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APPLICATION OF A SIMPLE METHYLATION PROCEDURE FOR THE ANALYSES OF GLYCOSPHINGOLIPIDS*

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ABSTRACT

Acid and neutral glycosphingolipids (0.01–1 μmol) were completely methylated in high yields and with little formation of by-products in 10 min at room temperature, using methyl sulphoxide, methyl iodide, and powdered NaOH. Re-methylation of methylated and $LiAlH_4$-reduced gangliosides gave a new derivative that was useful for the analysis of gangliosides by mass spectrometry.

INTRODUCTION

The structural characterisation of such glycoconjugates as glycosphingolipids, glycopeptides, and free oligosaccharides often involves Hakomori methylation[1] (methyl sulphoxide, methyl iodide, and methylsulphinyl carbanion). The use of potassium *tert*-butoxide instead of sodium hydride for preparing the methylation reagent gives less side-reactions[2] due to a more stable reagent. Several novel techniques for the methylation of carbohydrates have been described[3–5] recently, two of which[3,4] questioned the importance of the methylsulphinyl carbanion and introduced finely powdered NaOH/KOH as an alternative base. Powdered NaOH was used in 1964 for the methylation of polysaccharides with methyl sulphate in methyl sulphoxide[6].

The technique recently described by Ciucanu and Kerek[3] was reported to give complete methylation of D-glucose, with high yields and no by-products, within minutes at room temperature using optimal proportions of methyl iodide, powdered NaOH, and methyl sulphoxide. The speed and simplicity of this technique prompted its application to glycosphingolipids and a comparison with the Hakomori procedure[1]. In addition, the technique has been used to re-methylate methylated and $LiAlH_4$-reduced gangliosides[7], thereby giving a new and useful derivative for analysis by mass spectrometry.

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EXPERIMENTAL

Globoside and glycolipids with globotetraose carbohydrate chains bound to *N*-acetylsphingosine and to *N*-acetylphytosphingosine were prepared from human erythrocytes[8], globosides with hydroxylated and non-hydroxylated ceramides and a globoside-like tetraglycosylceramide having a terminal GlcNAc residue and GM3 from human meconium[9,10], cerebroside from rat small intestine[11], and GM1 from human brain[12]. Stearic acid was a gift from Dr. N. Dinh-Nguyen.

Procedure for methylation. — Dry samples were sonicated with methyl sulphoxide (0.5 mL) for 1 min. Methyl iodide (0.1 mL) and finely powdered NaOH (20–30 mg) were added, each mixture was shaken gently and magnetically stirred at room temperature for 10 min, and then distilled water (2 mL) and chloroform (1 mL) were added. After centrifugation, the clear chloroform phase was washed with water (4 × 2 mL) until the aqueous phase was neutral.

G.l.c. — A column (20 m × 0.22 mm) of fused silica with poly(arylene-methylphenyl)siloxane[13] as the stationary phase (d_f 0.2 μm) was used in a Carlo-Erba 4160 gas chromatograph with flame-ionisation detection. Heptadecanoic acid was used as the internal standard.

Mass spectrometry. — A VG ZAB-HF instrument with a VG 11-250 data system was used with e.i., the in-beam technique, positive mode, and calibration with Fomblin (Dow Chemicals). The spectra reproduced were drawn with absolute masses, but the peaks are given nominal masses in order to simplify the interpretations. Mass fragmentograms of terminal sugar ions were used to calculate the yields of methylated glycolipids. For cerebroside, *m/z* 187 (Hex − MeOH) was measured at an ion-source temperature of 250°, *m/z* 344 (NeuAc − MeOH) for GM3 at 280°, and *m/z* 260 (HexNAc) for globoside at 285°.

RESULTS

Methylation of glycosphingolipids. — The yields of glycosphingolipids upon methylation at room temperature are shown in Table I. High yields were obtained when 0.01–1 μmol of glycosphingolipids were methylated; when these amounts were exceeded, as exemplified with GM1, the recovery was considerably lower. Also, a large intermediate phase appeared in the chloroform–water partition. The recovery was not critically dependent upon the time of reaction, as illustrated by the methylation of globoside for either 10 min or 2 h.

