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A procedure for the preparation of GM3 ganglioside from GM1-lactone

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A simple procedure is described for preparing GM3 ganglioside, from a few milligrams to grams, from GM1-lactone (Sonnino *et al.,* (1985) *Glycoconjugate J* 2: 343–54) [1]. The synthesis was carried out under the following optimal conditions: 30 mM GM1-lactone in 0.25 M H_2SO_4 in DMSO, 30 min, 70°C, nitrogen atmosphere, strong stirring. The yield of GM3 was 55%. The procedure applied to milligram amounts of GD1b-dilactone gave GD3 ganglioside.

Keywords: **gangliosides, synthesis, GM3, GD3, lactones**

Abbreviations: **Ganglioside and glycosphingolipid nomenclature is in accordance with Svennerholm (1980) [29], and the IUPAC-IUBMB recommendations (1997, 1998) [30]. LacCer, β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM3, II³-α-Neu5AcLacCer, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD3, II³(-α-Neu5Ac)$_2$LacCer, α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM2, II³-α-Neu5AcGg$_3$Cer, β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; Gg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1, II³-α-Neu5AcGg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GM1-lactone, β-Gal-(1,3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3,1-2)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1b, II³(-α-Neu5Ac)$_2$ Gg$_4$Cer, β-Gal-(1-3)-β-GalNAc-(1-4)-[Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer; GD1b-dilactone, β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8, 1-9)-α-Neu5Ac-(2-3, 1-2)]-β-Gal-(1-1)-Cer**

Introduction

Gangliosides occur ubiquitously in vertebrate cell plasma membrane where they are located in the outer layer with the oligosaccharide moiety facing the external medium. Gangliosides appear to play an important role in the modulation of cell functionality [2–4]. Like many gangliosides the GM3 ganglioside, α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer, has been described as being involved in different cellular processes: modulation of growth factors, receptor activities, cell proliferation and modulation of immunosystem [5–9]. Thus, in order to investigate the GM3 role in such processes, large amounts of GM3 in the homogeneous form are needed to develop experimental systems.

GM3 is the major ganglioside in mammals, being widely distributed throughout extranervous tissues and fluids. However it is often difficult and time consuming to prepare it from these sources due to the low content per mg of protein, the presence of other amphiphilic compounds, and the heterogeneity of the sialic acid and ceramide structures [10–12]; even the most recent purification procedures yield only small amounts of homogeneous GM3 [13]. A larger amount of GM3 ganglioside can be prepared chemically by the total synthesis of both the oligosaccharide and ceramide moieties [14–15], but many reactions are required and specialized chemical laboratories are needed.

This makes GM3 ganglioside a very expensive compound, currently quoted at over USA $300 per mg (Sigma, 1998 catalog).

A good source of GM3 could be the readily available complex gangliosides of the nervous system. The problem lies in the partial chemical hydrolysis of such gangliosides, all attempts to produce adequate amounts of GM3 having been hindered by the rapid release of sialic acid residues and the subsequent sequential detachment of the neutral sugars [16].

In this paper we present a rapid, simple and cheap procedure that allows the preparation of GM3 ganglioside by the partial acidic hydrolysis of GM1-lactone [1].

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Materials and Methods

Materials

Commercial chemicals were of analytical grade or the highest grade available. Common solvents were redistilled before use and water for routine use was deionized and freshly distilled in a glass apparatus. LiChroprep RP18, 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) were purchased from Merck GmbH. DEAE-sepharose C1-6B from Pharmacia; Chelex-100 (100–200 mesh, sodium form) and styrene-type G50 (100–200 mesh, H^+ form) from Bio-Rad. *Clostridium perfrigens* sialidase (EC 3.2.1.18) from Boehringer AG; *Vibrio chloerae* sialidase and sialic acid from Sigma. All major standard gangliosides and glycosphingolipids were available in the laboratory.

Ganglioside preparation

Gangliosides were extracted from 25 kg of bovine brain and purified by partitioning [17]. Further purification of the ganglioside mixture by alkaline treatment [18] removed a slight glycerolipid contamination.

