Amphoteric Glycosphingolipids in Parasitic Nematode (I) : Chemical Structures of Glycosphingolipid Series Containing Cholinephosphoryl($\rightarrow$ 6)-*N*-Acetylglucosamine as an Amphoteric Group from Porcine Roundworm, *Ascaris suum**

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Abstract : A novel series of glycolipids containing cholinephosphoryl($\rightarrow$ 6)-*N*-acetylglucosamine as an amphoteric group was found present in whole tissues of the porcine parasitic nematode, *Ascaris suum*. Thin layer chromatography of the total amphoteric glycolipids indicated at least seven components to react positively with orcinol-sulfuric acid (hexose), Hanes-Isherwood (phosphate) and Dragendorff (choline) spray reagents. Four of these components (PGL₁~PGL₄) were purified and their chemical structures completely characterized as PGL₁ : cholinephosphoryl $\rightarrow$ 6GlcNAc β 1-3Man β 1-4Glc β 1-Cer, PGL₂ : GalNAc β 1-4(cholinephosphoryl $\rightarrow$ 6)GlcNAc β 1-3Man β 1-4Glc β 1-Cer, PGL₃ : Gal α 1-3GalNAc β 1-4(cholinephosphoryl $\rightarrow$ 6)GlcNAc β 1-3Man β 1-4Glc β 1-Cer and PGL₄ : a mixture of Gal β 1-3Gal α 1-3GalNAc β 1-4(cholinephosphoryl $\rightarrow$ 6)GlcNAc β 1-3Man β 1-4Glc β 1-Cer and Gal β 1-6Gal α 1-3GalNAc β 1-4(cholinephosphoryl $\rightarrow$ 6)GlcNAc β 1-3Man β 1-4Glc β 1-Cer. All characterizations were based on compositional sugar, fatty acid and sphingoid analyses, hydrogen fluoride degradation, acid hydrolysis, methylation analysis, exoglycosidase degradation, thin layer chromatography-immunostaining and matrix-assisted laser desorption ionization time-of-flight mass spectrometry. The ceramide moiety was found to consist of 2-hydroxydocosanoic acid and 2-hydroxytetracosanoic acid as the major fatty acids, and 15-methylhexadecasphing-4-enine and 15-methylhexadecasphinganine as the main sphingoids.

Key words : Amphoteric glycolipid, phosphoglycosphingolipid, parasite phosphoglycolipid, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (*Ascaris suum*)

1 Introduction

In our laboratory, the structural investigation of

neutral glycolipids of the porcine parasitic nematode, *A. suum* has been performed on mono- to pentaglycosylceramides, which are characterized

*The previous paper : *J. Jpn. Oil Chem. Soc.* 43, 495 (1994)

Abbreviations : Cer, ceramide ; Glc, glucose ; Man, mannose ; Gal, galactose ; GlcNAc, *N*-acetylglucosamine ; GalNAc, *N*-acetylgalactosamine ; TLC, thin-layer chromatography ; HPLC, high-performance liquid chromatography ; GC, gas-liquid chromatography ; GC-MS, gas chromatograph-mass spectrometry ; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

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as Glc β 1-Cer, Man β 1-4Glc β 1-Cer, GlcNAc β 1-3 Man β 1-4Glc β 1-Cer, GalNAc β 1-4GlcNAc β 1-3 Man β 1-4Glc β 1-Cer, Gal α 1-3GalNAc β 1-4 GlcNAc β 1-3Man β 1-4Glc β 1-Cer containing 2-hydroxydocosanoic and 2-hydroxytetracosanoic acids, and branched heptadecaspheganine and heptadeca-4-sphingenine as their aliphatic components¹⁾. By the presence of a core sugar structure, GlcNAc β 1-3Man β 1-4Glc, the neutral glycolipid series in the porcine nematode has been categorized to Arthro series (a name derived from Arthropod) which has so far found in the class Insecta, Crustacea and Diplopoda, as members of the phylum Arthropoda^{2)~4)}. In our previous paper, in addition, preliminary results indicated the occurrence of a more complex glycolipid series that lacked GlcNAc as a sugar component¹⁾. Subsequently, however, GlcNAc was recognized in the methanolysates of these complex glycolipids after treatment with HF, a finding that suggested the occurrence of cholinephosphoryl-*N*-acetylglucosamine^{5),6)}. This was also confirmed by two spray reagents, Hanes-Isherwood reagent for phosphate and Dragendorff reagent for choline, on TLC. These findings encouraged us to elucidate the structures of the complex glycolipid series, that is, a new amphoteric glycolipid series.

We report here on the structures of four amphoteric glycolipids from *A. suum*.

2 Experimental

2.1 Fractionation and Purification of Amphoteric Glycolipids from the Parasitic Nematode

Whole tissues (1.2 kg of adult male and female porcine roundworm, *A. suum*, collected from the local abattoir and stored at -70°C until use) dried with acetone, powdered and extracted twice with 3 L of chloroform/methanol, 2:1 (v/v) and once with 3 L of chloroform/methanol, 1:1 (v/v) at room temperature. The chloroform-methanol extracts were combined and subjected to mild alkaline hydrolysis in 0.5 M KOH in methanol/water, 9:1 (v/v) at 37°C for 6 h to eliminate glycerolipids. The hydrolysate was acidified to pH 1.0 with 2 M HCl, kept for 1 h at 20°C in that condition, and dialyzed against tap-water for 2 d. The inner fluid was concentrated to near dryness *in vacuo* at 40°C , and precipitated with acetone. The resulting 7.2 g of alkaline-stable product was taken up in