The e.i. mass spectra obtained for globoside methylated for 10 min (Fig. 1) or 2 h were both virtually identical to that of the product obtained by methylation using the Hakomori procedure[1,9]. The intense peaks at *m/z* 260 (HexNAc–), 464 (HexNAc–Hex–), 722 (phytosphingosine and hydroxy 24:0 fatty acid), 945 (HexNAc–Hex–Hex–Hex–O–C_3H_7N), and 1369 (M − $C_{16}H_{33}O$), and the lack of ions 14 mass units below, except for fragments containing fatty acids of various chain-length, clearly indicated complete methylation of hydroxyl groups and amido

TABLE I

YIELDS OF METHYLATED GLYCOSPHINGOLIPIDS[a]

Substances	*Sample amount*		*Yield (%)*
	(μg)	*(μmol)*	
Cerebroside	10	0.011	—
	100	0.11	78
	500	0.55	83
	1,000	1.1	100
GM3	15	0.01	87
	150	0.1	63
	750	0.5	82
	1,500	1.0	100
GM1	10,000	6.5	10[b]
Globoside	100	0.08	96
	100	0.08	100[c]

[a]With methyl sulphoxide (0.5 mL), methyl iodide (0.1 mL), and finely powdered NaOH (~25 mg) for 10 min. [b]Estimated by weight. [c]Methylation for 2 h.

nitrogens of amino sugars and long-chain bases. The peak at *m/z* 182 corresponds to a fragment that correlates empirically with a methylated HO-3 of GlcNAc residues in glycosphingolipids[14] and should not be used for determining the level of methylation.

G.l.c. showed that the amount of low-molecular-weight by-products formed increased with increase in duration of the methylation procedure (Fig. 2, A and B). The major contaminant was phthalate, eluting at 23.5 min, which was present also in a blank methylation (Fig. 2C) and in methylated GM3 (Fig. 2D) and was a contaminant in the methyl iodide. The amount of contaminants differed considerably in reagents from different commercial sources.

Methylation of stearic acid. — On methylation of 1, 10, 50, and 100μg of stearic acid (corresponding to 3.5 nmol to 0.35 μmol), the yields were 82, 100, 97, and 88% respectively, but for 500 μg and 1 mg, the yields were only 42 and 17%, respectively. These lower yields were accompanied by an increasingly larger intermediate phase in the chloroform–water partition. By increasing the amount of reagents four times for the 500-μg sample, the yield became 84% and the two phases were clearly separated. At no time was incompletely methylated stearic acid detected in the chloroform phase.

Derivatives of GM3 analysed by mass spectrometry. — The e.i. mass spectrum of the product obtained by methylation of GM3 for 10 min is shown in Fig. 3. Complete methylation of sialic acid at the carboxyl group, the acetamido group, and all the hydroxyl groups was evident from the peaks at *m/z* 376 and 344 (376 − 32). The ions at *m/z* 660 (sphingosine and non-hydroxy 24:0 fatty acid), 690 (sphingosine and hydroxy 24:0 fatty acid), and 722 (phytosphingosine and hydroxy 24:0 fatty acid) pointed to a complex ceramide composition of this ganglioside.