GM1 and GD1b gangliosides

GM1 was prepared from the bovine brain ganglioside mixture [19]; 50 g of ganglioside mixture were dissolved in prewarmed (36°C) 2.5 L of 0.05 M sodium acetate, 1 mM $CaCl_2$ buffer, pH 5.5. *Clostridium perfrigens* sialidase (5 Units) was added to the solution every 12 hours. Incubation at 36°C was maintained for two days under stirring. The sialidase treated ganglioside mixture was applied to a LiChroprep RP18 column (6 × 4 cm) and, after washing with water (2 L) to remove salts and free sialic acid, the gangliosides were eluted with 2 L of methanol. The methanolic solution was dried, dissolved in chloroform-methanol-water, 60:35:8 by vol, and applied to a silica gel 100 column (100 cm × 3 cm) chromatography, equilibrated and eluted with the same solvent system; the chromatography elution profile was monitored by TLC (see below). Fractions containing GM1 were collected, dried, and the residue dissolved in 40 mL of propan-1-ol/water, 7:3 by vol, and precipitated by adding 4 volumes of cold acetone. After centrifugation (15.000g) the pellet was separated from the acetone and dried under high vacuum. By this procedure 20.5 g of GM1 with homogeneity >99.9% (by TLC and NMR analyses, see below) were prepared.

GD1b ganglioside was prepared by fractionating the bovine brain ganglioside mixture on DEAE-sepharose column chromatography [20] followed by silica gel 100 column chromatography.

GM1-lactone and GD1b-dilactone gangliosides

GM1-lactone was prepared from GM1 according to the quantitative procedure previously described [1]. 20 g of GM1 dissolved in 200 mL dehydrated dimethylsulfoxide were converted into the acid form by passing through an anhydrous styrene-type ion exchange column (100 cm × 6 cm) chromatography, equilibrated and eluted with dimethylsulfoxide. The eluted solution was treated with 3 g of dicyclohexylcarbodiimide at room temperature for one hour under stirring. After filtration to remove the formed dicyclohexylurea, the GM1-lactone was precipitated with cold acetone and dried under high vacuum. The final yield of GM1-lactone was 90% and the homogeneity 98%. GD1b-dilactone was prepared as reported [21] following the above procedure.

Preparation of GM3 and GD3 gangliosides from GM1-lactone and GD1b-dilactone

Between 50 mg to 10 g of GM1-lactone as a dried powder was added to an anhydrous balloon provided with reflux apparatus and nitrogen flux, and warmed to 70°C under stirring. A prewarmed (70°C) solution of 0.25 M H_2SO_4 in DMSO, previously maintained three hours under nitrogen bubbling, was then added very rapidly under vigorous stirring, giving a 30 mM ganglioside concentration. After 30 min at 70°C and vigorous stirring the reaction balloon was cooled in an ice bath. The reaction mixture was brought to pH 9 with 1 M NaOH and precipitated at 4°C by addition of cold CH_3CN. The precipitate was filtered on a Buchner filter, dried, dialyzed for 4 days and lyophilized. The lyophilized mixture containing GM3 and a number of byproducts (Fig. 1) was dissolved in chloroform-methanol-water, 60:35:8 by vol, and applied to a silica gel 100 column chromatography, equilibrated and eluted with the same solvent system. The elution profile was monitored by TLC (see below). Fractions containing GM3 were collected and dried and the residue was dissolved in a small volume of chloroform-methanol 2:1 by vol, and precipitated by adding 4 volumes of cold acetone. After centrifugation (15.000*g*) the pellet was separated from the acetone and dried under high vacuum. This procedure resulted in GM3 ganglioside with homogeneity >99.9% (by TLC and NMR analyses, see below).

GD3 ganglioside was prepared as above starting from 10 mg of GD1b-dilactone.

Analytical procedures

Ganglioside-bound sialic acid was determined by the resorcinol-HCI method [22–23], pure Neu5Ac being used as the reference standard.

