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Structural characterization of glycosylinositolphospholipids with a blood group type B sugar unit from the edible mushroom, *Hypsizygus marmoreus*

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Edible fungi, mushrooms, are a popular food in Japan and over 15 cultured mushroom species are available at the food markets. Recently, constituents or ingredients of edible mushrooms have drawn attention because possibilities have been seen for their medical usage. Mycoglycolipids (basidiolipids) of higher mushrooms have been characterized as glycosylinositolphosphoceramides, having a common core structure of Manα1-2Ins1-[PO_4]-Cer and extensions of Man, Gal, and/or Fuc sugar moieties. Seven mycoglycolipids were purified from the edible mushroom *Hypsizygus marmoreus* by successive column chromatography on ion exchange Sephadex (DEAE-Sephadex) and silicic acid (Iatrobeads). Their structures were characterized to be Ins1-[PO_4]-Cer (AGL0), Manα1-2Ins1-[PO_4]-Cer (AGL1), Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL2), Fucα1-2Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL3), Galα1-3(Fucα1-2)Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL4), Galα1-2Galα1-3(Fucα1-2)Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL5), and Galα1-2Galα1-2Galα1-3(Fucα1-2)Galβ1-6Manα1-2Ins1-[PO_4]-Cer (AGL6) by sugar compositional analysis, methylation analysis, periodate oxidation, partial acid hydrolysis, enzymatic hydrolysis, immunochemical analysis, gas–liquid chromatography (GC), gas chromatography-mass spectrometry (GC-MS), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), and ^{1}H-nuclear magnetic resonance spectroscopy (NMR). Ceramide constituents of their mycoglycolipids were composed of phytosphingosine as the sole sphingoid, and mainly 2-hydroxy C22:0 and C24:0 acids as the fatty acids. By immunochemical detection, the terminal structure of AGL4, Galα1-3(Fucα1-2)Galβ-, was shown to have blood group type B activity. Galα1-2 and its repeating sequence in AGL5 and AGL6 are novel structures on the nonreducing sugar end in mycoglycolipids. These two mycoglycolipids in *H. marmoreus* distinguish it from other basidiomycetes.

Keywords: blood group/glycosphingolipid/*Hypsizygus marmoreus*/mushroom/mycoglycolipid

Introduction

Edible fungi, mushrooms, are a popular food in Japan and about 15 cultured mushroom species are available in Japanese food markets. These edible mushrooms mostly belong to agaricales, basidiomycete. Chemical structures of free ceramide, cerebroside, and glycosphingolipids from a wide variety of basidiomycetes have been reported. Glycosphingolipids of the edible fungi have been reported as glucosylceramide (glucocerebroside) with 9-methyl-4,8-octadecasphingadienine or trihydroxysphinganine (phytosphingosine) and 2-hydroxy fatty acids (Fujino and Ohnishi 1976; Ohnishi et al. 1996). As acidic glycosphingolipids (AGL), mycoglycolipids (basidiolipids) of higher mushrooms have been characterized as glycosylinositolphosphoceramides, and they have a common core structure of Manα1-2Ins-[PO_4]-ceramide (MIPC) and extension of Man, Gal, and/or Fuc sugar moieties (Jennemann, Bauer, Bertalanffy, Geyer, et al. 1999; Jennemann, Geyer, et al. 2001; Heise et al. 2002; Arigi et al. 2007). Interestingly, the ceramide composition of mycoglycolipid has been characterized solely as phytoceramide consisting mainly of phytosphingosine (t18:0) and 2-hydroxylignoceric acid (h24:0) (Weiss and Stiller 1972; Weete 1974; Lester and Dickson 1993).

So far, a limited number of investigations have been carried out on the physiological activity of fungal glycolipid. The fungal cerebroside or structural analogues showed fruiting body-inducing activity in *Schizophyllum commune* (Kawai and Ikeda 1983), and cerebroside extracted from the rice pathogen *Magnaporthe grisea* exhibited elicitor activity with hypersensitive cell death of the plant (Koga et al. 1998; Umemura et al. 2002). Also mycoglycolipid extracted from filamentous fungi *Acremonium* sp. was found to exhibit the induction of cultured rice cell death (Aoki et al. 2004). In the mammal, the basidiolipids from Agaricus showed immune adjuvanticity without toxic or hemolytic properties (Jennemann, Bauer, Bertalanffy, Selmer, et al. 1999). On the other hand, it has been reported that basidiolipids that were recognized by human heterophile antibodies had either terminal Galα1-6Gal or Galβ1-6Man epitopes (Jennemann, Sandhoff, et al. 2001).