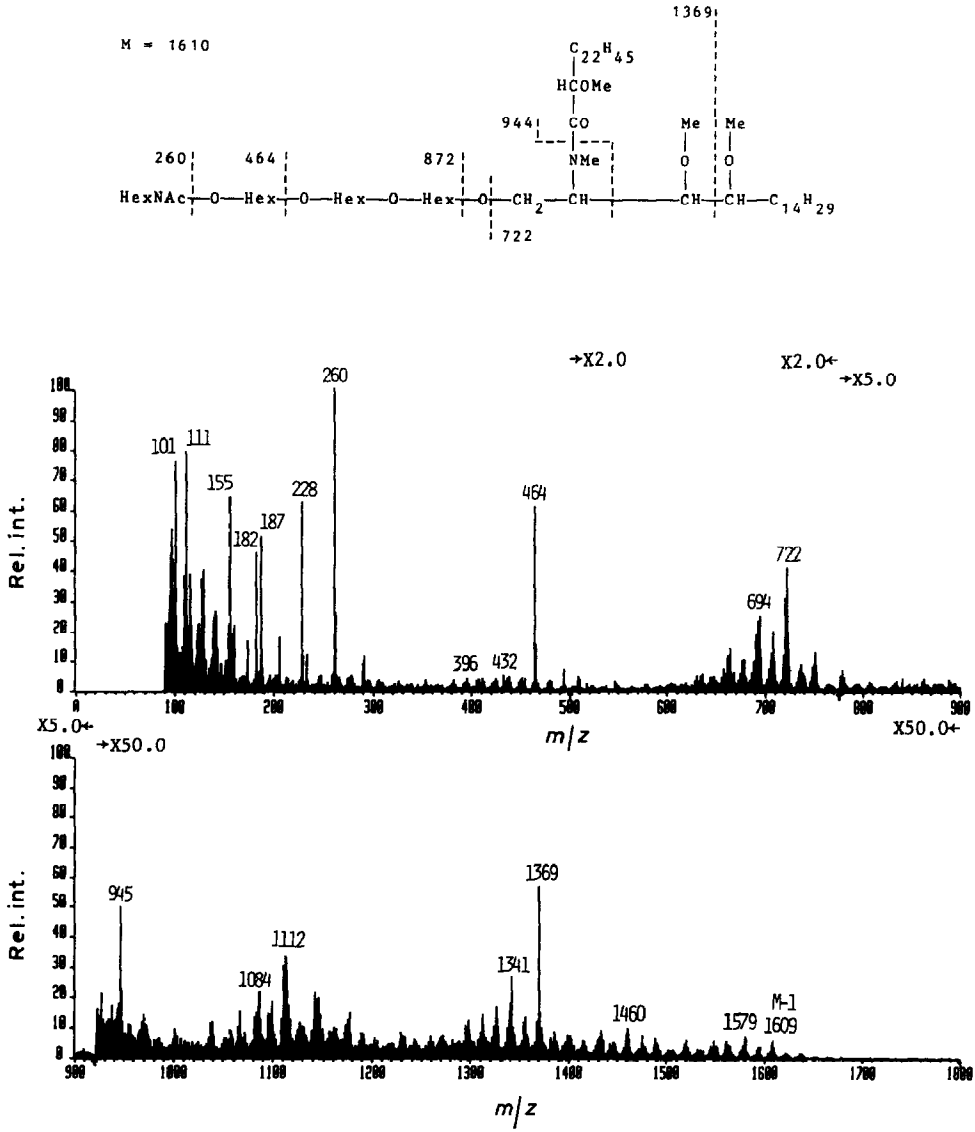

Fig. 1. Average e.i. mass spectrum of the product of methylation of globoside with hydroxylated ceramide[9] (1): trap current, 500 μA; electron energy, 44 eV; acceleration voltage, 8 kV; and ion-source temperature, 260–300°.

Reduction[7] of GM3 with $LiAlH_4$ converted the amide groups into amines, and the molecular ion, formed by ionisation at the ceramide nitrogen, readily fragmented into the primary immonium ion $[imm]^+$ through an α-cleavage of the sphingosine moiety. In the e.i. mass spectrum of methylated-reduced GM3 (Fig. 4), these ions appeared as two series of peaks at *m/z* 1039–1151 (non-hydroxy 16:0–24:0 fatty acids) and at *m/z* 1069–1181 (hydroxy 16:0–24:0 fatty acids). The series of peaks

