PHOSPHONOSPHINGOGLYCOLIPIDS, A NEW CLASS OF IONIC SPHINGOGLYCOLIPIDS

Akira Hayashi and Toshiko Matsubara

Department of Chemistry, Faculty of Science and Technology, Kinki University

Since the discovery of the direct carbon-phosphorus bond in nature as aminoethylphosphonate(ciliatine) in bovine rumen protozoa by Horiguchi and Kandatsu (1), the existence of a carbon-phosphorus bond as aminoalkylphosphonate has been found successively in lipids (2), glycoproteins (3), antibiotics (4) and peptides (5).

In the field of lipids, three kinds of phosphonolipids, namely, sphingophosphonolipids (2,6), glycerophosphonolipids (7) and ciliatocholic acid (8), have been found. Rouser *et al.* (2) and Hori *et al.* (6) have found sphingophosphonolipids containing AEP, and we have found MAEP containing one (9).

The fourth type of lipid containing a C-P linkage, the animoalkylphosphonate (AEP and MAEP) derivatives of galactosyl ceramide, were found in our laboratory in 1975 (10), and they are named phosphonosphingoglycolipids. They are the first ionic glycolipids to be shown to be contained in the tissues of marine animals belonging to the Protostomia. As ionic glycolipids, gangliosides usually found in vertebrates are well known, and recently Hori *et al.* (11) have reported the presence of glucuronic acid containing glycolipid in the spermatozoa of a fresh-water bivalve.

Abbreviations: PnSGL, phosphonosphingoglycolipid; SGL, sphingoglycolipid; CMH, ceramide monohexoside; CDH, ceramide dihexoside; CTH, ceramide trihexoside; CTeH, ceramide tetrahexoside; CPH, ceramide pentahexoside; CPG, ceramide polyglycoside; Pn-, phosphono-; AEP, aminoethylphosphonate; MAEP, N-methylaminoethylphosphonate.

As for PnSGL, Araki *et al.* (12) reported the occurrence of PnCPG in *Aplysia kurodai*, however, its structure has not been determined yet.

In this paper we report the occurrence and distribution of PnSGL with various sugar chains in marine Gastropoda (*Turbo cornutus*, *Monodonta labio*, and *Chlorostoma argyrostoma turbinatum* (*C.a.t.*)) and Polyplacophora (*Liolophura japonica*) and also the partial characterization of the structure of PnSGL with a complex sugar chain (PnCPG) obtained from *C.a.t.*

MATERIALS AND METHODS

Isolation and purification of phosphonosphingoglycolipids:

Extraction of total lipids and separation of PnSGL and SGL were performed as outlined in Fig. 1.

Quantitative analysis:

Phosphorus was determined by the methods of King (13) and Bartlett (14). Total sugar was determined with the phenol-sulphuric acid reagent (15), hexosamine by the method of Elson and Morgan (16), and fucose by the method of Dische (17).

