Carbohydrate Research, 221 (1991) 49–61
Elsevier Science Publishers B.V., Amsterdam

The resolution into molecular species on desorption of glycolipids from thin-layer chromatograms, using combined thin-layer chromatography and fast-atom-bombardment mass spectrometry

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(Received February 5th, 1991; accepted for publication May 4th, 1991)

ABSTRACT

Using a specially designed, motorised t.l.c.–f.a.b.-m.s. probe with continuous desorption and scanning over a moving t.l.c. plate, it was shown that glycolipids with identical carbohydrate sequences were well resolved into molecular species with differences in long-chain base and fatty acid. There was no serious diffusion of the glycolipids into the matrix. The technique is demonstrated for sulphatides (one and two sugar residues) isolated from human kidney, GM3 ganglioside isolated from human malignant melanoma, and chemically modified gangliotetraosylceramide from mouse intestine. T.l.c.–f.a.b.-m.s. is convenient for sequencing and composition analysis of receptor-active glycolipids, the biological activity of which can be monitored in parallel by overlay on the t.l.c. plate with proteins, viruses, bacteria, or animal cells.

INTRODUCTION

There is, at present, rapid progress within glycobiology[1,2]. Specific protein–carbohydrate interactions are being defined for both normal and pathological phenomena. Of special importance is the abundance of glycoconjugates on animal-cell surfaces that participate in immunological recognition processes and cellular interactions.

The glycolipid is amenable to analysis since it has one oligosaccharide moiety per molecule in contrast to glycoproteins, which may carry several different oligosaccharides. One efficient analytical technique involves t.l.c. of glycolipids followed by a binding assay using an overlay of protein reagents[3,4], viruses[5,6], bacteria[7,8], or eukaryotic cells[9] on the t.l.c. plate. In this way, several glycolipid receptors for bacteria[10] have been characterised. Therefore, t.l.c. is of crucial importance in the characterisation of protein–carbohydrate specificities.

E.i.-mass spectrometry has been used to sequence derivatives of glycolipids[11,12]. Information on sequence could be obtained[13] from the relatively intense peaks of the components of complex mixtures, which allowed characterisation of different animal tissues[14–16]. With the development of the t.l.c. overlay-binding assay, it became important to improve the methods for identification of an unknown receptor substance detected as a component in a mixture of glycolipids. The combination of t.l.c. and mass

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0008-6215/91/$ 03.50

spectrometry (t.l.c.–m.s.), reported by Unger *et al.*[17], was first used for glycolipids by Handa and co-workers[18,19]. An essential prerequisite for t.l.c.–m.s. is the preparation of a mixture of glycolipids free from non-glycolipid contaminants[20].

Our first applications[16,21–23] of t.l.c.–m.s. for the sequence analysis of glycolipids indicated that much developmental work was needed to make the technique reliable on a routine basis. However, the power of the method was demonstrated by the characterisation[22,23] of the glycolipid receptor in the small intestine of piglets for diarrhoea-inducing *E. coli* K99. One important question concerned the possible diffusion of separated spots into the t.l.c. matrix. T.l.c. effected good resolution of glycolipids based on differences in the long-chain base and fatty acid. A partial separation of such species can be effected[16] by direct-probe distillation e.i.-m.s. We now report on the good resolution in t.l.c.–m.s. of mixtures of relatively simple glycolipids; namely, sulphatides with one and two sugar residues, which are glycolipids involved in specific interactions with several proteins[24]; the GM3 ganglioside, which is a tumor-associated glycolipid of human melanoma[25]; and a chemically modified receptor tetrasaccharide glycolipid for bacteria[10].

EXPERIMENTAL

Glycolipids. — The mixture of gangliosides was the total acid glycolipid fraction prepared by ion-exchange chromatography[20] from a liver metastasis of human malignant melanoma. The mixture of human-kidney sulphatides was isolated from a total acid glycolipid fraction[20] by acetylation, chromatography, and deacetylation[26]. Gangliotetraosylceramide was isolated by chromatography on silicic acid of a total non-acid glycolipid fraction[20] of the faeces of a germ-free mouse. The catalytically hydrogenated glycolipid was subjected to trifluoroacetolysis[27] followed by purification.

T.l.c. — Nanoplates (Merck) with a layer of silica gel on alumina were developed with chloroform–methanol–water (65:35:8). For the t.l.c. plates illustrated in Figs. 2, 5, and 8 (anisaldehyde detection), 30–40-μg samples were used but, for t.l.c.–m.s., good spectra were obtained with $\sim$50 μg of each mixture.

T.l.c.–m.s. — A ZAB-2F/HF mass spectrometer was used with a special f.a.b. probe (t.l.c.–f.a.b.-m.s.) and an extended solid inlet port (VG Analytical). The mounted t.l.c. strip was moved by a motor over a maximum distance of 60 mm at 0.25 mm per scanning cycle, which allowed >200 scans per plate. Xe atoms at 8 kV were used. The matrix, triethanolamine, was applied with a soft-surfaced roller in order to produce a uniform coating. Spectra were plotted from a Mach-3 work station (Kratos). General conditions for the m.s. of glycolipids are summarised elsewhere[16].

RESULTS

Figure 1 shows examples of the ceramide compositions of the glycolipids studied. R may be a sulphated mono- or di-saccharide (Figs. 2–4), a trisaccharide (Figs. 5–7), or a modified tetrasaccharide (Figs. 8 and 9).

