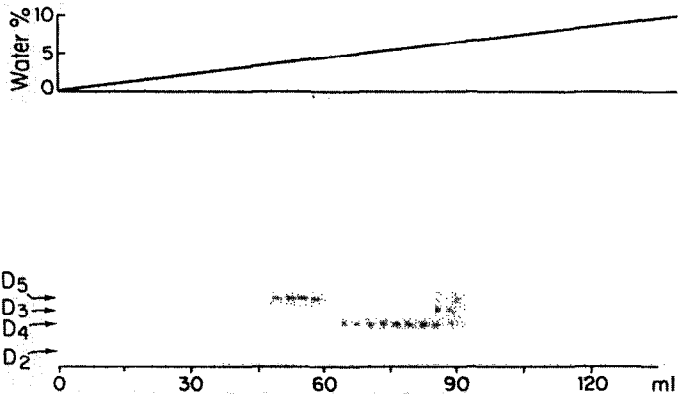


Fig. 3. Products of carbodiimide-catalyzed *N*-acetylation of D_2 in micellar dispersion. The mixture was desalted by hydrophobic chromatography and eluted from a column (4 × 300 mm) of Iatrobeads with a gradient of 2-propanol–hexane–water, 55:45:0 → 55:35:10. Aliquots were analyzed by h.p.t.l.c. as in Fig. 2.

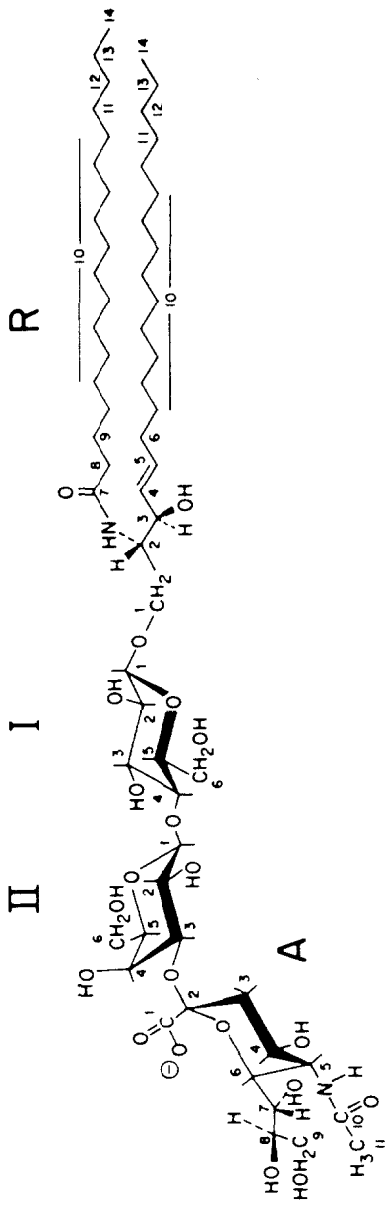


Fig. 4. Proton labeling scheme for GM_3.

