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Two-dimensional mapping by high-performance liquid chromatography of pyridylamino oligosaccharides from various glycosphingolipids

Kanako Ohara*, Mutsumi Sano, Akihiro Kondo and Ikunoshin Kato

Biotechnology Research Laboratories, Takara Shuzo Co., Ltd., 3-4-1 Seta, Otsu, Shiga 520-21 (Japan)

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ABSTRACT

A method to map sugars two-dimensionally for the analysis of the structures of oligosaccharides from glycosphingolipids is described. Nine neutral and ten acidic oligosaccharides were obtained from glycosphingolipids by endoglycoceramidase digestion and labelled with 2-aminopyridine. The pyridylamino oligosaccharides were clearly separated by high-performance liquid chromatography on commercially available C_{18}-silica and amide-silica column. All compounds tested were mapped without any overlapping. The separation of the pyridylamino oligosaccharides on the C_{18}-silica column depended on the numbers and positions of sialic acid and N-acetylhexosamine residues; on the amide-silica column, the separation depended on the total number of sugar residues.

INTRODUCTION

The oligosaccharides on cell surfaces change in type and amount when the cells differentiate or become malignant, so the involvement of oligosaccharides in cell recognition is of much interest [1]. A glycosphingolipid consists of a hydrophobic ceramide moiety and a hydrophilic oligosaccharide moiety, making it amphipathic. Many ceramides are only slightly different from other ceramides, so analysis of the stuctures of the oligosaccharides in glycosphingolipids can be difficult, especially when chromatography is used. Analytical methods for the identification of the structures of glycosphingolipids involve thin-layer chromatography, mass spectrometry, and nuclear magnetic resonance (NMR). However, more than a trace amount of purified sample is needed in these methods, and only certain structures can be identified [2–5].

Hase *et al.* [6] developed a method for a kind of pyridylamination in which there is fluorescence labelling of oligosaccharides with 2-aminopyridine. The combination of pyridylamination with high-performance liquid chromatography (HPLC) makes it possible to analyse oligosaccharides sensitively, with a detection limit of 50 fmol of pyridylamino (PA) oligosaccharides. PA-oligosaccharides from glycoproteins have been analysed by HPLC on reversed-phase and normal-phase columns, and each elution position has been plotted two-dimensionally by Hase *et al.* [7] and Tomiya *et al.* [8]. Higashi *et al.* [9] have reported a method for analysis in which oligosaccharides from glycosphingolipids are labelled with UV-absorbing reagent, *p*-aminobenzoic acid ethyl ester (ABEE), and analysed by HPLC. The sensitivity of detection of the ABEE derivatives was inadequate, and at least 500 pmol of oligosaccharides were needed for a typical analysis. Large ABEE-oligosaccharides, such as ABEE-G_{T1b}, were not mapped with the reversed-phase column, since they were eluted in the void volume.

Kondo *et al.* [10] reported an improved method in which sialic acid residues are not released by pyridylamination. We used this method for the analysis of the structures of oligosaccharides obtained from glycosphingolipids. PA-oligosaccharides were ana-

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TABLE I

ELUTION POSITIONS OF PA-OLIGOSACCHARIDES FROM PALPAK TYPE R AND S COLUMNS

Elution positions on HPLC columns are expressed as the numbers of glucose units of the corresponding standard glucose oligomers. The conditions for HPLC are described in Experimental. The abbreviations of Svennerholm [16] for gangliosides are used for the oligosaccharides of the corresponding gangliosides. The other oligosaccharides are abbreviated as recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [17]. Glc = glucose; Gal = galactose; GlcNAc = N-acetylglucosamine; GalNAc = N-acetylgalactosamine; SA = N-acetylneuraminic acid; NeuGc = N-glycolylneuraminic acid.