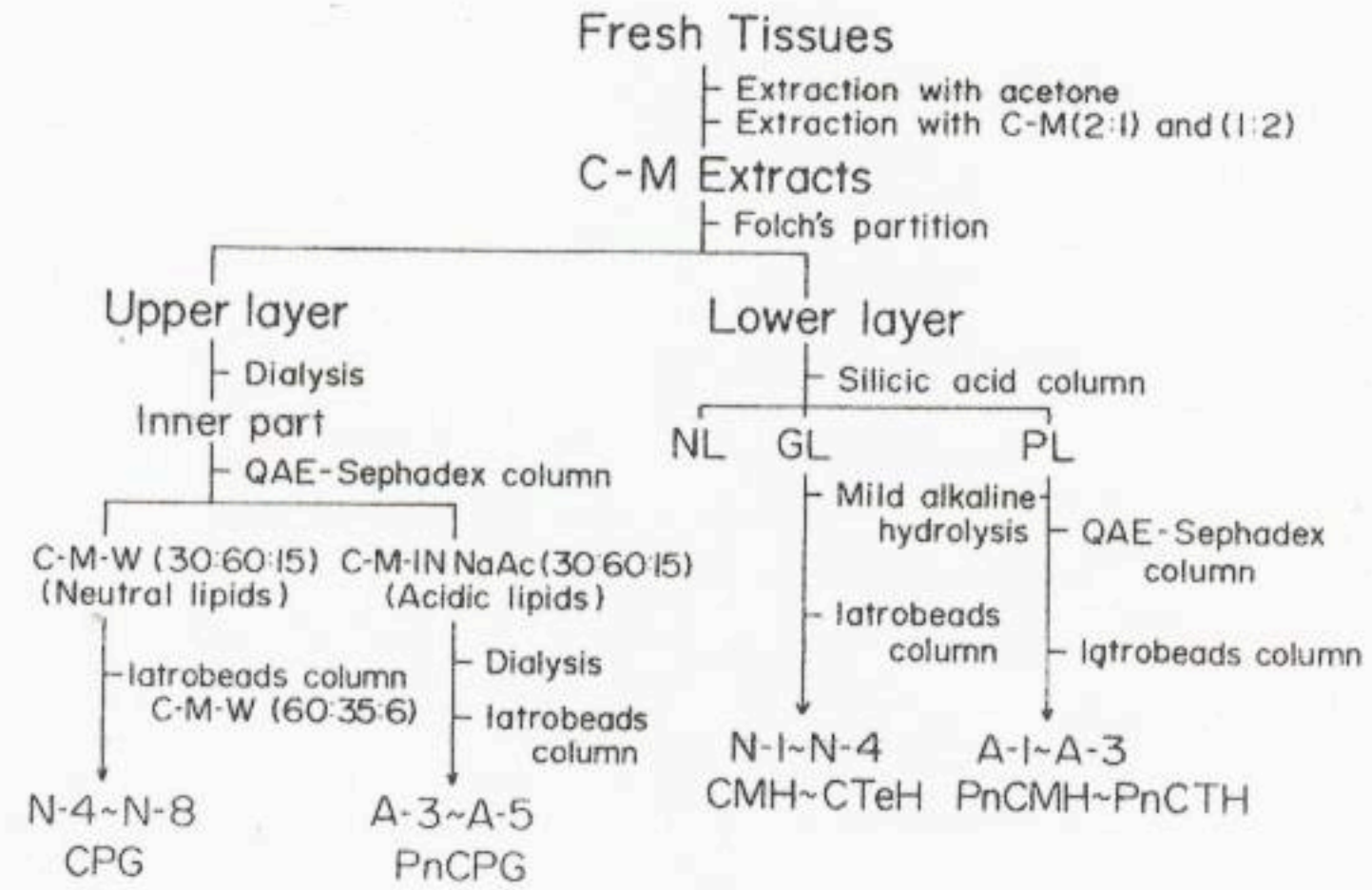

Fig. 1. Procedure of extraction and isolation of sphingoglycolipids and phosphonosphingoglycolipids.

Analysis of hydrolysis and degradation products of PnSGL by GLC and GC-MS:

Sugar aminoalkylphosphonate was liberated from PnSGL by partial acidic hydrolysis with 2N HCl at 100°C for 100 min., and the sugar moiety was released from the sugar aminoalkylphosphonate by hydrolysis with 2N HCl at 100°C for 15 h. Also partial hydrolysis of PnSGL with 0.05N HCl at 100°C for 5 h was carried out.

The sugar component was analyzed as trimethylsilyl (TMS) methyl glycoside (18) and also as alditol acetates (19).

For linkage analysis of the sugar moiety perdeuteromethylation was carried out by the method of Hakomori (20).

Parent SGL of PnSGL was obtained by hydrogen fluoride degradation of PnSGL according to the method used by Fischer *et al.* (21).

Aminoalkylphosphonate, long chain bases (LCB) and fatty acids were obtained by the methods described in the previous paper (18).

GC or GC-MS of TMS-derivatives of sugar aminoalkylphosphonate, aminoalkylphosphonate, methyl glycoside and LCB, and fatty acid methyl esters were performed as described previously (18).

Alditol acetates and partially methylated alditol acetates were analyzed on a 3% OV-225 column (2m) programmed from 150 to 190°C at 1°C/min and also on a 3% ECNSS-M column (1m) programmed from 150 to 210°C at 2°C/min.

RESULTS

PnSGL in marine gastropods:

TLC of SGL and PnSGL obtained from *C.a.t.*, PnCMH and PnCTH from *T. cornutus*, and reference SGLs is shown in Fig. 2. The structures of PnCMH and PnCTH of *T. cornutus* are characterized as 1-O-[6'-O-(N-methylaminoethylphosphonyl) galactosyl] ceramide (18) and 6'-O-MAEP-Gal(1→6)Gal(1→6)Gal(1→1)ceramide (22), respectively. We also determined that the structures of PnCMHs obtained from *M. labio* (23) and *C.a.t.* (24) are the same as PnCMH of *T. cornutus*.

Sphingoglycolipids of C.a.t.:

SGLs, N-1 through N-4 in Figs. 1 and 2, are considered to be CMH through CTeH from comparison with the Rf values of reference SGLs in the extreme right lane in Fig. 2, and also with those of CMH through CTH, which consist of only galactose, obtained from *T. cornutus* (25). GC analysis of the sugar composition as alditol acetates showed that N-1 is ceramide galactoside, N-2 ceramide