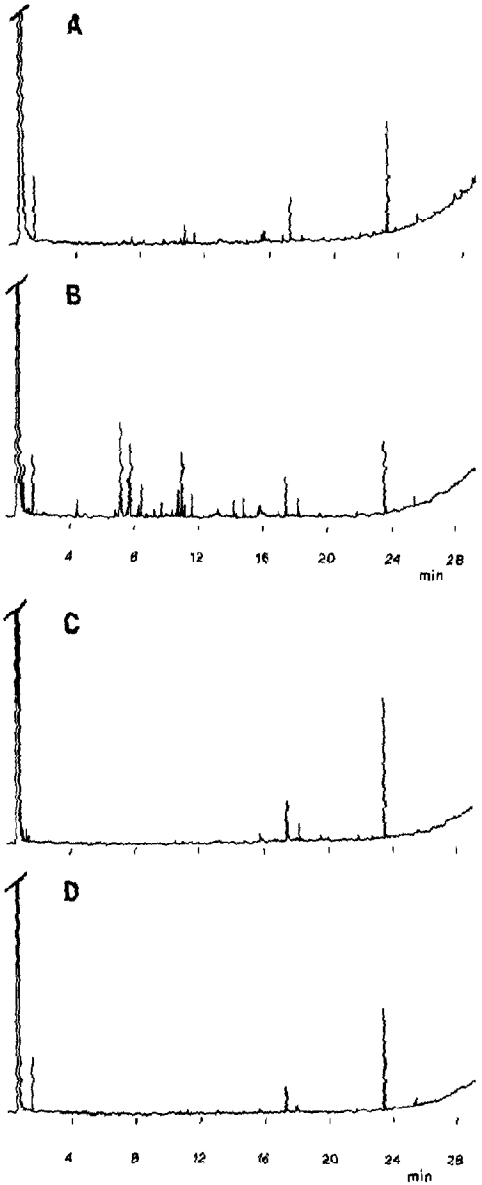

Fig. 2. Gas chromatograms of a globoside fraction methylated for 10 min (A) and 2 h (B), a blank methylation (C), and GM3 after methylation for 10 min (D). Split injection at 70°, programmed at 8°/min up to 300°. Carrier gas, H_2; linear velocity, 50 cm/s.

