J. Biochem. 129, 93–100 (2001)

Characterization of a Novel Triphosphonooctaosylceramide from the Eggs of the Sea Hare, *Aplysia kurodai*[1]

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Received October 16, 2000; accepted October 25, 2000

We have reported the existence of a triphosphonoglycosphingolipid, EGL-I, in the eggs of a sea gastropod, *Aplysia kurodai* [Yamada, S., Araki, S., Abe, S., Kon, K., Ando, S., and Satake, M. (1995) *J. Biochem.* 117, 794–799]. We have now isolated a novel glycosphingolipid, named EGL-II, from the eggs of *Aplysia*. By component analysis, sugar analysis, permethylation studies, fast atom bombardment–mass spectrometry, secondary ion mass spectrometry, and proton magnetic resonance spectrometry, its structure was revealed to be as follows: Galα1→3(GlcNAcα1→2)Galα1→3(3-*O*-MeGalα1→2)Galα1→3[6′-*O*-(2-aminoethylphosphonyl)Galα1→2](2-aminoethylphosphonyl→6)Galβ1→4(2-aminoethylphosphonyl→6)Glcβ1→1ceramide. The major aliphatic components of the ceramide are palmitic acid, stearic acid, and anteisononadeca-4-sphingenine.

Key words: 2-AEP*n*, *Aplysia kurodai*, egg mass, phosphonoglycosphingolipid, SIMS.

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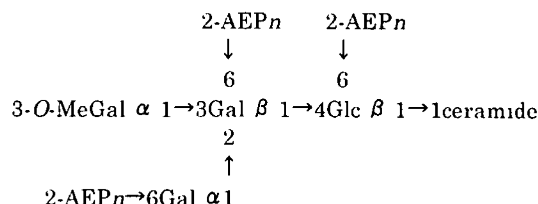
In our systematic studies on the structures of glycolipids in tissues of a sea hare, *Aplysia kurodai*, a new group of glycolipids containing 2-aminoethylphosphonate (2-AEP*n*), which belongs to a family of phosphonoglycosphingolipids (P*n*GSL), has been detected (*1*). P*n*GSLs seem to be unique to mollusca (*2–13*) and crustaceans (*14*), and have not yet been found in Deuterostomia. On the other hand, sialic acid-containing glycosphingolipids, gangliosides, have never been detected in the invertebrate phyla of Protostomia.

In *Aplysia* tissues, each tissue shows a characteristic pattern of P*n*GSLs on two-dimensional TLC (*1*). We have reported that the egg mass contains two main egg-specific glycolipids, EGL-I and EGL-II, which are not found in the adult *Aplysia* (*2*). Gangliosides are known to play important roles in early cell differentiation and the developmental processes of mammalian cells (*15, 16*). Therefore, egg-specific glycolipids may be involved in the differentiation and development in *Aplysia*. In order to understand the physiological roles of these new species of glycolipids, a detailed elucidation of their chemical structure is required. Previously, we reported the structure of the egg glycolipid, EGL-I, as follows (*2*).



In the present study, we determined the structure of a novel egg glycolipid, EGL-II, in the eggs of *Aplysia*, and found that its carbohydrate structure is distinct from EGL-I and the common carbohydrate structures of glycolipids isolated from the tissues of an adult sea hare.

MATERIALS AND METHODS

Fractionation and Purification of Egg Glycolipid, EGL-II—Egg masses of *Aplysia kurodai* were collected after they were laid in early summer, frozen immediately and stored at −80°C until use. From the acetone powder made from 1 kg of egg mass, total water-soluble glycolipids (273 mg) were obtained using the method described previously (*3*). EGL-II was further purified by successive chromatography on an Iatrobeads column. The total glycolipid fraction (140 mg/run) was dissolved in 2 ml of chloroform:methanol: water (60:35:8, v/v) and applied to a column of Iatrobeads (6RS-8060, Iatron Lab., Tokyo; 1.6 × 36.5 cm) previously

[1] This work was supported by a Grant-in-Aid for Scientific Research (C) (to S.A. and S.A) from the Ministry of Education, Science, Sports and Culture of Japan (10680563).

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Abbreviations: 2-AEP*n*, 2-aminoethylphosphonate; F-21, 3-*O*-MeGalβ1→3(2-aminoethylphosphonyl→6)GalNAcα1→3[6′-*O*-(2-aminoethylphosphonyl→6)Galα1→2](2-aminoethylphosphonyl→6)Galβ1→4Glcβ1→1Cer; FAB-MS, fast atom bombardment–mass spectrometry; HPTLC, high-performance thin-layer chromatography; HF, hydrogen fluoride; PBS, phosphate-buffered saline; P*n*GSL, phosphonoglycosphingolipid; PVP, polyvinylpyrrolidone; SIMS, secondary ion mass spectrometry; SGL-I, 4-*O*-MeGlcNAcα1→4GalNAcα1→3[6′-*O*-(2-aminoethylphosphonyl)Galα1→2](2-aminoethylphosphonyl→6)Galβ1→4(2-aminoethylphosphonyl→6)Glcβ1→1Cer; SGL-I′, 3-*O*-MeGalβ1→3GalNAcα1→3[6′-*O*-(2-aminoethylphosphonyl)Galα1→2]Galβ1→4Glcβ1→1Cer; SGL-II, 3-*O*-MeGalβ1→3GalNAcα1→3[6′-*O*-(2-aminoethylphosphonyl)Galα1→2](2-aminoethylphosphonyl→6)Galβ1→4Glcβ1→1Cer.