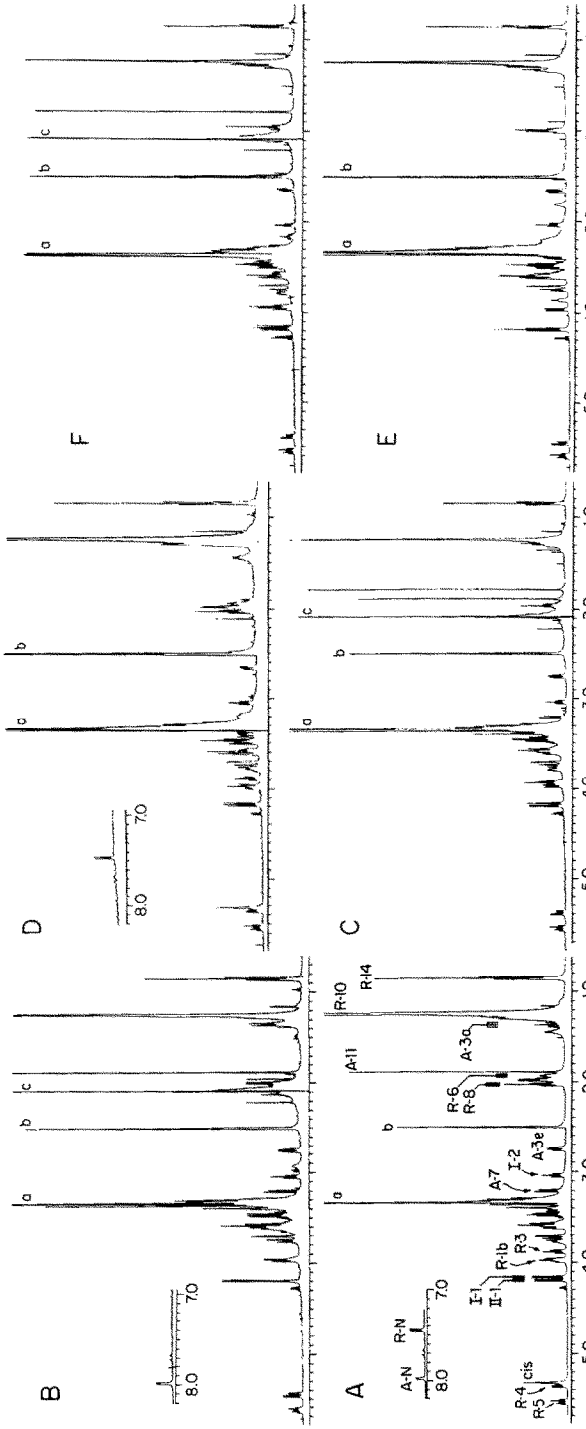


Fig. 5. Resolution-enhanced ^{1}H-n.m.r. spectra: A, GM_3; B, lyso-GM_3 (D_3); C, GM_3 with *N*-acetylsphingosine (D_5); D, de-*N*-acetyl-GM_3 (D_1); E, de-*N*-acetyl-lyso-GM_3 (D_2); and F, de-*N*-acetyl-GM_3 with *N*-acetylsphingosine (D_4). The resonance assignments given in A follow those of Koerner *et al.*[10]. The peaks a–c are due to HOD, Me_2SO, and acetone, respectively.

D_2 in dpPC liposomes followed by treatment with acetate and DEC, thus avoiding the use of acetic anhydride (see Discussion). The product was characterized by the mobilities in h.p.t.l.c. and h.p.l.c. (Figs. 2 and 3, respectively).

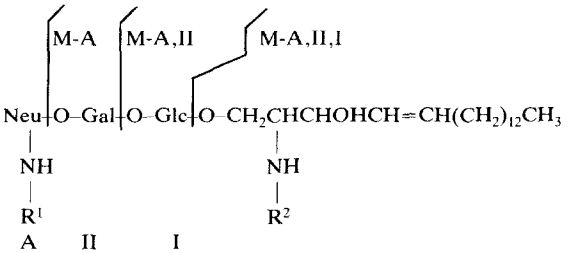
*Preparation of de-*N*-acetyl-GM_3 containing* N*-acetylsphingosine (D_4) and GM_3 containing* N*-acetylsphingosine (D_5).* — D_4 was prepared from D_2 by treating an aqueous micellar solution with acetate and DEC. Preferential *N*-acetylation occurred at the sphingosine amino group rather than the neuraminosyl amino group (see Fig. 1). D_5 was obtained by *N*-acetylation of D_2 by acetic anhydride in sodium hydrogencarbonate (see Fig. 1). The mobilities of these compounds in h.p.t.l.c. and h.p.l.c. are shown in Figs. 2 and 3.

N.m.r. spectroscopy. — The structures of D_1–D_5 were verified by ^{1}H-n.m.r. spectroscopy. The structure and proton labeling scheme for GM_3 and its derivatives are shown in Fig. 4. Fig. 5 shows the ^{1}H-n.m.r. spectra of GM_3 and D_1–D_5, along with selected resonance assignments.

F.a.b.-mass spectrometry. — Using the negative ion mode, the mass spectra of the parent GM_3 and D_1–D_5 were obtained in both triethanolamine and glycerol matrices. The major fragments observed are summarized in Table I and the spectra are shown in Figs. 6–11.

TABLE I

MASS (*m/z*) OF RELEVANT FRAGMENTS PRODUCED BY NEGATIVE ION F.A.B.-MASS SPECTROMETRY OF GM_3 AND SYNTHETIC DERIVATIVES



	R^1	R^2	$M-H$	$M-A$	$M-A,II$	$M-A,II,I$
GM_3	$COCH_3$	$COC_{23}H_{45}$	1261	970	808	646
D_1	H	$COC_{23}H_{45}$	1219	970	808	646
D_2	H	H	871	622	460	298[a]
D_3	$COCH_3$	H	913	622	460	298[a]
D_4	H	$COCH_3$	913	664	502	340
D_5	$COCH_3$	$COCH_3$	955	664	502	340

[a]Obscured by the triethanolamine matrix cluster ion at *m/z* 297.