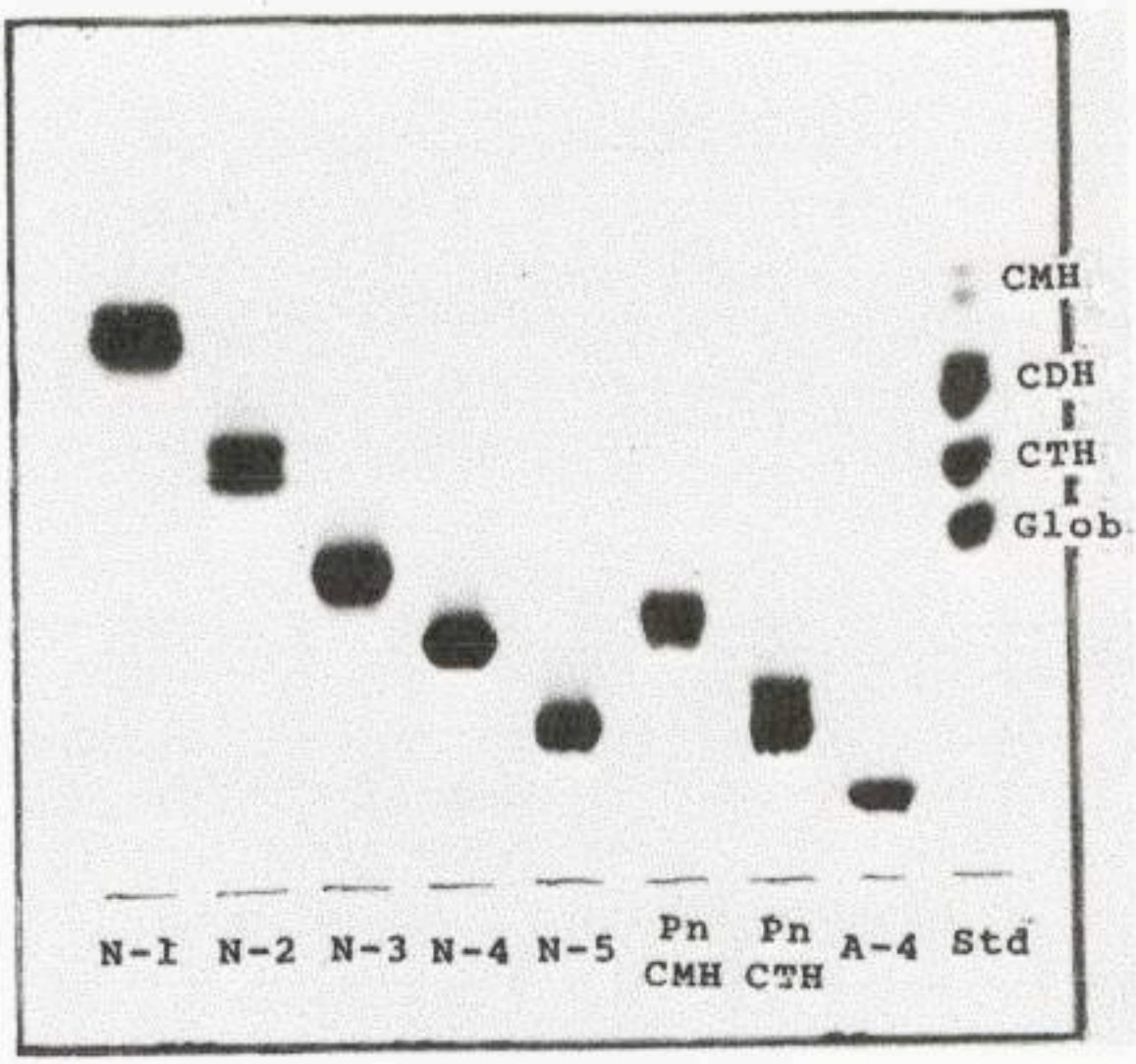

Fig. 2. Thin-layer chromatogram of neutral sphingoglycolipids (N-1 to N-5) and phosphonosphingoglycolipids (A-4) obtained from *C.a.t.*, PnCMH and PnCTH from *T. cornutus*, and reference SGLs (CDH, CTH, and globoside) from human erythrocytes except for CMH from ox brain. The solvent system used was chloroform-methanol-water (56:38:10), and detection of spots was carried out with an orcinol-sulfuric acid spray.

digalactoside, N-3 ceramide trigalactoside and N-4 ceramide tetragalactoside since these lipids contained only galactose. However, N-5 consists of not only galactose but also other sugars. It is clear that the attachment of only one aminoalkylphosphonyl group to SGL remarkably lowers the Rf value of PnSGL compared to the parent SGL. Then A-4 (PnCPG obtained from *C.a.t.*) must have a sugar chain consisting of more than four sugars.

Structural studies of PnCPG (A-4) of C.a.t.:

Quantitative analysis of phosphorus by King's and Bartlett's methods clarified that phosphorus of A-4 is all the C-P type and the molar ratio of phosphorus:fucose:hexosamine is 1.00:1.87:1.10 (Table 1). Analytical results for the sugar composition as alditol acetates (Fig. 3) and TMS-Me-glycoside indicate that the sugar moiety of A-4 consists of glucose, galactose, fucose, galactosamine, and 3-O-methyl-galactose, in the molar ratio of about 1:3:2:1:1. The presence of 3-O-Me-Gal was determined by comparison of its retention time and mass spectrum (Fig. 4) with those of an authentic sample.

Table 1. Quantitative analysis of A-4

		%	Molar ratio
P	King's method	1.49	1.00
	Bartlett's method	0.06	---
Sugar		45.5	5.55
Fucose		14.8	1.87
Hexosamine		11.8	1.10

TLC shown in Fig. 5 shows that the Rf value of the product of HF degradation of A-4 is almost the same as that of N-5. This fact indicates that the parent SGL of A-4 may have nearly the same sugar chain as N-5.

