Preparation of lyso-GM1 (II^{3}Neu5AcGgOse$_4$-long chain bases) by a one-pot reaction

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Abstract A simple procedure is described for preparing lyso-GM1, a GM1 derivative that lacks the fatty acid moiety, starting from GM1 ganglioside using a one-pot reaction. Ganglioside deacylation was carried out in KOH/propan-1-ol in the absence of oxygen. The yield of lyso-GM1 under optimal conditions (6 h, 90°C, 0.2 N KOH, 1 mM GM1) was 54%. The chemical structure of lyso-GM1 was determined by ^{1}H-NMR and FAB-MS analyses, thus proving that the acetamide groups of galactosamine and sialic acid units were not affected during the deacylation reaction.—**Sonnino, S., D. Acquotti, G. Kirschner, A. Uguaglianza, L. Zecca, F. Rubino, and G. Tettamanti.** Preparation of lyso-GM1 (II^{3}Neu5AcGgOse$_4$-long chain bases) by a one-pot reaction. *J. Lipid Res.* 1992. **33:** 1221–1226.

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Gangliosides, sialic acid-containing glycosphingolipids, are components of the cell membranes of vertebrates. They are particularly abundant in the nervous system where they could play an important functional role due to their involvement in a number of interaction processes with cell external ligands and cell membrane components (1).

Ganglioside derivatives carrying the appropriate probe in the lipid moiety (2–5) and ganglioside molecular species containing fatty acids with different chemical features (6) have been synthesized and used (2, 4, 7, 8) to elucidate some physico-chemical properties of gangliosides and the modalities of the interactions occurring among the gangliosides and a variety of ligands. The synthetic work was performed starting from deacylated gangliosides and from an appropriate activated acyl chain (2–4, 6). Ganglioside deacylation was performed under different experimental conditions, all using alkaline media (6, 9–12). The main drawback of these procedures was the production of ganglioside derivatives lacking both the fatty acid chain and one or more acetyl groups in the oligosaccharide chain.

In the present work we present a simple and convenient procedure developed on GM1 ganglioside for the preparation of deAcyl-GM1, a derivative lacking only the fatty acid chain. This compound is designated as "lyso-GM1 ganglioside."

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. Propan-1-ol was dehydrated before use by refluxing over metallic magnesium. Potassium hydroxide was maintained under freeze-drying conditions for 1 day before use. 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 (Darmstad, Germany). *N*-acetylneuraminic acid was from Sigma Chemical Co. (St. Louis, MO). Ganglioside GM1 was extracted from calf brain (13), purified, and structurally characterized (14). Ganglioside GM1 was maintained under high vacuum and in the presence of dehydrating agents before use. DeAc-GM1 and deAc-deAcyl-GM1, used as reference standards, were prepared from GM1 (6).

Abbreviations: This report follows the ganglioside nomenclature of Svennerholm (22) and the IUPAC-IUB recommendations (23). GM1, II^{3}Neu5AcGgOse$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; deAc-GM1, II^{3}NeuGgOse$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; deAc-deAcyl-GM1, II^{3}NeuGgOse$_4$LCB, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-LCB; Lyso-GM1, deAcyl-GM1, II^{3}Neu5AcGgOse$_4$LCB, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-LCB: Neu5Ac, *N*-acetylneuraminic acid; Neu, neuraminic acid; Cer, ceramide; LCB, long chain bases.

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De-acylation of GM1 ganglioside

The optimal conditions for the alkaline reaction on GM1 in propan-1-ol were determined in preliminary experiments, varying the time (up to 24 h) and temperature (from 50 to 100°C) of the reaction at different concentrations of both potassium hydroxide and the ganglioside, in the presence or absence of oxygen. Optimal conditions were found to be as follows.