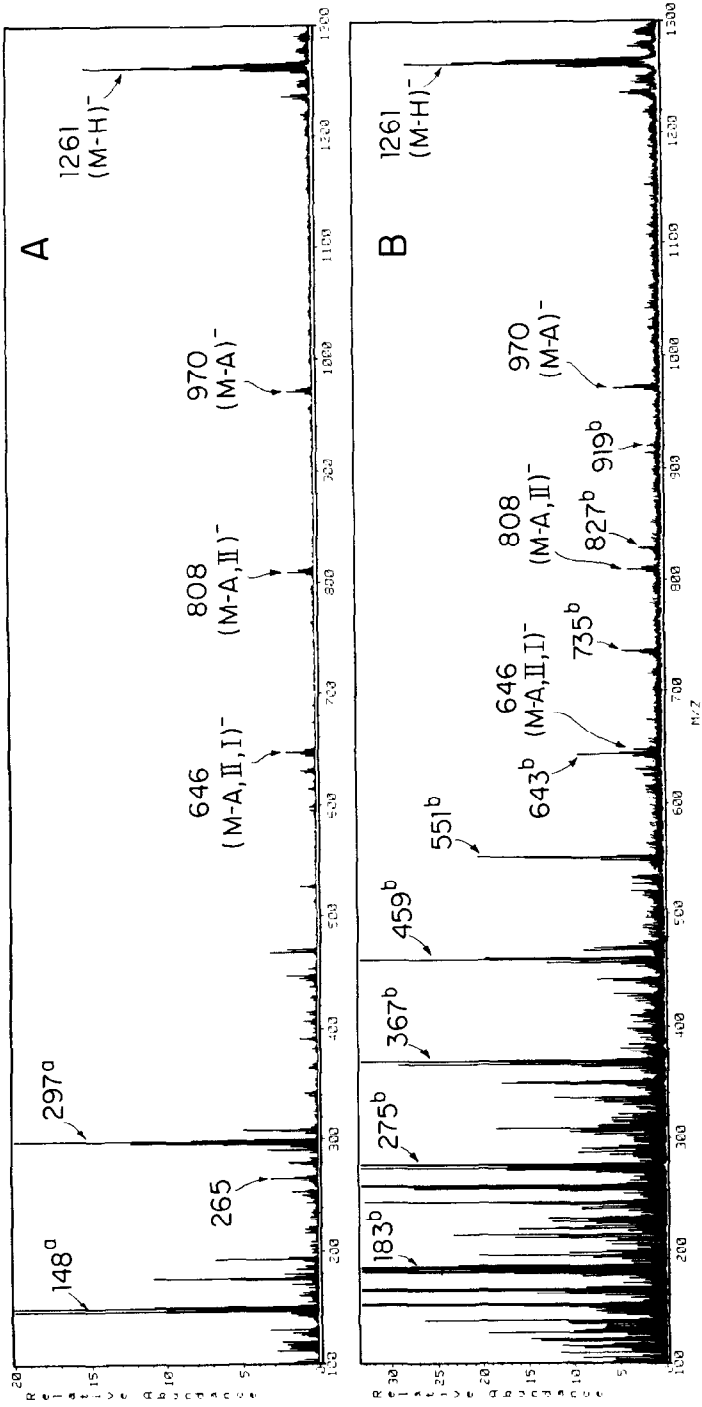


Fig. 6. Negative ion f.a.b.-mass spectra of dog erythrocyte GM_3 in A, triethanolamine (TEA) matrix; B, glycerol matrix. Ions are designated as in Table I: a, triethanolamine matrix cluster ions of formula $(n[TEA] - 1)^-$; b, glycerol matrix cluster ions of formula $(nG - 1)^-$.