In this study we have isolated acidic glycosphingolipids (provisionally named AGL) containing inositol phosphate from a popular Japanese mushroom *Hypsizygus marmoreus* (Shimeji). Six mycoglycolipid structures were characterized using a variety of chemical structural analyses; these were glycosphingolipids

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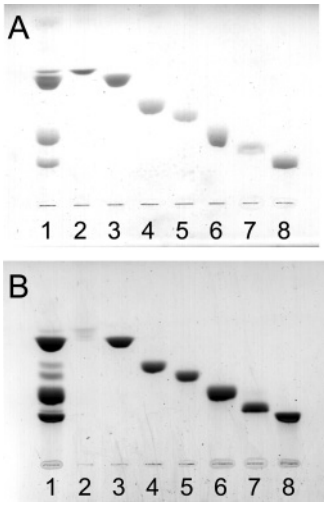

Fig. 1. Thin layer chromatograms of the mycoglycolipids isolated from *Hypsizygus marmoreus*. Lane 1, AGL fraction obtained from DEAE-Sephadex column chromatography; lanes 2 to 8, isolated AGL0, AGL1, AGL2, AGL3, AGL4, AGL5, and AGL6. The plates were developed in 1-propanol/water/ammonia (75:30:5, v/v) for 2 h, and the spots were visualized with Hanes–Isherwood reagent for Panel A and with orcinol–H_2SO_4 reagent for Panel B.

having a core motif, Manα1-2Ins-[PO_4]-ceramide. Two of these structures were novel and characteristic of *H. marmoreus*.

Results

Isolation of AGLs from Hypsizygus marmoreus

Lipids extracted from the dried powder (131.5 g) of the Shimeji mushroom, *H. marmoreus*, were submitted to mild alkali hydrolysis, and the resulting alkali-stable substances (1.3 g) were applied to a DEAE-Sephadex column. The acidic glycolipid fraction (476 mg) free of neutral glycolipids was recovered from the DEAE-Sephadex column with 0.45 M ammonium acetate in methanol. Major components of this fraction were obtained by subsequent Iatrobeads gradient elution column chromatography with chloroform/methanol/3 M NH_4OH. Minor contaminants in each of these components were removed by Iatrobeads column chromatography using 1-propanol/water/15 M NH_4OH. Elution was monitored by thin-layer chromatography. As shown in Figure 1, purified acidic glycolipids, namely AGL0 to AGL6, showed positive reactions with Hanes–Isherwood reagent, while AGL0 did not react with the orcinol/H_2SO_4 reagent. The yields were AGL0, 9.0 mg; AGL1, 126.6 mg; AGL2, 6.3 mg; AGL3, 7.4 mg; AGL4, 111.6 mg; AGL5, 2.0 mg; and AGL6, 58.7 mg, respectively.

Carbohydrate constituent analysis of AGLs

Inositol substituted with a phosphate residue in the inositolphosphoceramide could not be determined as the free carbohydrate using typical methanolysis. AGL was hydrolyzed with 2 M HCl at 100°C for 20 h and the carbohydrate components in the hydrolysates were converted to alditol acetate derivatives for GC analysis. As shown in Figure 2, the alditol acetate derivatives were identified, from their retention times on GC, as Ins for AGL0; 1 mol each of Ins and Man for AGL1; 1 mol each of Ins, Man, and Gal for AGL2; 1 mol each of Ins, Man, Gal, and Fuc for AGL3; 1 mol each of Ins, Man, and Fuc, and 2 mol of Gal for AGL4; 1 mol each of Ins, Man, and Fuc, and 3 mol of Gal for AGL5; and 1 mol each of Ins, Man, and Fuc, and 4 mol of Gal for AGL6, respectively.