Propan-1-ol was deoxygenated by 30-min bubbling with a stream of argon (20–30 l/min) and maintained in a closed bottle under moderate argon pressure. A solution of ganglioside (1.4 mM) in deoxygenated propan-1-ol, warmed at 90°C, was mixed in a macro-vial provided with an open-top screw-cap and a natural rubber septum, with a 90°C prewarmed deoxygenated propan-1-ol potassium hydroxide solution (1 N) to obtain a final KOH concentration of 0.2 N. Two stainless-steel needles were introduced in the vial septum, one connected to the argon bottle, and the reaction mixture was maintained for 30 min under an argon flux of 5 ml/min (under these conditions and with one-fifth of the vial immersed in the heating bath, we observed only a slight loss of the solvent, in the range of 5–10% of the total volume). Then the needles were removed and the reaction was continued at 90°C and under continuous stirring for 6 h. The reaction mixture was then dried, and the residue was dissolved in water (5 mg starting ganglioside/0.1 ml), dialyzed for 3 h, and freeze-dried. Purification of the lyso-ganglioside was carried out by silica gel column chromatography (60 × 1.5 cm/50 mg of starting ganglioside) using chloroform–methanol–water 60:35:5 (by vol) as the eluting solvent; the elution profile was monitored by TLC (see below).

Analytical procedures

^{1}H-NMR spectra of lyso-GM1 were obtained at 500 MHz on a Bruker AM500 spectrometer equipped with the ASPECT 3000 computer, a process controller, and an array processor. Samples (2–3 mg) were carefully dried under vacuum and then dissolved in DMSO-d_6 (0.5 ml) or DMSO-d_6/D_2O 20:1 (v/v). Chemical shift assignments were obtained by correlated spectroscopy (COSY) and were indirectly referred to $(CH_3)_4Si$ by setting the residual $(CHD_2)_2SO$ signal at 2.49 ppm.

Mass spectrometric measurements of lyso-GM1 were performed on a Finningan MAT90 instrument, fitted with its own FAB source and IonTech atom gun (Xe, 5×10^{-5} mbar, 8 KeV, 20 A). Calibration in the positive-ion mode was accomplished with cesium fluoride, and resolution in the normal scan mode (100 to 2000 u, 20 sec/decade) was kept at better than 2500, as judged by the instrument's own software on the calibrant peak at *m/z* 1500. Precise mass measurement of the MH^+ peaks of the analyzed substances was accomplished by linear magnetic scan of the mass range between the calibrant peaks at *m/z* 1196 and 1348 at a resolution about 6000. Several 20-sec scans of both the analyzed sample and of the calibration standard were software-summed (10 mmu merge window) and the masses of the unknowns were assigned after linear realignment of the calibrant peaks. Metastable daughter-ion spectra were recorded with a computer-controlled linked scan at B/E = const. Lyso-GM1 (100 μg per run) was dissolved in chloroform–methanol 1:1 (by vol) (50 μl), and an aliquot of the solution (5–10 μl) was added to a 10% solution of thioglycerol (3-mercapto-1,2-propanediol) in methanol (50 μl). After evaporation of the volatile solvents, the sample was transferred to the copper FAB target.

Thin-layer chromatography of gangliosides and ganglioside derivatives was performed on HPTLC plates at room temperature using the solvent system chloroform–methanol–30 mM aqueous $CaCl_2$–100 mM aqueous KCl 50:50:4:8 (by vol). Gangliosides and ganglioside derivatives were made visible by treatment with a *p*-dimethylaminobenzaldehyde spray reagent, followed by heating at 120°C for 10 min (15). Quantification of ganglioside and ganglioside-derivative spots was performed by total densitometry of the TLC plate (16).

Ganglioside-bound sialic acid was assayed by the resorcinol–HCl method (17, 18), pure Neu5Ac being used as the reference standard.

RESULTS AND DISCUSSION

The optimal conditions for the deacylation reaction of GM1 were established by TLC of the reaction products followed by densitometric quantification of the separated compounds. Four main compounds were present in the reaction mixtures. They were identified as GM1, lyso-GM1 (deAcyl-GM1), deAc-GM1, and deAc-deAcyl-GM1 by means of thin-layer chromatographic comparison with reference standards, and spectroscopic and spectrometric analyses (see below). The reaction conditions of absence of oxygen, 6 h of reaction at 90°C, 0.2 N KOH and 1 mM ganglioside led to 60% of lyso-GM1 among the four above-mentioned final products, as determined by TLC. At the end of the reaction the recovery of Neu5Ac was 90%. Thus, the yield of lyso-GM1 was 54%. These results were confirmed for starting amounts of GM1 varying from 5 to 50 mg.