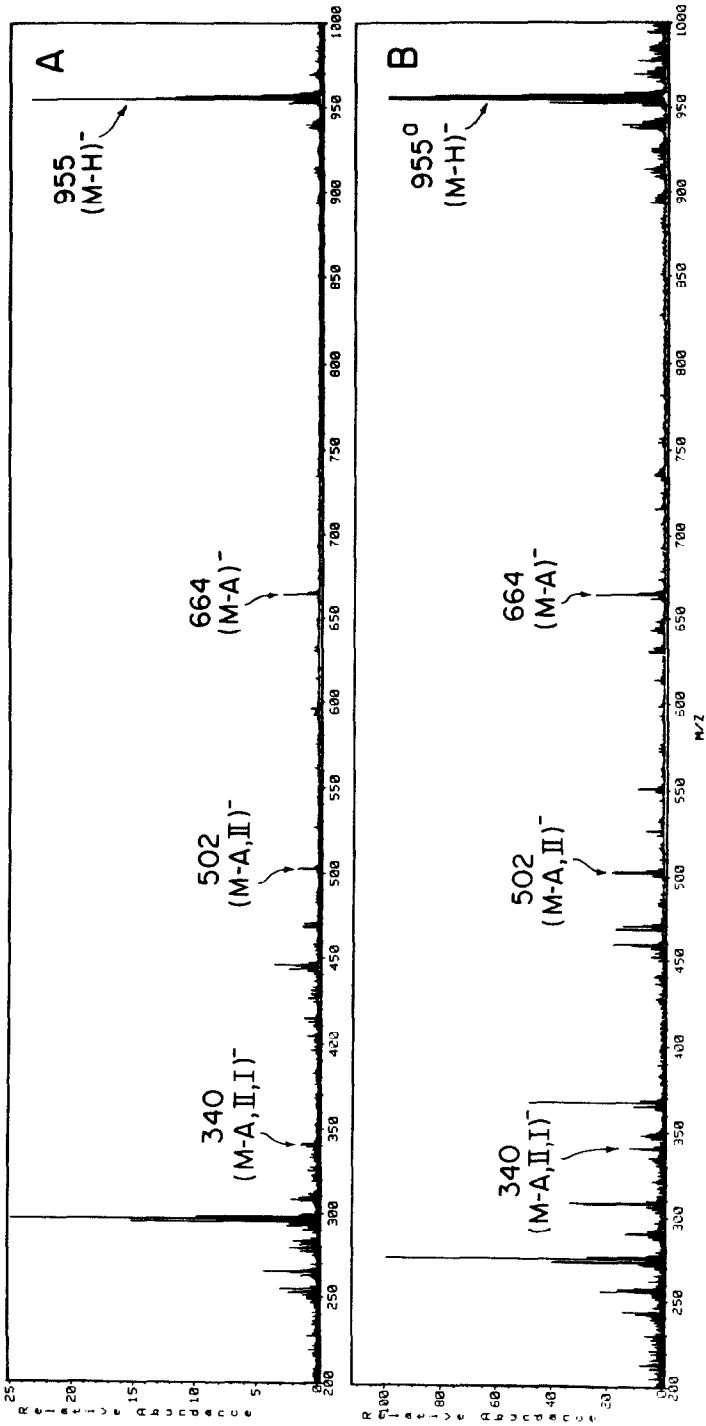


Fig. 7. Negative ion f.a.b.-mass spectra of D_5 in A, triethanolamine matrix; B, glycerol matrix (a is the pseudomolecular ion at m/z 955 saturated electron multiplier).

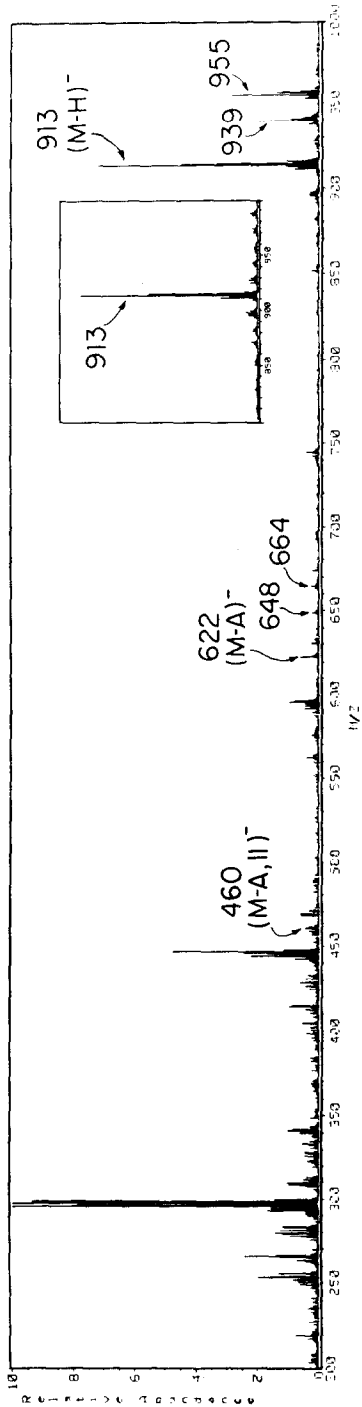


Fig. 8. Negative ion f.a.b.-mass spectrum of D_3 in a triethanolamine matrix. Inset: molecular ion region of spectrum in a glycerol matrix.

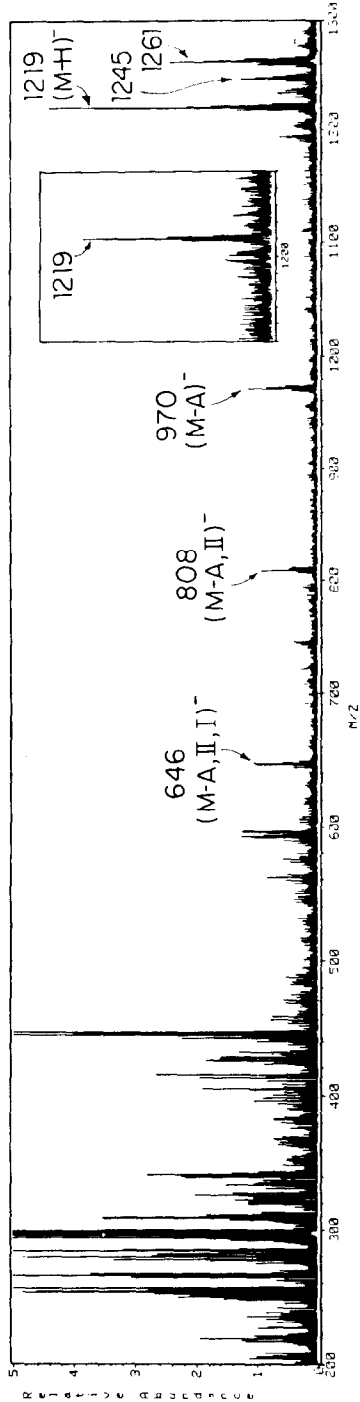


Fig. 9. Negative ion f.a.b.-mass spectrum of D_1 in a triethanolamine matrix. Inset: molecular ion region of spectrum in a glycerol matrix.