Abbreviation	Structure of oligosaccharides	No. of Glc units	
		Type R	Type S
Lac	Galβ1-4Glc	0.9	2.1
Gg_3	GalNAcβ1-4Galβ1-4Glc	1.1	2.6
Gg_4	Galβ1-3GalNAcβ1-4Galβ1-4Glc	0.9	3.8
G_{M3}-A	SAα2-3Galβ1-4Glc	3.2	2.1
G_{M3}-G	NeuGcα2-3Galβ1-4Glc	2.9	2.5
G_{M2}	GalNAcβ1-4Galβ1-4Glc	3.3	2.6
	3 | SAα2		
G_{M1}	Galβ1-3GalNAcβ1-4Galβ1-4Glc	3.3	3.4
	3 | SAα2		
G_{D3}	SAα2-8SAα2-3Galβ1-4Glc	4.5	2.7
G_{D2}	GalNAcβ1-4Galβ1-4Glc	4.5	3.2
	3 | SAα2-8SAα2		
G_{D1a}	Galβ1-3GalNAcβ1-4Galβ1-4Glc	5.2	3.5
	3 3 | | SAα2 SAα2		
G_{D1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glc	4.3	4.0
	3 | SAα2-8SAα2		
G_{T1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glc	6.3	4.0
	3 3 | | SAα2 SAα2-8SAα2		
G_{Q1b}	Galβ1-3GalNAcβ1-4Galβ1-4Glc	10.0	4.3
	3 3 | | SAα2-8SAα2 SAα2-8SAα2		
Gb_3	Galα1-4Galβ1-4Glc	1.2	2.8
Gb_4	GalNAcβ1-3Galα1-4Galβ1-4Glc	2.6	3.4
Gb_5	GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glc	3.6	4.0
Lc_3	GlcNAcβ1-3Galβ1-4Glc	2.2	2.7
Lc_4	Galβ1-3GlcNAcβ1-3Galβ1-4Glc	2.7	3.5
nLc_4	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	2.4	3.6

lysed by HPLC on an C_{18} silica column and also on an amide-silica column. We plotted the elution positions of the PA-oligosaccharides on a two-dimensional sugar map. This mapping is useful for the analysis of the structures of the oligosaccharides in glycosphingolipids.

EXPERIMENTAL

Oligosaccharides and glycosphingolipids

The structure of and abbreviations for the oligosaccharides used are listed in Table I. The oligosaccharides were released from the various glycosphingolipids by digestion with endoglycoceramidase from *Rhodococcus* sp. strain G-74-2 (Seikagaku Kogyo, Tokyo, Japan). Glycosphingolipids G_{M1}, G_{M2}, G_{M3}-A, G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b} were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Glycosphingolipids Gb_3, Gb_4, Gb_5, Gg_4, G_{M3}-G, and G_{D3} were purchased from BioCarb Chemicals (Lund, Sweden). Oligosaccharides Lc_4 and nLc_4 were purchased from Seikagaku Kogyo. PA-Gg_3 was prepared by acid hydrolysis of PA-G_{M2} in 20 m*M* HCl for 1 h at 90°C. PA-Lc_3 and PA-G_{D2} were prepared from PA-Lc_4 and PA-G_{D1b}, respectively, by digestion with β-galactosidase from an *Aspergillus* sp. as described below.

Enzyme reaction

The glycosphingolipids (10–300 nmol) were digested at 37°C for 15 h with endoglycoceramidase (0.5–10 mU) in 10–300 μl of 50 m*M* sodium acetate buffer (pH 6.0) containing 5–150 μg of sodium taurodeoxycholate [11]. The oligosaccharides released were detected by thin-layer chromatography (HPTLC aluminium sheets, Silica Gel 60, E. Merck, Darmstadt, Germany) developed in 1-butanol–acetic acid–water (2:1:1), and made visible with orcinol–H_2SO_4 reagent [12].

Pyridylamino derivatization

PA-oligosaccharides were obtained by the method of Kondo *et al.* [10] with use of a Palstation apparatus (Takara Shuzo, Kyoto, Japan). Then 100 nmol of the oligosaccharide obtained were aminated with 20 mg of the fluorescent reagent 2-aminopyridine in 10 μl of acetic acid at 90°C for 1 h. To the reaction mixture, 10 μl of acetic acid containing 1.95 mg of dimethylamine borane was added, and a reductive reaction was allowed to proceed at 80°C for 1 h. The reaction mixture was evaporated at reduced pressure with a stream of N_2 gas, and the residue was put on a Sephadex G-15 column (200 × 8 mm I.D.; Pharmacia, Uppsala, Sweden) to remove excess reagents [7]. The fractions in each peak were hydrolysed in 4 *M* trifluoroacetic acid at 100°C for 3 h, and the hydrolysate was analysed with a Beckman Ultrasphere ODS column (250 × 4.6 mm I.D.; 5 μm particle size) by the method of Takemoto *et al.* [13]. The fractions in which the residue of the reduced end was PA-glucose were regarded as containing PA-oligosaccharides.