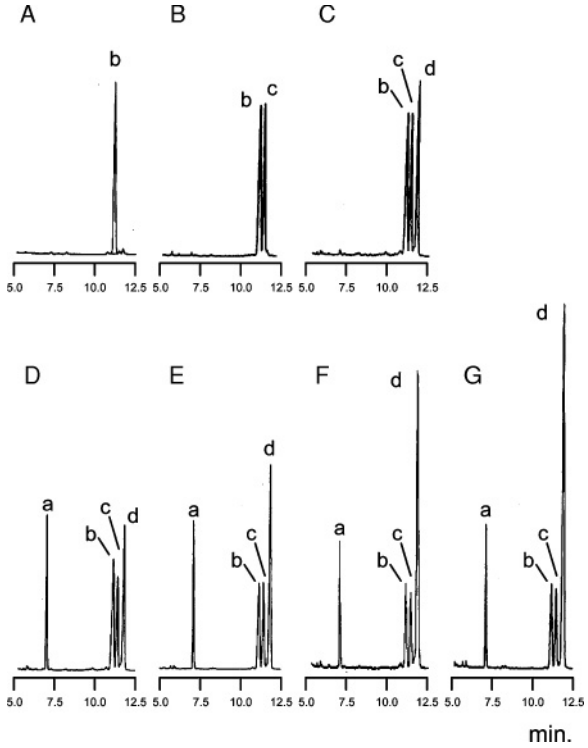

Fig. 2. Gas chromatograms of alditol acetate derivatives derived from AGL0 to AGL6. (**A**) AGL0; (**B**) AGL1; (**C**) AGL2; (**D**) AGL3; (**E**) AGL4; (**F**) AGL5; (**G**) AGL6; peak a, fucitol pentaacetate; peak b, inositol hexaacetate; peak c, mannitol hexaacetate; peak d, galactitol hexaacetate.

Table I. Methylated alditol acetate analysis of AGL1 to AGL6 A from *H. marmoreus*

	Relative amount					
Linkage	AGL1	AGL2	AGL3	AGL4	AGL5	AGL6
t-Man	+					
t-Gal		0.9		1.0	1.0	1.0
6-Man		1.0	1.0	1.0	1.0	1.0
t-Fuc			0.6	0.6	0.7	0.7
2-Gal			0.7		0.9	1.5
2,3-Gal				1.0	1.0	1.0

Results were calculated from the area of each peak by FID of GC with reference to standards for response factors. The area of 6-Man was set to 1.0.

Methylation linkage analysis for neutral sugar

To determine the sugar linkages, the partially methylated alditol acetate derivatives of AGLs and de-fucosylated AGL were analyzed by GC and GC/MS, and the results are listed in Table I. The methylation analysis demonstrated the presence of terminal Man (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, *t*-Man) for AGL1; 6-linked Man (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylmannitol, 6-Man) and terminal Gal (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, *t*-Gal)

for AGL2; 6-Man, 2-linked Gal (1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol, 2-Gal), and terminal Fuc (1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol, *t*-Fuc) for AGL3; 6-Man, 2,3-linked Gal (1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylgalactitol, 2,3-Gal), *t*-Fuc, and *t*-Gal for AGL4; 6-Man, 2,3-Gal, *t*-Fuc, 2-Gal, and *t*-Gal for AGL5 and 6. The peak area of 2-Gal of AGL6 was significantly larger than that in the case of AGL5. De-fucosylated AGL4 yields 6-Man, 3-linked Gal (1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, 3-Gal), and *t*-Gal. This means that de-fucosylated AGL4 has the Gal1-3Gal1-6Man sugar sequence, and that the glycosidic substitution on Gal by Fuc was at the C-2 position of the internal Gal in AGL4.

Identification of inositol phosphate and linkage of sugar and inositol

To identify the inositol phosphate, the trimethlsilyl (TMS) derivative of the phosphorus compound was prepared from the methanolysate of AGL0. It showed one major peak with the same retention time on its gas chromatogram as the derivative from authentic *myo*-inositol 1-phosphate. Its mass spectrum was consistent with that of the heptatrimethylsilyl derivative prepared from the same standard (data not shown).

