ORIGINAL

Glycosphingolipids with Gal α 1-6Gal and Gal β 1-6Gal Sequences in the Leech, *Hirudo nipponia*Mutsumi SUGITA^{*1}, Aki MORIKAWA^{*1,*3}, John T. DULANEY^{*2},
and Akiko OKADA^{*1}^{*1} Department of Chemistry, Faculty of Liberal Arts and Education, Shiga University
(2-5-1, Hiratsu, Otsu-shi, Shiga-ken, 〒520)^{*2} Department of Medicine, Division of Nephrology, University of Tennessee
(951 Court Avenue, 649D, Memphis, Tennessee, 38163, U.S.A.)^{*3} Current address; Bioassay Division, Japan Clinical Laboratories, Inc.
(17-18, Nakahata-cho, Nishiwaki-shi, Hyogo-ken, 〒677)

Abstract : Glycosphingolipids containing two gala-6 core series were shown to be present in the leech, *Hirudo nipponia*. Their structures were determined as Gal β 1-1Cer, Gal α 1-6Gal β 1-1Cer, Gal α 1-6Gal α 1-6Gal β 1-1Cer and Gal α 1-6Gal α 1-6Gal α 1-6Gal β 1-1Cer as neutral glycolipids and cholinephosphate→6Gal β 1-1Cer and cholinephosphate→6Gal β 1-6Gal β 1-1Cer as amphoteric glycolipids, based on the results of compositional and methylation analyses and exogalactosidase treatments. Ceramides mainly contained C_{16:0}, C_{18:0}, C_{22:0} and C_{24:0} fatty acids and dihydroxy- (C_{18:1}, C_{19:1} and C_{22:3}) and trihydroxy- (C_{18:0} and C_{19:0}) sphingoids. The authors tentatively designated the disaccharide structure, Gal α 1-6Gal, as the "isoneogala-series", which may represent a new type of glycosphingolipid core series.

Key words : gala-6 series glycolipid, leech glycolipid, glycosphingolipid

1 Introduction

The phylum Annelida is a phylogenetically interesting organism intermediate between Mollusca and Arthropoda, and is divided into five classes, namely Archiannelida, Oligochaeta, Polychaeta, Hirudinea and Echiuroidea. Our previous studies paid special attention to two classes, Oligochaeta and Polychaeta^{1)~5)}, and the major glycosphingolipids found were a series of neogalalipids, Gal β 1-1Cer (galactosylceramide), Gal β 1-6Gal β 1-1Cer (neogalabiaosylceramide), Gal β 1-6Gal β 1-6Gal β 1-1Cer (neogalatriaosylceramide), Man α 1-4Gal β 1-6Gal β 1-1Cer (mannosylneogalabiaosylceramide), Gal β 1-6Gal β 1-6Gal β 1-6Gal β 1-1Cer (neogalatetraosylceramide) and Gal α 1-4 (Man α 1-4) Gal β 1-6Gal β 1-1Cer (galactosylmannosylneogalabiaosylceramide) as neutral glycolipids, and cholinephosphate→6Gal β 1-1Cer (cholinephosphorylgalactosylceramide), cholinephosphate→6Gal β 1-6Gal β 1-1Cer (cholinephosphorylneogalabiaosylceramide), cholinephosphate→6 (Man α 1-4) Gal β 1-6Gal β 1-1Cer (cholinephosphorylmannosylneogalabiaosylceramide) and cholinephosphate→6Gal β 1-6Gal β 1-6Gal β 1-1Cer (cholinephosphorylneogalatriaosylceramide) as amphoteric glycolipids.

We have initiated an investigation into the glycolipids of the leech, *Hirudo nipponia*, which belongs to the class Hirudinea, and have found that neutral triglycosylceramide and amphoteric monoglycosylceramide are the major components of leech glycosphingolipids, minor ones being neutral mono-, di- and tetraglycosylceramides together with amphoteric di- and triglycosylceramides^{6),7)}. Isolation of these glycolipids has allowed their structural charac-

Corresponding author : Mutsumi SUGITA

terization as members of the gala-series. Furthermore, two novel glycolipids, NGL₃ and NGL₄ containing a Gal α 1-6Gal linkage, were discovered in neutral tri- and tetraglycosylceramides.

This paper deals with the molecular structures of four neutral glycolipids, mono- (NGL₁), di- (NGL₂), tri- (NGL₃) and tetraglycosylceramides (NGL₄), and of two amphoteric glycolipids, mono- (PGL₁) and diglycosylceramides (PGL₂).