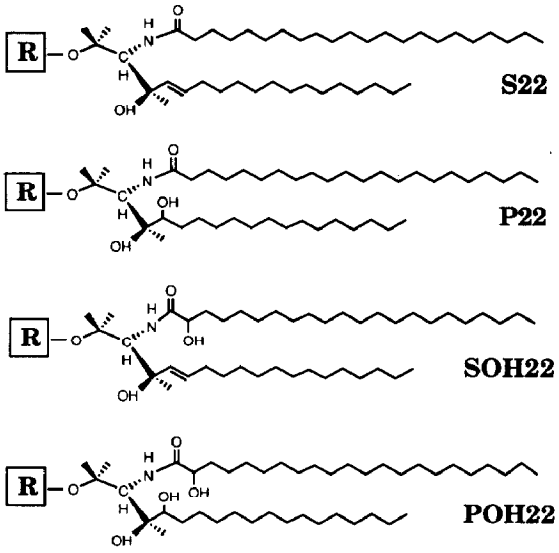

Fig. 1. Molecular species of glycolipids based on the variation of the long-chain base [sphingosine (S), dihydrosphingosine, or phytosphingosine (P) usually have 18 carbon atoms as shown] and the fatty acid (may vary in chain length, as shown in Figs. 2–7, but only a 22-carbon fatty acid is shown). The fatty acid may be 2-hydroxylated and mono-unsaturated. R is 3-sulphated galactose and 3-sulphated lactose (Figs. 2–4), sialyl-lactose (Figs. 5–7), or gangliotetraose.

Analysis of the sulphatides. — With the solvent system used, the mono- and di-saccharide sulphatides were not well separated in t.l.c. (Fig. 2). The slower-moving disaccharide species was partially separated into two bands. This separation was based on the length of the fatty acid (longer species moving faster) or the level of hydroxylation (hydroxyl groups reduce mobility).

Altogether, 46 mass spectra were collected over a distance of 10 mm, as indicated in Fig. 2. The molecular ion regions from eleven of these spectra are reproduced in Figs. 2–4. Of these spectra, six reflect the monosaccharide species (scans 20–46), two contain both species (scans 52–54), and three contain the disaccharide species (scans 58–65). Clearly, scan 20 (Fig. 2) is dominated by molecular ions of the ceramide species with sphingosine in combination with 22-, 23-, and 24-carbon fatty acids. Scan 24 revealed a relative increase in the proportion of the shorter species and the appearance of probably phytosphingosine combined with 24- and 25-carbon fatty acids. In scan 32, unsaturated 24:1 fatty acid was detected, and, in scan 39, unsaturation in combination with phytosphingosine. Alternatively, as for scan 44, the heavier species contained hydroxy fatty acid combined with sphingosine (same molecular weight, see Fig. 1, and therefore cannot be differentiated). Scan 46 revealed unsaturated hydroxy fatty acids. When the first disaccharide peaks appeared (scan 52), the monosaccharide glycolipid was dominated by shorter-chain hydroxy fatty acid. The disaccharide sulphatide was dominated by non-hydroxy fatty acids and sphingosine, which shifted successively by analogy with the monosaccharide species (scans 52–56). The partially separated t.l.c. bands of the

disaccharide glycolipid (Fig. 2), therefore, were based on sphingosine in combination with mainly 22–25-carbon fatty acids (scans 52–60) and with 16-carbon fatty acid (scans 60 and 65). For the monosaccharide sulphatide, there was no visual separation in t.l.c. (Fig. 2), probably due to a higher complexity with also phytosphingosine and hydroxy fatty acids (*cf.* discussion of Fig. 5 below). The ceramide compositions obtained from these analyses are in agreement with earlier results[28] from direct-probe e.i. analysis of derivatised monoglycosylceramides in relation to mobility in t.l.c.

Analysis of the tumor ganglioside. — For the mixture of human-melanoma gangliosides, the ten mass spectra of the molecular region selected for illustration are from 15 scans that covered a narrow zone over two partially separated bands (Fig. 5). The resolution in t.l.c. was similar to that of the two sulphatides (Figs. 2–4). Scan 106 was dominated by sphingosine combined with the 24-carbon fatty acid. In scan 108, the 22-carbon fatty acid was present also, and, in scans 110–115, the unsaturated 24:1 fatty acid. Hydroxy fatty acids with 24–20 carbons appeared in scans 114–119, and shorter-chain non-hydroxy acids successively increased in relative intensity in scans 115–120. Thus, the front part of the rapidly moving band (Fig. 5) contained saturated and longer fatty acids, the end part contained the hydroxy acids with 20–24 carbons, and the middle part contained the unsaturated 24:1 acid. The mass spectra of the second band are dominated by 16- and 18-carbon fatty acids. Thus, compared to the one-sugar sulphatide (Figs. 2–4), this ganglioside was relatively free of phytosphingosine and 16–18-carbon hydroxy fatty acids. The same good resolution was obtained with two different types of glycolipids, monosaccharide sulphatide, and trisaccharide ganglioside.

Analysis of the chemically modified gangliotetraosylceramide. — In investigating structure–activity relations for bacterial receptors by chemical modification of natural glycolipids, although the starting material usually is homogenous in t.l.c., the chemical treatment often results in a number of bands. The product expected after hydrogenation and trifluoroacetolysis of gangliotetraosylceramide has the *N*-acetyl group of GalNAc and the fatty acid replaced with trifluoroacetyl groups (Fig. 8). However, several additional products were formed which were subjected to t.l.c.-m.s. (spectra not shown). The spectra in Fig. 9 (scans 70 and 75, respectively) show that two closely moving bands are species based on dihydrosphingosine and phytosphingosine (a mass difference of 16 units). This finding is not unexpected since the glycolipid originated in the small intestine of the mouse and has a complex ceramide structure with phytosphingosine as the major long-chain base[29]. Repeated t.l.c.–m.s., using the same plate the next day, showed an identical result.