TLC of gangliosides was carried out on silica gel HPTLC plates using the solvent system chloroform/methanol/0.4% aqueous CaCl2/50 mM KCl, 50:50:4:8 by vol. After TLC the

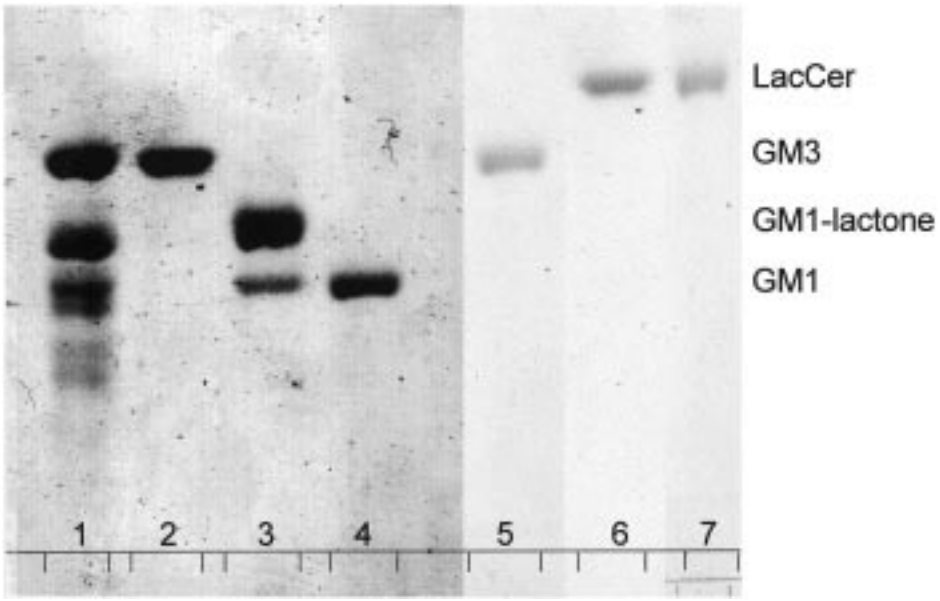

Figure 1. TLC of the reaction products obtained by chemical hydrolysis of GM1-lactone. 1, reaction mixture; 2, standard GM3; 3, starting GM1-lactone (partial hydrolysis to GM1 is due to the chromatographic conditions, as reported in Sonnino *et al.,* 1985; under optimal TLC conditions for ganglioside lactones, the GM1-lactone was homogeneous; 4, standard GM1; 5, GM3 purified from the reaction mixture shown in 1; 6, purified GM3 after sialidase treatment; 7, standard LacCer.