2 Experimental

2.1 Preparation of neutral and amphoteric glycolipid fractions from the leech.

Dried tissues (400 g, commercial origin for Chinese medicine) were powdered and extracted two times with 1.5 L of chloroform-methanol, 2 : 1 (vol/vol) and then with 1.2 L of chloroform-methanol, 1 : 1 (vol/vol). The chloroform-methanol extracts were combined and subjected to mild alkaline hydrolysis in 0.5 M KOH in methanol-water, 95 : 5 (vol/vol) at 37 °C for 6 h. The hydrolysate was neutralized with 2 M HCl, dialyzed against tap water for 2 d, concentrated to near dryness, and precipitated by addition of acetone. The resulting 6 g of alkali stable product (acetone powder) was taken up in chloroform-methanol-water, 30 : 60 : 8 (vol/vol) and applied to a column (2.5×100 cm) packed with QAE-Sephadex A-25 (OH⁻ form, Pharmacia LKB Biotechnology AB, Uppsala) equilibrated with the same solvent. The column was eluted successively with the same solvent (5 column volumes), with pure methanol (2 volumes), and with 0.45 M ammonium acetate in methanol (5 volumes) as a polar solvent⁸⁾. The sample of 3.9 g from the glycolipid fraction eluted in the first solvent (chloroform-methanol-water) was acetylated and then fractionated on a Florisil column (2.0×55 cm, 60~100 mesh, Floridin, Pittsburgh) by the slightly modified method of Saito and Hakomori⁹⁾. The column was eluted successively with 200 mL of hexane-dichloroethane (1 : 4, vol/vol), 200 mL of dichloroethane, 900 mL of dichloroethane-methanol (9 : 1, vol/vol), 500 mL of dichloroethane-methanol (3 : 1, vol/vol), 900 mL of dichloroethane-methanol-water (2 : 8 : 1, vol/vol), 200 mL of methanol, and chloroform-methanol-water (6 : 4 : 1, vol/vol). The solutions of acetylated glycolipids eluted by dichloroethane-methanol (9 : 1 and 3 : 1), dichloroethane-methanol-water (2 : 8 : 1) and chloroform-methanol-water (6 : 4 : 1) were evaporated to dryness, and reacted with 50 mL of 0.5 M KOH in methanol at 37°C for 6 h. Yields of each deacetylated product were 1 g (Fraction I), 13 mg (Fraction II), 120 mg (Fraction III), and 500 mg (Fraction IV). Of these fractions, Fractions I and IV were used for isolation of neutral and amphoteric glycolipids in this study.

2.2 Purification of neutral glycolipids.

The sample of 500 mg from the deacetylated neutral glycolipid fraction (Fraction I) eluted with dichloroethane-methanol (9 : 1, vol/vol) on the Florisil column was applied to a column of porous silica gel (2×90 cm, Iatrobeads 6RS-8060, Iatron Lab.) equilibrated with chloroform-methanol-water, 80 : 20 : 1 (vol/vol). The column was then treated with two consecutive linear gradient systems of chloroform-methanol-water (80 : 20 : 1, vol/vol, 730 mL to 50 : 50 : 5, 800 mL and 50 : 50 : 5, 620 mL to 20 : 80 : 10, 750 mL) to obtain mono-, di-, tri- and tetraglycosylceramide fractions. Further purification of each ceramide di-, tri- and tetrasaccharide fraction was carried out on an Iatrobeads column rechromatography. The columns were severally eluted with solvent mixtures of chloroform-methanol-water (85 : 15 : 0.5, vol/vol for elution of ceramide disaccharide fraction and 65 : 25 : 4, vol/vol for ceramide trisaccharide fraction), and 1-propanol - water - 28% NH₄OH (75 : 20 : 5, vol/vol) for ceramide tetrasaccharide fraction.

2.3 Purification of amphoteric glycolipids.

The sample of 500 mg from the deacetylated amphoteric glycolipid fraction (Fraction IV) eluted with chloroform-methanol-water (60 : 40 : 10, vol/vol) on the Florisil column was dissolved in chloroform-methanol-water, 60 : 40 : 10 (vol/vol) and subjected to Iatrobeads col-

umn chromatography (2×90 cm) equilibrated with the same solvent. The column was treated with a linear gradient of chloroform-methanol-water (60 : 40 : 10, vol/vol, 350 mL to 40 : 70 : 15, 400 mL). The flow rate was 0.8 mL/min and the effluent was collected in 5-mL fractions. An aliquot from alternate tubes was analyzed by TLC (described in 2.4), and it was found that two major amphoteric glycolipid fractions were obtained as an appreciably purified glycolipid fraction (PGL₁) and a mixed fraction containing two components (PGL₂ and PGL₃). Purification of PGL₂ and PGL₃ was achieved by rechromatography of the mixed fraction using an Iatrobeads column (2×60 cm). The column was treated with a linear gradient of chloroform-methanol-water (55 : 45 : 4, vol/vol, 650 mL to 30 : 70 : 15, 750 mL).

2.4 Thin-layer chromatography (TLC).

Silica gel 60 precoated plates (E. Merk, Darmstadt) were developed with the following solvents : chloroform-methanol-water, 60 : 40 : 10 (vol/vol), 65 : 25 : 4, and 30 : 30 : 10 ; 1-propanol - water - 28% NH₄OH, 75 : 20 : 5 ; and 1-butanol - acetic acid - water, 40 : 20 : 10. Detection was performed with orcinol-H₂SO₄ reagent for sugars, Dittmer-Lester reagent for phosphorus, ninhydrin reagent for the amino groups, Dragendorff reagent for choline, and I₂ vapor and 50% H₂SO₄-charring for lipids.