The purified PA-oligosaccharides were checked by ^1H NMR measurements, digestion with various exoglycosidases, and gas chromatography by the method of Mega and Ikenaka [14].

Exoglycosidase digestion

To prepare PA-Lc_3 or PA-G_{D2}, 10 pmol of PA-Lc_4 or PA-G_{D1b} were digested with 0.2 U of β-galactosidase (E.C. 3.2.1.23) from an *Aspergillus* sp. (Toyobo, Osaka, Japan) in 0.1 *M* acetate buffer (pH 5.0) at 37°C for 15 h. The structures of PA-G_{D2} and PA-Lc_3 were checked by exoglycosidase digestion and HPLC analysis only, because the amounts of the purified samples were small. By HPLC with the two columns described below, an acid hydrolysate of PA-G_{D2} was eluted at the same position as PA-Gg_3. Next, 10 pmol of PA-nLc_4 were digested at 37°C for 15 h with 2 mU of β-galactosidase from bovine testes (Seikagaku Kogyo) in 0.1 *M* sodium citrate–phosphate buffer (pH 4.3) containing 1% bovine serum albumin and 10% glycerol. PA-Lc_3 was eluted at the same position as PA-nLc_4 digested with β-galactosidase from bovine testes. Then, 10 pmol of PA-Lc_3 were digested at 37°C for 15 h with 2 mU of β-N-acetylhexosaminidase (E.C. 3.2.1.52) from jack beans (Seikagaku Kogyo) in 0.1 *M* citrate–phosphate buffer (pH 5.0), and the digest was eluted at the same position as PA-Lac during HPLC.

Analysis of PA-oligosaccharides by HPLC

PA-oligosaccharides were separated with a Shimadzu LC-6A HPLC system with the two kinds of columns.

Column I was a C_{18} silica column, Palpak Type R column (250 × 4.6 mm I.D.; 5 μm particle size;

Takara Shuzo), used in reversed-phase HPLC. The flow-rate was 1.0 ml/min at 40°C. Solvent A was 50 m*M* acetic acid, adjusted to pH 5.0 with triethylamine, and solvent B was solvent A containing 0.5% 1-butanol. After injection of a sample into the column equilibrated with solvent A, the ratio of solvent B was increased on a linear gradient to 50% over a 50-min period. PA-oligosaccharides were detected by their fluorescence, with excitation and emission wavelengths of 320 and 400 nm, respectively.

Column II was an amide-silica column, Palpak Type S column (250 × 4.6 mm I.D.; 5 μm particle size; Takara Shuzo), used in size-fractionation HPLC. The flow-rate was 1.0 ml/min at 40°C. Solvent C was a 25:75 mixture of 200 m*M* acetic acid, adjusted to pH 7.3 with triethylamine, and acetonitrile, and solvent D was a 50:50 mixture of the same two components. After injection of a sample into the column equilibrated with solvent C, the ratio of solvent D was increased on a linear gradient to 50% over a 25-min period. PA-oligosaccharides were detected by their fluorescence, with excitation and emission wavelengths of 310 and 380 nm, respectively.

Construction of two-dimensional maps

To standardize the elution positions of PA-oligosaccharides on HPLC columns, PA-isomaltooligosaccharides (Takara Shuzo) prepared from a dextran hydrolysate were analysed on the same columns and with the same solvents and conditions. Glucose units were defined by comparison of the elution position of each PA-oligosaccharide with that of the standard glucose oligomers. Glucose unit numbers obtained by reversed-phase HPLC (Palpak Type R column) were expressed on the abscissa, and those obtained by size-fractionation HPLC (Palpak Type S column) were expressed on the ordinate, when two-dimensional maps were plotted.