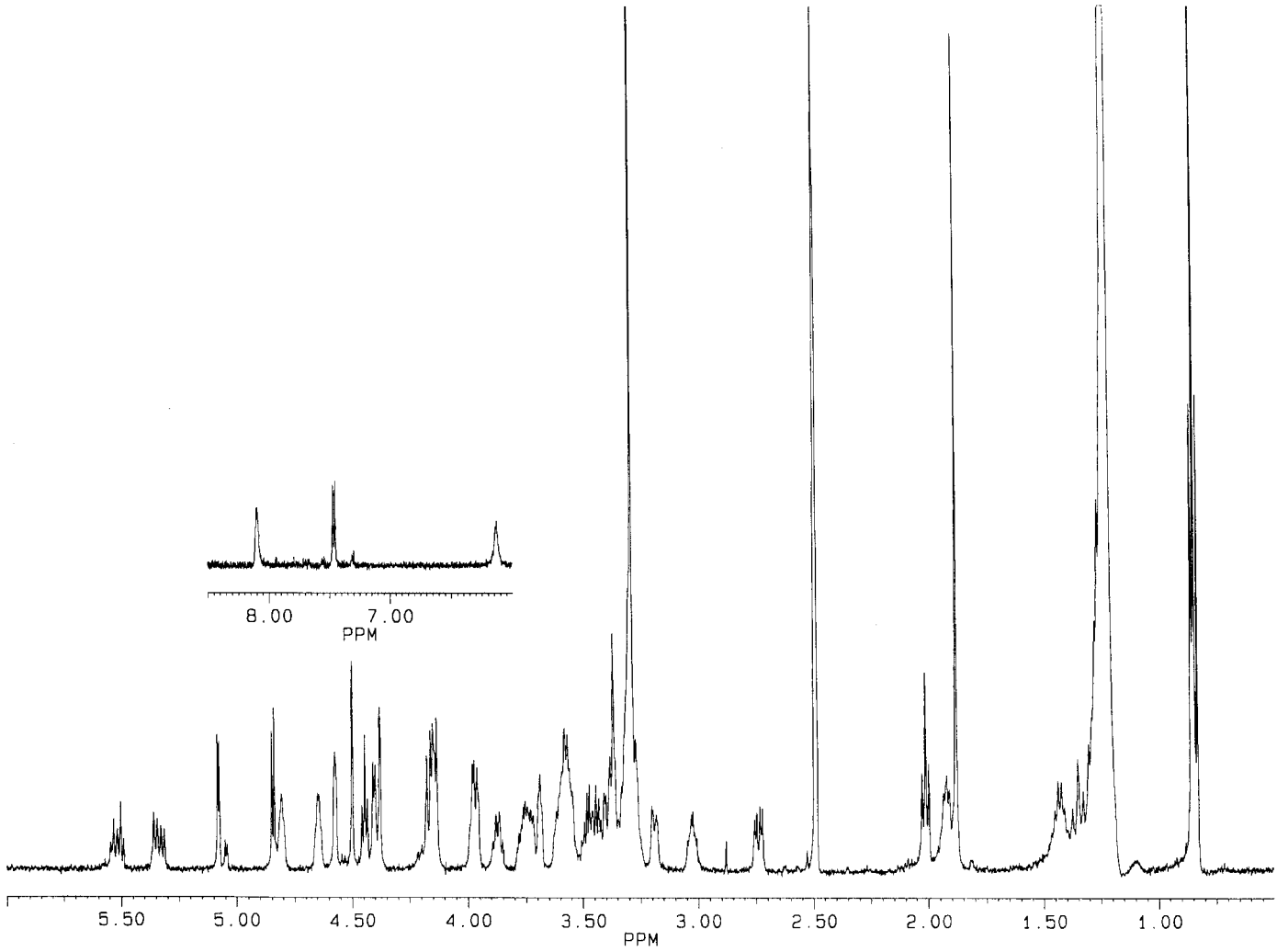

Figure 2. 500 MHz ^{1}H-NMR spectrum in DMSO-$_{d6}$ at 303°K of GM3 ganglioside prepared by chemical hydrolysis of GM1-lactone.

gangliosides were made visible by treatment with an anisaldehyde reagent followed by heating at 140°C for 15 min, with a *p*-dimethylaminobenzaldehyde reagent followed by heating at 120°C for 20 min, and with 10% ammonium sulfate followed by heating up to 160°C. Quantification of the ganglioside spots was done with a Biorad 700 imaging densitometer.

Structural characterization of GM3 was carried out by ^{1}H-NMR at 500 MHz [24] and by ESI MS/MS [25].

Enzymatic hydrolysis of gangliosides was carried out with *Vibrio cholerae* sialidase and the formed products analysed by TLC [26].

Results and discussion

Treatment of GM1-lactone with sulphuric acid in dimethylsulfoxide gave GM3 ganglioside and a number of byproducts (Fig. 1), however optimal conditions (0.25 M H_2SO_4 in DMSO, 30 min, 70°, 30 mM GM1-lactone, nitrogen atmosphere, strong stirring) were needed. These were first established processing 50 mg GM1-lactone amounts. The yield of GM3 was 55% ± 5%, a value that did not change on increasing the starting amount of GM1-lactone up to 10 g. To have this yield of GM3 it was essential to have a long bubbling of sulphuric acid-DMSO solution with nitrogen, and that the GM1-lactone and glass were anhydrous. Under optimal conditions the GM1-lactone dissolved in sulphuric acid-DMSO solution in less than 5 min, and at the end of the reaction the solution was still clear. When the drying and bubbling procedures were not done with great care, the solution turned brown rapidly and the GM3 yield dropped to a few percent. Thus it is reasonable to believe that less water and oxygen in the reaction mixture the higher the final reaction yield, although it is quite difficult to have no water in the solution because of the high hydrophilicity of sulphuric acid.