GC-MS of partially deuteromethylated alditol acetates of SGL obtained from A-4 by HF degradation produced acetates of 2,3,4-trideuteromethyl-fucitol, 2,4,6-trideuteromethyl-galactitol, 4,6-dideuteromethyl-galactitol, 2,3,6-trideuteromethyl-glucitol, 4,6-O-

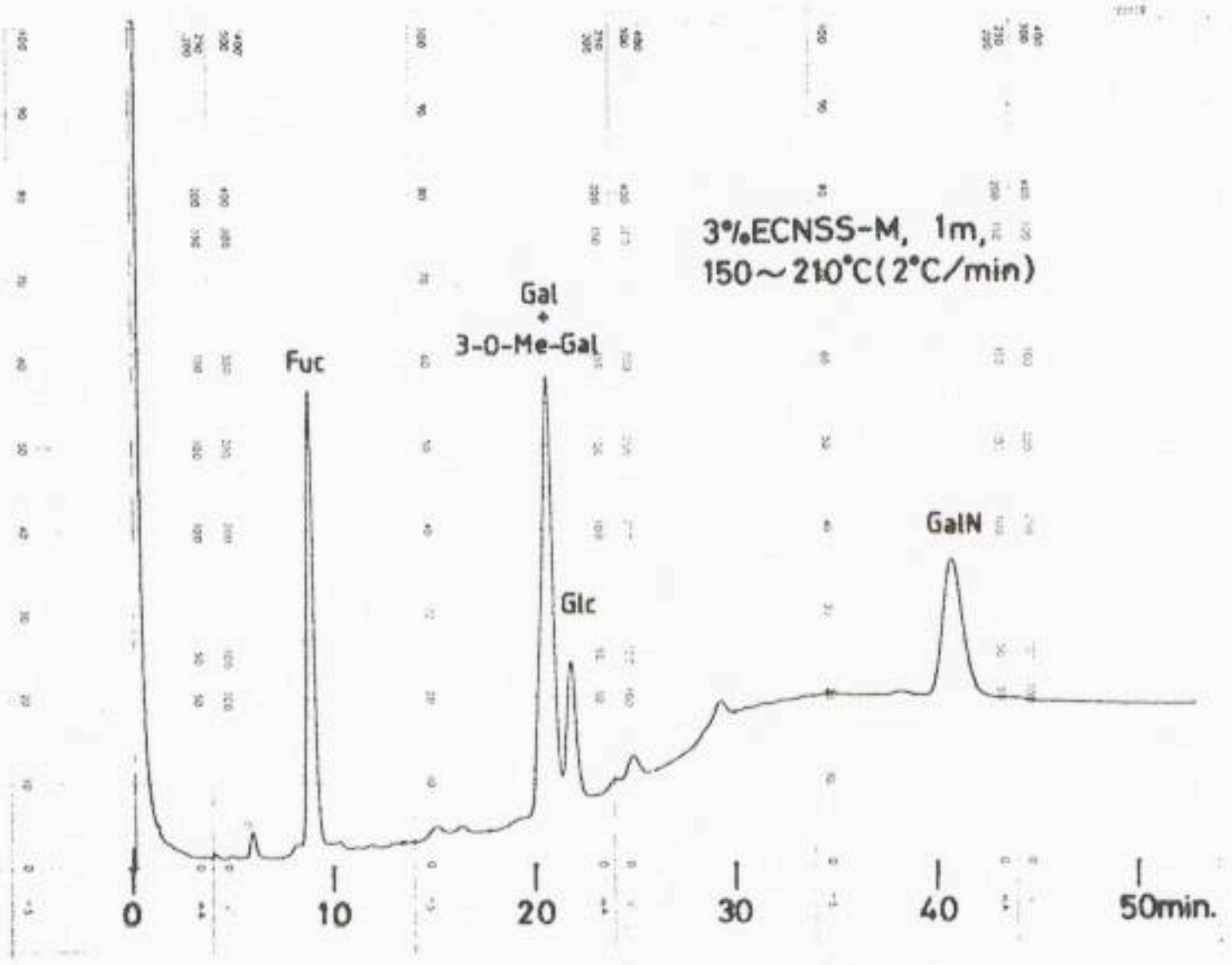

Fig. 3. Gas chromatographic analysis of alditol acetates obtained from A-4.