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equilibrated with the same solvent. Glycolipids were eluted with a discontinuous gradient of chloroform:methanol: water as follows: 60:35:8, v/v, 160 ml; 55:38:10, v/v, 103 ml; and 50:45:10, v/v, 105 ml. Fractions of 2 ml were analyzed by high-performance thin-layer chromatography (HPTLC) with Solvent II, as described below, and the fraction containing EGL-II was re-chromatographed on an Iatrobeads column (1.0 × 45 cm) using 180 ml of a linear gradient of 1-propanol:concentrated ammonium hydroxide:water (70:10: 10 to 60:10:20, v/v). Fractions of 1 ml were collected and monitored by HPTLC with Solvent II.

Thin-Layer Chromatography Analysis—TLC was performed using the following solvents: Solvent I, chloroform: methanol:water (60:35:8, v/v); Solvent II, 1-propanol:concentrated ammonium hydroxide:water (75:5:25, v/v); and Solvent III, 1-propanol:water (7:3, v/v). HPTLC plates, Silica Gel 60 (Merck), were used. The location of the glycolipids was determined using the anthrone–sulfuric acid reagent for sugar (*17*), Dittmer-Lester's reagent for phosphorus (*18*), or ninhydrin reagent for amino groups.

Analysis of Chemical Composition—Ninhydrin-positive compounds were analyzed qualitatively and quantitatively using an amino acid analyzer (Hitachi 835) after hydrolysis with 4 M HCl at 100°C for 8 h. The phosphorus content was determined by the method of King (*19*). The hexosamine content was determined by the method of Elson-Morgan with a slight modification after hydrolysis with 3 M HCl at 100°C for 15 h (*20*).

For the analysis of carbohydrate composition, glycolipids were methanolyzed in 5% anhydrous methanolic HCl at 100°C for 3 h; trimethylsilyl or trimethylsilyl *N*-acetyl derivatives of methylglycoside were analyzed by GLC in a Shimadzu GC-14A apparatus using a fused silica capillary column (ULBON HR-1, 0.32 mm × 30 m) at a temperature programmed to increase from 140 to 230°C at a rate of 2°C/ min. Methylesters of fatty acid were extracted from the methanolysate with petroleum ether and analyzed on the same column at a temperature programmed to increase from 170 to 240°C at a rate of 2°C/min.

Long-chain bases were obtained by hydrolysis of EGL-II in 2 M aqueous methanolic HCl at 85°C for 3 h, separated as their aldehyde derivatives on a column of 2% OV-1 at 180°C by GLC and GC-MS (*21*), and identified by their relative retention times and mass spectra (*22*).

Dephosphorylation—EGL-II or permethylated EGL-II was treated with hydrogen fluoride (HF), which selectively cleaves phosphate ester bonds (*23*). HF (47%, w/v) was added to EGL-II or permethylated EGL-II in a polyallomer tube fitted with a cap. The reaction mixture was left to stand at 15°C for 20 h, and then neutralized to pH 6.5 with saturated LiOH. The precipitate was removed by centrifugation. The supernatant was dialyzed against water and lyophilized.

Mild Acid Hydrolysis—HF-treated EGL-II was partially hydrolyzed with 0.2 M HCl at 100°C for 1 h. The hydrolysate was mixed with 5 volumes of chloroform:methanol (2:1, v/v); the lower layer thus obtained was condensed and the glycolipid fragments were separated by preparative TLC with Solvent I.

Methylation Analysis—EGL-II, HF-treated EGL-II, and glycolipid fragments were permethylated by the method of Imanari and Tamura (*24*) with modification (*25*). The partially methylated alditol acetates obtained were analyzed by GLC on a fused silica capillary column of ULBON HR-1 (0.32 mm × 30 m) as described previously (*8*) and identified by mass spectrometry on a capillary column of HiCap-CBP5 (0.22 mm × 25 m: Shimadzu., Kyoto) (*26*).

Mass Spectrometry—Fast atom bombardment–mass spectrometry (FAB-MS) of EGL-II was recorded in the negative-ion mode using a JEOL DX 304/DX 304 mass spectrometer equipped with a JMA-DA 5000 computer system. The spectrum was recorded at an accelerating voltage of 3.0 kV. A mixture of triethanolamine and 15-crown ether-5 (2:1, v/v) was used as a matrix. Secondary ion mass spectrometry (SIMS) analysis of HF-treated EGL-II was performed in the negative-ion mode on a Fourier transform ion cyclotron resonance mass spectrometer BioApex47E (Bruker Instruments) fitted with a cesium gun. HF-treated EGL-II dissolved in chloroform:methanol (1:2, v/v) was mixed with triethanolamine as the matrix. Spectra were recorded at an accelerating voltage of 10 kV.

^{1}H-NMR Spectroscopy—^{1}H-NMR spectra were recorded with a Varian XL-400 spectrometer (Varian Associates) at 400 MHz in a Fourier transform mode. EGL-II was dissolved in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) and the spectrum was recorded at 50°C. HF-treated EGL-II was dissolved in $[D]_6$ dimethylsulfoxide containing 2% D_2O, and the spectrum was recorded at 60°C.

Immunostaining of Glycolipids with Anti–EGL-I and Anti–SGL-II Antisera on TLC Plates—Anti–EGL-I and anti–SGL-II antisera were raised in rabbits as described previously (*2*, *27*). EGL-II and other phosphonoglycolipids (5 μg each) were applied to plastic plates (Polygram, silG/ UV254, Macherey-Nagel, Germany), and the plates were developed with Solvent II. Enzyme-immunostaining on plastic TLC plates was performed by the method of Higashi *et al.* (*28*) with slight modification (*2*), and the chromatograms were stained by the method of Mierendorf *et al.* (*29*).