chloroform/methanol/water, 30:60:8 (v/v) and applied to a column (3.0 $\times$ 45 cm) packed with QAE-Sephadex A-25, OH⁻ form (Pharmacia LKB Biotechnology, Sweden) equilibrated with the same solvent. The column was eluted successively with the same solvent (5 column volumes) and pure methanol (2 volumes) as neutral solvents, and with 0.45 M ammonium acetate in methanol (5 volumes) as a polar solvent. The sample of 1.1 g from the fraction (neutral and amphoteric glycolipid fraction) eluted in the first solvent (chloroform/methanol/water) was acetylated and then fractionated on a Florisil column (2.3 $\times$ 60 cm, 60~100 mesh, US Silica Co., USA) by the slightly modified method of Saito and Hakomori⁷⁾. The column was eluted successively with hexane/dichloroethane, 4:1 (v/v), dichloroethane/acetone, 1:1 (v/v), dichloroethane/methanol/water, 2:8:1 (v/v) and chloroform/methanol/water, 6:4:1 (v/v). The solution of acetylated glycolipids eluted by chloroform/methanol/water was evaporated to dryness and reacted with 0.5 M KOH in methanol at 37°C for 3 h. The reaction mixture was dialyzed against tap-water, and lyophilized (yield, 280 mg). The deacetylated product was applied to a column of porous silica gel (2.0 $\times$ 60 cm, Iatrobeads 6 RS-8060, Iatron Lab., Japan) and individual glycolipids were eluted with a linear gradient system of 1-propanol/water/ammonia, 85:15:1 (v/v, 500 mL)~50:50:5 (v/v, 465 mL). The effluent was collected in 5 mL-fractions and an aliquot from alternate tubes was analyzed by TLC. The final purification of individual components was performed using HPLC with a Shimadzu LC-10A system equipped with an Iatrobeads column (Iatrobeads 6 RS-8010, 4.6 mm $\times$ 25 cm) at 25°C . The column was treated with a solvent mixture of 1-propanol/water/ammonia, 85:15:1~75:25:3, in consideration of polarities in each glycolipid fraction. The flow rate was 0.8 mL/min and the effluent was collected in 0.8 mL-fractions.

2.2 Thin-Layer Chromatography (TLC)

Silica gel 60 precoated plates (E. Merck, Germany) were developed with the following solvents: neutral solvents, chloroform/methanol/water (60:40:10 and 65:25:4, v/v); basic solvent, 1-propanol/water/ammonia (75:35:5, v/v); and acidic solvent, 1-butanol/acetic acid/water (4:2:1, v/v). Detection was performed with orcinol-sulfuric acid reagent for hexose, Hanes-Isherwood reagent

(molybdate reagent) for phosphate⁹⁾ and Dragendorff reagent for choline⁹⁾.

2·3 Fatty Acid and Sugar Analyses

For fatty acid and sugar analyses, 100~200 μg of glycolipids were methanolized with 0.1 mL of 1 M anhydrous methanolic HCl at 100°C for 3 h. The resultant fatty acid methyl esters were extracted 3 times with 0.3 mL of 1-hexane and analyzed by GC (Shimadzu GC-17 A) on a capillary column of Shimadzu HiCap-CBP 5 (0.22 mm $\times$ 25 m) programmed at 2°C/min from 170°C to 240°C and GC-MS. GC-MS spectra by using a Shimadzu GCMS-QP 5050 gas chromatograph-mass spectrometer were recorded by both chemical ionization (CI) with isobutane and electron-impact ionization (EI) under the following conditions: ionizing voltage, 100 eV (CI) and 70 eV (EI); ionizing current, 200 μA (CI) and 60 μA (EI); oven temperature, 80°C (2 min) $\rightarrow$ 180°C (20°C/min) $\rightarrow$ 240°C (4°C/min), 60 min; interface temperature, 250°C; injection port temperature, 240°C; helium gas pressure, 100 kPa. The methanolic phase that remained was neutralized by adding silver carbonate and evaporated to dryness. The residue containing methyl glycosides was *N*-acetylated in 0.4 mL of methanol/acetic acid, 3:1 (v/v) containing about 5 mg of silver acetate and *O*-trimethylsilylated with a TMS-reagent (a mixture of pyridine/ hexamethyldisilazane/trimethylchlorosilane, 9:3:1, v/v). The reaction mixture was injected into the same capillary GC programmed at 2°C/min from 140°C to 230°C.

2·4 Sphingoid-Base (Long-Chain Base) Analysis

For sphingoid analysis, 200~500 μg of intact glycolipids were hydrolyzed according to Gaver and Sweeley¹⁰⁾ with 0.2 mL of 1 M aqueous methanolic HCl at 70°C for 18 h. The hydrolysate was alkalized to pH 11.0 with 1 M NaOH and then sphingoids were extracted with chloroform after fatty acid methyl esters were extracted with 1-hexane. The sphingoids were converted to their *O*-trimethylsilyl (*N*-free) derivatives and analyzed by GC and GC-MS on the same capillary column described above. For a detailed characterization of the paraffin chain branching in sphingoids, sphingoids were subjected to catalytic hydrogenation from which saturated long-chain bases were obtained. The saturated bases were oxidized with lead tetraacetate in benzene at 50°C and the

aldehydes produced were further reduced with LiAlH_4 for 5 min in ether. The resulting alcohols were derivatized as their methyl ethers and then analyzed by the capillary GC and GC-MS^{(11), (12)}. Under these conditions, saturated bases led to fatty alcohols with alkyl-chains lacking two carbon atoms.