The time course of the deacylation reaction performed under optimal conditions is presented in **Fig. 1**. The amount of lyso-GM1 increased progressively with time. In the first 2 h a small amount of deAc-GM1, amounting to 5% of the total sialic acid content, was formed. Then it disappeared, probably being transformed into deAc-deAcyl-GM1. Byproducts of unknown structure, showing high mobility under the chromatographic conditions that were used, were present in low amount only during the first 2 h of reaction. After 6 h of reaction the distribution

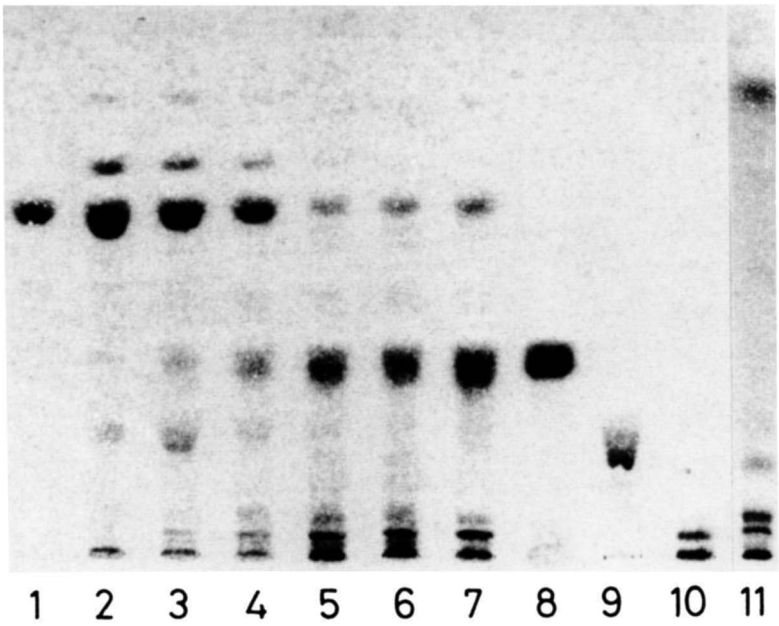

Fig. 1. Time course of the deacylation reaction of GM1 as determined by TLC performed with the solvent system chloroform–methanol–27 mM aqueous $CaCl_2$–100 mM aqueous KCl 50:50:4:8. 1, Standard GM1; 2–7, reaction conditions: 90°C, 0.2 N KOH, 1 mM ganglioside, absence of oxygen, 0, 1, 2, 3, 6, 21, and 24 h reaction time; 8, column chromatography-purified lyso-GM1; 9, standard deAc-GM1; 10, standard deAc-deAcyl-GM1; 11, reaction conditions: 90°C, 0.2 N KOH, 1 mM ganglioside, presence of oxygen, 24 h reaction time.

within the reaction products was constant. The presence of oxygen in the reaction mixture resulted in a low recovery of lipid-bound Neu5Ac and the main components were very polar derivatives (Fig. 1). The effect of oxygen on GM1 ganglioside is not understood and cannot be explained, as the reaction products were of unknown structure.

The chromatographic behavior of lyso-GM1 was unexpected. Lyso-GM1 should be more hydrophilic than deAc-GM1, but under our thin-layer chromatographic conditions (Fig. 1) its mobility was higher than that of the deacetylated ganglioside. This suggests that different modalities of interaction between the double-chain and the single-chain ganglioside derivatives and the silica gel and/or the solvent system occur.

Lyso-GM1 was purified by silica gel column chromatography and structurally characterized by ^{1}H-NMR and FAB-MS analyses.

The main proton chemical shifts of lyso-GM1 are reported in **Table 1**. The data are very similar to those of GM1 (19). Two signals were present at 7.60 and 7.91 ppm in the COSY spectrum (**Fig. 2**). They correlated to GalNAc H-2 (3.94 ppm) and Neu5Ac H-5 (3.35 ppm), respectively, and were identified as the amide protons of GalNAc and Neu5Ac residues. This, together with the occurrence of the two signals at 1.76 and 1.88 ppm, belonging to the GalNAc and Neu5Ac acetyl protons, confirm that the *N*-acetyl groups were not lost during the deacylation reaction. On the other hand, the signals present in GM1 at 7.49 and 2.02 ppm (19), which are characteristic for the presence of the fatty acid moiety belonging to the ceramide amide and the methylene protons α to the carbonyl, were absent in the lyso-GM1 spectrum, proving the lack of the fatty acid moiety in lyso-GM1.