RESULTS

Isolation of Glycolipid EGL-II—EGL-II was prepared from the total water-soluble glycolipid fraction of an egg mass by Iatrobeads column chromatographies as described in "MATERIALS AND METHODS." EGL-II migrated as a single band in HPTLC in three solvent systems (Fig. 1, lanes 3–5), and was stained with anthrone, Dittmer-Lester's and ninhydrin reagents. The yield of EGL-II was 9 mg /100 g dry weight of egg mass of *Aplysia*.

Chemical Composition of EGL-II—Amino acid analysis (Fig. 2) showed the presence of 2-AEP*n* and glucosamine in a molar ratio of 3:1. The contents of 2-AEP*n* and glucosamine determined using the amino acid analyzer are equivalent to those of phosphorus and glucosamine, respectively, estimated by colorimetric analysis (data not shown).

The ceramide contained 16:0 (43.9%), 17:0 (8.1%), and 18:0 (48.0%) as the fatty acids, and d 18:1 (19.1%), anteiso d 19:1 (50.5%), anteiso d 21:0 (13.6%), and anteiso d 22:0 (16.7%) as the long-chain base components.

Carbohydrate Composition of EGL-II—When EGL-II was treated with HF, which selectively cleaves phosphate ester bonds (*23*), the complete carbohydrate backbone of EGL-II could be detected as methylglycosides by GLC, because the phosphate ester linkage between the carbohydrate and 2-AEP*n* cannot be cleaved by usual methanolysis (*3*). After treatment with HF, EGL-II yielded glucose, galac-

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tose, *N*-acetylglucosamine, and 3-*O*-methylgalactose in a molar ratio of 1:5:1:1 (Table I). The carbohydrates to which 2-AEP*n* is attached were determined by comparison of the molar ratio of carbohydrates both before and after the removal of 2-AEP*n* by HF treatment. On GLC of methyl-glycosides derived from EGL-II by methanolysis, galactose, *N*-acetylglucosamine, and 3-*O*-methylgalactose in a molar ratio of 3:1:1 were detected. The above results indicate that EGL-II is composed of glucose/galactose/*N*-acetylglucosamine/3-*O*-methylgalactose in a molar ratio of 1:5:1:1, and 2-AEP*n* is attached to each of 2 mol of galactose and 1 mol of glucose.

Carbohydrate Linkage and Sequence—Figure 3 shows the negative-ion FAB-mass spectra of EGL-II and negative-ion SIMS of HF-treated EGL-II. The ions (a) at *m/z* 536, 550, 564, and 578 in HF-treated EGL-II correspond to the ceramide species containing 16:0-d 18:1, 16:0-anteiso d 19:1, 18:0-d 18:1, and 18:0-anteiso d 19:1, respectively (Fig. 3B).

The pseudomolecular ions, $[M-H]^-$, of HF-treated EGL-II at *m/z* 1,887, 1,901, 1,915, and 1,929 correspond to an octaglycosyl ceramide with 6 mol of hexose, 1 mol of *O*-methylhexose and 1 mol of *N*-hexosamine (Fig. 3B). On the other hand, the pseudomolecular ions, $[M-H]^-$, of EGL-II at *m/z* 2,208, 2,222, 2,236, and 2,250 correspond to those of HF-treated EGL-II plus 3 mol of 2-AEP*n* (Fig. 3A). Thus, the results of the mass spectrometry confirmed the carbohydrate composition of EGL-II determined by GLC (Table I) and the presence of 3 mol of 2-AEP*n* in EGL-II.

Table II summarizes the results of permethylation analyses of EGL-II, HF-treated EGL-II, and glycolipid fragments. The presence of 2,3,4,6-tetra-*O*-methylgalactitol (1-linked galactose), 4,6-di-*O*-methylgalactitol (1,2,3-linked galactose), and 3,4,6-tri-*O*-methyl-2-*N*-methylacetamidoglucitol (1-linked *N*-acetylglucosamine) in HF-treated EGL-II shows that 5 mol of galactose are positioned at the branching point or at the nonreducing end, and EGL-II has terminal 3-*O*-methylgalactose and *N*-acetylglucosamine.

The extended carbohydrate sequence of the EGL-II oligosaccharide was constructed from the characteristic fragment ions of negative-ion SIMS of HF-treated EGL-II (Fig. 3B). The fragment ions at *m/z* 698, 712, 726, and 740 were assigned to $[Glc-Cer]^-$ (b), because glucosylceramide was found in a mild acid hydrolysate of HF-treated EGL-II by GLC (Fig. 4 and Table I). The fragment ions, $[M-Gal]^-$ (e) or $[M-GlcNAc]^-$ (f), produced by the elimination of terminal galactose or terminal *N*-acetylglucosamine were detected at *m/z* 1,725, 1,739, 1,753, and 1,767 or *m/z* 1,684, 1,698, 1,712, and 1,726, respectively. The fragment ions $[M-(Gal\text{-}GlcNAc)]^-$ (e′), that arise from the elimination of both terminal galactose and *N*-acetylglucosamine, were detected at *m/z* 1,522, 1,536, 1,550, and 1,564 with weaker intensity than (e). The fragment ions at *m/z* 1,360, 1,374, 1,388, and 1,402 were assigned to $[M-(Gal\text{-}Gal\text{-}GlcNAc)]^-$ (d) and the fragment ions at *m/z* 1,184, 1,198, 1,212, and 1,226 with weaker intensity than (d) were assigned to $[M-(Gal\text{-}Gal\text{-}GlcNAc\text{-}3\text{-}O\text{-}MeGal)]^-$ (d′). The fragment ions at *m/z* 1,022, 1,036, 1,050, and 1,064 and those with relatively weak intensity at *m/z* 860, 874, 888, and 902 correspond to $[Gal\text{-}Gal\text{-}Glc\text{-}Cer]^-$ (c) and $[Gal\text{-}Glc\text{-}Cer]^-$ (c′), respectively. Thus, the sequence of EGL-II oligosaccharide was concluded to be (Gal)(GlcNAc)Gal→(3-*O*-MeGal)Gal→(Gal)Gal→Glc.