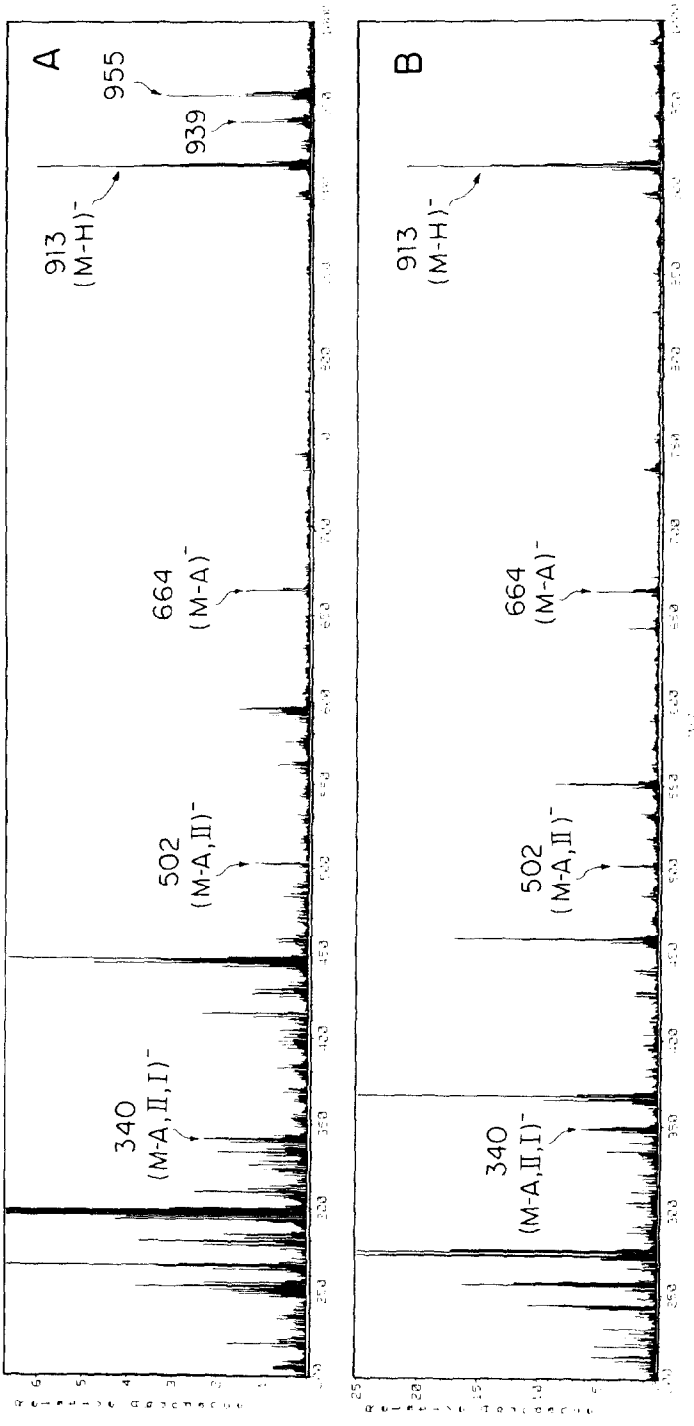


Fig. 10. Negative ion f.a.b.-mass spectra of D_4 in A, a triethanolamine matrix; B, a glycerol matrix.