For determination of the substitution site on the inositol ring by mannose in AGLs, AGL1 was subjected to periodate oxidation followed by $NaBH_4$ reduction, hydrolysis, anion-exchange chromatography, dephosphorylation, and acetylation, and the resulting acetylated alcohol product was analyzed by gas–liquid chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). The GC-MS analysis of the final acetylated alcohol product derived from the inositol ring of AGL1 gave one peak, the mass spectrum of which corresponded to a tetriol. The tetriol derived from AGL1 was found to have an identical retention time with that of acetylated authentic erythritol. These results indicate that Man is linked to the C-2 position of the inositol ring (data not shown).

Aliphatic composition of AGLs

The aliphatic components of AGLs were determined by GC and GC-MS (Table II). The main fatty acids were 2-hydroxyoctadecanoic, 2-hydroxydocosanoic, and 2-hydroxytetracosanoic acids, except for AGL0, and the sphingoid was composed entirely of 4-hydroxyoctadecasphinganine (phytosphingosine) in all AGLs. On the other hand, the fatty acid composition of AGL0 contained a considerable amount of the nonhydroxy C16 and C18 acids, as well as hydroxyl C16, C18, C22, and C24 acids; the ratio of these two groups was about 1:1. The phytoceramide composition of fungal cells and higher mushrooms usually consists of phytosphingosine and 2-hydroxy fatty acid. Nonhydroxy saturated fatty acids in AGLs was a characteristic of their fatty acid constituents. Besides these fatty acids, 2-hydroxytetracosenoic acid was also detected at about 10% in AGL1, 4, and 5.

MALDI-TOF MS analysis

The putative structures of AGLs were confirmed by negative-ion mode MALDI-TOF MS analysis as shown in Figure 3 and Table III. Their mass spectra have several different pseudomolecular ions because of the presence of different fatty acid species. The mass spectrum of AGL0 has several different pseudomolecular species, $[M-H]^-$ ions at *m/z* 796.4, 824.4, 812.4,