The mass spectrum of EGL-II shows molecular ion species at *m/z* 805, 819, 833, and 847 that can be ascribed to $[2\text{-}AEPn\text{-}Glc\text{-}Cer]^-$. Furthermore, the fragment ions at *m/z* 1,357, 1,371, and 1,385 were assigned to $[(2\text{-}AEPn\text{-}Gal)(2\text{-}AEPn\text{-}Gal)\text{-}(2\text{-}AEPn\text{-}Glc)\text{-}Cer]^-$ (Fig. 3A). These fragment ions were also detected in the FAB-mass spectrum of SGL-I

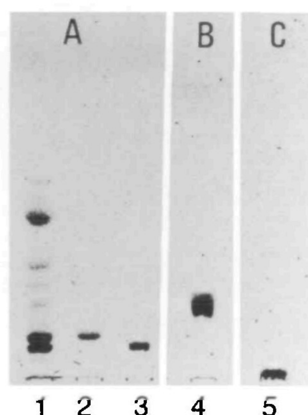

Fig. 1. **Thin-layer chromatograms of glycolipids obtained from the eggs of *Aplysia kurodai*.** The plates were developed with (A) 1-propanol:concentrated ammonium hydroxide:water (75:25:5, v/v), (B) 1-propanol:water (7:3, v/v), and (C) chloroform:methanol:water (60:35:8, v/v), and sprayed with anthrone reagent. Lane 1, total glycolipid from eggs; lane 2, EGL-I; and lanes 3–5, purified EGL-II.

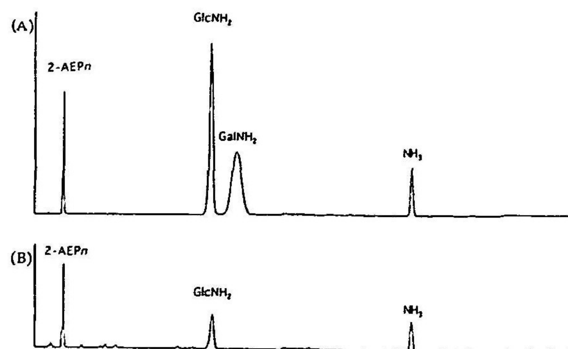

Fig. 2. **Amino acid analysis of EGL-II.** 2-AEP*n* was determined using an amino acid analyzer with authentic standards after hydrolysis in 4 M HCl at 100°C for 8 h. (A) Standards. (B) EGL-II.

TABLE I. **Carbohydrate composition of the acid methanolysates of EGL-II, HF-treated EGL-II, and glycolipid fragments, A to F, as determined by GLC.**

		3-*O*-MeGal	Gal	Glc	GlcNAc
EGL-II		1	3	–	1
HF-treated EGL-II		1	5	1	1
Glycolipid fragment[a]	A	–	–	1	–
	B	–	1	1	–
	C	–	2	1	–
	D	1	3	1	–
	E	1	4	1	1
	F	1	5	1	1

[a]Glycolipid fragments, A to F, were prepared from a mild acid hydrolysate of HF-treated EGL-II as described in "MATERIALS AND METHODS."

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(data not shown).

For determining the location of 2-AEP*n*, the permethylated EGL-II was treated with HF, which selectively cleaves phosphate ester bonds (*23*), and then subjected to acetolysis. This treatment produced new peaks of 2,3,4-tri-*O*-methylgalactitol, 2,3-di-*O*-methylglucitol, and 4-*O*-methylgalactitol, indicating the substitution of 2-AEP*n* at the C-6 of glucose, C-6 of galactose at the nonreducing end and C-6 of galactose at the branching point (Table II).

Figure 4 shows thin-layer chromatogram of glycolipid fragments, A to F, obtained from a mild acid hydrolysate of HF-treated EGL-II. Each glycolipid fragment was isolated by preparative TLC, and its carbohydrate composition and linkage positions were analyzed (Tables I and II). Glycolipid fragments A and B were identified as Glc1-1Cer and Gal1-4Glc1-1Cer. GC-MS of the partially methylated alditol acetates obtained from glycolipid fragment C showed the presence of 2,3,4,6-tetra-*O*-methylgalactitol, 2,4,6-tri-*O*-methylgalactitol, 3,4,6-tri-*O*-methylgalactitol, and 2,3,6-tri-*O*-methylglucitol. These results show that glycolipid fragment C consists of two species of trihexosylceramide, Gal1-2Gal1-4Glc1-1Cer and Gal1-3Gal1-4Glc1-1Cer. As indicated in Table I, glycolipid fragments D and E were characterized as pentaosylceramide containing 1 mol of glucose and 3-*O*-methylgalactose, and 3 mol of galactose, and heptaosylceramide containing glucose, galactose, *N*-acetylglucosamine, and 3-*O*-methylgalactose in a molar ratio of 1:4:1:1. The results of permethylation analysis of glycolipid fragments D and E show that glycolipid fragment D consists of two species of pentaosylceramide, 3-*O*-MeGal1-2Gal1-3(Gal1-2)Gal1-4Glc1-1Cer and Gal1-3(3-*O*-MeGal1-2)Gal1-3Gal1-4Glc1-1Cer, and fragment E consists of two species of hep-