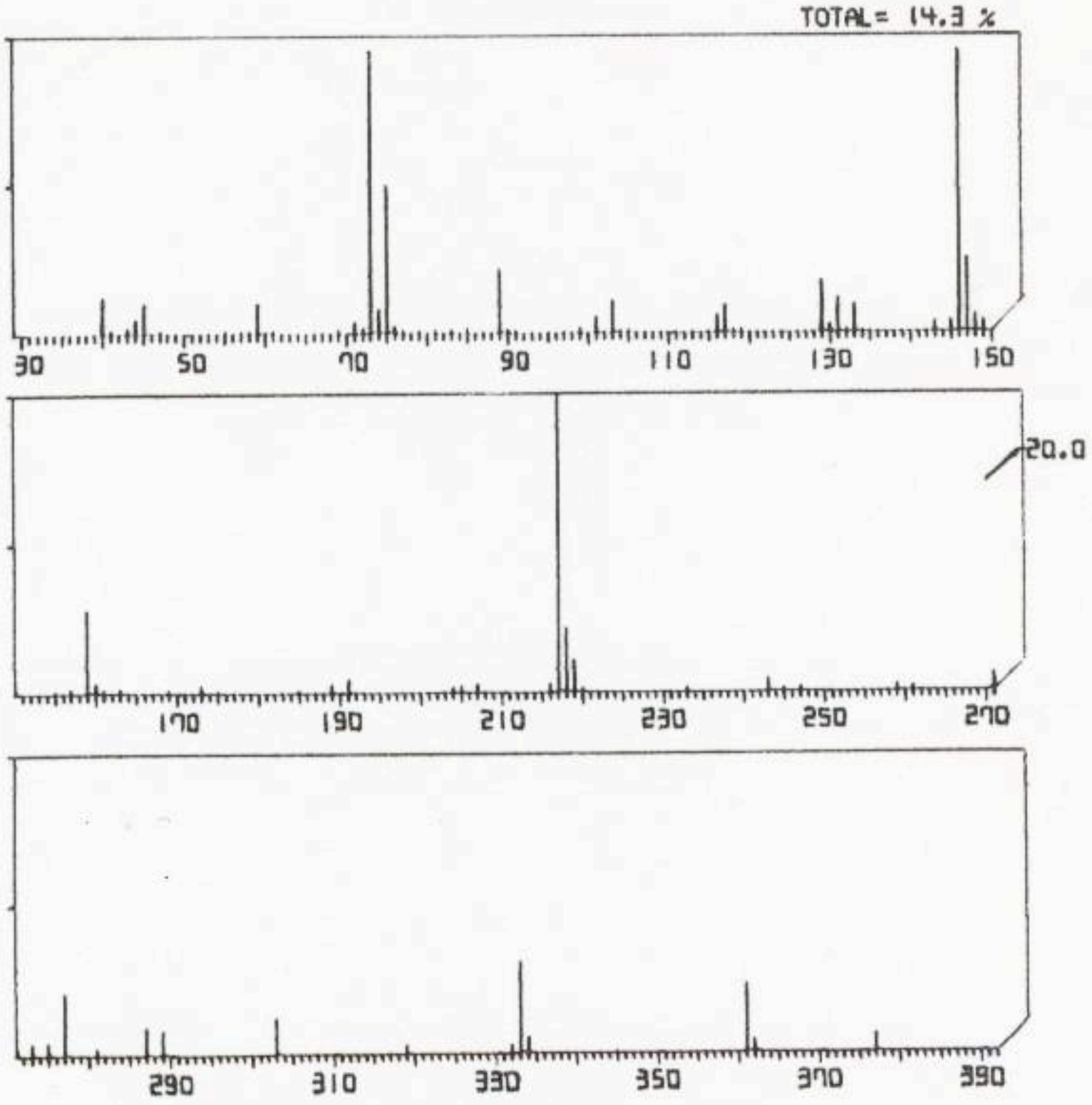

Fig. 4. Mass spectrum of TMS-methyl-3-O-methyl-galactoside. The ionization energy was 70eV.

dideuteromethyl-2-deoxy-2-N-deuteromethyl-acetamidogalactitol, and 4.6-dideuteromethyl-3-O-methyl-galactitol (Fig. 6). From the results we assume that 3-O-Me-Gal is located on the non-reducing terminal of the sugar chain and fucose may be attached to its 2-position.

GC-MS of the water soluble component of A-4 revealed that the C-P component was MAEP and this was shown to be attached to the 6-position of aldohexose from its mass spectrum shown in Fig. 7. This hexose was in turn determined as galactose after hydrolysis of MAEP-hexose followed by GC.

TLC of the products obtained by partial acid hydrolysis of A-4 with 0.05N HCl showed spots of SGL corresponding to CMH and of PnSGL which corresponds to PnCDH and not PnCMH as shown in Fig. 8. Then it was confirmed that this CMH is glucosyl ceramide by analysis of its component sugar, and that the PnSGL of the lower spot is PnCDH since the Rf value of the HF degradation product of the PnSGL coincided with that of the reference CDH. The former fact