DISCUSSION

The advantage of t.l.c.–m.s., in relation to m.s. combined with gas chromatography (g.l.c.–m.s.), liquid chromatography (l.c.–m.s.), or supercritical-fluid chromatography (s.f.c.–m.s.), is the potential for direct comparison with the results from the overlay of biological reagents[3–9]. With the present progress of glycobiology[2], improved techniques for carbohydrate sequencing are needed urgently. T.l.c.–m.s. may be highly

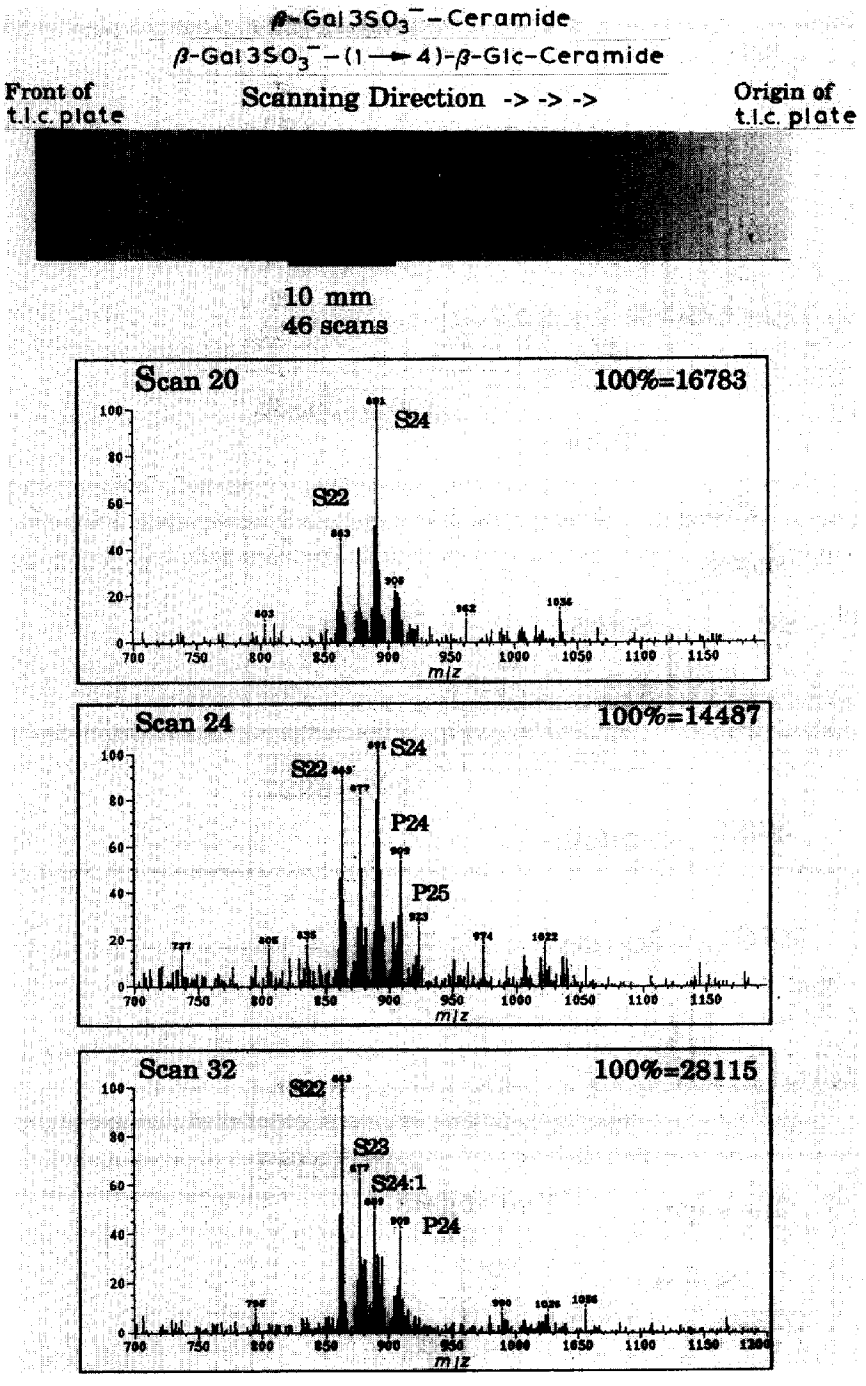

Fig. 2. T.l.c. plate (top) and molecular-ion region of selected scans from t.l.c.–m.s. of two sulphatides isolated from human kidney; 46 scans were recorded over a distance of 10 mm, of which scans 20, 24, and 32 are shown.