2·5 Acid Hydrolysis

Samples of the purified amphoteric glycolipids (500 μg ~1 mg) were hydrolyzed with 3 M HCl for 3 h at 100°C for estimation of the choline. The hydrolysates were partitioned into the two layers of Folch system¹³⁾, and the upper aqueous layers were dried under a stream of nitrogen. The residues were subjected to TLC analysis^{(14)~(16)}.

2·6 Dephosphorylation with Hydrogen Fluoride

Isolated amphoteric glycolipids were treated with HF, which selectively cleaves phosphate bonds^{(14), (17)~(19)}. In this method, 3.5 mL of 47% HF was added at 15°C to 1 mg of the amphoteric glycolipids dissolved in 0.5 mL of dimethylsulfoxide in a polyethylene tube fitted with a cap. After incubation at 15°C for 20 h, the reaction mixture was dialyzed against water to remove HF, and the tube contents were lyophilized. The dephosphorylated lipid produced was purified by Iatrobeds column chromatography using a gradient system of chloroform/methanol/water, 60:40:4 (v/v, 150 mL) $\sim$ 30:70:7 (v/v, 180 mL) as an eluting solvent.

2·7 Methylation Analysis

Permethylation of the samples was performed, using 300 μg each of the isolated and dephosphorylated amphoteric glycolipids, with NaOH and CH_3I in dimethylsulfoxide according to the method of Ciucanu and Kerek²⁰⁾. The partially methylated glycolipids were acetylated with 0.6 M HCl in 80% acetic acid at 80°C for 18 h, hydrolyzed, reduced with NaBH_4 , and then acetylated with a mixture of acetic anhydride/pyridine, 1:1 (v/v) at 100°C for 15 min. The partially methylated alditol acetates thus obtained were analyzed by GC and GC-MS on the same HiCap-CBP 5 capillary column as described above. The peaks on gas chromatograms were identified by comparison of the mass spectra and the retention times with those of reference partially methylated alditol acetates prepared from the neutral glycolipids of *A. suum*¹⁾. Furthermore, for determination of the sugar attachment site of

the phosphate group, isolated amphoteric glycolipids were subjected to methylation analysis followed by dephosphorylation with HF. Permethylation of the amphoteric glycolipids (about 1 mg) was carried out as described above, and the permethylated product was purified on a Iatrobeads column equilibrated with chloroform as usual. The purified permethylated amphoteric glycolipid recovered from the column with 1 volume of chloroform/methanol, 90:10 (v/v) was dissolved in a small amount of dimethylsulfoxide and treated with HF under the same conditions as described above. The dephosphorylated compound was subjected to acetolysis, hydrolysis, reduction, and acetylation as described previously.

2.8 TLC/Enzyme-Immunostaining

Sensitive TLC/enzyme-immunostaining was performed by the method described previously^{21),22)}. A dephosphorylated amphoteric glycolipid was chromatographed on the precoated plastic plate (Polygram Sil G, Macherey-Nagel, Germany) with the solvent system of chloroform/methanol/water, 60:40:10 (v/v) and was reacted with ArOse₃Cer (Amino-CTH containing mannose, GlcNAc β 1-3Man β 1-4Glc β 1-Ceramide, purified from the green bottle fly, *Lucilia caesar*)-specific antibody, which was prepared as described previously^{21),22)}.

2.9 Treatment with Exoglycosidases

α -Galactosidase [EC 3.2.1.22] from green coffee beans (Sigma Chemical Co. USA), β -galactosidase [EC 3.2.1.23] from jack beans (Sigma Chemical Co.) and β -N-acetylhexosaminidase from jack beans (Seikagaku Corporation, Japan) were used¹⁾. Samples of 30~50 μ g were suspended in 0.1 mL of 0.1 M Tris buffer (pH 6.5) for α -galactosidase, of 0.1 M citrate buffer (pH 3.5) for β -galactosidase or of 0.1 M citrate buffer (pH 5.0) for β -N-acetylhexosaminidase, containing 0.1 mg of sodium taurodeoxycholate. The reaction was carried out with 0.25 units of α -galactosidase at 25°C for 24 h, with 0.5 units of β -galactosidase at 37°C for 48 h or with 0.1 units of β -N-acetylhexosaminidase at 37°C for 3 h. Each incubation was stopped by adding 0.5 mL of a mixture of chloroform/methanol, 2:1 (v/v). The hydrolysate, after extraction into the lower layer, was dried under a nitrogen stream, and then analyzed by TLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

2.10 Matrix-Assisted Laser Desorption

Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS analysis of purified amphoteric glycolipids was performed using a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer equipped with a Workstation SPARC station, operating in positive-ion linear mode. Ions were formed by a pulsed ultraviolet laser beam (N₂ laser, 337 nm; 3-nanosecond-wide pulses/sec). The matrix used was 7-amino-4-methylcoumarin (coumarin 120; Sigma Chemical Co.). External mass calibration was provided by the [M+H]⁺ ions of leucine enkephalin (M.W. 555.6; Sigma Chemical Co.), angiotensin II (M.W. 1046.2), neurotensin fragment 1-11 (M.W. 1446.6) and insulin chain A (M.W. 2531.6)²³⁾.