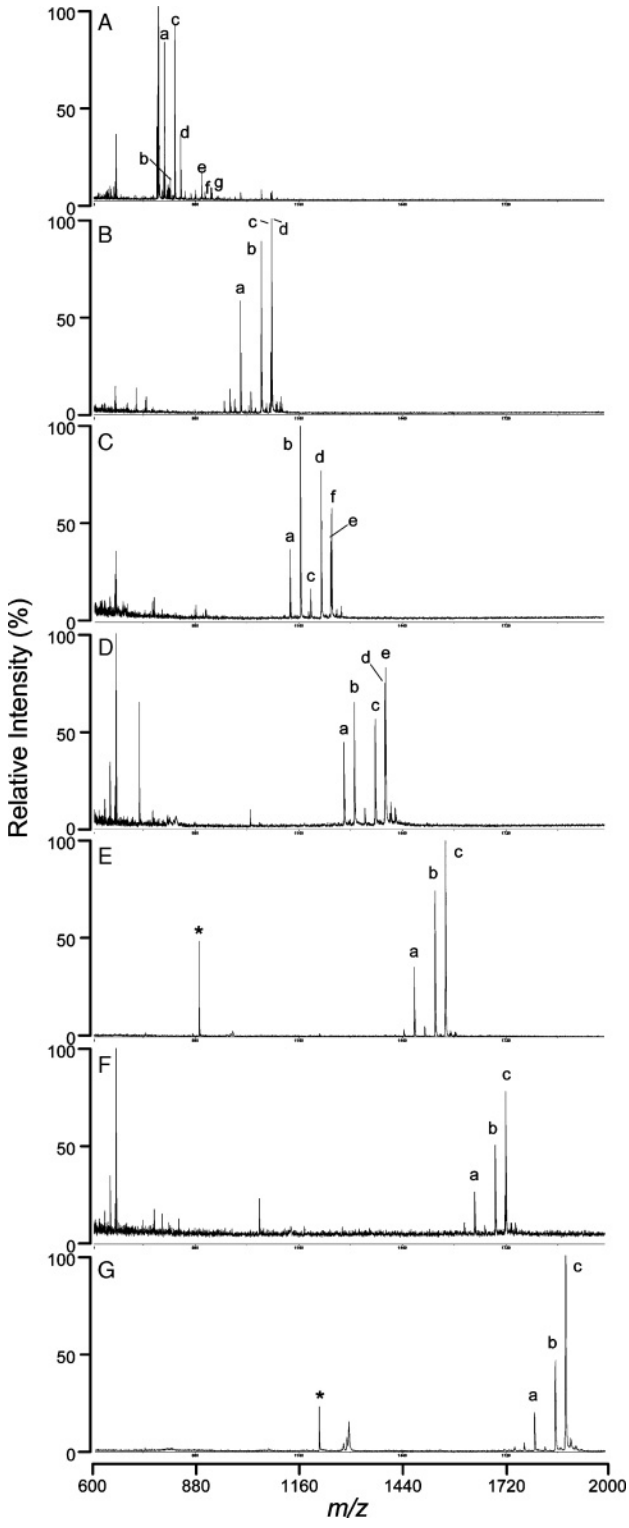

Fig. 3. Negative-ion reflector mode MALDI-TOF MS spectra of AGL0 to AGL6. (**A**) AGL0; a, $[M-H]^-$ ion at *m/z* 796.4; b, *m/z* 812.4; c, *m/z* 824.4; d, *m/z* 840.4; e, *m/z* 896.5; f, *m/z* 922.5; g, *m/z* 924.6; (**B**) AGL1; a, *m/z* 1002.6; b, *m/z* 1058.7; c, *m/z* 1084.7; d, *m/z* 1086.7; (**C**) AGL2; a, *m/z* 1136.6; b, *m/z* 1164.6; c, *m/z* 1192.7; d, *m/z* 1220.7; e, *m/z* 1246.7; f, *m/z* 1248.7; (**D**) AGL3; a, *m/z* 1282.5; b, *m/z* 1310.6; c, *m/z* 1366.6; d, *m/z* 1392.7; e, *m/z* 1394.7; (**E**) AGL4; a, *m/z* 1472.4; b, *m/z* 1528.5; c, *m/z* 1556.5; (**F**) AGL5; a, *m/z* 1635.0; b, *m/z* 1691.0; c, *m/z* 1719.1; (**G**) AGL6; a, *m/z* 1796.9; b, *m/z* 1853.2; c, *m/z* 1881.1. *, presumed glycosylinositolphosphate ions at *m/z* 891.0 (**E**) and 1215.3 (**G**).

Table II. Aliphatic compositions (ceramide compositions) of mycoglycolipids from *H. marmoreus*

Composition	AGL0	AGL1	AGL2	AGL3	AGL4	AGL5	AGL6
Fatty acid (%)							
Nonhydroxy acid							
16:0	27.0	tr.	3.2	tr.	7.1	4.9	tr.
18:0	21.0	tr.	tr.	tr.	tr.	tr.	tr.
22:0	tr.	tr.	3.5	5.3	tr.	tr.	tr.
	48.0	–	6.7	5.3	7.1	4.9	–
2-Hydroxy acid							
16:0	11.4	tr.	5.3	tr.	tr.	tr.	3.7
18:0	7.3	14.1	19.7	15.4	11.7	11.1	12.2
20:0	tr.	tr.	3.6	tr.	tr.	tr.	tr.
22:0	12.9	31.2	35.2	35.3	28.8	28.5	30.3
24:0	20.4	40.6	29.5	44.0	41.0	46.2	53.8
24:1	tr.	14.1	tr.	tr.	11.4	9.3	tr.
	52.0	100.0	93.3	94.7	92.9	95.1	100.0
Sphingoid (%)							
t18:0	100	100	100	100	100	100	100

Results were calculated from the area of each peak by FID of GC with reference to standards for response factors.
t, trihydroxy sphingoid (phytosphingosine); tr., trace.