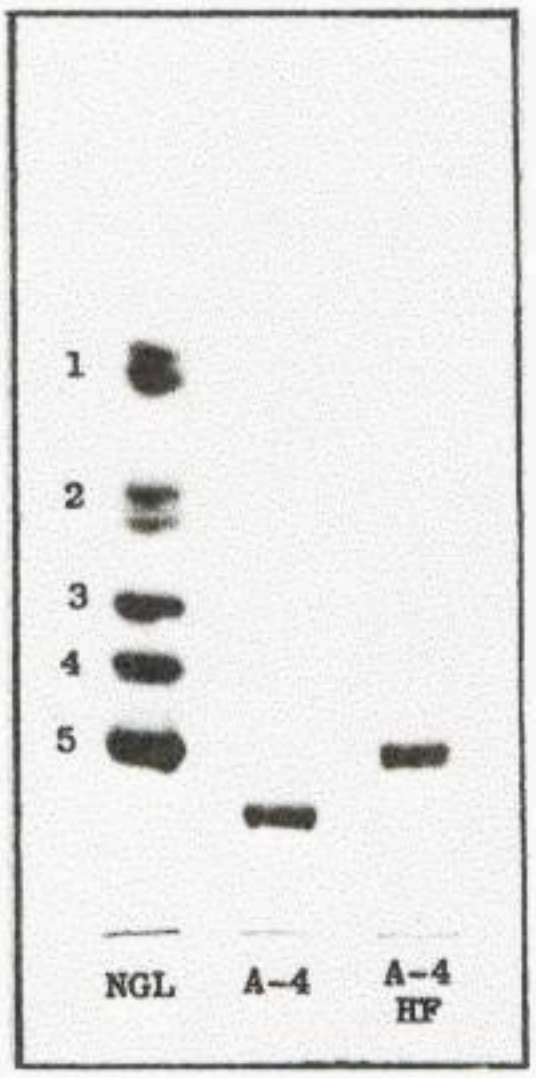

Fig. 5. Thin-layer chromatogram of the degradation product of A-4 with HF. Conditions used were the same as in Fig. 2.

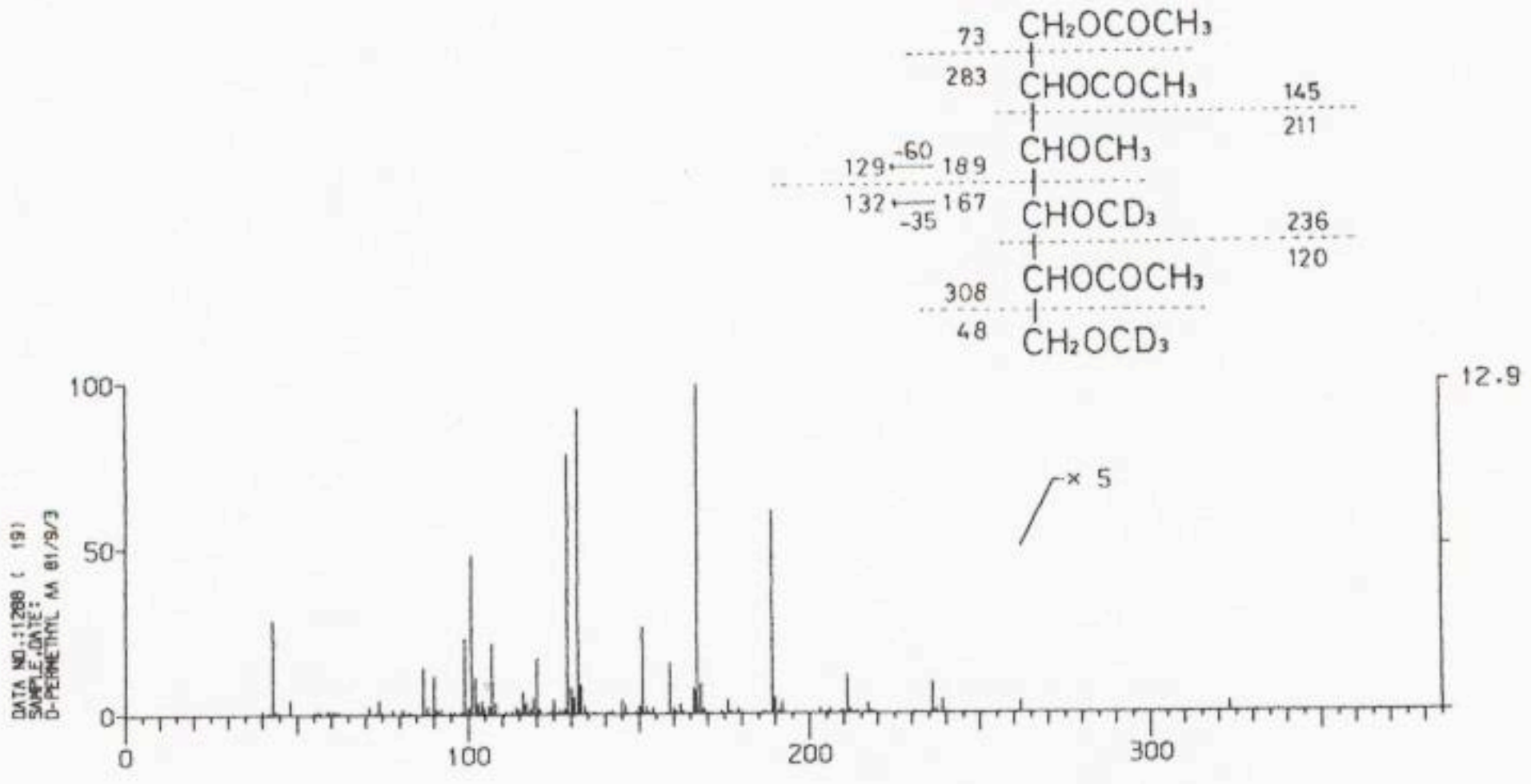

Fig. 6. Mass spectrum of 4,6-deuteromethyl-3-O-methyl-galactitol 1,2,5-triacetate. The ionization potential was 22.5eV.