2.5 Compositional analysis.

Compositional sugar analysis of the purified glycolipids, as their trimethylsilyl (TMS) methyl glycosides was carried out by GC on a capillary column of chemical bonded type, Hi-Cap-CBP5 (0.2 mm×25 m, Shimadzu, Kyoto) programmed at 2°C/min from 140°C to 230°C. Glycolipid fatty acid and sphingoid compositions were determined by GC and GC-MS, using their methyl esters and trimethylsilyl derivatives, on the same capillary column programmed at 2°C/min for 170 to 240°C for fatty acids and at 2°C/min from 200 to 240°C for sphingoids²⁾.

2.6 Methylation analysis.

Samples (100~200 µg) of the isolated glycolipids were permethylated with NaOH and CH₃I in dimethylsulfoxide according to the method of Ciucanu and Kerek¹⁰⁾. The partially methylated glycolipids were acetolyzed with 0.6 M HCl in 80% acetic acid at 80°C for 18 h, hydrolyzed, reduced with NaBH₄, and then acetylated with a mixture of acetic anhydride-pyridine, 1 : 1 (vol/vol) at 100°C for 10 min. The partially methylated alditol acetates thus obtained were analyzed by GC on the same HiCap-CBP5 capillary column as described above at 200°C. The peaks on the gas chromatograms were identified by comparison of the retention times with those of the following reference partially methylated alditol acetates prepared from the authentic oligosaccharides, Gal β1-6Gal, Gal α1-4Gal and Gal β1-3Gal (Sigma Chemical Co., St. Louis) and confirmed by comparing their physicochemical data with those reported by Björndal *et al.*¹¹⁾ and Jansson *et al.*¹²⁾.

2.7 Acid hydrolysis of amphoteric glycolipids.

Samples of the purified amphoteric glycolipids (500 µg~1 mg) were hydrolyzed with 6 M HCl for 3 h at 100°C for estimation of sugar, sugar phosphate and choline²⁾. The hydrolyzates were partitioned into the two layers of a Folch system¹³⁾, and the upper aqueous layers were dried under a stream of nitrogen. The residues were subjected to TLC analysis²⁾.

2.8 Dephosphorylation of intact and permethylated amphoteric glycolipids.

Isolated amphoteric glycolipids were treated with HF, which selectively cleaves phosphate bonds^{2),3),14)~16)}. In this procedure, 2 mL of 47% HF was added at 18°C to 1~2 mg of the amphoteric glycolipids dissolved in 0.2 mL of dimethylsulfoxide in a polyethylene tube fitted with a cap. After incubation at 18°C for 18 h, the reaction mixture was dialyzed against water to remove HF, and the tube contents were lyophilized. The dephosphorylated lipid produced was purified by Iatrobeads column chromatography as described above. 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 1~2 mg) was carried out as described above, and the permethylated product was purified on a Iatrobeds column equilibrated with chloroform as usual. The purified permethylated amphoteric glycolipid recovered from the column with 1 volume of chloroform-methanol, 90 : 10 (vol/vol) 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.

2.9 Enzymatic hydrolysis.

α -Galactosidase [EC 3.2.1.22] from green coffee beans (Sigma Chemical Co.) and β -galactosidase [EC 3.2.1.23] from jack beans (Seikagaku Corporation, Tokyo) were used¹⁷⁾. Samples of 20~30 μ g were suspended in 0.1 mL of 0.1 M Tris buffer (pH 6.5) for α -galactosidase or in 0.1 mL of 0.1 M citrate buffer (pH 3.5) for β -galactosidase, containing 0.1 mg of sodium taurodeoxycholate. The reaction was carried out with 0.25 units of α -galactosidase or 0.5 units of β -galactosidase at 37°C for 20 h. Incubation was stopped by adding 0.5 mL of a mixture of chloroform-methanol, 2 : 1 (vol/vol). The hydrolysate, after extraction into the lower layer, was dried under a nitrogen stream and then analyzed by TLC.

3 Results

3.1 Isolation of neutral and amphoteric glycolipids from the leech.

Lipids extracted from whole tissues of the leech, *H. nipponia*, were submitted to mild alkaline hydrolysis, and the resulting alkali-stable substances (6 g) were applied to a QAE-Sephadex column. A mixture of the neutral and amphoteric glycolipids (3.9 g) was recovered from the QAE-Sephadex column with chloroform-methanol-water and could be separated into four subfractions (Fractions I, II, III and IV) by Florisil column chromatography.

Fig. 1 Thin Layer Chromatogram of Purified Neutral Glycolipids (NGLs) Obtained from *H. nipponia*.

Lane 1, neutral glycolipid fraction eluted with dichloroethane-methanol (9 : 1, vol/vol) using Florisil column chromatography; lanes 2, 3, 4 and 5, purified NGL₁, NGL₂, NGL₃ and NGL₄, respectively. The separation was performed on a precoated silica gel 60 plate (Merck, Darmstadt) developed in chloroform-methanol-water (60 : 40 : 10, vol/vol), and the spots were visualized with orcinol-H₂SO₄ reagent.