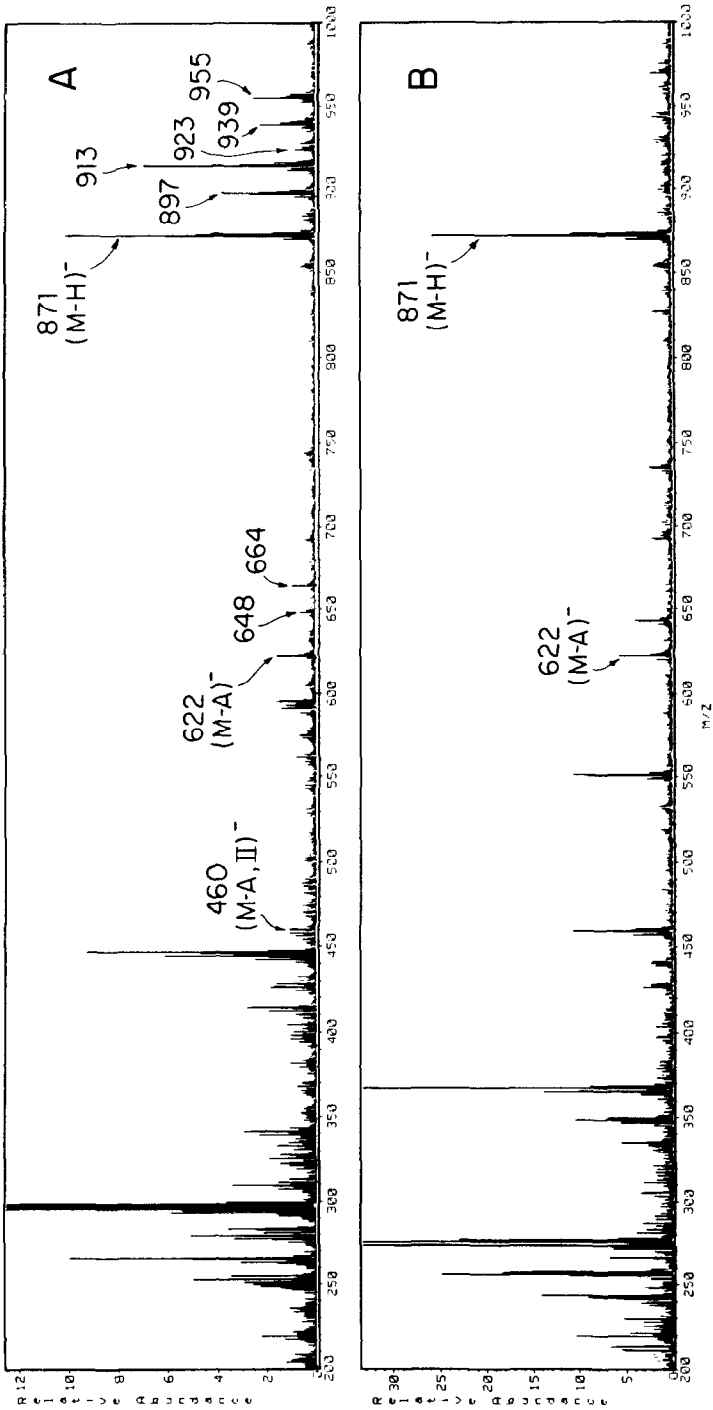


Fig. 11. Negative ion f.a.b.-mass spectra of D_2 in A, a triethanolamine matrix; B, a glycerol matrix.

DISCUSSION

The functional role of membrane gangliosides has been thought to be modulation of membrane proteins such as receptors and transporters, although the gangliosides have been implicated as receptors for cell–cell and cell–microbial recognition. GM_3 and GM_1 have been well documented[1–3] to modulate the epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors, respectively. Addition of GM_3 to cell cultures modifies cell growth and inhibits phosphorylation of the tyrosine in the EGF receptor[1,2]. A derivative of GM_3, present in trace quantities in A431 cells, can modulate the kinase activity of the EGF receptor in a manner significantly different from that of GM_3, both qualitatively and quantitatively[3]. Although this bioactive derivative of GM_3 has not been characterized, it was suspected to be de-*N*-acetyl-GM_3 or lyso-GM_3. For this reason, reference compounds were urgently needed. Previously, "lyso-hematoside" was obtained[4,8] by treatment of GM_3 with M KOH in 1-butanol, but the compound contained de-*N*-acetyl-sialic acid (neuraminic acid) and de-*N*-acyl-sphingosine. An unambiguous synthesis of lyso-derivatives of GM_3, GM_2, GM_1, and GD_{1a} has been accomplished in several steps[6], but the yield was poor.

We now present a method for the synthesis of five GM_3 derivatives, as shown in Fig. 1, based on (*a*) preferential hydrolysis of the *N*-acetyl group of sialic acid, (*b*) de-*N*-acylation of both *N*-acetylsialic acid and *N*-acylsphingosine, followed by selective *N*-acetylation of sialic acid by protection of the amino group of sphingosine in dpPC liposomes, and (*c*) preferential *N*-acetylation of the sphingosine amino group by catalytic *N*-acetylation in an aqueous micellar solution. The structure of each derivative was verified by ^{1}H-n.m.r. and by f.a.b.-mass spectrometry.

When D_2 was treated with acetic anhydride and sodium hydrogencarbonate, the amino groups of both neuraminic acid and sphingosine were *N*-acetylated. *N*-Acetylation with acetic anhydride may occur preferentially at the more hydrophobic amino group in sphingosine, followed by *N*-acetylation at the relatively hydrophilic neuraminic amino group, because of the hydrophobicity of acetic anhydride. In contrast, carbodiimide-catalyzed *N*-acetylation favored the neuraminic acid amino group when the sphingosine amino group was protected in a liposome. Carbodiimide-catalyzed *N*-acetylation applied to an aqueous micellar solution of D_2 resulted in preferential reaction of the sphingosine amino group. These higher-yielding procedures do not require a covalently-linked protecting group and are simpler than the methods described previously[6].

The procedures described for GM_3 can be applied to any ganglioside to give derivatives which may be useful for the study of various cell physiological and pharmacological effects.

N.m.r. spectra of GM_3 and derivatives. — The ^{1}H-n.m.r. spectra of GM_3 and D_1–D_5 are shown in Fig. 5. The spectrum of GM_3 (Fig. 5A) is identical to that reported[10] and the assignments follow those of these authors, except for those of

the amide protons, which were not assigned[10]. The amide assignments were made by comparison with the spectra of D_3 and D_1, and agree with the assignments[11] for GM_3 in $(CD_3)_2SO$ at 35°. Of special note for the identification of GM_3 derivatives are the resonances for the *cis* olefinic protons of the unsaturated fatty acid (δ 5.3), the anomeric protons of glucose (I-1, δ 4.17) and galactose (II-1, δ 4.19), NAc of the sialic acid (A-11, δ 1.88), and the amide protons R-N and A-N (δ 7.45 and 7.98, respectively).