840.4, 896.5, 922.5, and 924.6 in good accordance with the values calculated from the proposed structures, that is, 1 mole each of inositol phosphate, fatty acid (C16:0, C18:0, 2-hydroxy-16:0, 18:0, 22:0, 24:1, 24:0), and sphingoid (t18:0). The mass spectrum of AGL1 has several different pseudomolecular species, $[M-H]^-$ ions at *m/z* 1002.6, 1058.7, 1084.7, and 1086.7 in good accordance with the values calculated from the proposed structures, that is, 1 mole each of Man, inositol phosphate, fatty acid (2-hydroxy-18:0, 22:0, 24:1, 24:0), and sphingoid (t18:0). The $[M-H]^-$ ions of AGL2 at 1136.6, 1164.6, 1192.7, 1220.7, 1246.7, and 1248.7 coincided with the mass values of compounds having 1 mole each of Gal, Man, inositol phosphate, fatty acid (2-hydroxy-16:0, 18:0, 20:0, 22:0, 24:1, 24:0), and sphingoid (t18:0); and those of AGL3 at 1282.5, 1310.6, 1366.6, 1392.7, and 1394.7 coincided with the mass values of compounds having 1 mole each of Fuc, Gal, Man, inositol phosphate, fatty acid (2-hydroxy-16:0, 18:0, 22:0, 24:1, 24:0), and sphingoid (t18:0). Sequentially, the $[M-H]^-$ ions of AGL4 at 1472.4, 1528.5, and 1556.5 coincided with the mass values of compounds having 2 mole of Gal and 1 mole each of Fuc, Man, inositol phosphate, fatty acid (2-hydroxy-18:0, 22:0, 24:0), and sphingoid (t18:0); and those of AGL5 at 1635.0, 1691.0, and 1719.1 coincided with an additional 1 mole of Gal on AGL4. The $[M-H]^-$ ions of AGL6 at 1796.9, 1853.2, and 1881.1 coincided with an additional 1 mole of Gal on AGL5.

Anomeric configuration analysis of sugar components in AGLs

Anomeric configurations of the sugar residue in AGLs were determined by a ^{1}H-NMR spectrometer (Figure 4) and assignments are listed in Table IV. In the anomeric region of the spectrum for each AGL, the following anomeric proton resonances were observed: at 5.03 ppm (singlet) for α-Man (Figure 4A, AGL1); at 4.96 ppm (broad singlet) for α-Man and at 4.24 ppm ($J_{1,2} = 7.3$ Hz) for β-Gal (Figure 4B, AGL2); at 4.96 ppm (broad singlet) for α-Man, at 4.28 ppm ($J_{1,2} = 7.3$ Hz) for β-Gal, and at 5.05 ppm ($J_{1,2} = 3.7$ Hz) for α-Fuc (Figure 4C, AGL3); at 4.97 ppm (broad singlet) for α-Man, at 4.37 ppm ($J_{1,2} = 7.3$ Hz) for β-Gal, at 5.15 ppm ($J_{1,2} = 4.3$ Hz) for α-Fuc, and at