Fig. 2 Thin Layer Chromatogram of Purified Amphoteric Glycolipids (PGLs) and Their Degradation Products (Dep-PGLs) Obtained by HF Treatment for Dephosphorylation.

Lane 1, a mixture of neutral glycolipids, NGL₁ (a), NGL₂ (b) and NGL₃ (c); lane 2, amphoteric glycolipid fraction eluted with chloroform-methanol-water (6 : 4 : 1, vol/vol) using Florisil column chromatography; lanes 3, 4 and 5, purified PGL₁, PGL₂ and PGL₃; lanes 6, 7 and 8, Dep-PGL₁, Dep-PGL₂ and Dep-PGL₃. TLC conditions were the same as described in Fig. 1.

They were stained by orcinol- H_2SO_4 spray reagent and, in addition, Fraction IV reacted with both Dittmer-Lester and Dragendorff reagents on a TLC plate. These observations indicated that Fractions I to III were neutral glycolipid fractions and that Fraction IV was an amphoteric glycolipid fraction having the choline phosphate residue as part of its constituents. From Fraction I (the major neutral glycolipid fraction), four substances, NGL_1 , NGL_2 , NGL_3 and NGL_4 which were obtained by Iatrobeads column chromatography and which migrated on TLC in the region of standard mono- to tetraglycosylceramides were purified and analyzed in detail to determine their chemical structures. The yields of the purified neutral glycolipids obtained from 400 g dry weight of the leech were 63 mg (NGL_1), 130 mg (NGL_2), 420 mg (NGL_3) and 18 mg (NGL_4), respectively (Fig. 1). From Fraction IV (the amphoteric glycolipid fraction) three substances, PGL_1 , PGL_2 and PGL_3 , were isolated which migrated on TLC in the region of standard amphoteric glycolipids prepared in this laboratory from the earthworm, *P. hilgendorfi*^{2).5)}. The yields of the isolated amphoteric glycolipids obtained from 400 g dry weight of the leech were 130 mg (PGL_1), 8 mg (PGL_2) and <3 mg (PGL_3), respectively (Fig. 2).

3.2 Chemical characterization of neutral mono-, di-, tri- and tetraglycosylceramides.

The sugar composition of NGL_1 was determined by GC as glucose and galactose in a molar ratio of 1.00 : 7.14, being a mixture of glucosylceramide and galactosylceramide. The partially methylated alditol acetates derived from NGL_1 were identified as 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl) glucitol (1-linked glucose) and 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl) galactitol (1-linked galactose) as shown in Fig. 3. NGL_2 , NGL_3 and NGL_4 con-

Fig. 3 Gas Chromatograms of Partially Methylated Alditol Acetates Derived from NGL_1 , NGL_2 , NGL_3 and NGL_4 for Glycosidic Linkage Analysis (Substituted Positions of Sugars).

A, NGL_1 ; B, NGL_2 ; C, NGL_3 ; D, NGL_4 ; Gal1-, 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl)galactitol (1-linked galactose); Glc1-, 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl) glucitol (1-linked glucose); -6Gal1-, 1,5,6-tri(*O*-acetyl)-2,3,4-tri(*O*-methyl) galactitol (1,6-linked galactose).

Fig. 4 Thin Layer Chromatogram of the Reaction Products from NGL_4 by Treatment with α -Galactosidase.

Lane 1, intact NGL_4 ; lanes 2, 3 and 4, NGL_4 incubated for 6 h, 12 h and 24 h; a~d, corresponding to mono~tetraglycosylceramides. TLC conditions were the same as described in Fig. 1.

tained only galactose, all three being gala-series glycolipids, and their methylation analysis showed the presence of 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl) galactitol (1-linked galactose) and 1,5,6-tri(*O*-acetyl)-2,3,4-tri(*O*-methyl) galactitol (1,6-linked galactose) in a molar ratio of 1 : 1 for NGL₂, 1 : 2 for NGL₃ and 1 : 3 for NGL₄ (Fig. 3). Anomeric configurations of the galactose residues in the four neutral glycolipids were determined by enzymatic analysis using α - and β -galactosidases (from green coffee beans and jack beans, respectively) ; NGL₁ was degraded with β -galactosidase to ceramide, and NGL₂, NGL₃ and NGL₄ were degraded with α -galactosidase in a stepwise fashion to mono-, di- and trigalactosylceramides corresponding to each glycolipid (Fig. 4). From these findings, the structures of neutral glycolipids were identified as Gal β 1-1Cer for NGL₁, Gal α 1-6Gal β 1-1Cer for NGL₂, Gal α 1-6Gal α 1-6Gal β 1-1Cer for NGL₃ and Gal α 1-6Gal α 1-6Gal α 1-6Gal β 1-1Cer for NGL₄, respectively. Their aliphatic components were determined by GC and GC-MS (Tables 1 and 2). The main fatty acids were C_{22:0} and C_{24:0}, which accounted for more than 60~70% of the total acids, and the sphingoids were composed of C_{18:1} (17~34%), C_{19:1} (20~60%) and C_{22:3} (5~30%). In NGL₁, furthermore, 23% of the sphingoids were trihydroxy bases.