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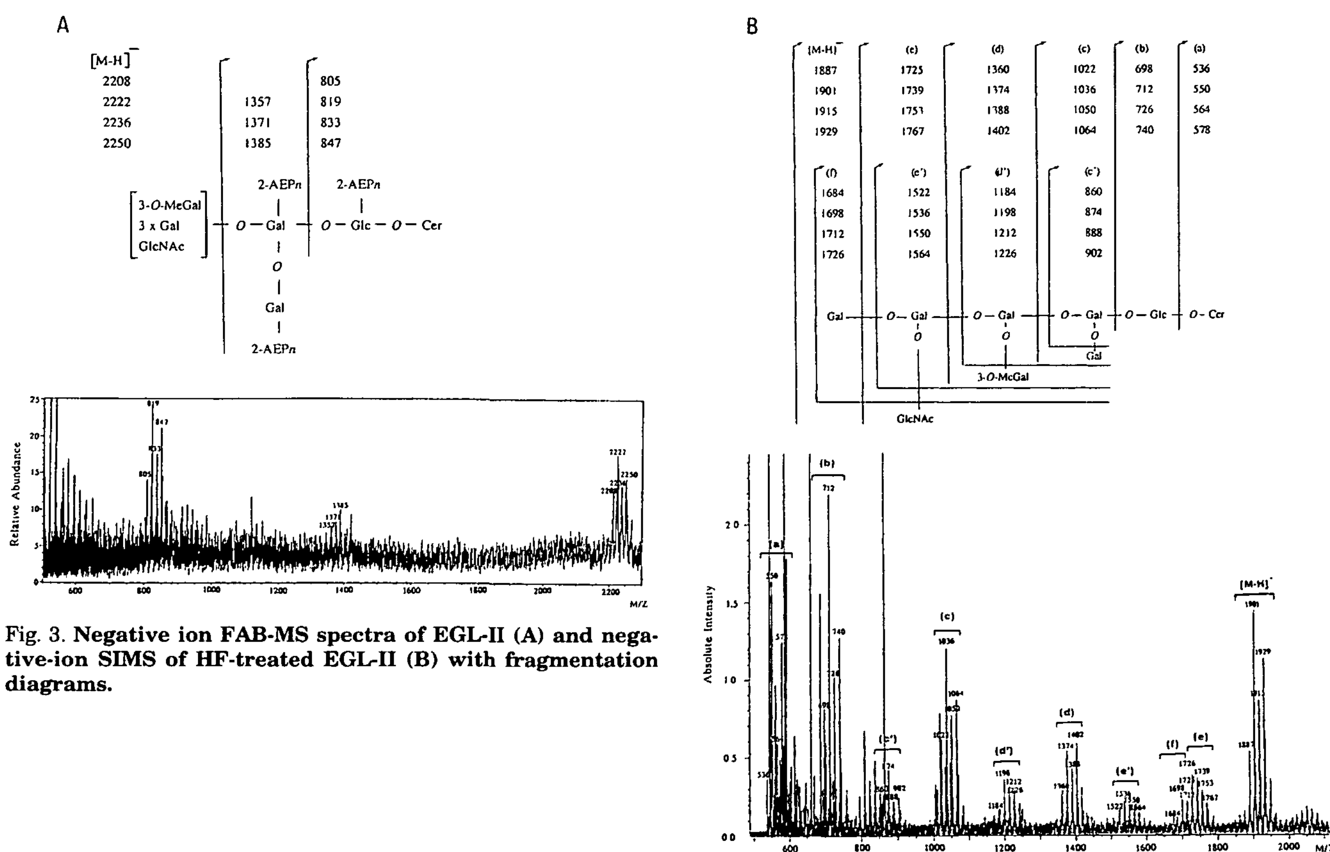


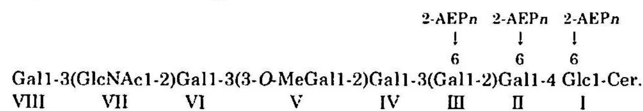
Fig. 3. **Negative ion FAB-MS spectra of EGL-II (A) and negative-ion SIMS of HF-treated EGL-II (B) with fragmentation diagrams.**

TABLE II. **Partially methylated alditol acetates derived from EGL-II, HF-treated EGL-II, and glycolipid fragments.**