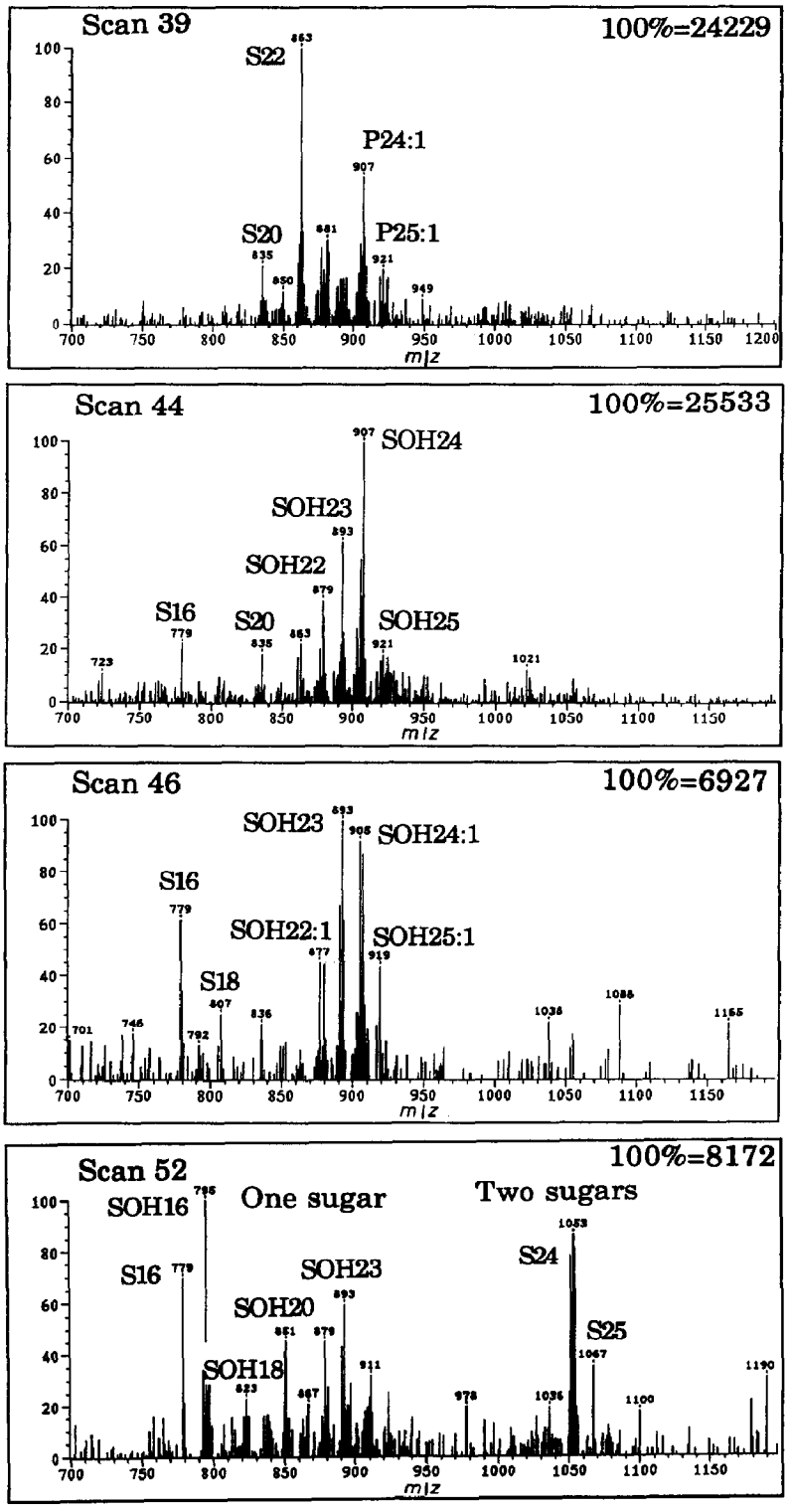

Fig. 3 Scans 39, 44, 46, and 52 from t.l.c.–m.s. of the sample described in Fig. 2; P24:1 of scan 39 and SOH24 of scan 44 have the same molecular weight and are assigned arbitrarily.