3.3 Chemical characterization of amphoteric mono- and diglycosylceramides.

Dephosphorylation of the three amphoteric glycolipids, PGL₁, PGL₂ and PGL₃ with HF yielded products that no longer stained with Dittmer-Lester or Dragendorff reagents and which migrated to new positions on TLC corresponding to those of three neutral glycolipids,

Table 1 Fatty Acid Compositions of the Leech Neutral and Amphoteric Glycolipids.

FA ^a	Neutral				Amphoteric	
	NGL ₁	NGL ₂	NGL ₃	NGL ₄	PGL ₁	PGL ₂
16 : 0	17.9	15.3	12.2	8.4	13.2	11.3
17 : 0	5.9	—	—	—	5.4	—
18 : 0	9.9	6.2	7.3	11.2	19.1	7.3
22 : 0	28.9	28.2	34.3	44.2	24.0	27.3
24 : 0	32.3	42.2	37.5	27.8	23.0	41.4
Unknown	5.8	8.1	8.7	7.5	7.1	8.8

^a Expressed as percentage of total fatty acids.

Table 2 Sphingoid Compositions of the Leech Neutral and Amphoteric Glycolipids.

Sphingoid ^a	Neutral				Amphoteric	
	NGL ₁	NGL ₂	NGL ₃	NGL ₄	PGL ₁	PGL ₂
d17 : 0	2.5	—	—	—	—	—
br,d18 : 1	6.3	5.2	4.0	3.4	9.6	—
d18 : 1	27.6	23.0	23.8	13.5	48.5	6.0
d18 : 0	7.8	6.8	8.6	5.0	—	—
t18 : 0	12.4	—	—	7.1	—	24.7
br,d19 : 1	16.7	51.5	43.2	18.5	18.4	13.5
d19 : 1	4.9	8.5	7.8	29.9	—	6.8
br, t19 : 0	10.6	—	—	—	—	29.5
d22 : 3	11.2	5.0	5.5	29.7	23.5	19.5

br, branched sphingoid ; d, dihydroxysphingoid ;
t, trihydroxysphingoid.

^a Expressed as percentage of total sphingoids.

NGL₁, NGL₂ and NGL₃ (**Fig. 2**). The molar ratios of phosphorus/sugar of the three amphoteric glycolipids were 1.00 : 1.02 for PGL₁, 1.00 : 1.98 for PGL₂ and 1.00 : 2.89 for PGL₃. Their sugar constituents were analyzed quantitatively as methylglycosides-TMS derivatives, after methanolysis of the dephosphorylated products obtained from PGL₁, PGL₂ and PGL₃. The dephosphorylation was required because the residue substituted with choline phosphate could not be determined as the free neutral sugar under the conditions of methanolysis employed here (5% methanolic HCl at 100°C for 3 h). The following appropriate molar ratios were determined ; PGL₁ contained 1 mol of Gal, PGL₂ contained 2 mol of Gal, and PGL₃ contained Gal : Glc : GlcNAc, 3.20 : 1.00 : 0.95. PGL₃ was thus concluded to be a mixture of amphoteric glycolipids containing galactose, glucose and *N*-acetylglucosamine. Due to the small quantities of PGL₃ remaining after this analysis, it was not possible to further purify this glycolipid ; its purification and characterization will be reported in detail elsewhere. PGL₁ and PGL₂ were respectively hydrolyzed with 6 M HCl at 100°C for 3 h, yielding galactose, galactose 6-phosphate and choline as shown by TLC (**Fig. 5**) ; it should be noted production of some galactose is expected from galactose 6-phosphate under these hydrolytic conditions. For determination of the galactose linkage and the attachment site of a choline phosphate residue to galactose in PGL₁ and PGL₂, partially methylated alditol acetates were prepared 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 as listed in **Table 3**, as are also, for comparison, those derived from NGL₂, the nonphosphorylated analogue of PGL₂. Application to the intact amphoteric glycolipids (Method A in **Table 3**) yielded no detectable galactitol derivative from either PGL₁ or the terminal galactose of PGL₂, since both of the expected galactitol products remain phosphorylated and are hence not observable under the conditions used here for GC analysis. The only product observed from PGL₂ was therefore 1,5,6-tri(*O*-acetyl)-2,3,4-tri(*O*-methyl)galactitol (1,6-linked galactose) derived from the internal galactose. Dephosphorylation with HF prior to methylation (Method B in **Table 3**) yielded 1,6-linked galactose from internal galactose of PGL₂ and 1,5-di(*O*-acetyl)-2,3,4,6-tetra(*O*-methyl)galactitol (1-linked galactose) from the terminal galactose of both PGL₁ and PGL₂. However, if the amphoteric glycolipids were first permethylated and the products, still substituted with choline phosphate, were then dephosphorylated (Method C in **Table 3**), subsequent acetylation produced 1,6-linked galactose from both PGL₁ and PGL₂. These results indicated that

Fig. 5 Thin Layer Chromatograms of the Acidic Hydrolyzates of PGL₁.