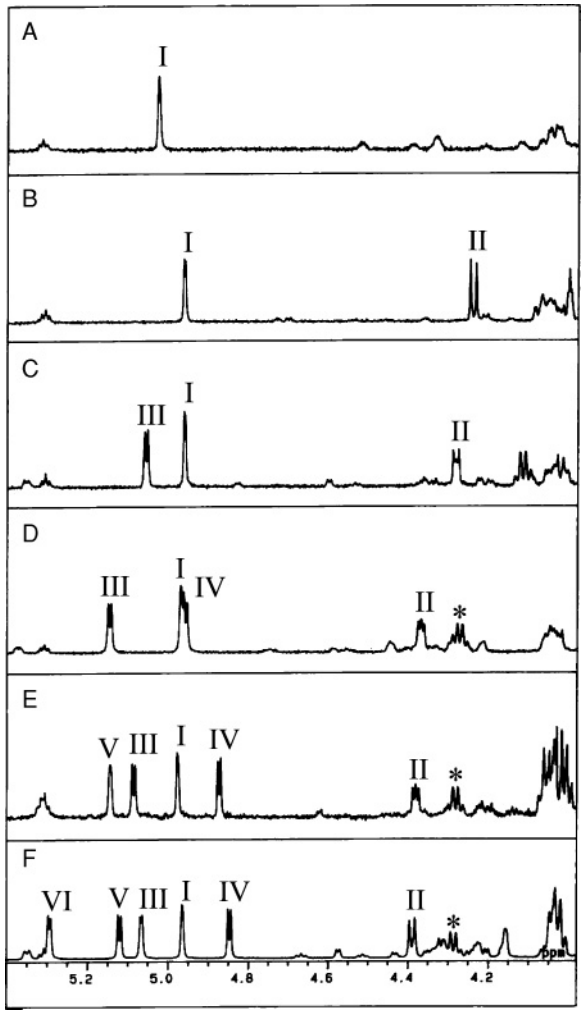

Fig. 4. Anomeric proton regions of the ^{1}H-NMR spectra. (**A**) AGL1; (**B**) AGL2; (**C**) AGL3; (**D**) AGL4; (**E**) AGL5; (**F**) AGL6; I, Manα(H-1); II, Galβ(H-1); III, Fucα(H-1), IV, Galα(H-1); V, Galα(H-1); VI, Galα(H-1). *Fucα(H-5).

4.96 ppm ($J_{1,2} = 4.3$ Hz) for α-Gal (Figure 4D, AGL4); at 4.98 ppm (broad singlet) for α-Man, at 4.38 ppm ($J_{1,2} = 7.3$ Hz) for β-Gal, at 5.08 ppm ($J_{1,2} = 3.7$ Hz) for α-Fuc, at 4.87 ppm ($J_{1,2} = 3.7$ Hz) and at 5.14 ppm (broad singlet) for 2 mol of α-Gal (Figure 4E, AGL5); at 4.98 ppm (broad singlet) for α-Man, at 4.39 ppm ($J_{1,2} = 6.1$ Hz) for β-Gal, at 5.06 ppm (broad singlet) for α-Fuc, at 4.84 ppm ($J_{1,2} = 3.7$ Hz), 5.12 ppm ($J_{1,2} = 3.7$ Hz), and at 5.30 ppm (broad singlet) for 3 mol of α-Gal (Figure 4F, AGL6).

Thin-layer chromatography (TLC) immunostaining

Monoclonal antibody against blood group type B clearly recognized the spot corresponding to AGL4 in the acidic fraction, as shown in Figure 5. These results show that the terminal structure of AGL4, Galα1-3(Fucα1-2)Galβ-, has blood group type B activity.

Table III. Summary of MALDI-TOF MS analysis of AGL0 to AGL6 from *H. marmoreus*

Fatty acid	AGL0		AGL1		AGL2		AGL3		AGL4		AGL5		AGL6	
	Measured	Calculated	Measured	Calculated	Measured	Calculated	Measured	Calculated	Measured	Calculated	Measured	Calculated	Measured	Calculated
C16:0	796.4	796.5												
C18:0	824.4	824.6												
h16:0	812.4	812.5	974.6	974.6	1136.6	1136.6	1282.5	1282.7	1444.4	1444.7			1768.9	1768.9
h18:0	840.4	840.6	1002.6	1002.6	1164.6	1164.7	1310.6	1310.7	1472.4	1472.8	1635.0	1634.8	1796.9	1796.9
h20:0			1030.6	1030.6	1192.7	1192.7			1500.4	1500.8				
h22:0	896.5	896.6	1058.7	1058.7	1220.7	1220.7	1366.6	1366.8	1528.5	1528.8	1691.0	1690.9	1853.2	1852.9
h24:1	922.5	922.6	1084.7	1084.7	1246.7	1246.7	1392.7	1392.8	1554.5	1554.9	1717.0	1716.9	1879.2	1879.0
h24:0	924.6	924.7	1086.7	1086.7	1248.7	1248.8	1394.7	1394.8	1556.5	1556.9	1719.1	1718.9	1881.1	1881.0

$[M-H]^-$ ions are calculated using the monoisotropic mass. Sphingoid compositions are used as t18:0.