Linkage	Permethylation followed by acetolysis						Permethylation, HF-treatment then acetolysis	Alditol acetates
	EGL-II	HF-treated EGL-II	Glycolipid fragment E	D	C	B	EGL-II	
Gal1-	+	+	+	+	+	+	+	2,3,4,6-Tetra-*O*-methylgalactitol
-4Glc1-	−	+	+	+	+	+	−	2,3,6-Tri-*O*-methylglucitol
-3Gal1-	−	−	+	+	+	−	−	2,4,6-Tri-*O*-methylgalactitol
-2Gal1-	−	−	+	+	+	−	−	3,4,6-Tri-*O*-methylgalactitol
-6Gal1-	−	−	−	−	−	−	+	2,3,4-Tri-*O*-methylgalactitol
-2,3Gal1-	+	+	+	+	−	−	+	4,6-Di-*O*-methylgalactitol
-4,6Glc1-	−	−	−	−	−	−	+	2,3-Di-*O*-methylglucitol
GlcNAc1-	+	+	+	−	−	−	+	3,4,6-Tri-*O*-methyl-*N*-methyl acetamidoglucitol
-2,3,6Gal1-	−	−	−	−	−	−	+	4-Mono-*O*-methylgalactitol

taosylceramide, GlcNAc1-2Gal1-3(3-*O*-MeGal1-2)Gal1-3-(Gal1-2)Gal1-4Glc1-1Cer and Gal1-3(GlcNAc1-2)Gal1-3(3-*O*-MeGal1-2)Gal1-3Gal1-4Glc1-1Cer.

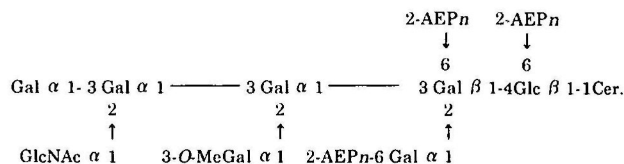
From the above results of the carbohydrate composition and permethylation studies, and the fragmentation diagram of the FAB-MS and SIMS spectra, the carbohydrate sequence and linkages of EGL-II are concluded to be



The Roman numerals correspond to the individual carbohydrates and were also used for the assignment of the NMR spectrum (see below).

^{1}H-NMR Spectroscopy—The proton magnetic resonance data of HF-treated EGL-II dissolved in DMSO-d_6:D_2O (98:2, v/v) revealed two β- and six α-protons (Table III and Fig. 5). The chemical shift of two β-protons at 4.389 and 4.157 ppm was in good agreement with the corresponding chemical shifts of β-galactose and β-glucose in HF-treated EGL-I (2). The chemical shift of α-protons at 4.959 and 4.866 ppm was similar to the corresponding chemical shifts of α-3-*O*-methylgalactose in HF-treated EGL-I (2) and that of the terminal α-4-*O*-methylglucosamine at 4.842 ppm in HF-treated SGL-I (5), respectively. Accordingly, the four remaining α-protons at 5.413, 5.358, 5.320, and 5.099 ppm are ascribed to galactose, although the H1 signals corresponding to the individual galactose residues were not identified. Similarly, the one-dimensional ^{1}H-NMR spectrum of EGL-II dissolved in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) and recorded at a probe temperature of 50°C shows two β- and six α-protons in the anomeric region (Fig. 5 and Table III). In this experiment, a difference of 0.189 ppm in the ^{1}H chemical shift between *N*-acetyl methyl proton resonance in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) and DMSO-d_6:D_2O (98:2, v/v) was observed. The chemical shifts of EGL-II dissolved in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) were corrected for 0.189 ppm, and the difference in the H1 chemical shifts between EGL-II and HF-treated EGL-II are presented as (C)–(B) (Table III). Upfield shifts following the removal of 2-AEP*n* were observed in β-glucose, β-galactose, and α-galactose. The H1 shift of β-glucose is due to the spacial interaction of 2-AEP*n* bound to C6 of the branched chain galactose (III), and the H1 shift of the internal β-galactose (II) is explained by the interaction of 2-AEP*n* bound to C6 of the glucose (I) (2). As H1 of individual α-galactose residue could not be assigned, the α-galactose residue that exhibited the H1 shift due to 2-AEP*n* linked to C6 of the internal galactose (II) also could not be assigned.

Taken together, the structure of EGL-II was concluded to be as follows:



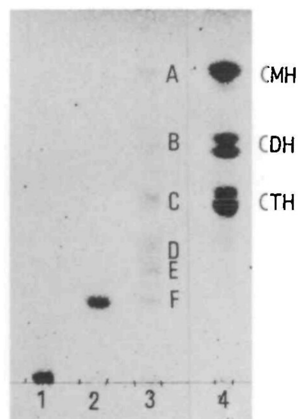


Fig. 4. **Thin-layer chromatogram of glycolipid fragments, A to F, derived from HF-treated EGL-II.** HF-treated EGL-II was hydrolyzed with 0.2 M HCl at 100°C for 1 h, and the hydrolysate was mixed with 5 volumes of chloroform:methanol (2:1, v/v). The lower chloroform layer was concentrated and analyzed by HPTLC with chloroform:methanol:water (60:35:8, v/v) (lane 3). Spots were detected with anthrone reagent. Lane 1, EGL-II; lane 2, HF-treated EGL-II; and lane 4, standard glycolipids: glucocerebroside (CMH) from human (Gaucher's) spleen, ceramide dihexoside (CDH) from bovine red blood cells, and ceramide trihexoside (CTH) from porcine red blood cells.

Immunochemical Findings—Anti–EGL-I antiserum, which recognizes both α- and β-isomers of the terminal 3-*O*-methylgalactose (2), immunoreacted with EGL-II on TLC (Fig. 6). However, the anti–SGL-II antiserum, which is specific to β-3-*O*-methylgalactose linked glycosidically at the nonreducing end of glycolipid (2), did not immunoreact with EGL-II on TLC (Fig. 6). These observations indicate that EGL-II has a nonreducing α-3-*O*-methylgalactose.