Panel A : lane 1, intact PGL₁ ; lane 2, authentic galactose ; lane 3, authentic galactose 6-phosphate ; lane 4, acidic hydrolyzate of PGL₁. Panel B : lane 1, authentic choline; lane 2, acidic hydrolyzate of PGL₁. The plate were developed with chloroform-methanol-water (30 : 30 : 10, vol/vol) for A and with 1-butanol - acetic acid - water (40 : 20 : 10, vol/vol) for B. The spots were visualized with orcinol-H₂SO₄ reagent for A and with Dragendorff reagent for B.

Table 3 Glycosidic Linkage Analysis (Substituted Positions of Sugars) of PGL₁ and PGL₂.

Methylation method	PGL ₁	PGL ₂
A	N.D. ^a	1,6 ^b Gal
B	1Gal	1Gal and 1,6Gal
C	1,6Gal	1,6Gal

Method A, alditol acetates obtained from intact PGLs ;

Method B, from the dephosphorylated PGLs ;

Method C, from the permethylated and dephosphorylated PGLs.

^a Not detected.

^b Numbers express the substituted positions of sugar residues.

Fig. 6 Thin Layer Chromatogram of the Reaction Products from Dep-PGL₂ and NGL₂ by Treatment with α - and β -Galactosidases.

Lane 1, a mixture of NGL₁ (a) and NGL₂ (b) ; lane 2, intact Dep-PGL₂ ; lane 3, Dep-PGL₂ incubated with α -galactosidase ; lane 4, Dep-PGL₂ incubated with β -galactosidase ; lane 5, intact NGL₂ ; lane 6, NGL₂ incubated with α -galactosidase ; lane 7, NGL₂ incubated with β -galactosidase. The plate was developed with chloroform-methanol-water (65 : 25 : 4, vol/vol) and the spots were visualized with orcinol-H₂SO₄ reagent.

the galactose linkage in PGL₂ was Gal1-6Gal and that a choline phosphate was attached to the 6-hydroxy group of the terminal galactose in both PGL₁ and PGL₂. For determination of anomeric configurations of the galactose residues, the amphoteric glycolipids were subjected to enzymatic hydrolysis by α - and β -galactosidases. Neither of the intact amphoteric glycolipids were acted upon by either galactosidase. However, after dephosphorylation of PGL₁ and PGL₂ by HF, the resulting neutral glycolipids were degraded with β -galactosidase to ceramide for PGL₁ and to ceramide as well as some residual monogalactosylceramide for PGL₂ (Fig. 6). From these findings, the structures of amphoteric glycolipids were identified as cholinephosphoryl $\rightarrow$ 6Gal β 1-1Cer for PGL₁ and cholinephosphoryl $\rightarrow$ 6Gal β 1-6Gal β 1-1Cer for PGL₂, respectively. Their aliphatic components are summarized in **Tables 1** and **2**. The fatty acyl moieties in PGL₁ and PGL₂ mainly consisted of C_{22:0} and C_{24:0} acids (47~69%), which were very similar fatty acid compositions to those of neutral glycolipids. On the other hand, the sphingoid patterns of PGL₁ and PGL₂ were quite different. The sphingoids in PGL₁ were composed of C_{18:1} (58%), C_{19:1} (18%) and C_{22:3} (24%), while in PGL₂ more than 54% of the total sphingoids were C₁₈- and C₁₉-trihydroxy bases.

Table 4 Gala-Series Glycolipids Found in Protostomia phyla.

Prefix of series	Structure	Organism
Gala	Gal α 1-4	Cestoda (Plathelminthes) ²³⁾
Isogala ^a	Gal β 1-4	Pelecypoda (Mollusca) ²⁴⁾
Neogala	Gal β 1-6	Gastropoda (Mollusca) ^{25),26)} Oligochaeta (Annelida) ^{1),2),4),5)} Cestoda (Plathelminthes) ²³⁾
Isonogala	Gal α 1-6	Gastropoda (Mollusca) ²²⁾ Hirudinea (Annelida)

^a Not isolated.

4 Discussion

Galactose linkages found in nature have been classified into three core types, Gal α 1-4Gal (gala), Gal β 1-4Gal (isogala) and Gal β 1-6Gal (neogala), and glycosphingolipids containing these and other core structures have been reviewed¹⁸⁾. In this study, we found that neutral tri- (NGL₃) and tetraglycosylceramides (NGL₄) isolated from *H. nipponia* contain a fourth galactose linkage, Gal α 1-6Gal (tentatively designated isoneogala) in their sugar chains, which structures may represent a new type of glycosphingolipid core series (Table 4).