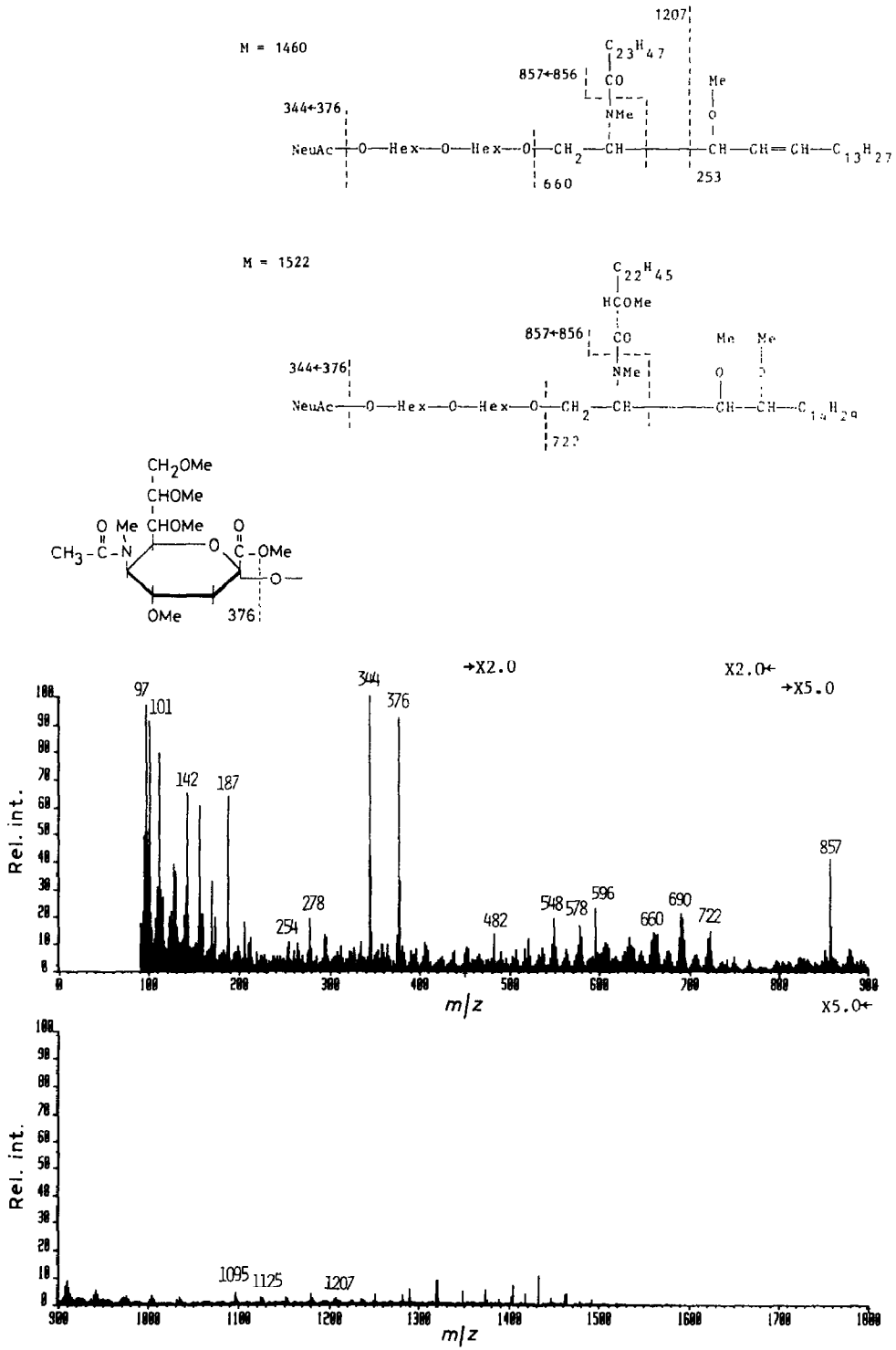

Fig. 3. Average e.i. mass spectrum of methylated GM3: trap current, 500 μA; electron energy, 42 eV; acceleration voltage, 8 kV; and ion-source temperature, 230–310°.