TABLE III. **Chemical shifts (δ, ppm) and coupling constants ($J_{1,2}$, Hz) of anomeric protons of EGL-II and HF-treated EGL-II.**

	(A) EGL-II		(B) HF-treated EGL-II		(C)[a] (A)–0.189	(C)–(B)
	ppm	($J_{1,2}$)	ppm	($J_{1,2}$)	(ppm)	
α-Gal	5.590	3.7	5.413	3.7	5.401	−0.012
α-Gal	5.560	3.7	5.358	4.0	5.371	0.013
α-Gal	5.519	3.4	5.320	4.4	5.330	0.010
α-Gal	5.500	3.4	5.099	3.7	5.311	0.212
α-3-*O*-MeGal	5.174	3.7	4.959	4.0	4.985	0.026
α-GlcNAc	5.017	3.7	4.866	3.7	4.828	−0.038
β-Gal	4.767	7.6	4.389	7.7	4.578	0.189
β-Glc	4.505	7.8	4.157	8.1	4.316	0.159
N-Acetyl methyl	2.016		1.827			

[a] ^{1}H chemical shift of *N*-acetyl methyl proton resonance in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) and DMSO-d_6:D_2O (98:2, v/v) show a difference of 0.189 ppm. The chemical shifts of EGL-II dissolved in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) were corrected by 0.189 ppm.

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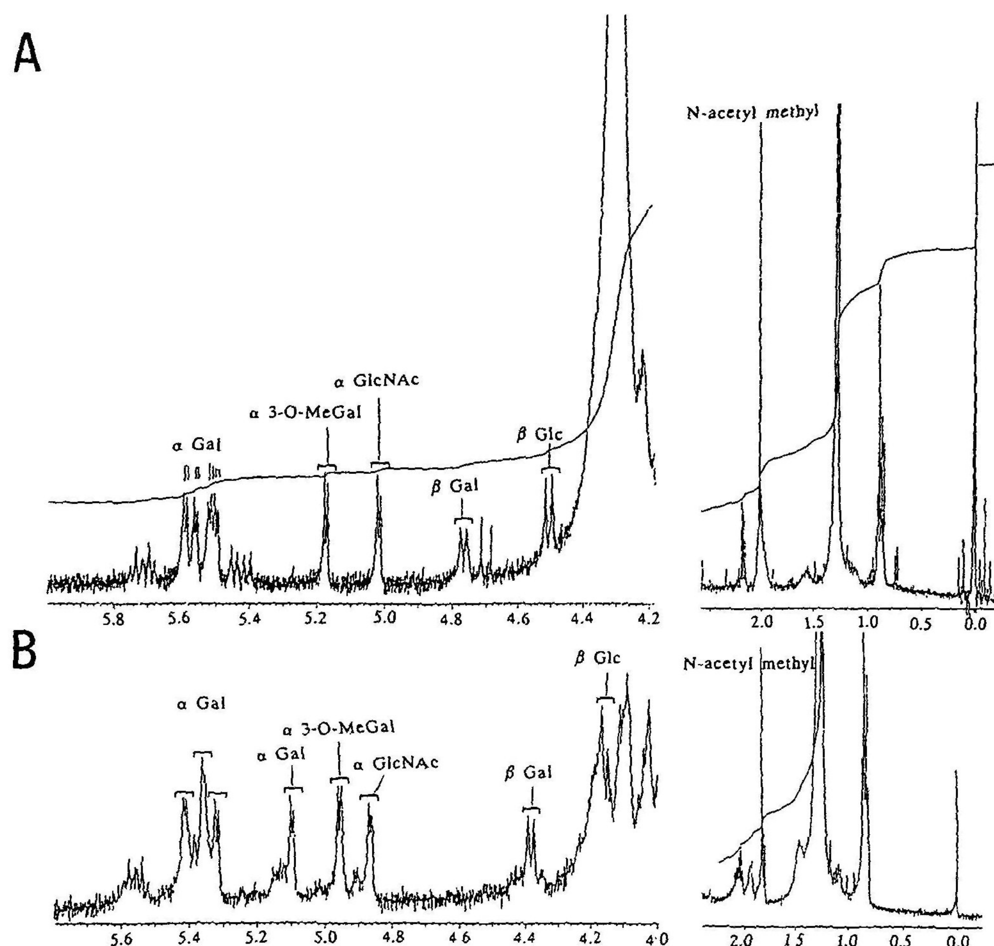

Fig. 5. **^{1}H-NMR spectrum of EGL-II.** EGL-II was dissolved in $CDCl_3$:CD_3OD:D_2O (43:43:14, v/v) and measured at 400 MHz in the Fourier transform mode at a probe temperature of 50°C (A). HF-treated EGL-II was dissolved in DMSO-d_6:D_2O (98:2, v/v) and the spectrum was recorded at 60°C (B).