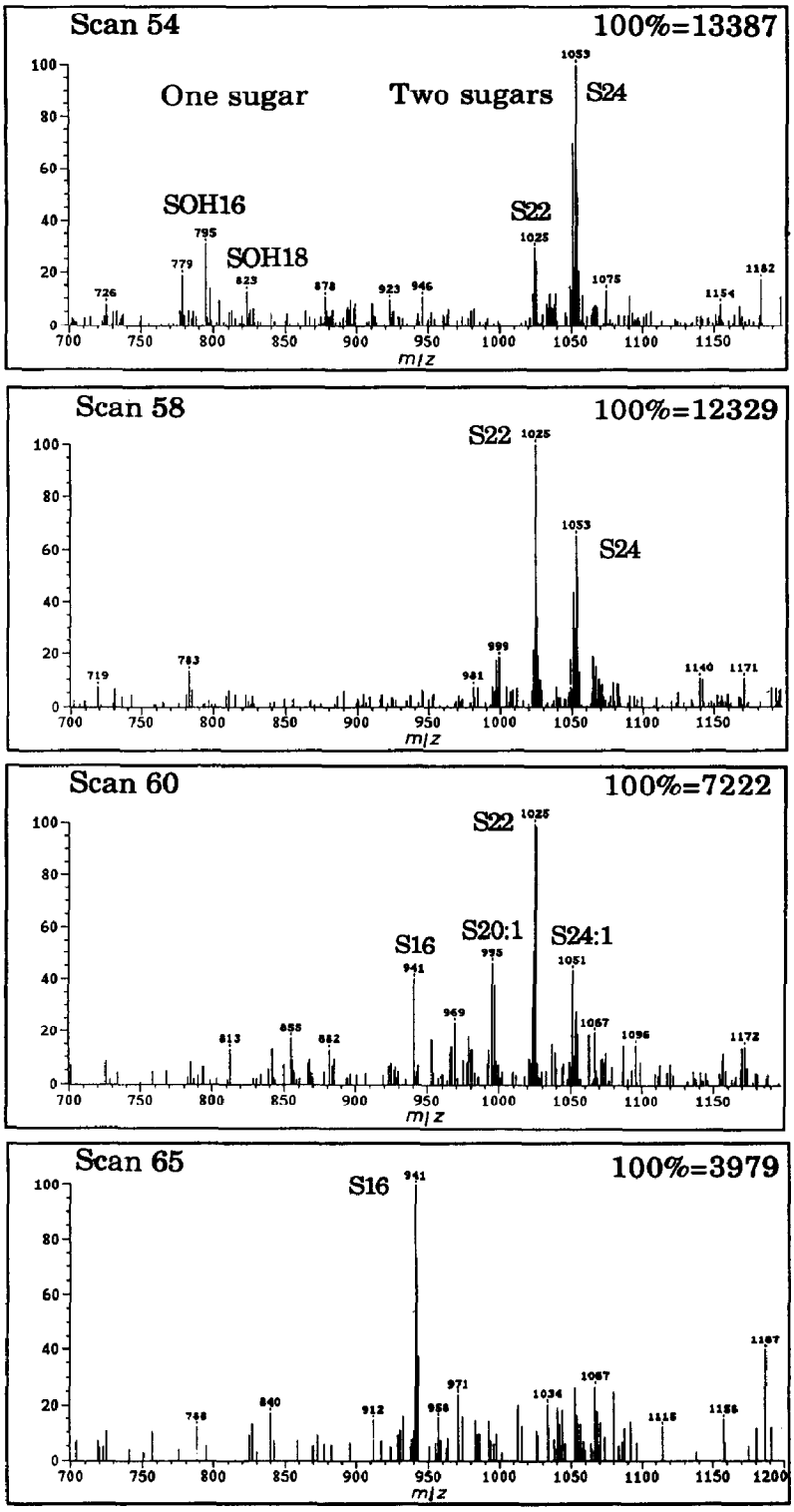

Fig. 4. Scans 54, 58, 60, and 65 from t.l.c.–m.s. of the sample described in Fig. 2.

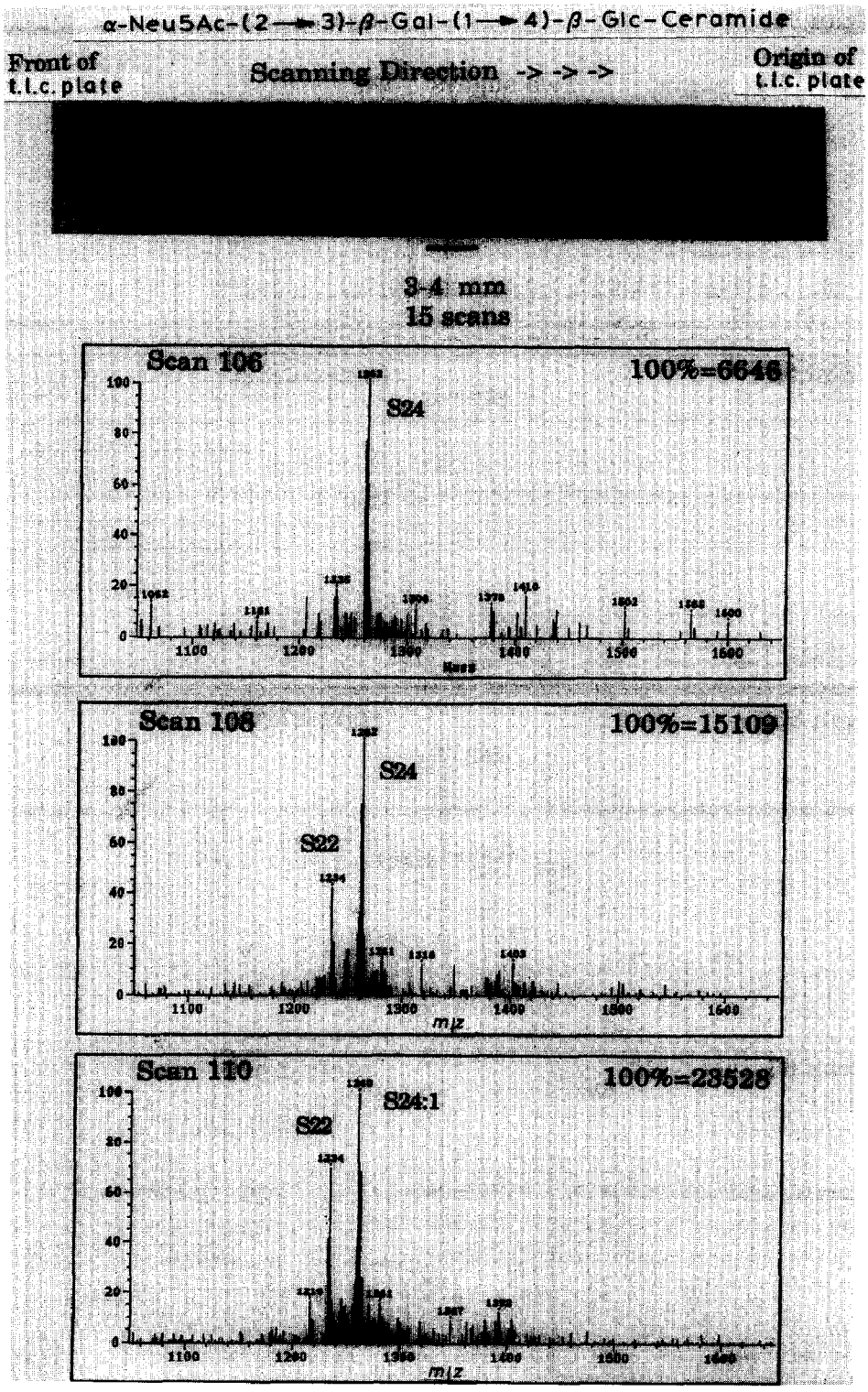

Fig. 5. T.l.c. plate (top) and molecular-ion region of selected scans from t.l.c.–m.s. of the ganglioside GM3 in a mixture of gangliosides isolated from human malignant melanoma; 15 scans were recorded over the indicated distance of 3–4 mm, of which scans 106, 108, and 110 are shown.