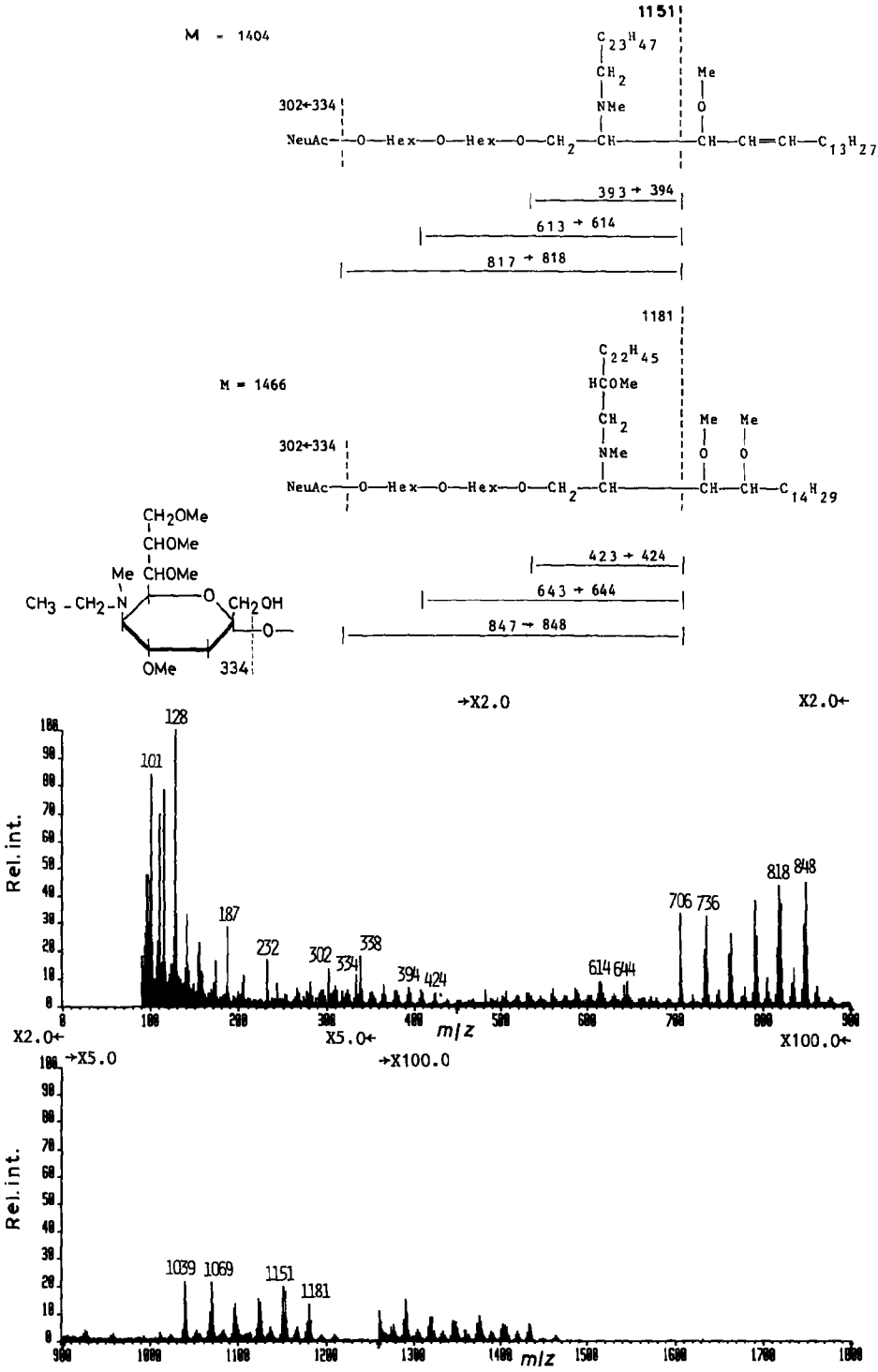

Fig. 4. Average e.i. mass spectrum of methylated $LiAlH_4$-reduced GM3: trap current, 500 μA; electron energy, 42 eV; acceleration voltage, 8 kV; and ion-source temperature, 225–280°.