3 Results and Discussion

3.1 Isolation of Four Amphoteric Glycolipids (PGL₁ to PGL₄)

Lipids extracted from whole nematode tissues were subjected to mild alkaline hydrolysis, and the resulting alkali-stable substances (sphingolipids), 7.2 g were applied to a QAE-Sephadex column. A neutral fraction was recovered from the QAE-Sephadex column with chloroform/methanol/water and was further subdivided into a neutral glycolipid fraction and an amphoteric glycolipid fraction by a peracetylation:Florisil fractionation-procedure. Separation of the deacetylated amphoteric glycolipid fraction (280 mg) by Iatrobeads column chromatography yielded seven subfractions (these were designated PGL₁ to PGL₇ according to their TLC migration properties.). Four of these amphoteric glycolipid fractions, PGL₁ to PGL₄ in Fig. 1, upon TLC or subsequent sugar analysis, showed no evidence of the heterogeneity that we observed in other fractions, PGL₅ to PGL₇. In this paper, therefore, we describe the structural elucidation only of PGL₁ to PGL₄ in the amphoteric glycolipid series. The clear resolution of the remaining three amphoteric glycolipids, PGL₅ to PGL₇ is in progress, and their complete structural determination will be reported elsewhere. PGL₁ to PGL₄ in each fraction were further purified by HPLC with a single elution system of 1-propanol/water/ammonia. The yields of four purified amphoteric glycolipid components were: PGL₁, 10 mg; PGL₂, <1 mg; PGL₃, 27 mg; and PGL₄, 5 mg (Fig. 1).

Fig. 1 Thin Layer Chromatogram of the Amphoteric Glycolipids from the Parasitic Nematode, *A. suum*.

Lane 1, amphoteric glycolipid fraction obtained from QAE-Sephadex and Florisil column chromatography; lanes 2 to 5, isolated PGL₁ to PGL₄, respectively. The plate was developed in 1-propanol/water/ammonia (75:35:5, v/v) for 2 h, and the spots were visualized with orcinol-H₂SO₄ spray reagent.

3.2 Properties of Intact and Dephosphorylated Glycolipids

As a first step in visualizing their structures, the amphoteric glycolipid compounds, PGL₁ to PGL₄ were hydrolyzed with HF to remove phosphate groups, and were then subjected to TLC analysis. Dephosphorylation of the four glycolipids with HF yielded products that no longer stained with either Hanes-Isherwood or Dragendorff reagents, and which migrated to different positions on TLC from the parent glycolipids. The chromatographic mobilities of the dephosphorylated glycolipids derived from PGL₁, PGL₂ and PGL₃ were identical with those of three neutral glycolipids from the present organism already reported on (Fig. 2)¹⁾. The sugar compositions of the intact (PGLs) and dephosphorylated glycolipids (Dep-PGLs) were analyzed as *N*-acetyl-*O*-TMS derivatives of their methyl glycosides; molar ratios are given in Table 1. Dep-PGL₁ contained Glc, Man and GlcNAc in a molar ratio of 1:1:1. Compositions of the other lipids of this series are as follows. Dep-PGL₂: Glc, Man, GlcNAc and GalNAc (1:1:1:1); Dep-PGL₃: Glc, Man, GlcNAc, GalNAc and Gal (1:1:1:1:1); and Dep-PGL₄: Glc, Man, GlcNAc, GalNAc and Gal (1:1:1:1:2). Furthermore, glycolipid products obtained by mild acid hydrolysis (0.1 M HCl for 60 min at 100 °C)²⁾ of Dep-PGL₃ and Dep-PGL₄ were Glc-Cer, Man-Glc-Cer, GlcNAc-Man-Glc-Cer,

Fig. 2 Thin Layer Chromatogram of the Products Obtained from Amphoteric Glycolipids, PGL₁ to PGL₄ by Treatment with HF for Dephosphorylation.

Lane 1, an authentic mixture of *A. suum* neutral glycolipids (a, GlcNAc β 1-3Man β 1-4Glc β 1-Cer; b, GalNAc β 1-4GlcNAc β 1-3Man β 1-4Glc β 1-Cer; c, Gal α 1-3GalNAc β 1-4GlcNAc β 1-3Man β 1-4Glc β 1-Cer)¹⁾; lanes 2 to 5, dephosphorylated PGL₁ (Dep-PGL₁) to PGL₄ (Dep-PGL₄), respectively. The plate was developed in chloroform/methanol/water (60:40:10, v/v) for 15 min, and the spots were visualized with orcinol-H₂SO₄ reagent.

Table 1 Sugar Compositions of Intact and Dephosphorylated PGL₁ to PGL₄.

	Glc	Man	GlcNAc	GalNAc	Gal
PGL ₁	1.00	0.93	—	—	—
Dep-PGL ₁	1.00	0.91	0.69	—	—
PGL ₂	1.00	1.01	—	1.10	—
Dep-PGL ₂	1.00	1.15	0.70	1.00	—
PGL ₃	1.00	0.86	—	0.88	0.79
Dep-PGL ₃	1.00	0.93	0.75	0.79	0.73
PGL ₄	1.00	1.01	—	1.05	1.87
Dep-PGL ₄	1.00	0.95	0.81	0.80	1.65

GalNAc-GlcNAc-Man-Glc-Cer (hydrolysates from Dep-PGL₃ and Dep-PGL₄), and Gal-GalNAc-GlcNAc-Man-Glc-Cer (hydrolysate from Dep-PGL₄), as identified by comparison with authentic neutral glycolipid samples from the present organism¹⁾.

3.3 Identification of a Choline Residue

Each amphoteric glycolipid was hydrolyzed with 3 M HCl at 100 °C for 3 h, yielding hexoses, glucosamine-phosphate and choline (results not shown).