Annelida is divided taxonomically into five classes, but up to now published information on glycolipids is available for only two, *P. hilgendorfi*^{1),2),4),5)} and *Pheretima asiatica*¹⁹⁾ (land annelida in class Oligochaeta), and *Marphysa sanguinea*²⁰⁾, *Tylorhynchus heterochaeta*³⁾ and *Neanthes diversicolor*²¹⁾ (marine annelida in class Polychaeta). Neutral and amphoteric glycolipids isolated from these organisms have mainly the neogala linkage, Gal β 1-6Gal as the core sequence.

In the present study, four neutral and two amphoteric glycolipids from the leech, *H. nipponia* belonging to the class Hirudinea were isolated and characterized by compositional and methylation analyses and exogalactosidase treatments as follows; NGL₁, Gal β 1-1Cer (galactosylceramide); NGL₂, Gal α 1-6Gal β 1-1Cer (isonogalabiosylceramide); NGL₃, Gal α 1-6Gal α 1-6Gal β 1-1Cer (isonogalatriasylceramide); and NGL₄, Gal α 1-6Gal α 1-6Gal α 1-6Gal β 1-1Cer (isonogalatetraosylceramide) for neutral glycolipids, and PGL₁, cholinephosphate→6Gal β 1-1Cer (cholinephosphorylgalactosylceramide); and cholinephosphate→6Gal β 1-6Gal β 1-1 Cer (cholinephosphorylneogalabiosylceramide) for amphoteric glycolipids. In these isoneogala glycolipids, NGL₂ has already been described by Matsubara and Date²²⁾ in a marine mollusc, *Nerita albicilla* together with 3-*O*-methyl-Gal α 1-6Gal β 1-1Cer. However, isoneogalatriasyl- (NGL₃) and isoneogalatetraosylceramides (NGL₄) characterized here have so far not been found in any other animals. The present findings demonstrate the coexistence of isoneogala- and neogala- glycosphingolipids in one animal species, and raise questions about the biosynthetic processes involved in the formation of these amphoteric glycolipids, especially the identity of the donor and the enzyme in the phosphocholine:galactolipid phosphocholinetrasferase reaction. It is also noteworthy that in the sphingoid composition of the leech glycolipids, a polyunsaturated base, C_{22:3}, has been observed.

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日本油化学会誌本号掲載 論文要旨

[総説]

機能性タンパク質の配向制御

小山 行一

新技術事業団「さきがけ研究 21」

(〒300-26 つくば市東光台 5-9-4 筑波研究コンソーシアム)

機能性タンパク質の人為的配向制御は将来のバイオデバイス開発に必要な不可欠な技術である。本報告では、まずこれまで試みてこられた感光性タンパク質の一般的な配向制御技術を概観した後、バクテリオロドプシンというきわめて安定なタンパク質を BS 抗体という一分子に 2 種の異なった結合サイトを有する特殊な抗体を用いて配向を制御したわれわれの結果を紹介し、さらに免疫電顕法による配向状態を解析する手段についても述べる。

(連絡者：小山行一) Vol. 45, No. 8, 715 (1996).

[報文]

熱帯植物由来のデンプンをベースにしたポリカルボン酸塩の
生分解性、加水分解性及びビルダー性能

Isananto WINURSITO・松村 秀一

慶應義塾大学 理工学部

(〒223 横浜市港北区日吉 3-14-1)

部分的ジカルボキシル化多糖をコーン、サゴ及びタピオカデンプンより合成し、それらの生分解性、加水分解性及び洗剤配合物中でのビルダー性能について比較を行った。ジカルボキシル化デンプンナトリウム塩 (DC-starch) の生分解性はジカルボキシル化度に依存した。約 75-85 mol% 以上の未反応グルコピラノース部分をポリマー鎖中に有する DC-starch は優れた生分解性を示した。一方、ジカルボキシル化度の高い DC-starch は生分解性に劣ったが、洗剤組成物中で優れたビルダー性能を示した。コーン、サゴ及びタピオカデンプンから得られた DC-starch の中では、加水分解性及びビルダー性能に顕著な差異は認められなかった。

(連絡者：松村秀一) Vol. 45, No. 8, 723 (1996).

[報文]

ヒル, *Hirudo nipponia* における Gal α 1-6 Gal 及び
Gal β 1-6 Gal 構造を有するスフィンゴ糖脂質杉田 陸海^{*1}・森川 亜紀^{*1,*3}・John T. Dulaney^{*2}・岡田 亜希子^{*1}^{*1} 滋賀大学教育学部化学教室 (〒520 滋賀県大津市平津 2-5-1)^{*2} テネシー大学医学部腎臓学教室 (951 Court Avenue, 649 D Memphis, TN 38163, USA)^{*3} 現住所 日本医学臨床検査所バイオアッセイ事業部 (〒677 兵庫県西脇市中畑町 17-18)