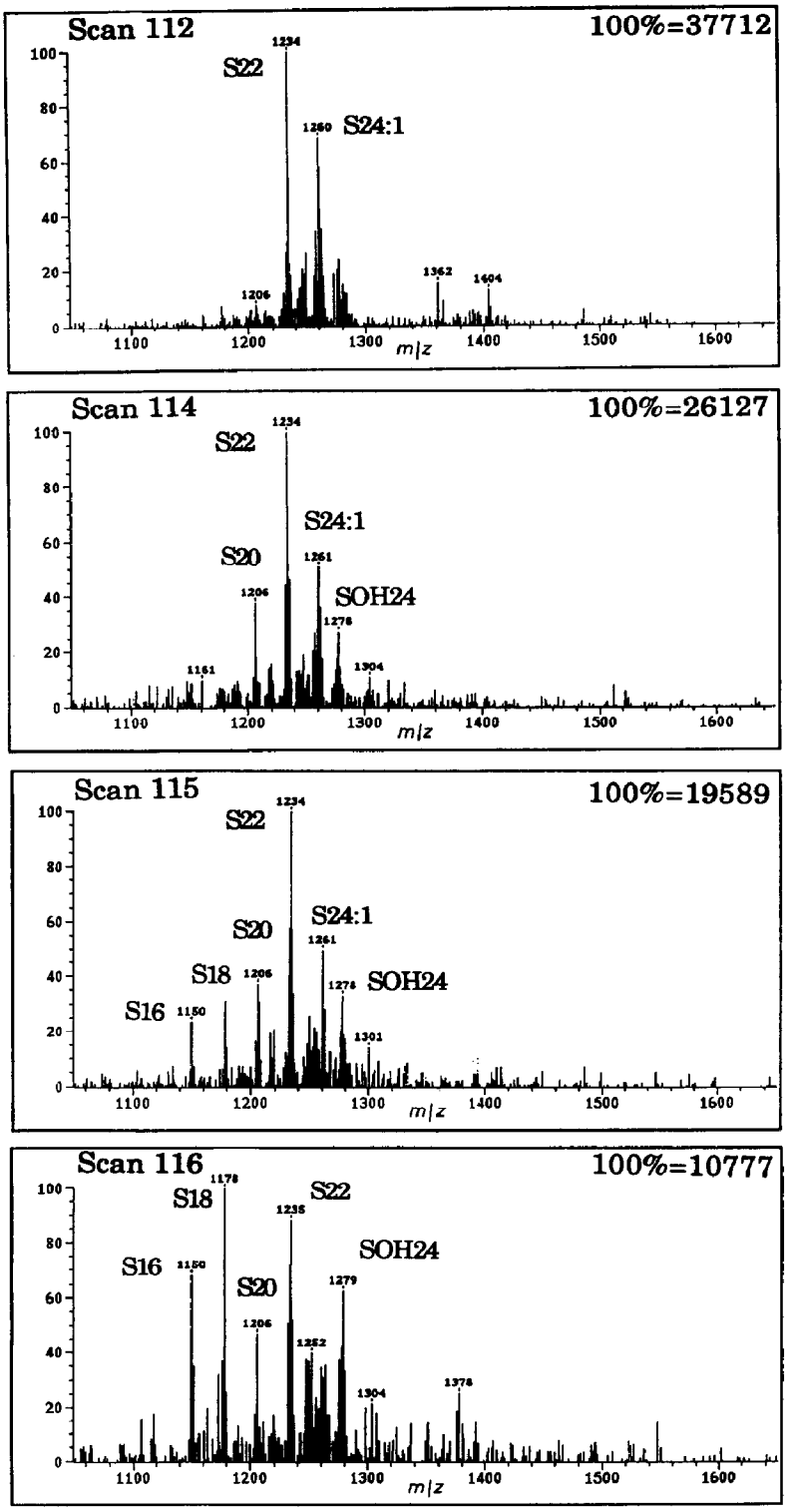

Fig. 6. Scans 112, and 114–116 from t.l.c.–m.s. of the ganglioside GM3 described in Fig. 5.

informative and time-saving, although conditions are still needed for the desorption of larger molecules, to increase the sensitivity, and to minimise contamination of the ion source by the matrix. The receptor glycolipid for the diarrhoea-inducing *E. coli* K99 has been identified[23] by t.l.c.–m.s. The mixed glycolipids prepared from the target epithelial cells of piglet small intestine were used for overlay with the ^{35}S-methionine-labelled bacteria and the detected receptor-active doublet band was shown to be the trisaccharide ganglioside GM3 with *N*-glycolylneuraminic acid. The two bands showed identical saccharide sequences but differed in the structure of the ceramide. T.l.c.–m.s. is especially useful for the identification of mixtures of products formed by chemical modification