3·4 Methylation Analysis

Evidence concerning the sugar sequences and linkages, and the attachment site of a cholinephosphoryl residue to the amphoteric glycolipids, was gained by studying the partially methylated alditol acetates obtained from intact glycolipids, as well as from glycolipids subjected either to prior dephosphorylation or to permethylation followed by dephosphorylation. The products were analyzed by GC and GC-MS, and are listed in **Table 2**, as are also, for comparison, those derived from pentaglycosylceramide, Gal α 1-3GalNAc β 1-4GlcNAc β 1-3Man β 1-4Glc β 1-Cer, purified from the present organism, the non-phosphorylated analogue of PGL₃. Application to the intact amphoteric glycolipids (**A** in **Table 2**) yields 1,4,5-tri-(*O*-acetyl)-2,3,6-tri-(*O*-methyl) glucitol (1,4 Glc) and 1,3,5-tri-(*O*-acetyl)-2,4,6-tri-(*O*-methyl) mannitol (1,3 Man) from PGL₁, 1,4 Glc, 1,3 Man and 1,5-di-(*O*-acetyl)-3,4,6-tri-(*O*-methyl)-*N*-acetylgalactosaminitol (1 GalNAc) from PGL₂, 1,4 Glc, 1,3 Man, 1,3,5-tri-(*O*-acetyl)-4,6-di-(*O*-methyl)-*N*-acetylgalactosaminitol (1,3 GalNAc) and 1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl) galactitol (1 Gal) from PGL₃, and 1,4 Glc, 1,3 Man, 1,3 GalNAc, 1 Gal, 1,3,5-tri-(*O*-acetyl)-2,4,6-tri-(*O*-methyl) galactitol (1,3 Gal) and 1,5,6-tri-(*O*-acetyl)-2,3,4-tri-(*O*-methyl) galactitol (1,6 Gal) from PGL₄, respectively, since all the expected *N*-acetylglucosamine products remain phosphorylated and are hence not observable under the conditions used here for GC analysis. In the case of PGL₄, two partially methylated galactitol acetates assigned to be 1,3- and 1,6-substituted galactoses were observed (underlined in **Table 2**). This means that PGL₄ is a mixture of

Table 2 Glycosidic Linkage Analysis (Substituted Positions of Sugars) of PGL₁ to PGL₄.

	PGL ₁	PGL ₂	PGL ₃	PGL ₄
A	1,4Glc	1,4Glc	1,4Glc	1,4Glc
	1,3Man	1,3Man	1,3Man	1,3Man
		1GalNAc	1,3GalNAc	1,3GalNAc
			1Gal	<u>1,3Gal</u> <u>1,6Gal</u> 1Gal
B	1GlcNAc	1,4GlcNAc	1,4GlcNAc	1,4GlcNAc
C	1,6GlcNAc	1,4,6GlcNAc	1,4,6GlcNAc	1,4,6GlcNAc

Alditol acetates obtained from intact PGLs, A; from dephosphorylated PGLs, A+B; from permethylated and dephosphorylated PGLs, A+C. Numbers expressed the substituted positions of sugar residues.

the amphoteric glycolipids with two different sugar sequences. Dephosphorylation with HF prior to methylation (**A+B** in **Table 2**) yields additionally 1,5-di-(*O*-acetyl)-3,4,6-tri-(*O*-methyl)-*N*-acetylglucosaminitol (1 GlcNAc) from the terminal *N*-acetylglucosamine with a cholinephosphoryl residue of PGL₁, and 1,4,5-tri-(*O*-acetyl)-3,6-di-(*O*-methyl)-*N*-acetylglucosaminitol (1,4 GlcNAc) from the internal *N*-acetylglucosamine with a cholinephosphoryl residue of PGL₂, PGL₃ and PGL₄. However, if the amphoteric glycolipids are first permethylated and the products, still substituted with a cholinephosphoryl residue, are then dephosphorylated (**A+C** in **Table 2**), subsequent acetylation produces in addition 1,5,6-tri-(*O*-acetyl)-3,4-di-(*O*-methyl)-*N*-acetylglucosaminitol (1,6 GlcNAc) from PGL₁ and 1,4,5,6-tetra-(*O*-acetyl)-3-(*O*-methyl)-*N*-acetylglucosaminitol (1,4,6 GlcNAc) from PGL₂, PGL₃ and PGL₄. These results indicate that a cholinephosphoryl residue is attached to the 6-hydroxy groups of the terminal *N*-acetylglucosamine in PGL₁ and the internal *N*-acetylglucosamine in PGL₂, PGL₃ and PGL₄.

3·5 Detection of an Arthro Sugar Core Lipid by Immunochemical Method

Specific polyclonal antibody against mannose-containing triglycosylceramide, GlcNAc β 1-3Man β 1-4Glc β 1-Cer (ArOse₃Cer) was used as a "monospecific" probe for detection and deter-

Fig. 3 Detection of Arthro Sugar Core Lipid by TLC/Enzyme-Immunostaining.

Lane 1, authentic ArOse₃Cer (GlcNAc β 1-3Man β 1-4Glc β 1-Cer) obtained from insect, *L. caesar*; lane 2, Dep-PGL₁ (PGL₁ treated with HF). The plates (Polygram Sil G, precoated plastic sheet) were developed in chloroform/methanol/water (65:25:4, v/v) for 10 min, and the spots were visualized with orcinol-H₂SO₄ reagent for Panel A and with immunostaining for Panel B.

mination of the arthro sugar core lipid. A glycolipid antigen, Dep-PGL₁, reacted strongly with anti-ArOse₃Cer antibody as indicated by TLC/enzyme-immunostaining (Fig. 3). This result suggested the chemical structure to be GlcNAc β 1-3Man β 1-4Glc β 1-Cer for Dep-PGL₁.