Preparation of PA-oligosaccharides from bovine brain acetone powder

The gangliosides were extracted from 50 mg of bovine brain acetone powder by the method of Folch *et al.* [15]. The extract containing gangliosides was digested at 37°C for 24 h with 2.5 mU of endoglycoceramidase in 100 μl of 50 m*M* sodium acetate buffer (pH 6.0) containing 50 μg of sodium taurodeoxycholate. Then two 1 mU amounts of endoglycoceramidase were added, 24 h apart, and digestion was continued for 24 h after the second addition. The reaction mixture was evaporated, and the residue was directly labelled with 2-aminopyridine as described above. The reaction mixture was evaporated with a stream of N_2 gas under reduced pressure. The PA derivatives of the oligosaccharides were purified by gel filtration on a Sephadex G-15 column. Some of the fractions from HPLC were lyophilized and dissolved in 50 μl of 0.1 *M* sodium acetate buffer (pH 4.5). The solution was incubated with 50 mU of sialidase (E.C. 3.2.1.18) from *Arthrobacter ureafaciens* (Nacalai Tesque, Kyoto, Japan) at 37°C for 15 h.

RESULTS AND DISCUSSION

HPLC of standard glucose oligomers

When a single variety of PA-oligosaccharide is analysed by HPLC, it may elute at different times if the column is from different lots, or if the column contents have degraded, or if there are small differences in the preparation of the solvents, so it is necessary to standardize the elution position. The results of standardization are shown in Fig. 1. On both columns, the smaller oligomers were eluted sooner, and each oligomer was eluted separately.

HPLC analysis of PA-oligosaccharides

Nineteen kinds of PA-oligosaccharide prepared from glycosphingolipids were analysed by HPLC with the two types of column. Glucose units were defined by comparison of the elution position of each PA-oligosaccharide with that of the standard glucose oligomers (Table I). To check for reproducibility of the results, and for possible effects of differences in the lot or the number of times the column was used, three runs were done. Different columns were used for each run, with two new columns of different lots and one used column (several to dozens of hours of use) of the same lot as one of the new columns. For each PA-oligosaccharide, except PA-G_{Q1b}, the numbers of glucose units obtained from HPLC were exactly the same when rounded off from two to one decimal places. Thus, column degeneration did not affect the results, except for PA-G_{Q1b}. The numbers of glucose units of PA-G_{Q1b} were the same on the amide-silica column

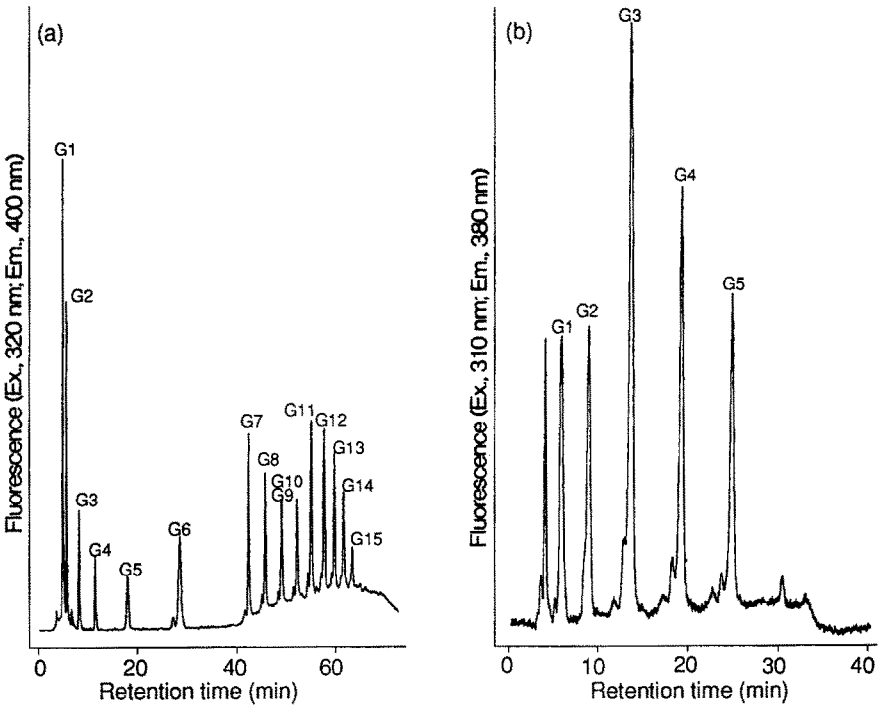

Fig. 1. HPLC profiles of pyridylaminoglucose oligomers (a) on a C_{18} silica column and (b) on an amide-silica column. The gradient starts at time 0. Glucose unit numbers are indicated.

in the three runs, but those on the C_{18} silica column became smaller as the column contents gradually degraded.