GM3 was purified to homogeneity by silica gel 100 column chromatography and its structure was determined by high resolution proton NMR, ESI MS/MS and enzymatic degradation with α-sialidase. Figure 2 shows the proton NMR spectrum of GM3 prepared from GM1-lactone. The signal assignment reported in Table 1 was in total agreement with previous data [12,24,27]. The Neu5Ac methylene equatorial and axial proton chemical shifts were exactly those reported for natural GM3, that contains Neu5Ac in the α configuration. It is known that the Neu5Ac methylene equatorial and axial proton chemical shifts are shifted moving from the α to the β configuration [28]. Moreover, exhaustive enzymatic treatment of GM3 with α-sialidase gave LacCer in quantitative yield (Fig. 1). All this excludes that the GM1-

Table 1. ^{1}H-NMR chemical shifts (ppm) for the GM3 ganglioside prepared by hydrolysis of GM1-lactone. 4 mg sample was analyzed at 303 K in DMSO-$_{d6}$. ax, axial; eq, equatorial; Sph, sphingosine; FA, fatty acid.

	Glc	*Gal*	*Neu5Ac*	*Sphingosine*	*Fatty acid*
H1	4.15	4.19		3.95	
				3.44	
H2	3.04	3.33		3.79	2.03
OH2	5.08	4.50			
H3	3.33	3.93	2.74eq	3.91	1.44
			1.37ax		
OH3	4.57			4.82	
H4	3.29	3.75	3.61	5.36	
OH4		4.42	4.85		
H5	3.26	3.32	3.40	5.54	
H6	3.62	3.46	3.61	1.93	
	3.75	3.51			
OH6	4.45	4.65			
H7			3.21		
OH7			4.65		
H8			3.34		
OH8			6.14		
H9			3.41		
			3.61		
OH9			4.17		
NH			8.10	7.45	
CH3CO-			1.88		
-CH2- of aliphatic chains				1.23	1.23
CH3-				0.85	0.85