From these results, the sugar sequences in each amphoteric glycolipid are as follows: cholinephosphoryl $\rightarrow$ 6GlcNAc1-3Man1-4Glc for PGL₁, GalNAc1-4(cholinephosphoryl $\rightarrow$ 6) GlcNAc 1-3Man1-4Glc for PGL₂, Gal1-3GalNAc1-4(cholinephosphoryl $\rightarrow$ 6)GlcNAc1-3Man 1-4Glc for PGL₃, and a mixture of Gal1-3Gal1-3 GalNAc1-4 (cholinephosphoryl $\rightarrow$ 6) GlcNAc 1-3 Man1-4Glc and Gal1-6Gal1-3GalNAc1-4 (cholinephosphoryl $\rightarrow$ 6)GlcNAc1-3Man1-4Glc for PGL₄.

3.6 Anomeric Configurations of the Sugar Residues in Amphoteric Glycolipids

For determination of anomeric configurations of the sugar residues, Dep-PGL₃ and Dep-PGL₄ were subjected to specific enzymatic hydrolysis. Dep-PGL₃ was degraded to diglycosylceramide in a

stepwise fashion through the consecutive actions of α -galactosidase from green coffee beans and β -N-acetylhexosaminidase from jack beans, and Dep-PGL₄ was degraded to pentaglycosylceramide with β -galactosidase from jack beans (Fig. 4). These enzymatic hydrolysates were also confirmed by MALDI-TOF MS analysis. MALDI-TOF MS of tetra- and diglycosylceramides produced from Dep-PGL₃ and pentaglycosylceramide from Dep-PGL₄ revealed pseudomolecular ions $[M+H]^+$ at m/z 1384 (main molecular species of ceramide: fatty acid-sphingoid, h 24:0-iso, d 17:0), at m/z 978 and at m/z 1546, which were consistent with HexNAc₂-Hex₂-Cer, Hex₂-Cer and Hex-HexNAc₂-Hex₂-Cer, respectively (data not shown). These findings suggest that with the exception of the terminal α -galactose linkage in PGL₃, and the penultimate α -galactose linkage in PGL₄, all of sugars in PGL₁ to PGL₄ are β -anomers.

3.7 Aliphatic Components (Fatty Acids and Sphingoid-Bases) of PGL₁ to PGL₄

The aliphatic components of PGL₁ to PGL₄ were determined by GC and GC-MS (Table 3). The main fatty acids were 2-hydroxydocosanoic acid and 2-hydroxytetraacosanoic acid, and the sphingoids were composed of *iso*-heptadeca-4-sphingenine (15-methylhexadecaspheing-4-enine) and *iso*-heptadecaspheinganine (15-methylhexadecaspheinganine).

Table 3 Ceramide Compositions of PGL₁ to PGL₄.

	PGL ₁	PGL ₂	PGL ₃	PGL ₄
Fatty acid				
h22:0	11.7	11.0	10.6	7.3
h24:0	88.3	89.0	89.4	92.7
Sphingoid				
iso, d17:1	35.7	33.3	28.1	17.1
iso, d17:0	64.3	66.7	71.9	82.9

h, 2-hydroxy fatty acid; d, dihydroxy sphingoid.

3.8 MALDI-TOF MS Analysis of PGL₁ and PGL₃

The putative sugar structures of PGL₁ and PGL₃ were confirmed by positive-ion MALDI-TOF MS analysis as shown in Fig. 5. The mass spectra revealed pseudomolecular ions $[M+H]^+$ at m/z 1346 for PGL₁ (c in Fig. 5 A, predicted molecular

Fig. 4 Thin Layer Chromatogram of the Reaction Products from Dep-PGL₃ and Dep-PGL₄ by the Treatments with Exoglycosidases.

Lane 1, an authentic mixture of *A. suum* neutral glycolipids (a, Man β 1-4Glc β 1-Cer; b, GlcNAc β 1-3Man β 1-4Glc β 1-Cer; c, GalNAc β 1-4GlcNAc β 1-3Man β 1-4Glc β 1-Cer; d, Gal α 1-3GalNAc β 1-4GlcNAc β 1-3Man β 1-4Glc β 1-Cer); lane 2, Dep-PGL₃; lane 3, Dep-PGL₃ incubated with α -galactosidase (green coffee beans); lane 4, Dep-PGL₃ incubated with α -galactosidase and then with β -N-acetylhexosaminidase (jack beans); lane 5, Dep-PGL₄; lane 6, Dep-PGL₄ incubated with β -galactosidase (jack beans). The plate was developed in chloroform/methanol/water (60:40:10, v/v) for 15 min, and the spots were visualized orcinol-H₂SO₄ reagent.

Fig. 5 MALDI-TOF MS Spectra of Amphoteric Glycolipids, PGL₁ and PGL₃.

Both amphoteric glycolipids were analyzed by MALDI-TOF MS in positive-ion linear mode with 7-amino-4-methylcoumarin as matrix. Pseudomolecular ions are given in accurate mass values rounded to the nearest mass units²⁹). A, PGL₁; B, PGL₃; a and b, presumable ions of [M-CH₃CO (acetyl)-CH₃ (methyl)+2H]⁺ and [M-3CH₃ (methyl)+Na+2H]⁺; c, [M+H]⁺.

species of ceramide: fatty acid-sphingoid, h24:0-iso,d17:0) and at *m/z* 1711 (c in **Fig. 5 B**, h24:0-iso,d17:0) for PGL₃, respectively, which corresponded to HexNAc-Hex₂-Cer and Hex-HexNAc₂-Hex₂-Cer having a cholinephosphoryl residue in their sugar chains. Furthermore, two more additional peaks (a and b in **Fig. 5 A,B**) were found in both mass spectra. At the present time these additional peaks are assumed to be [M-CH₃CO (acetyl)-CH₃ (methyl)+2H]⁺ at *m/z* 1288 and [M-3CH₃(methyl)+Na+2H]⁺ at *m/z* 1325 for PGL₁, together with corresponding peaks for PGL₃ at *m/z* 1653 and at *m/z* 1690. The detailed fragmentations resulting in these spectra are currently under study.