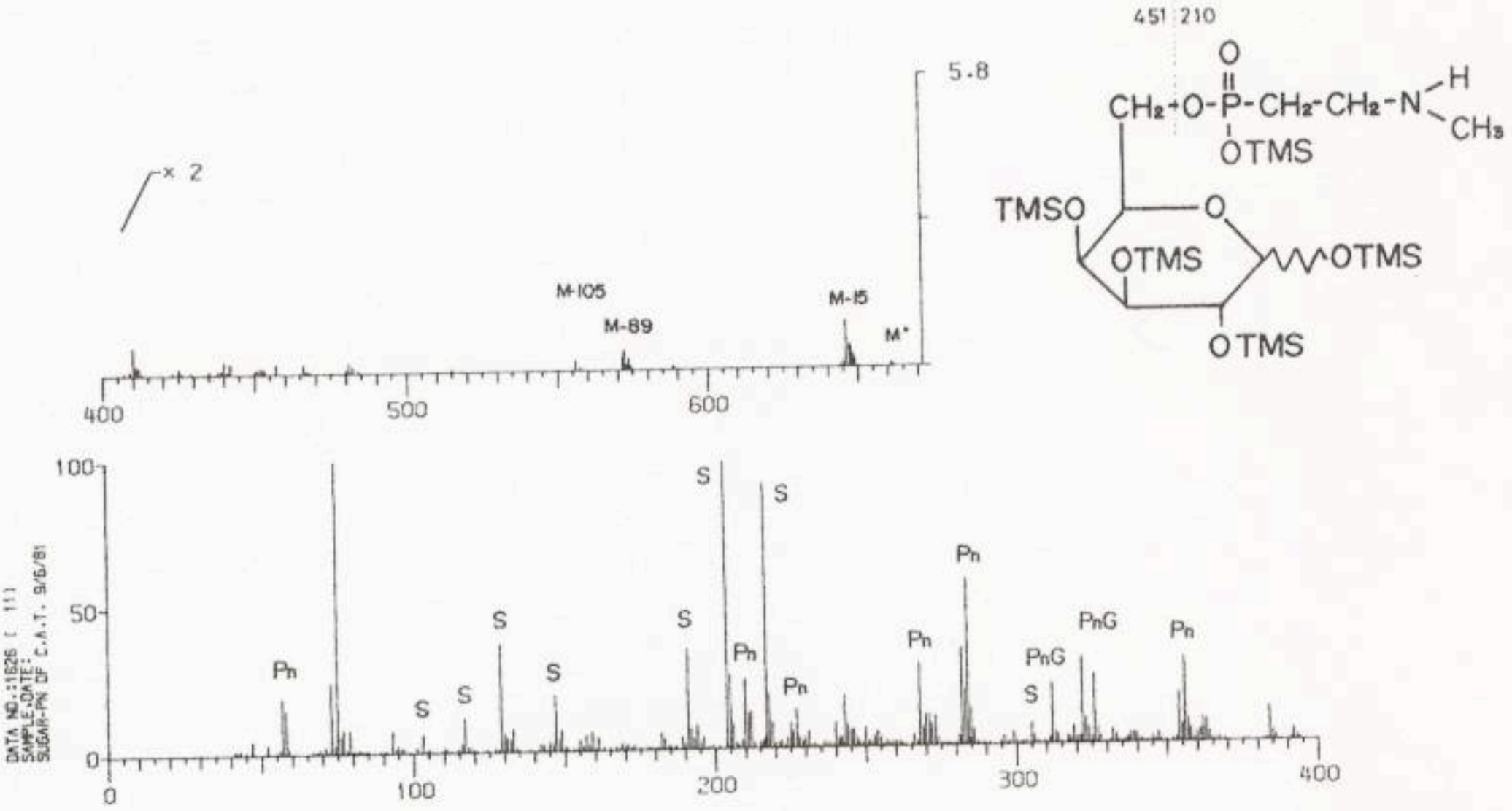

Fig. 7. Mass spectrum of TMS-hexose-N-methylaminoethyl-phosphonate. Pn means ions produced from the aminoalkylphosphonate portion, PnG from sugar-aminoalkylphosphonate, and S from the sugar moiety. The ionization potential was 22.5eV.