The positive-ion source spectrum of lyso-GM1 is shown in **Fig. 3**. The MH^+ species at *m/z* 1281 and 1309 (high-resolution measurement yielded 1280.6233 and 1308.5872) were the most intense sample-related signals, corresponding to the two molecular species containing C18- and

TABLE 1. ^{1}H-NMR chemical shifts (ppm) of lyso-GM1 at 323°K

	Gal(IV)	GalNAc	Gal(II)	Glc	Neu5Ac	LCB
H-1	4.23	4.91	4.27	4.18		3.62
H-2	3.35	3.94	3.18	3.05		2.92
H-3	3.32	3.51	3.75	3.30	1.64a	3.94
					2.56e	
H-4	3.63	3.74	3.97	3.30	3.77	5.45
H-5	3.48	3.64	3.47	3.36	3.35	5.62
H-6	nd	nd	3.49	3.65	3.13	2.00
	nd	nd	3.64	3.75		
H-7					3.18	
H-8					3.39	
$H\text{-}9_R$					3.19	
$H\text{-}9_S$					3.52	
$(\text{-}CH_2\text{-})_n$						1.23
CH_3-						0.85
CH_3CO-		1.76			1.88	
NH-		7.60			7.91	

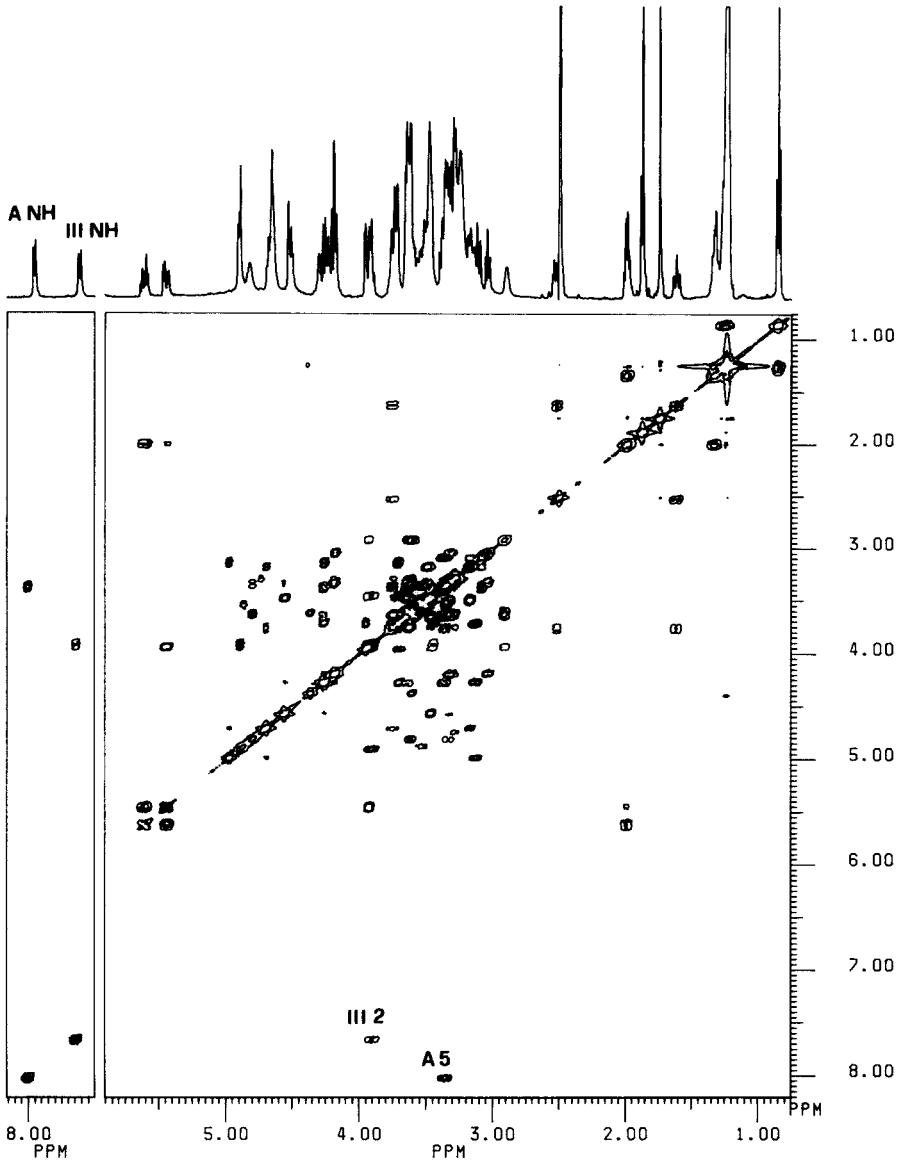

Fig. 2. 2D ^{1}H-NMR COSY (500 MHz) spectrum of lyso-GM1 in DMSO-d_6 at 323°K.

C20-LCB, respectively (calc. for $C_{55}H_{98}N_3O_{30}$: 1280.6235; calc. for $C_{57}H_{102}N_3O_{30}$: 1308.6548). These values are themselves consistent with a lyso-GM1 structure containing the *N*-acetyl groups linked both at the sialic acid and at the galactosamine units. Moreover, many other fragments, all spaced by 28 u, confirmed the lyso-GM1 structure (**Fig. 4**).

The peaks at *m/z* 328/356, 490/518, 944/972, and 1147/1175 were recognized as ^{1,5}X-type fragments (Fig. 4 and reference 20 for the nomenclature) produced by electrocyclic fission of the hexose rings. In the low-mass range of the source spectrum, the ion at *m/z* 292 was the B-type species of Neu5Ac, and the fragments of *m/z* 282 and 310 were characteristic of the LCB. Unimolecolar metastable decomposition of the protonated molecules gave rise to a prominent loss of sialic acid (−291 u; *m/z* 989/1017) and to a weaker one of the terminal Gal-GAlNAc portion (−364 u; *m/z* 916/944) and gave rise to Y-type fragments. Only the charge-initiated process leading to *m/z* 989/1017 was represented in the source spectrum, and lack of the ^{1,5}X-type fragments in the metastable spectrum confirmed that they were the result of charge-remote fragmentation, which requires a substantial activation energy (21).

CONCLUSION

The preparation of deacylated gangliosides by an alkaline reaction was introduced in 1970 by Taketomi and Kawamura (10) who used the hematoside as the starting