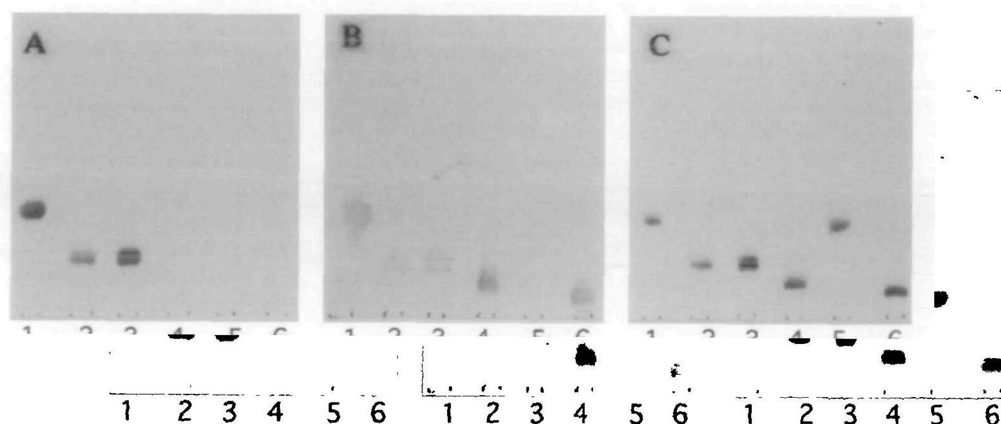

Fig. 6. **Enzyme immunostaining of EGL-II with anti-SGL-II (A) and anti-EGL-I (B) antisera on TLC plates.** Samples (5 μg each) of (1) SGL-I', (2) F-21, (3) SGL-II, (4) EGL-I, (5) SGL-I, and (6) EGL-II were chromatographed on plastic TLC plates with 1-propanol: concentrated ammonium hydroxide:water (75:5:25, v/v). (A) Enzyme immunostained with anti-SGL-II antisera. (B) Enzyme immunostained with anti-EGL-I antisera. (C) Sprayed with anthrone reagent.

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DISCUSSION

To date, we have isolated eleven glycolipids, including EGL-II, from the tissues of *Aplysia* and elucidated their structures. Nine glycolipids are PnGSLs containing 1 to 3 mol of 2-AEPn (*2–8*). The other two glycolipids contain 2-aminoethylphosphate (phosphoethanolamine), or phosphoethanolamine and 2-AEPn as phosphorus compounds linked to the carbohydrate moieties (*9*).

In this paper, we report the isolation of an egg-specific glycolipid, EGL-II, from an egg mass collected immediately after being laid in early summer, and the elucidation of its structure. The carbohydrate structure is distinct from those of glycolipids isolated from adult sea hare tissues.

With regard to the molecular species of the ceramide moiety of all GSLs that have been identified so far, the contents of the long-chain bases are similar. On the other hand, although the main fatty acid of GSLs containing 1 or 2 mol of 2-AEP*n* and/or phosphoethanolamine is palmitic acid (about 90%), the fatty acids of PnGSLs (SGL-I, F-21, EGL-I, and EGL-II) containing 3 mol of 2-AEP*n* are palmitic acid and stearic acid. These results suggest that PnGSLs containing 3 mol of 2-AEP*n* and GSLs containing

1 or 2 mol of 2-AEP*n* and/or phosphoethanolamine may have different biosynthetic pathways and play different biological functions.

In contrast to gangliosides, which are known to have a number of important cellular functions, the functional role of P*n*GSLs is unclarified. Previously, Arakane *et al.* (*30*) reported that P*n*GSLs isolated from *Aplysia kurodai* stimulates the activity of cAMP-dependent kinase by binding to the catalytic subunit of the enzyme at a site different from that for cAMP. However, the activity of cAMP-dependent protein kinase was irrespective of the difference in the carbohydrate chain and the presence of 2-AEP*n*.

Beitinger *et al.* (*31*) investigated the physicochemical surface properties of four GSLs, including SGL-II, a diphosphonopentaosylceramide from the skin of *A. kurodai*, (*3*) in a monolayer at an air/water interface. The addition of Ca^{2+} condensed the monolayers of all GSLs and increased the potential in the direction of more positive values, but SGL-II was not markedly effective. Compared with the negatively charged GSLs used in this experiment [gangliosides, sulfatide and glucuronic acid–containing GSL, Lipid IV (*32*)], SGL-II is a GSL containing 2-AEP*n* with electrically neutral but amphoteric characteristics. The amphoteric moieties of SGL-II may influence the membrane properties of *Aplysia*.

GSLs containing amphoteric substituents such as phosphocholine, phosphoethanolamine, or 2-AEP*n* have been structurally characterized from various members of the invertebrate phyla including arthropods, molluscs, and annelida (*33–38*). The diversity of amphoteric GSLs among various invertebrate phyla indicate their biological importance, but their functional significance is as yet unknown.

Lochnit *et al.* (*34*) isolated two amphoteric GSLs (termed Components A and C) from the pig parasitic nematode, *Ascaris suum*. Both GSLs contain phosphodiester substitutions: phosphocholine for component A, and phosphocholine and phosphoethanolamine for component C. Both GSLs are biologically active in inducing human peripheral blood mononuclear cells to release inflammatory monokines including tumor necrosis factor α, interleukin 1, and interleukin 6. Component A is the more bioactive molecule, and its biological activity is abolished upon removal of the phosphocholine by HF. The mechanism by which the *A. suum*–derived amphoteric GSLs induce cytokine production is, however, unknown.

In early summer, *Aplysia* lays egg masses that hatch about 12 days after they are laid. Posthatching development of *Aplysia* can be divided into four major phases: the veliger or planktonic, the metamorphic, the juvenile, and the adult (*39*). Developmental changes in the P*n*GSLs in egg masses are not observed during the embryonic phase (data not shown). This observation suggests that these glycolipids play some biological roles in early cell differentiation. However, to elucidate their possible involvement in differentiation and development, further studies on the composition of glycolipids in *Aplysia* during posthatching developmental stages are required.

We are grateful to Dr. M. Nozaki and Mr. K. Iwami for collecting *Aplysia kurodai* and Mr. S. Oyanagi for excellent photographs. We also thank Professor emeritus Mei. Satake for valuable suggestions and support throughout this work.

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