Comparison of the spectra of lyso-GM_3 (D_3) and GM_3 shows that the former lacks the resonances (δ 5.3) due to the *cis* olefinic protons of the unsaturated fatty acid and the amide proton (δ 7.45). Loss of the fatty acid from sphingosine was verified by integration of the methylene (R-10) and terminal methyl (R-14) resonances (δ 1.25 and 0.85, respectively). Another notable difference between the spectra of GM_3 and lyso-GM_3 is the downfield shift (0.032 p.p.m.) for the resonance of the glucose anomeric proton (I-1) in the spectrum of D_3. This significant substituent effect is most likely caused by loss of the anisotropic shielding of the fatty acid carbonyl. For D_5, which bears an acetyl group on the sphingosine N, anisotropic shielding again occurs, and the anomeric region of the spectrum is identical to that of GM_3 (Fig. 5C). The change in the chemical shift of the resonance of the glucose anomeric proton upon loss of the fatty acid implies a relatively close approach of the fatty acid carbonyl to the glucose anomeric proton in GM_3, and may have implications for the secondary structure of GM_3 (Neuenhofer *et al.*[7] attribute this downfield shift to steric perturbation caused by the free sphingosine amino group). Other differences between the spectra of Figs. 5A and 5B are evident and are compatible with the proposed structure of D_3; the sphingosine olefinic proton resonances R-4 and R-5 (δ 5.3 and 5.5, respectively) are shifted downfield in Fig. 5B compared to their positions in Fig. 5A. In addition, the multiplicities and chemical shifts of the resonances of the sphingosine protons R-1b and R-3 (δ 3.96 and 3.88, respectively) change upon loss of the fatty acid bound to sphingosine.

Fig. 5D shows the spectrum of de-*N*-acetyl-GM_3 (D_1) which, as expected, differs from that of GM_3 by the absence of the resonances for the sialic acid acetamido methyl group (A-11, δ 1.89) and the sialic acid amide proton (A-N, δ 7.98). In addition, the resonance for sialic acid H-7, which appears at δ 3.1 in the spectrum of GM_3, moves downfield in the spectrum of de-*N*-acetyl-GM_3, whereas that of H-3*e* moves upfield (−0.1 p.p.m.). These shifts may be explained by loss of the anisotropic effect of the carbonyl of the acetamido group formerly bound to neuraminic acid.

The spectra of de-*N*-acetyl-lyso-GM_3 (D_2) and D_1 with *N*-acetylsphingosine (D_4) appear as Figs. 5E and 5F, respectively. As expected, the spectrum of D_2 contains no resonance for NAc. The spectrum of D_4 contains an NAc resonance for *N*-acetylsphingosine at δ 1.795, the same chemical shift found for the methyl group resonance of *N*-acetylsphingosine in D_5 (Fig. 5C).

F.a.b.-mass spectrometry of GM_3 and derivatives. — GM_3 and D_5. In

agreement with the results of Arita *et al.*[12], the negative ion f.a.b.-mass spectrum of GM_3 in a triethanolamine matrix (Fig. 6A) was dominated by the pseudomolecular ions, some fragmentation occurring through loss of sugar residues from the non-reducing end, with charge retention on the ceramide-containing portion of the molecule. In contrast to GM_3 from bovine brain, however, the presence in canine erythrocyte GM_3 of ceramide composed almost exclusively of d18:1 sphingosine and 24:1 (+24:0) fatty acid was indicated by the abundant ion at *m/z* 1261 (1263). The presence in this spectrum of an ion at *m/z* 265 cannot be attributed to the fragment resulting from cleavage between C-2 and C-3 of sphingosine, as suggested[12], since the d20:1 sphingosine required to produce a fragment at this mass is not present in significant quantities in the sample, as shown in the subsequent spectra of derivatives synthesized therefrom. The analogous fragment expected from d18:1 sphingosine, at *m/z* 237, was not observed in the spectrum.

In the spectrum obtained using a glycerol matrix (Fig. 6B), the same ions were observed (*m/z* 1261, 970, 808, 646), along with a number of glycerol cluster ions $(nG - 1)^-$.

The spectra of D_5 in triethanolamine and glycerol matrices (Figs. 7A and 7B) each exhibits ions which reflect the replacement of the long-chain *N*-acyl group of ceramide with an *N*-acetyl moiety. The corresponding ions, containing the ceramide end of the molecule, were then found at 306 a.m.u. less than for the parent GM_3 (*m/z* 955, 664, 502, 340).

D_3, D_1, and D_4. The spectra of these mono-de-*N*-acyl compounds in a triethanolamine matrix were characterized by the unexpected presence, in addition to the abundant pseudomolecular $(M - H)^-$ ions, of ions 26 and 42 a.m.u. higher in mass. Since the $(M - H + 42)^-$ ions coincide with the mass of $(M - H)^-$ expected for other synthetic derivatives which are *N*-acetylated, this finding was first interpreted as an indication of mutual contamination. However, the preparation and purification schemes seemed to exclude this possibility and, in addition, the ^{1}H-n.m.r. spectra did not corroborate the presence of mixtures. Moreover, the production of $(M - H + 26)^-$ ions could not be explained simply as due to impurities, nor could the similarities in the relative abundances of these molecular weight region clusters. Finally, the occurrence of a second set of higher mass ions $[(M - H + 42 + 16)^-$ and $(M - H + 42 + 42)^-]$ in the spectrum of D_2 (discussed further below), which is deacylated at both nitrogens, seemed to indicate that the presence of these ions is related to the number of free amino functions in the molecule, and that they are the result of some reaction taking place during the bombardment process in a triethanolamine matrix. In order to assess this possibility, spectra were acquired in another matrix, glycerol. The results appear to confirm this hypothesis, as well as the identity and purity of the synthetic ganglioside derivatives.