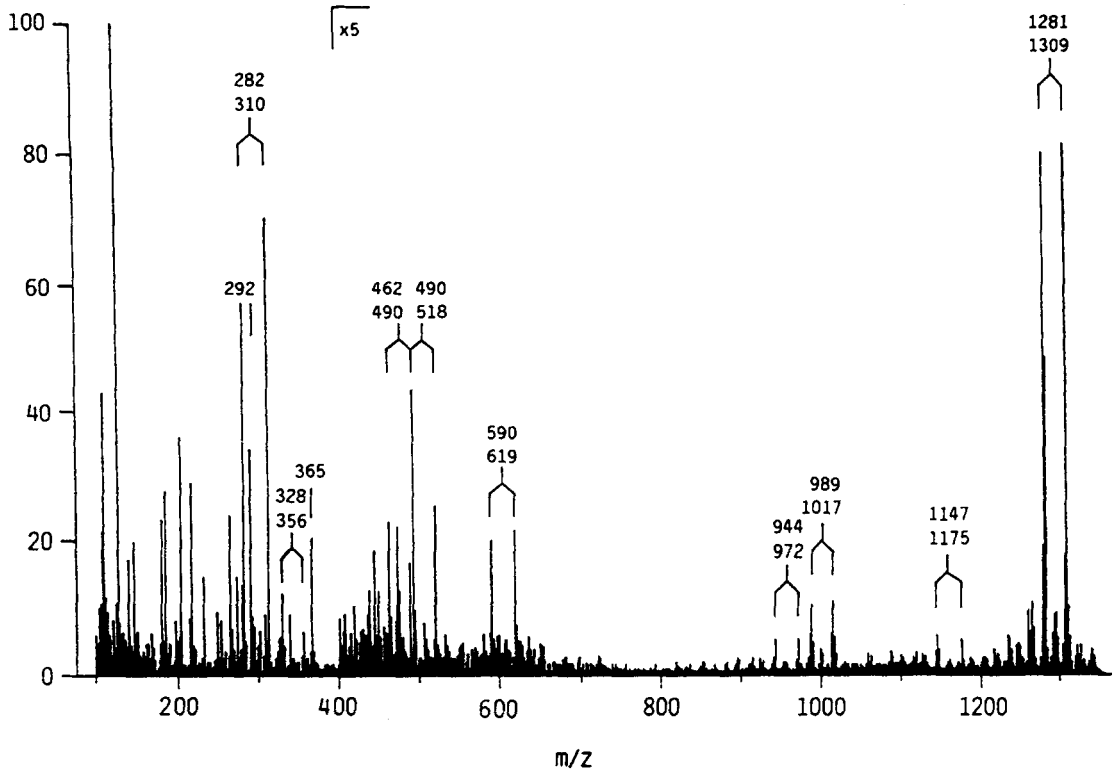

Fig. 3. FAB-MS positive-ion source spectrum of native lyso-GM1.

material. In the following years other groups applied, and in some cases improved, the procedure for the preparation of more complex deacylated gangliosides (6, 9, 11, 12). The alkaline conditions used gave rise to products lacking not only the fatty acyl group, but also the acetyl group of *N*-acetylneuraminic acid (6, 9, 10) or of both *N*-acetylneuraminic acid and *N*-acetylgalactosamine (11, 12). The preparation of lyso-gangliosides that lack only the fatty acyl group, was recently introduced (9) by a four-step synthesis.

In the present paper we report for the first time the preparation of lyso-GM1 using a one-pot reaction. The crucial point of this procedure was the removal of oxygen from the reaction mixture. Oxygen was removed by the solvent and the reaction mixture by bubbling inert gas (the procedure was developed using argon, but in some recent experi-

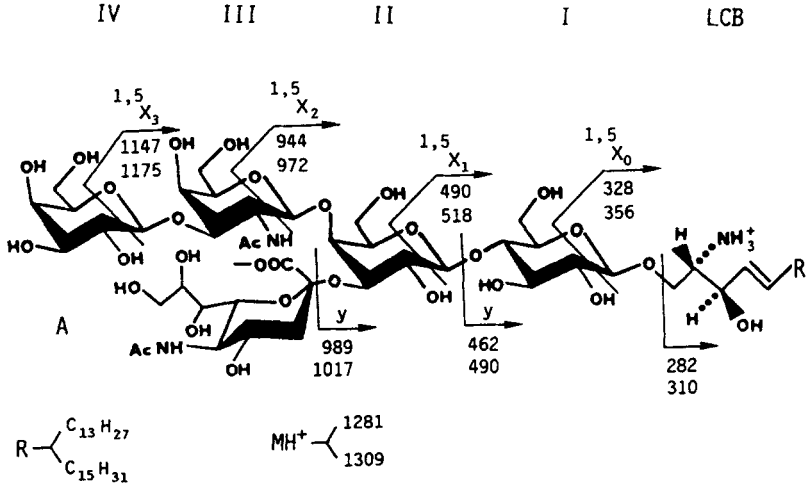

Fig. 4. FAB-MS positive-ion source proposed fragmentation pattern for native lyso-GM1. The fragments are coded according to Domon and Costello, 1988 (20).

ments using nitrogen we obtained similar results). In more than 10 experiments, performed under optimal conditions, this procedure was sufficient to obtain a constant yield of lyso-GM1. Temperature, time of reaction, and concentration of reagents over a large range of values gave a different amount of reaction products, but in the majority of cases lyso-GM1 was present. Thus we believe the procedure to be suitable for the preparation of other lyso-gangliosides. Work is in progress in our laboratory, but the determination of the optimal reaction conditions and the structural characterization of the different derivatives will require time before data can be presented.

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