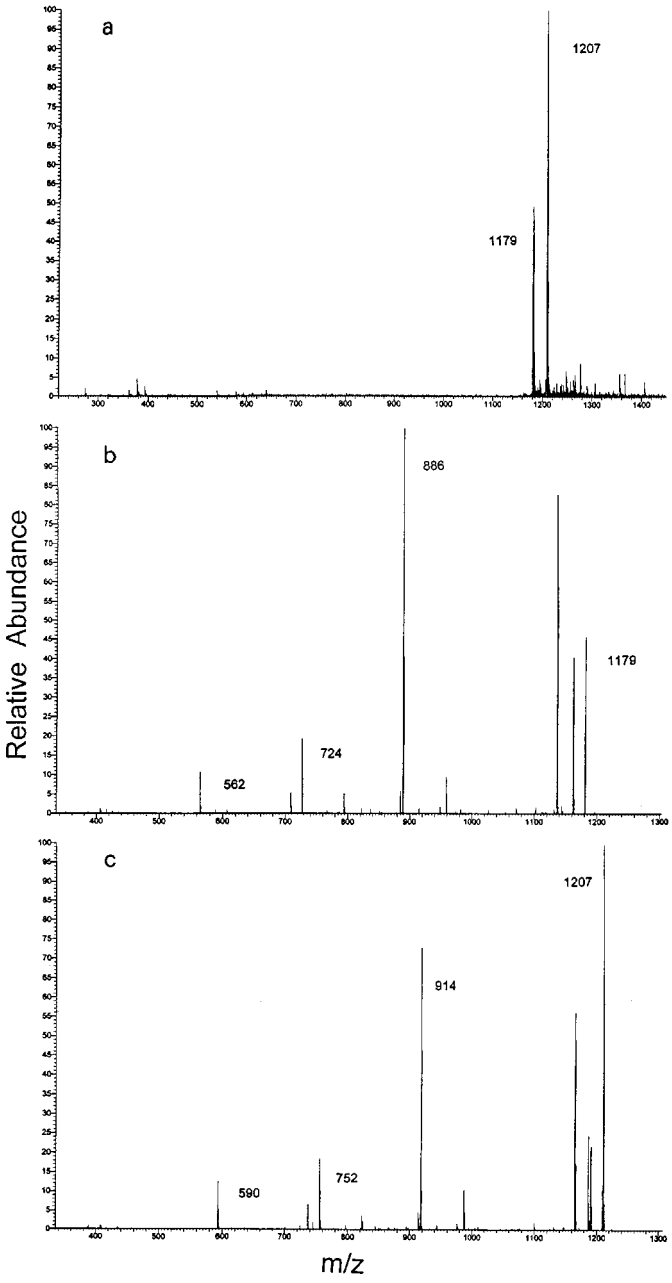

Figure 3. Negative-ion ESI mass spectrum of GM3 prepared by hydrolysis of GM1-lactone (a) and negative-ion ESI MS/MS spectra derived from ions at m/z 1179 (b) and 1207 (c).

lactone hydrolysis process led to α/β-anomerisation at the Neu5Ac residue of synthetic GM3.

The negative-ion ESI mass spectra of synthetic GM3 reported in Figure 3 confirmed the ganglioside structure and homogeneity. Figure 3a exhibits two pseudomolecular ions M-1 at *m/z* 1179 and 1207. They represent the two molecular species of GM3 that have stearic acid and either C18-sphingosine or C20-sphingosine as components of the ceramide moiety. According to the intensity of these two

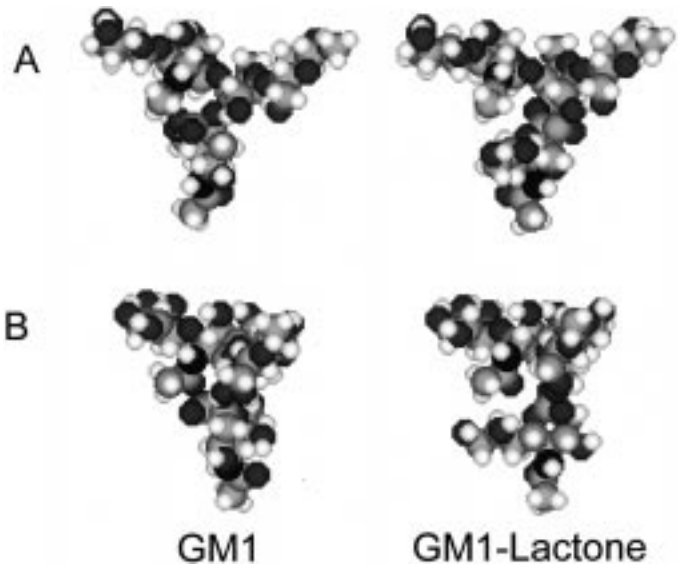

Figure 4. Minimum energy structures for GM1 and GM1-lactone as determined with HyperChem™ provided with the ChemPlus™ extension. Structures were calculated for the *O*-methyl-oligosaccharides. The upper and lower structures are rotated −60°.

ions the molar ratio between the two GM3 species was that of the GM1 species subjected to hydrolysis. The MS/MS spectrum related to the ion at *m/z* 1179 exhibited ions at *m/z* 886, 724 and 590 (Fig. 3b), and that related to the ion at *m/z* 1207, ions at *m/z* 914, 752 and 562 (Fig. 3c). The two sequences differ by 28 units and correspond to the ions produced by elimination of the negative ions of Neu5Ac, Neu5Ac-Gal and Neu5Ac-Gal-Glc.

Under acidic conditions the ketosidic linkage between sialic acid and galactose is not very stable in the GM1 ganglioside. In fact, several of our attempts to produce GM3 from GM1 under mild chemical conditions yielded first Gg_4Cer and then, sequentially, sphingolipids with shorter neutral oligosaccharide chains. Thus, increased stability of the sialic acid ketosidic linkage and/or reduced stability of the GalNAc-Gal glycosidic linkage are needed to ensure the formation of GM3 from GM1-lactone.