On the C_{18} silica column, HPLC of the nineteen kinds of PA-oligosaccharides took 50 min; on the amide-silica column, it took 25 min.

Two-dimensional mapping of PA-oligosaccharides

The glucose units obtained were plotted on a two-dimensional map (Fig. 2).

An NeuAc residue bonded to a galactose residue by an α2–3 linkage retarded elution from the C_{18} silica column by two glucose units, but did not retard elution from the amide-silica column. The following four pairs gave such results, with the second of each pair eluted later: PA-Lac and PA-G_{M3}-A; PA-Gg_3 and PA-G_{M2}; PA-G_{M1} and PA-G_{D1a}; and PA-G_{D1b} and PA-G_{T1b}.

An NeuAc residue bonded to an NeuAc residue by an α2–8 linkage retarded elution from the C_{18} silica column by one glucose unit, and it retarded elution from the amide-silica column by 0.5 glucose

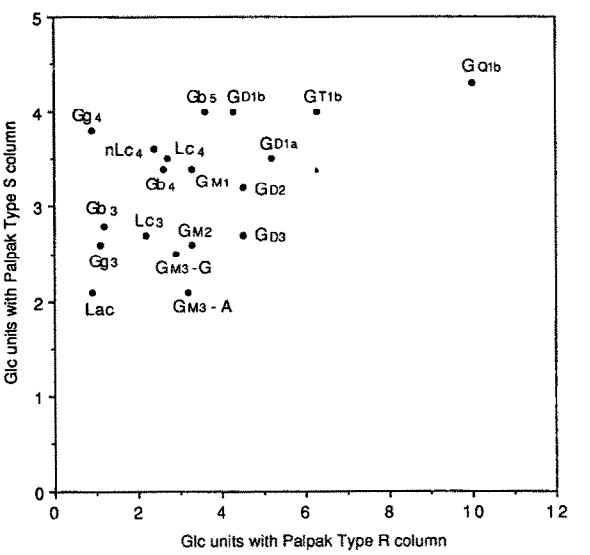

Fig. 2. Two-dimensional sugar map of PA-oligosaccharides. The numbers on the abscissa and ordinate indicate the glucose unit numbers obtained on the Palpak Type R and S columns, respectively.

units. The pairs giving such results were: PA-G_{M3}-A and PA-G_{D3}; PA-G_{M2} and PA-G_{D2}; PA-G_{M1} and PA-G_{D1b}; and PA-G_{D1a} and PA-G_{T1b}.

PA-G_{M3}-A was separated from PA-G_{M3}-G.

A GalNAc residue bonded by a β1–4 linkage did not retard elution from the C_{18} silica column, but it retarded elution from the amide-silica column by 0.5 glucose units. The pairs giving such results were: PA-Lac and PA-Gg_3; PA-G_{M3}-A and PA-G_{M2}; and PA-G_{D3} and PA-G_{D2}.

A galactose residue did not retard elution from the C_{18} silica column, but it retarded elution from the amide-silica column by one glucose unit. The pairs giving such results were: PA-G_{M2} and PA-G_{M1}; and PA-G_{D2} and PA-G_{D1b}.

PA-Gg_4, PA-Gg_3, PA-Lac, and PA-Gb_3 were eluted from the C_{18} silica column at a position close to the void volume, and they were not separated well. On the amide-silica column, their separation was satisfactory.

The elution positions of the PA-oligosaccharides did not overlap, and were as predicted, except for the number of glucose units of PA-Gg_4 on the amide-silica column (Fig. 2). To judge from the results with PA-Gg_3 and PA-G_{M2}, PA-G_{M1} and PA-G_{D1a}, and PA-Lac and PA-G_{M3}-A, PA-Gg_4 should have eluted at the same position as PA-G_{M1} on the amide-silica column; all of these pairs are different by the presence or absence of an NeuAc residue bonded to a galactose residue by an α2–3 linkage. The elution position suggested that the structure of PA-Gg_4 shown in Table I might be different from the correct one, so the structure was checked as follows: (i) the elution position of PA-Gg_4 was the same as that of the acid hydrolysate of PA-G_{M1}; (ii) the digests of PA-Gg_4 with β-galactosidase from the *Aspergillus* sp. were eluted at the same position as PA-Gg_3 on a two-dimensional map; (iii) ^{1}H NMR measurements were consistent with the structure in Table I. Therefore, the structure in Table I was correct. We do not know why PA-Gg_4 was retarded on the amide-silica column. Perhaps PA-Gg_4 interacted with the amide substituents or silica gel of the amide-silica column.