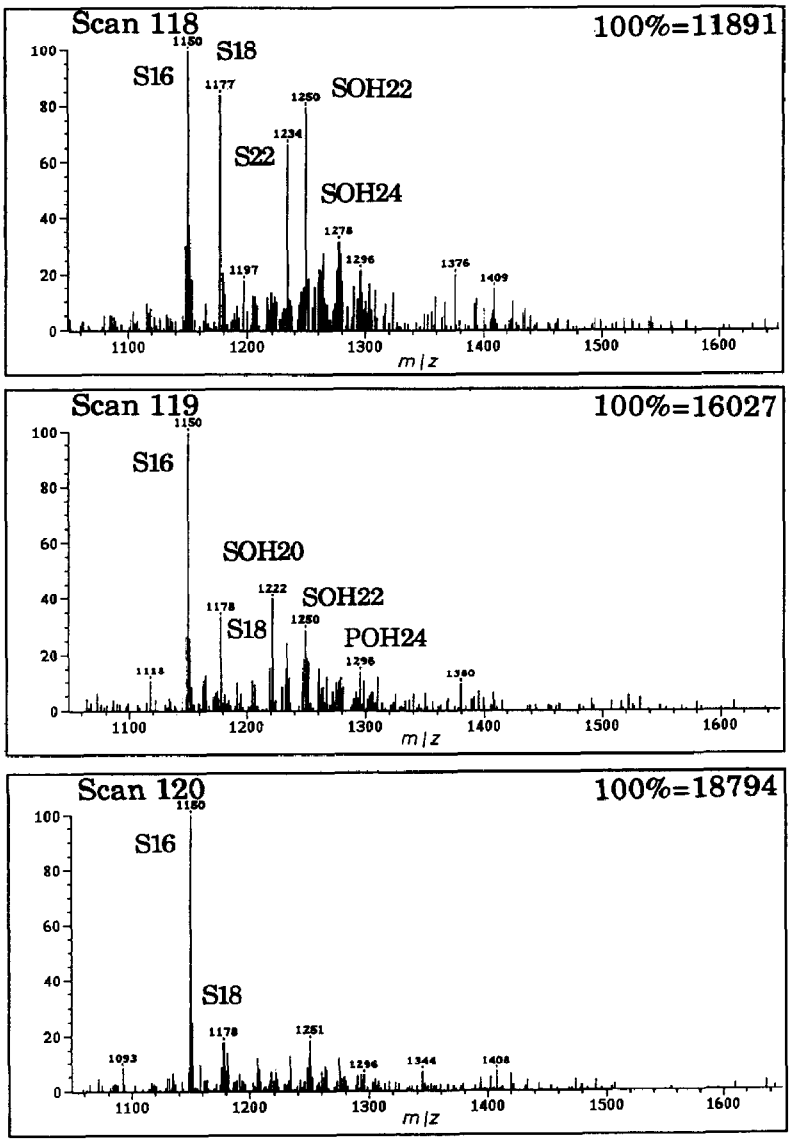

Fig. 7. Scans 118–120 from t.l.c.–m.s. of the ganglioside GM3 described in Fig. 5.