From all the results described above, we propose the structures of PGL₁ to PGL₄ to be as follows: PGL₁, cholinephosphoryl→6GlcNAcβ 1-3 Manβ 1-4Glcβ 1-Cer; PGL₂, GalNAcβ 1-4 (cholinephosphoryl→ 6)GlcNAcβ 1-3Manβ 1-4 Glcβ 1-Cer; PGL₃, Galα 1-3GalNAcβ 1-4(cholinephosphoryl→6)GlcNAcβ 1-3Manβ 1-4Glcβ 1-Cer; and PGL₄, a mixture of Galβ 1-3Galα 1-3GalNAcβ 1-4 (cholinephosphoryl→ 6) GlcNAcβ 1-3 Manβ 1-4

Glcβ 1-Cer and Galβ 1-6Galα 1-3 GalNAcβ 1-4 (cholinephosphoryl→ 6) GlcNAcβ 1-3 Manβ 1-4 Glcβ 1-Cer. Based on the structural similarity of the ceramide moieties it appears likely that a relationship joins these amphoteric glycolipids in one metabolic pathway. Additionally, when visualized on TLC plate, the amphoteric glycolipid fraction of the roundworm showed the existence of much more complex glycolipids (**Fig. 1**). In a preliminary analysis, we have found three more glycolipids seemingly containing the PGL₄-sugar chain, namely Gal-(Gal)-Gal-GalNAc-(cholinephosphoryl→)GlcNAc-Man-Glc-Cer, Fuc-Gal-(Gal)-Gal-GalNAc-(cholinephosphoryl→)GlcNAc-Man-Glc-Cer and GalNAc-(Fuc)-Gal-(Gal)-Gal-GalNAc-(cholinephosphoryl→)GlcNAc-Man-Glc-Cer^{5),6)} (the complete structural elucidation of these glycolipids is in progress).

Glycolipids containing cholinephosphate referred to as amphoteric glycolipids, have been most studied in earthworms (*Pheretima hilgendorfi*^{14),24)} and *Pheretima asiatica*^{25),26)}), lugworms (*Marphysa sanguinea*²⁷⁾, *Tylorhynchus heterochetus*¹⁵⁾ and *Neanthes diversicolor*²⁸⁾ and the leech (*Hirudo nipponica*)¹⁶⁾, which belong to the classes Oligochaeta, Polychaeta and Hirudinea of the phylum Annelida. These were neogala (Galβ 1-6Gal) series glycolipids having a cholinephosphoryl(→6)-galactose residue in their sugar chains. In the present study, however, a series of amphoteric glycolipids having an arthro sugar core (GlcNAcβ 1-3Manβ 1-4Glc) has been newly characterized from the roundworm, *A. suum*, including identification of a cholinephosphoryl(→6)-*N*-acetylglucosamine residue. The structure of the major component of *A. suum* amphoteric glycolipids, Galα 1-3GalNAcβ 1-4(cholinephosphoryl→ 6) GlcNAcβ 1-3Manβ 1-4Glcβ 1-Cer (PGL₃), has been published recently by Lochnit *et al.*³⁰⁾ and its biological activity, namely stimulation of the production of the human mononuclear cell-derived, inflammation-associated cytokines, tumor necrosis factor α (TNF-α), interleukin 1 (IL-1) and interleukin 6 (IL-6) has been reported.

(Received Sept. 21, 1999 ; Accepted Nov. 5, 1999)

References

- 1) M. Sugita, T. Mizunoma, C. Hayata, T. Hori, F. Nakatani, K. Aoki, *J. Jpn. Oil Chem. Soc.*, **43**, 495 (1994).