Application of the two-dimensional map for characterization of PA-oligosaccharides from bovine brain acetone powder

PA-oligosaccharides prepared from bovine brain acetone powder were analysed by HPLC with the C_{18} silica column (Fig. 3a). Portions of the fractions of peaks A to E were treated by acid hydrolysis, followed by HPLC with a Beckman Ultrasphere ODS column by the method of Takemoto *et al.* [13]. The peaks B to E, in which the residue of the reduced end was PA-glucose, were then analysed on the amide-silica column (Fig. 3b). Each fraction gave

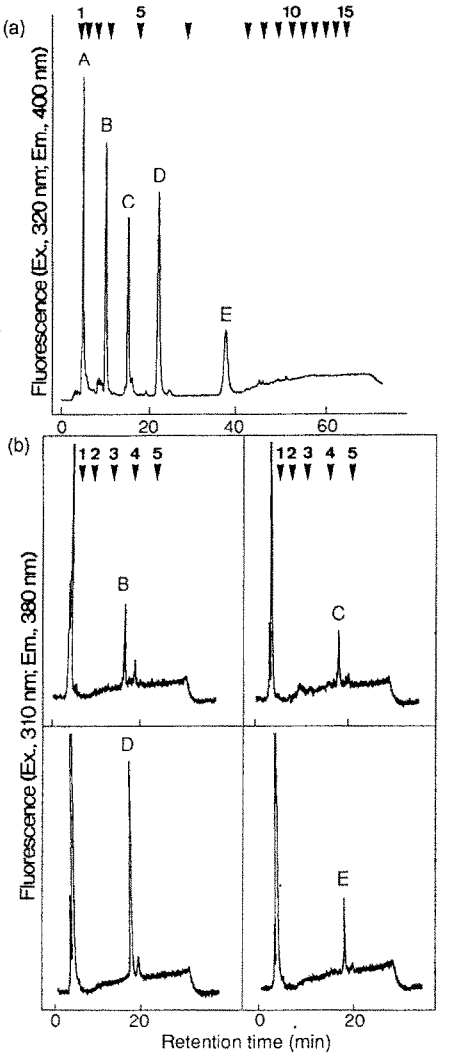

Fig. 3. HPLC profiles of PA-oligosaccharides released from bovine brain acetone powder. (a) PA-oligosaccharides obtained from 160 μg of bovine brain acetone powder were injected into a Palpak Type R column. The gradient starts at time 0. Glucose unit numbers are indicated. (b) Each peak B–E was chromatographed again on a Palpak Type S column.

a single peak, and each elution position on two HPLC columns was plotted on a two-dimensional map. The elution position of each fraction coincided with one of the four standard oligosaccharides, as follows: B and G_{M1}; C and G_{D1b}; D and G_{D1a}; and E and G_{T1b}. To check these results, the fractions of the peaks were digested with exoglycosidases, and the digests were analysed by HPLC. Then the elution positions were plotted on a two-dimensional map. We found that peak B was converted into PA-G_{M2} by β-galactosidase from an *Aspergillus* sp., and that peak C was converted into PA-G_{D2}. Both peaks D and E were converted into PA-Gg_4 by sialidase, but part remained as PA-G_{M1}. These results of exoglycosidase digestion are consistent with the structures we propose for the compounds corresponding to peaks B to E.

In this use of two-dimensional mapping, all of the major peaks correspond to points on the map. If new oligosaccharides that correspond to nothing on the map are found in a sample, the sample should be digested with exoglycosidases and then analysed by HPLC. The use of pyridylamination, together with two-dimensional mapping and ^1H NMR analysis, should make structural analysis of complicated sugar chains from small amounts of glycosphingolipids easier than before.

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