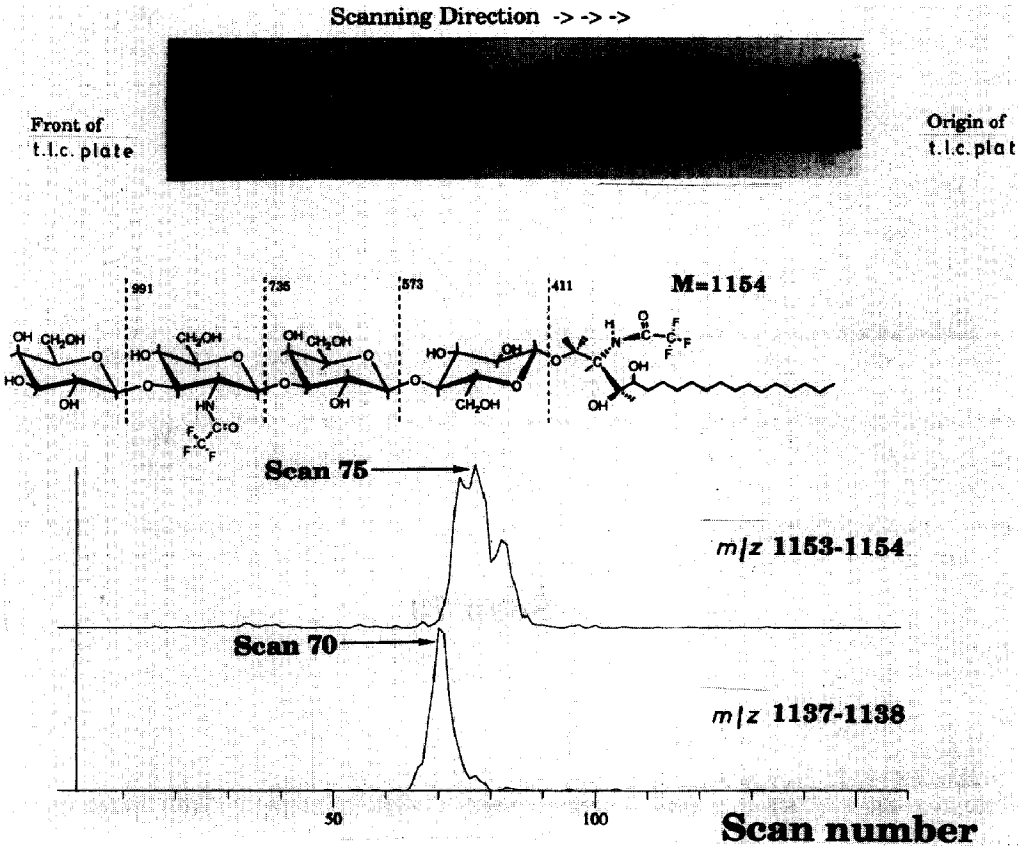

Fig. 8. Data from t.l.c.–m.s. of the chemically modified gangliotetraosylceramide β-Gal-(1→3)-β-GalNAc-(1→4)-β-Gal-(1→4)-β-Glc-ceramide. The glycolipid was catalytically hydrogenated and subjected to trifluoroacetolysis. A major and a minor (faster-moving) band were separated partially, and the selected ion curves recorded from the M region of the two spots differ by 16 mass units, which indicated the major band to contain phytosphingosine and the minor band to contain dihydrosphingosine (*cf.* Fig. 1).

of known receptor-active glycolipids, and most of the predicted products may be identified and compared with overlay results. The laborious alternative is a repeated fractionation by h.p.l.c. before m.s. and overlay, also requiring larger amounts of material.

One important aspect of t.l.c.–f.a.b.-m.s. is that the diffusion of substances in the matrix is so limited that it does not impair the original resolution in t.l.c. Distinct differentiation of glycolipids was possible based on long-chain bases and fatty acids. The order or mobility of such species was in good agreement with earlier data based on direct-probe e.i.-m.s. of derivatised monoglycosylceramides of different tissue origins and ceramide composition[28]. The negligible diffusion means that only the freshly released molecules from the silica gel are involved in the sputtering process, at the shortest distance from their site of origin. Probably only very small proportions are released from the spots, since the results were highly reproducible many hours after the initial application of the matrix.

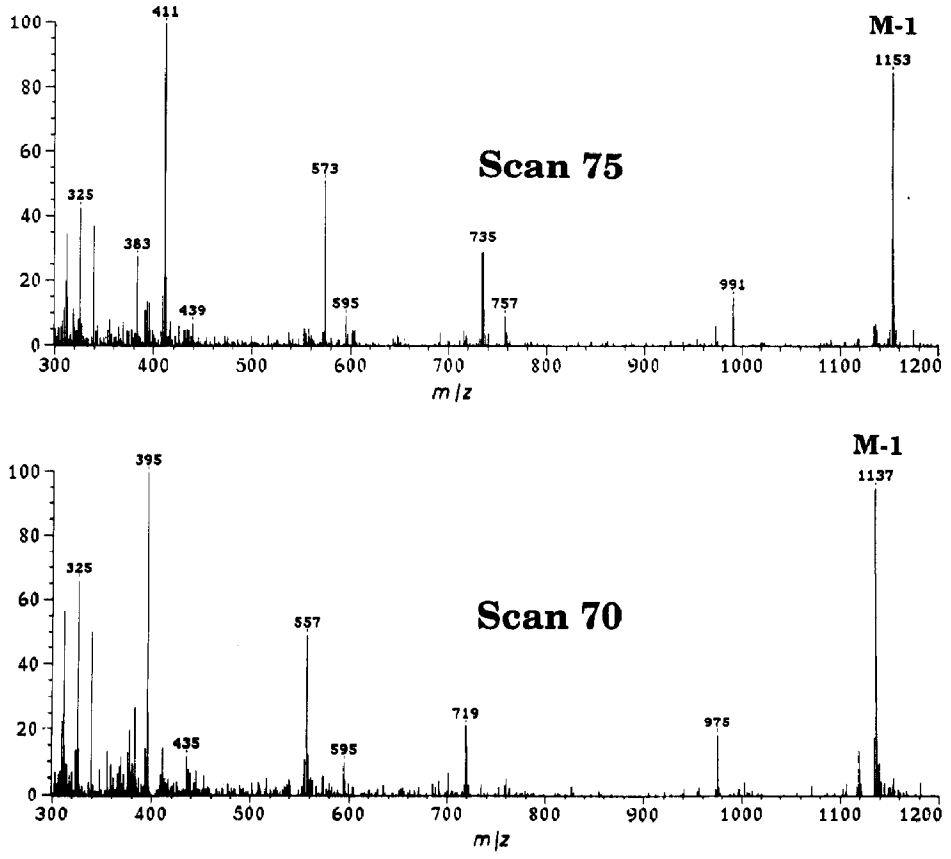

Fig. 9. Mass spectra from scans 70 and 75 in Fig. 8. Sequence ions differing by 16 mass units confirmed the presence of dihydrosphingosine and phytosphingosine (*cf.* Fig. 1.).

We have observed[22] occasionally a transient increase in sensitivity when the matrix is almost lost from the plate. Glycolipids that were almost undetectable suddenly gave relatively intense spectra. This finding may mean that desorption is more efficient with a thin layer of matrix, possibly because the xenon beam has direct access to the sample.

In a somewhat different approach (ref. 30 and references therein) substances separated in t.l.c. have been analyzed by l.s.i.-m.s. After separation, narrow strips were cut out from the alumina-backed plate and mounted on the target probe. Both matrix and extraction solvent were applied to the silica gel prior to analysis. In this way neoglycolipids with up to eight sugars were analyzed. However, the resolution into molecular species as shown here is not possible with this approach.

ACKNOWLEDGMENTS

We thank The Swedish Medical Research Council, The Swedish Council for Planning and Coordination of Research, The Wallenberg Foundation, and Symbicom Ltd. for financial support, and Jörgen Bergström for help with the illustrations.

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