- 2) M. Sugita, F. Inagaki, H. Naito, T. Hori, *J. Biochem.*, **107**, 899 (1990).
- 3) T. Hori, M. Sugita, *Prog. Lipid Res.*, **32**, 25 (1993).
- 4) R.D. Dennis, H. Wiegandt, *Adv. Lipid Res.*, **26**, 321 (1993).
- 5) A. Kurimoto, M. Sugita, "The 36th Ann. Meeting on Japan Oil Chem. Soc.", (Kyoto), Proceeding p.100 (1997).
- 6) M. Sugita, Y. Kawakami, A. Kurimoto, "The 71th Ann. Meeting on Japanese Biochemical Soc.", (Nagoya), Proceeding p.1065 (1998).
- 7) T. Saito, S. Hakomori, *J. Lipid Res.*, **12**, 2567 (1971).
- 8) C.S. Hanes, F.A. Isherwood, *Nature*, **164**, 1107 (1949).
- 9) E. Stahl, P. J. Schorn, "Thin-Layer Chromatography", p.873 (Reagent No.97), Springer-Verlag, New York (1969).
- 10) R.C. Gaver, C.C. Sweeley, *J. Am. Oil Chem. Soc.*, **42**, 294 (1965).
- 11) M. Sugita, K. Ohkawa, T. Hori, *Mem. of the Faculty of Ed. Shiga Univ.*, **24**, 40, (1974).
- 12) M. Sugita, O. Itasaka, T. Hori, *Chem. Phys. Lipids*, **16**, 1 (1976).
- 13) J. Folch, M. Lees, G.A. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).
- 14) M. Sugita, H. Fujii, F. Inagaki, M. Suzuki, C. Hayata, T. Hori, *J. Biol. Chem.*, **267**, 22595 (1992).
- 15) M. Sugita, T. Makino, C. Hayata, M. Suzuki, A. Suzuki, T. Hori, S. Miwa, F. Nakatani, *J. Jpn. Oil Chem. Soc.*, **42**, 935 (1993).
- 16) M. Sugita, A. Morikawa, J. T. Dulaney, A. Okada, *J. Jpn. Oil Chem. Soc.*, **45**, 731 (1996).
- 17) M. Sugita, S. Itonori, F. Inagaki, T. Hori, *J. Biol. Chem.*, **264**, 15028 (1989).
- 18) S. Itonori, K. Kamemura, K. Narushima, N. Sonku, O. Itasaka, T. Hori, M. Sugita, *Biochim. Biophys. Acta*, **1081**, 321 (1991).
- 19) S. Itonori, M. Nishizawa, M. Suzuki, F. Inagaki, T. Hori, M. Sugita, *J. Biochem.*, **110**, 479 (1991).
- 20) I. Ciucanu, F. Kerek, *Carbohydr. Res.*, **131**, 209 (1984).
- 21) M. Sugita, C. Hayata, T. Hori, A. Morikawa, S. Miwa, *J. Jpn. Oil Chem. Soc.*, **42**, 649 (1993).
- 22) M. Sugita, C. Hayata, T. Yoshida, M. Suzuki, A. Suzuki, T. Takeda, T. Hori, F. Nakatani, *Biochim. Biophys. Acta*, **1215**, 163 (1994).
- 23) K. Aoki, H. Hamana, H. Yamaoka, M. Sugita, *Mem. of the Faculty of Ed. Shiga Univ.*, **48**, 11 (1998).
- 24) M. Sugita, H. Fujii, J. T. Dulaney, F. Inagaki, M. Suzuki, A. Suzuki, S. Ohta, *Biochim. Biophys. Acta*, **1259**, 220 (1995).
- 25) N. Noda, R. Tanaka, K. Miyahara, T. Kawasaki, *Chem. Pharm. Bull.*, **41**, 1733 (1993).
- 26) R. Tanaka, K. Miyahara, N. Noda, *Chem. Pharm. Bull.*, **44**, 1152 (1996).
- 27) N. Noda, R. Tanaka, K. Miyahara, T. Kawasaki, *Tetrahedron Lett.*, **33**, 7527 (1992).
- 28) N. Noda, R. Tanaka, K. Miyahara, T. Kawasaki, *Biochim. Biophys. Acta*, **1169**, 30 (1993).
- 29) T. Taketomi, A. Hara, K. Uemura, E. Sugiyama, *J. Biochem.*, **120**, 573 (1996).
- 30) G. Lochnit, R. D. Dennis, A. J. Ulmer, R. Geyer, *J. Biol. Chem.*, **273**, 466 (1998).

日本油化学会誌本号掲載 論文要旨

[総説] 見逃されていたイソプレノイド生合成経路： 非メバロン酸経路とその特異的阻害剤

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近年、高等植物の色素体、緑藻、多くの真正細菌において、メバロン酸経路とは全く異なる新規経路、非メバロン酸経路の存在が明らかにされたがその詳細は不明であった。我々は、非メバロン酸経路における 1-deoxy-D-xylulose 5-phosphate (DXP) から 2-C-methyl-D-erythritol 4-phosphate への反応を触媒する酵素 DXP reductoisomerase を大腸菌からクローニングすることに成功し詳細に解析した。また、fosmidomycin がこの酵素を特異的に阻害することも明らかにした。

(連絡者：葛山智久) Vol.49, No.2, 119 (2000)

[報文] 寄生性線虫類の両性イオン型糖脂質 (第I報)： ブタカイチュウ (*Ascaris suum*) の N- アセチルグルコサミン -6- ホスホコリン含有スフィンゴ糖脂質群の化学構造

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寄生性線虫類、ブタカイチュウ (*Ascaris suum*) には、N- アセチルグルコサミン -6- ホスホコリンを含有する新しい両性イオン型糖脂質の一群が存在する。それらは、TLC 分析によって、糖、リンおよびコリン検出試薬のいずれにも陽性を示し、少なくとも7種類の成分から成る。これらの7種類のうち、TLC 上での移動度の大きい4種類 (PGL₁ ~ PGL₄) を単離、精製し、それらの化学構造を、PGL₁ : cholinephosphoryl → 6GlcNAc β 1-3Man β 1-4Glc β 1-Cer, PGL₂ : GalNAc β 1-4 (cholinephosphoryl → 6) GlcNAc β 1-3Man β 1-4Glc β 1-Cer, PGL₃ : Gal α 1-3GalNAc β 1-4 (cholinephosphoryl → 6) GlcNAc β 1-3Man β 1-4Glc β 1-Cer および PGL₄ : Gal β 1-3Gal α 1-3GalNAc β 1-4 (cholinephosphoryl → 6) GlcNAc β 1-3Man β 1-4Glc β 1-Cer と Gal β 1-6Gal α 1-3GalNAc β 1-4 (cholinephosphoryl → 6) GlcNAc β 1-3Man β 1-4Glc β 1-Cer の混合物、と決定した。化学構造の決定は、糖、脂肪酸、スフィンゴイド組成分析、フッ化水素酸分解、鉍酸分解、メチル化分析、酵素的加水分解、TLC- 免疫染色および MALDI-TOF MS (マトリックス支援レーザー脱離イオン化飛行時間型質量分析装置) 分析によって行った。これらの両性イオン型糖脂質のセラミド組成は、脂肪酸が2- ヒドロキシ-C22 : 0 酸および2- ヒドロキシ-C24 : 0 酸を、スフィンゴイド塩基がイソ-C17- スフィンゴシンおよびイソ-C17- スフィンガニンを主成分としていた。

(連絡者：杉田陸海) Vol.49, No.2, 127 (2000)