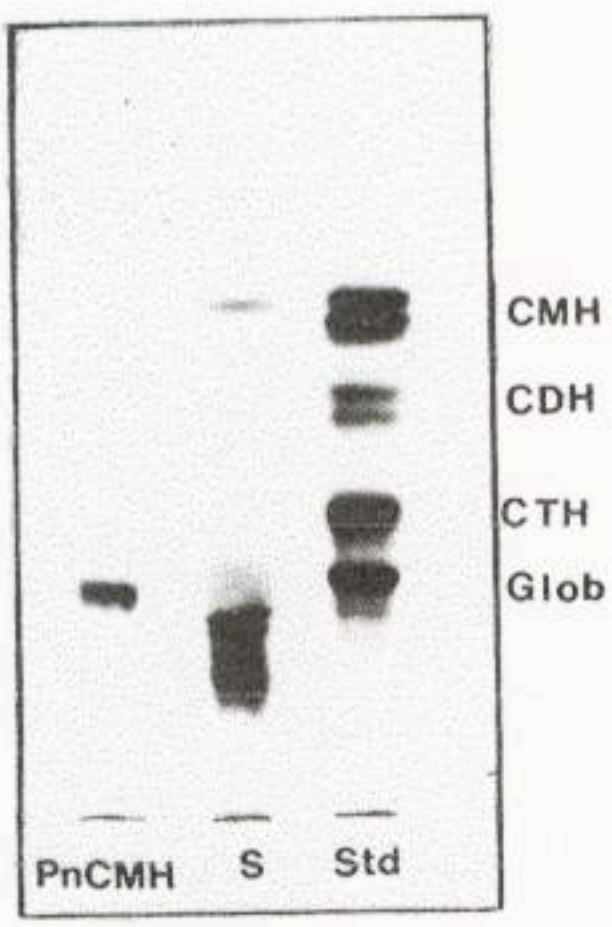

Fig. 8. Thin-layer chromatogram of partial hydrolysis products of A-4 with 0.05N HCl. Conditions used were the same as in Fig. 2.

indicates that the first sugar attached to the ceramide portion is glucose, and MAEP may be attached to the second sugar from the ceramide and the second sugar is galactose since Gal-MAEP was already confirmed.

From the results described above we assume the structure of A-4 to be as shown in Table 4.

The fatty acid and LCB composition of the ceramide moiety of A-4 are shown in Tables 2 and 3, respectively. Palmitic acid is the main fatty acid component and hexadecasphingenine, octadecasphingadienine and octadecasphingenine are the main components of LCB.

DISCUSSION

In Table 4, we summarize all PnSGLs that have been found so far in our laboratory and another laboratory.

From our structural studies on SGLs of marine animals over the past fifteen years and recent studies on PnSGLs, it is evident that there are at least two series of sugar chains in SGL. One is an all galactose series and the other is a fucose containing lactosyl ceramide series.

The former consists of ceramide monogalactoside through ceramide tetragalactoside, however, we have not detected caramide pentagalactoside yet. We consider that higher homologues than CPH of this series may occur in trace amounts if they exist at all. In marine animals, it has been shown that an SGL which has a more complex sugar chain than pentaglycosyl ceramide occurs in a con-

Table 2. Fatty acid composition of A-4 (%)

14:0	1.34
15:0	3.84
16:0	79.93
16:1	6.27
17:0	6.72
18:0	1.79

Table 3. Long chain base composition of A-4 (%)

d16:1	19.0
d17:1	3.8
---	3.5
br d18:1	9.3
d18:2	27.3
d18:1	25.3
---	4.7
---	7.2

Table 4. Phosphonosphingoglycolipids found in marine animals

PnSGL	Animal
MAEP→6Gal—Cer	. *Turbo cornutus* . *Monodonta labio* . *Chlorostoma argyrostoma turbinatum*
MAEP→6Gal1→6Gal1→6Gal—Cer	. *Turbo cornutus*
3-O-Me-Gal-(2↑1 Fuc) [Gal×2, GalN] →3Gal1→4Glc-Cer (MAEP↓6 at Gal1; 2↑1 Fuc at Glc)	. *Chlorostoma argyrostoma turbinatum*
AEP×2- [Glc, Gal×2, GalN, 3-O-Me-Gal] -Cer	. *Aplysia kurodai*

siderable amount. This complex SGL may belong to the fucose containing lactosyl ceramide series. The parent SGL of A-4 and N-5 of *C.a.t.*, SGLs reported by Karlsson *et al.* (26,27) and SGL obtained from abalone (28) are examples of this series and we named this group of lipids the fuco-lactosyl series. The parent SGL of PnSGL found in *Aplysia kurodai* has no fucose, but has a complex sugar composition and 2 moles of AEP.

In marine gastropods, aminoalkylphosphonate derivatives of these two series of SGL are present and they must have a close relationship with their parent SGLs in their biosynthesis and play important roles as ionic SGLs which were first found in marine animals belonging to the Protostomia. Recently, we revealed the presence of PnSGL in Folch's lower layer lipids obtained from *L. japonica*. The fact that PnSGL occurs in Polyplacophora indicates the possibility that PnSGL may occur widely not only in marine Mollusca but also in marine Protostomia.

Regarding aminoalkylphosphonate components of PnSGLs so far found, MAEP is a major one and AEP is a minor or trace one, though in the case of sphingophosphonolipids (SPnL), AEP containing SPnL is predominant in some marine animals (29).

The function of PnSGL should be considered bearing in mind these points, 1) this lipid is ionic, 2) it may be contained in biomembranes, and 3) the stiffness of the aminoalkylphosphonate portion, and then roles as receptors or transmitters of informations are predicted.

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