In the reaction mixture GM1, GM2 and $GgOse_4Cer$ were either absent or very scant; some NMR experiments suggested that the byproducts were modified sialic acid containing GM3 derivatives. Reducing the reaction time led only to minor changes in the reaction mixture, mainly referred to the GM3-lactone/GM3 ratio. This suggests that the release of β-Gal-(1-3)-β-GalNAc disaccharide from the GM1-lactone structure occurs quite rapidly, yielding GM3-lactone that is then partially converted to GM3. The total hydrolysis of the lactone ring is then obtained by alkaline treatment.

Figure 4 shows the minimum energy structures determined for GM1 and GM1-lactone: the higher accessibility

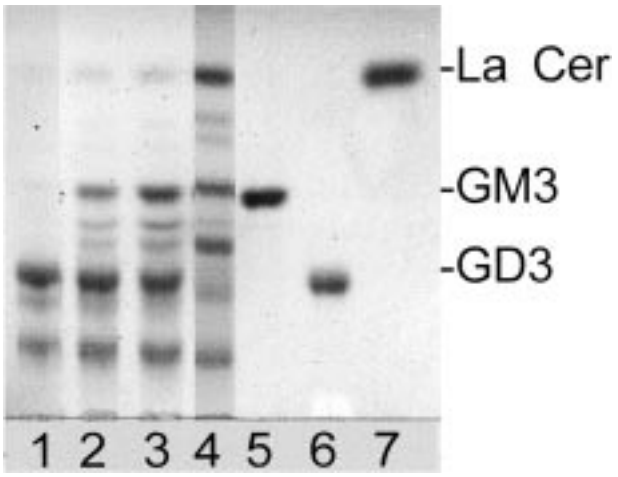

Figure 5. TLC of the reaction mixture obtained by chemical hydrolysis of GD1b-dilactone after sialidase treatment. 1, reaction mixture obtained by chemical hydrolysis of GD1b-dilactone; 2, 3 and 4, reaction mixtures after 15, 25 and 70 min treatment of 1, respectively, with 1 mU of *Vibrio cholerae* sialidase; 5, standard GM3; 6, standard GD3; 7, standard lactosylceramide.

of the GalNAc-Gal linkage is easily recognizable in GM1-lactone representation. This is supported by NMR data on GM1-lactone in DMSO solution [1,19,21]. In fact, the upfield shift by 0.42 and 0.29 ppm of H2 and NH protons and the downfield shift by 0.14 ppm of the acetyl group observed in GM1-lactone GalNAc suggest that the through-space interactions existing in GM1 between the acetamide group of GalNAc and the Neu5Ac carboxylate anion probably no longer occur in GM1-lactone.

Some confirmation of the role played by the lactonic structure in determining the stability of the glycosidic and ketosidic linkages of the oligosaccharide chains is given by the H_2SO_4-DMSO hydrolysis of small amounts of GD1b-dilactone. The main compound in the hydrolysis reaction behaved chromatographically like GD3, and was partially characterized by *Vibrio cholerae* sialidase treatment of the total reaction mixture. Figure 5 shows that GM3 and LacCer are sequentially formed by sialidase treatment. Thus, due to the Neu5Ac-(1-9)-Neu5Ac and Neu5Ac-(1-2)-Gal ester linkages, the Neu5Ac-(2-8)- Neu5Ac and Neu5Ac-(2-3)-Gal ketosidic linkages in GD1b-dilactone become more resistant than the GalNAc-(1-4)-Gal glycosidic linkage.

In conclusion, this paper shows how the GM3 ganglioside can be prepared in a few days, and in relatively large quantity, by hydrolysis of GM1-lactone ganglioside. Some years ago we developed a simple, rapid and quantitative procedure for the chemical preparation of GM1-lactone from GM1 [1]. Moreover, GM3 preparation in the manner described is very cost effective considering the rapid and low cost preparation of GM1 ganglioside through sialidase treatment of mammal brain gangliosides or through an anion exchange chromatography overloading technique [20]. Thus, for the first time, relatively large amounts of GM3 in homogeneous form are readly accessible, the cost being contained and the preparation times reduced considerably. Advantage can now be taken of the proposed procedure for further investigations.

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