チスイビル, *Hirudo nipponia* (環形動物, ヒル綱) の中性糖脂質には微量ながらグルコ及びガラクトセレブロシド (NGL₁) も存在するが、主要成分は Gal α 1-6 Gal β 1-1 Cer (NGL₂), Gal α 1-6 Gal α 1-6 Gal β 1-1 Cer (NGL₃), Gal α 1-6 Gal α 1-6 Gal α 1-6 Gal β 1-1 Cer (NGL₄) の gala-6 系糖脂質である。これらの糖鎖構造は、アノマー配置において、今までに他の環形動物から見いだしている neogala 系列 (Gal β 1-6 Gal) とは異なっており, isoneogala 系列 (Gal α 1-6 Gal) と称することができよう。一方、現在のところ環形動物を特色づ

けるリン糖脂質と考えている両性イオン型糖脂質は、neogala 系列の糖鎖を基本骨格とする cholinephosphate→6 Gal β 1-1 Cer, cholinephosphate→6 Gal β 1-6 Gal β 1-1 Cer である。この糖脂質のセラミド部分の分子種は、脂肪酸: C_{16:0}, C_{18:0}, C_{22:0}, C_{24:0} 酸; スフィンゴイド塩基: C_{18:1}⁻, C_{19:1}⁻, C_{22:3}⁻ ジヒドロキシスフィンゴイド, C_{18:0}⁻, C_{19:0}⁻ トリヒドロキシスフィンゴイドが主要成分であった。

(連絡者: 杉田陸海) Vol. 45, No. 8, 731 (1996).

[報文]

油脂分解微生物の利用 (第1報)

Candida famata US-238 株によるドコサヘキサエン酸の相対的濃縮

南 憲二^{*1}・山本浩志^{*1}・辻脇義一^{*1}・樺本五男^{*2}

^{*1} 植田製油株式会社 (〒658 兵庫県神戸市東灘区魚崎浜町 17)

^{*2} 大阪府立大学農学部 (〒593 大阪府堺市学園町 1-1)

油脂を分解資化する微生物の中で相対的にドコサヘキサエン酸 (DHA) を資化する能力の低いものについてスクリーニングし、一菌株を得た。本菌は(財)日本食品分析センターによって *Candida famata* US-238 と同定された。本菌を用い、魚油中の DHA の相対的濃縮に適する培養条件について検討した。さらに、大量の DHA 高含有油脂を調製するという目的から、ジャーフェーマンターを用いた培養を試みた。その結果、DHA 約 50% を含有する油脂を得ることができた。

(連絡者: 南憲二) Vol. 45, No. 8, 741 (1996).

[報文]

エポキシ環を有する両親媒性リン酸エステルによる

抗体の修飾と抗体保持リポソームの作製

宋 溪明^{*2}・中村幹彦^{*1}・山本隆斉^{*1}・故黒崎富裕^{*1}・辻井 薫^{*1}

^{*1} 花王(株)基礎科学研究所, 〒321-34 栃木県芳賀郡市貝町赤羽 2606

^{*2} 現住所: 遼寧大学化学系 (中国遼寧省瀋陽市皇姑区崇山中路 66 号)

二種類のリン酸二水素アルキル (C₁₂-MAP, C₁₆-MAP, Fig. 1, 2 を参照) のモノナトリウム塩と 1,2-epoxy-3-chloropropane (ECP) の反応により, Sodium alkyl 2-hydroxy-3-chloropropylphosphate (C₁₂-AHCP-Na, C₁₆-AHCP-Na) を合成した。この二種類の AHCP-Na ともそれぞれの適当なアルコール-水混合溶液中、室温で当量の NaOH で処理することにより、非常に速やかにかつ定量的に、エポキシ環を有する両親媒性リン酸エステルである Sodium alkyl 2,3-epoxypropyl phosphate (C₁₂-AGP-Na, C₁₆-AGP-Na, Fig. 1 を参照) が生成した。

AGP-Na のエポキシ環と求核剤 (-NH₂ および -SH) の反応性を利用する、AGP-Na によるマウス IgG および抗 DNP モノクローナル抗体の修飾反応を行い、修飾実験後の遊離のアミノ酸の減少より IgG 分子の修飾率を算出した。その結果、0.05 M ホウ酸緩衝液 (pH=9.0) 中で C₁₂-AGP, C₁₆-AGP とも抗体の全遊離アミノ基の約 15% を修飾した。より疎水性の高い C₁₆-AGP による修飾前後の抗 DNP モノクローナル抗体の活性の検討から、抗体の修飾前後の活性変化は殆ど無く、抗 DNP モノクローナル抗体が活性を保持したまま C₁₆-AGP で修飾されたことが分かった。したがって、C₁₆-AGP は他のタンパク質修飾剤に比べ非常に良いタンパク質修飾剤と言え、より疎水性の高い修飾剤として抗体保持リポソームの作製に利用できる可能性があることが分かった。

更に、透析法で C₁₆-AGP 修飾抗 DNP モノクローナル抗体を活性を保持したままリポソーム表面へかなりの高効率 (修飾抗体使用量の 30%) で導入できた。従来のカップリング剤は反応後、分離が困難で問題であったが、AGP は水溶液中で分解し、分離容易な dodecylglycerylphosphate を与えるので、抗体保持リポソームのより容易な作製が可能となった。

(連絡者: 中村幹彦) Vol. 45, No. 8, 747 (1996).