Thus, whereas the spectrum of D_3 in a triethanolamine matrix (Fig. 8) contained abundant ions at *m/z* 939 and 955 in addition to that expected for $(M - H)^-$ at *m/z* 913, these extra ions were absent in the spectrum obtained in a glycerol

matrix (Fig. 8, inset). The spectrum of D_3 in a triethanolamine matrix also contained ions corresponding to loss of Neu5Ac (*m/z* 622) and of Neu5Ac·Hex (*m/z* 460; *cf.* the spectrum obtained by Neuenhofer *et al.*[6]). The ion produced by loss of Neu5Ac and both hexoses, corresponding to (sphingosine-H)$^-$ and expected at *m/z* 298, was obscured by the large matrix cluster ion at *m/z* 297. Inspection of the spectrum of D_3 in a triethanolamine matrix revealed ions at *m/z* 648 and 664, corresponding to the loss of NeuAc from the extra ions at *m/z* 939 and 955, respectively. This result might be expected from a chemical reaction involving a free amino group on sphingosine. The f.a.b.-mass spectrum for a glycerol matrix showed little useful fragmentation (*cf.* the positive ion f.a.b.-mass spectra of gluco- and galactopsychosines obtained by Hara and Taketomi[13]).

The de-*N*-acetylation of GM_3 Neu5Ac to produce D_1 should reduce the mass of the pseudomolecular ions by 42 a.m.u. In the mass spectrum of D_1 in a triethanolamine matrix (Fig. 9), the expected predominant pseudomolecular ion was found at *m/z* 1219 (1221) for 24:1 (+24:0) fatty acid containing ceramides, accompanied, as for D_3, by associated $(M - H + 26)^-$ and $(M - H + 42)^-$ ions at *m/z* 1245 (1247) and 1261 (1263). As with D_3, the mass spectrum of D_1 in a glycerol matrix (Fig. 9, inset) exhibited only the pseudomolecular ions (*m/z* 1219, 1221), again with little useful fragmentation. The abundant fragment ions seen with the triethanolamine matrix (*m/z* 970, 972, 808, 810, 646, 648) were unaccompanied by additional higher mass clusters, since the terminal neuraminic acid possessing the free amino function was eliminated in the production of these fragments. The fact that the difference between $(M - H)^-$ and $(M - A)^-$ is 249 a.m.u., instead of 291 a.m.u. expected for loss of Neu5Ac, confined the missing 42 a.m.u. to that residue, confirming its de-*N*-acetylation.

The pattern for D_4 was similar, except that the replacement of the 24:1/24:0 *N*-acyl chain with an *N*-acetyl group again produced fragments 306 a.m.u. less than the corresponding ions for D_1 (see Fig. 10A). With the glycerol matrix, there was useful fragmentation accompanying the pseudomolecular ion (*m/z* 913) (Fig. 10B).

D_2. In the spectrum of the di-de-*N*-acylated ganglioside taken in a triethanolamine matrix (Fig. 11A), the expected pseudomolecular ion at *m/z* 871 was accompanied by a series of abundant ions representing $(M - H + 26)^-$, $(M - H + 42)^-$, $(M - H + 42 + 26)^-$, and $(M - H + 42 + 42)^-$. A less abundant ion, representing $(M - H + 26 + 26)^-$, could also be detected. With a glycerol matrix, only the pseudomolecular ion could be seen (Fig. 11B). The ion representing elimination of $NeuNH_2$ was present in the spectra for both the triethanolamine and glycerol matrices at *m/z* 622, and in the former only was this accompanied by the higher mass ions *m/z* 648 and 664, as found for D_3. The fragment representing loss of $NeuNH_2$·Hex (*m/z* 460) was detectable for the triethanolamine matrix, but its presence in the glycerol matrix was obscured by a matrix cluster ion at *m/z* 459.

At present, the identity and mechanisms for formation of the amine-associated adduct ions encountered with the triethanolamine matrix are unknown and, apparently, there has been no report of this phenomenon. The possibility that

the appearance of these ions was due to reactions of the sample with impurities in the matrix, perhaps catalysed by triethanolamine, cannot be excluded. On the other hand, the products of f.a.b. of glycerol have been studied[14,15] and contrasted with those arising from γ-radiolysis[15]. These studies demonstrated extensive formation of reactive species during the f.a.b. process, the products of which were identifiable by g.l.c.–m.s. The formation of unique reactive species from the triethanolamine matrix during f.a.b. seems to be a likely possibility. Although such reactions introduce complications into the interpretation of the spectra, they may be of use for detecting and locating amine functions. Studies of these reactions are in progress.

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