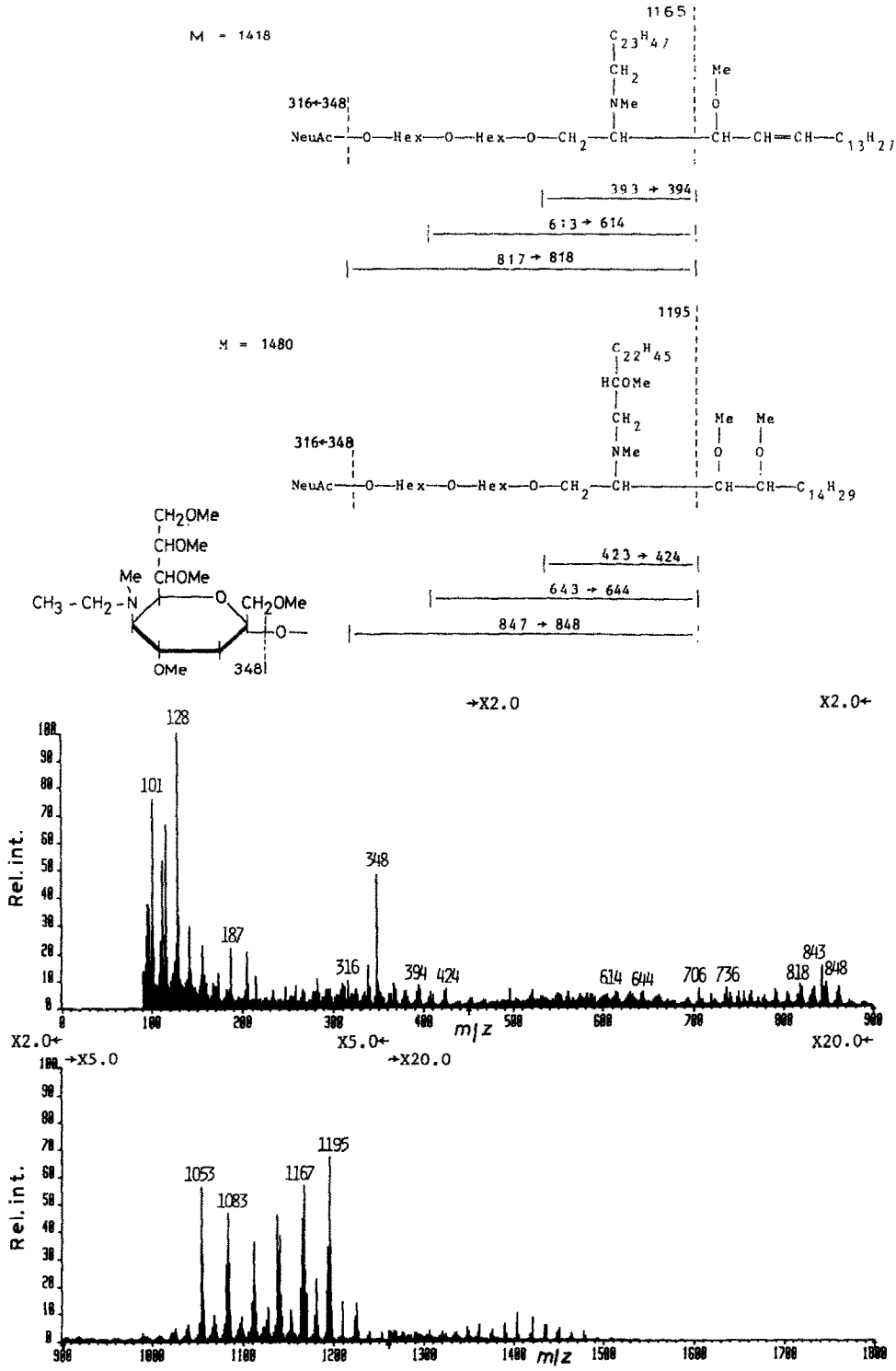

Fig. 5. Average e.i. mass spectrum of re-methylated methylated-reduced GM3: trap current, 500 μA; electron energy, 42 eV; acceleration voltage, 8 kV; and ion-source temperature, 225–300°.

that appeared at *m/z* 706–818 and 736–848 corresponded to a loss of sialic acid [imm − NeuAc + H]$^+$. Loss of an additional hexose [imm − NeuAc − Hex + H]$^+$ gave rise to the series of peaks at *m/z* 502–614 and 532–644.

After re-methylation of the methylated-reduced GM3, the e.i. mass spectrum (Fig. 5) was similar to that of the reduced molecule with the exception that decomposition of the primary immonium ion was not so extensive. The two series at *m/z* 1053–1165 and 1083–1195 corresponded to the immonium ions with non-hydroxy and hydroxy 16:0–24:0 fatty acids. The strong peak at *m/z* 348 indicated complete methylation at C-1 of the terminal sialic acid residue.

Methylated-reduced GM3 was also re-methylated with trideuteriomethyl iodide for 10 min and, as expected, intense peaks in the mass spectrum (*cf.* Fig. 5) of the product appeared at *m/z* 351 for the sialic acid residue and at *m/z* 1056–1168 for the primary immonium ions with non-hydroxy 16:0–24:0 fatty acids. However, an additional series of peaks at *m/z* 1059–1171 was as intense as that at *m/z* 1056–1168, and to the series of ions at *m/z* 706–818 for non-hydroxy 16:0–24:0 fatty acids was added another series at *m/z* 709–821. This result suggested a mixture of deuterated and non-deuterated methyl groups on the amino group of sphingosine in addition to the deuterated sialic acid residue. The series for the hydroxylated fatty-acid fragments of GM3 showed a lower proportion of the deuterated sphingosine analogue. Comparisons of re-methylated methylated-reduced GM3, globoside, and globoside lacking fatty acid showed a variable ratio of deuterated and non-deuterated methyl groups at the amino groups on the sphingosines, *N*-acetylhexosamines, and sialic acid.

DISCUSSION

The new methylation technique described by Ciucanu and Kerek[3] for carbohydrates works well for the analysis of glycosphingolipids. It is simple, fast, reliable with good yields, completely methylates all hydroxyl and carboxyl groups and amido nitrogens, and gives few by-products. In our laboratories, it has replaced the Hakomori[1] procedure and has now been used successfully for glycosphingolipids with up to eight sugars including tetrasialogangliosides. Stearic acid was originally chosen as a model compound for studying the yields and completeness of methylation of carboxyl-containing substances. Despite the addition of water to the alkaline reaction mixture, no hydrolysis was observed.

Re-methylation of methylated and $LiAlH_4$-reduced GM3 gave a stable and clean derivative which, mass spectrometrically, was stabilised at the sialic acid glycosidic bond and gave intense immonium ions with the intact carbohydrate chain and part of the ceramide. This stabilisation may be important for the analysis of polysialogangliosides and may be a practical alternative to trimethylsilylation[7]. Methylation of a tertiary amine gives a quaternary derivative[14] which, in e.i.-mass spectrometry, is reconverted in the ion source into the tertiary amine. The presence of such quaternary amines was detected (data not shown) by positive f.a.b.-mass

spectrometry of the re-methylated methylated-reduced GM3. Due to the formation of such quaternary amines by re-methylation of tertiary amines, it is not possible to locate the sialic acid residues of gangliosides by using trideuteriomethyl iodide.

During the preparation of this manuscript, the application of the Ciucanu and Kerek methylation technique to a trisialoganglioside was reported[15].

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