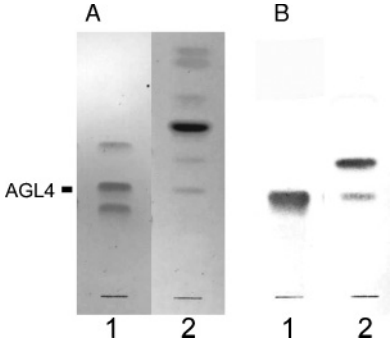

Fig. 5. Detection of the mycoglycolipids having a human blood type B activity by TLC-immunostaining. Lane 1, AGL fraction of *H. marmoreus*; lane 2, neutral glycosphingolipid fraction of type B human red blood cell. The plates were developed with chloroform–methanol–water (65:35:10, v/v). The spots were visualized with orcinol–H_2SO_4 (**A**) and immunostaining by anti-human blood group type B antibody (**B**).

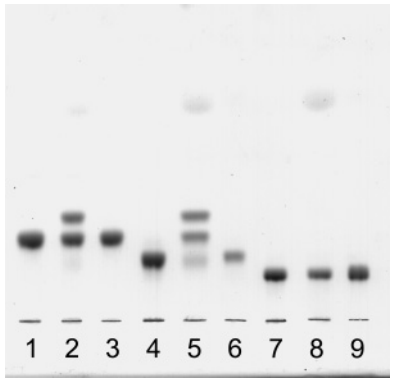

Fig. 6. Thin-layer chromatogram of the reaction products from the mycoglycolipids, AGL4, AGL5, and AGL6 resulting from treatment with α- and β-galactosidases. Lane 1, AGL4; lane 2, AGL4 incubated with α-galactosidase; lane 3, AGL4 incubated with β-galactosidase; lane 4, AGL5; lane 5, AGL5 incubated with α-galactosidase; lane 6, AGL5 incubated with β-galactosidase; lane 7, AGL6; lane 8, AGL6 incubated with α-galactosidase; and lane 9, AGL6 incubated with β-galactosidase. The plate was developed in 1-propanol/water/ammonia (75:30:5, v/v) for 2 h, and the spots were visualized with orcinol–H_2SO_4.

Glycosidase treatment of AGLs

The terminal Gal residues of AGL4, 5, and 6 were treated with galactosidases (Figure 6). AGL4, 5, and 6 were not hydrolyzed with β-galactosidase (lanes 3, 6, and 9). The hydrolyzed product of AGL4 was detected after treatment with α-galactosidase, corresponding to AGL3 on TLC (lane 2). AGL3 and AGL4 were obtained from AGL5 after treatment with α-galactosidase (lane 5). Under the same hydrolyzing conditions, no degraded product was detected from AGL6 with α-galactosidase

Table IV. Chemical shifts and $J_{1,2}$ coupling constants of the protons of AGL1 to AGL6 in the anomeric regions

AGL1						Man1-I	Ins-P-Cer
Chemical shifts (ppm)						5.03	
Coupling constants (Hz)						*	
AGL2					Gal1-II	6Man1-I	Ins-P-Cer
Chemical shifts (ppm)					4.24	4.96	
Coupling constants (Hz)					7.3	**	
AGL3				Fuc1-III	2Gal1-II	6Man1-I	Ins-P-Cer
Chemical shifts (ppm)				5.05	4.28	4.96	
Coupling constants (Hz)				3.7	7.3	**	
AGL4			Gal1-IV	(Fuc1-2) III	3Gal1-II	6Man1-I	Ins-P-Cer
Chemical shifts (ppm)			4.96	5.15	4.37	4.97	
Coupling constants (Hz)			4.3	4.3	7.3	**	
AGL5		Gal1-V	2Gal1-IV	(Fuc1-2) III	3Gal1-II	6Man1-I	Ins-P-Cer
Chemical shifts (ppm)		5.14	4.87	5.08	4.38	4.98	
Coupling constants (Hz)		**	3.7	3.7	7.3	**	
AGL6	Gal1-VI	2Gal1-V	2Gal1-IV	(Fuc1-2) III	3Gal1-II	6Man1-I	Ins-P-Cer
Chemical shifts (ppm)	5.30	5.12	4.84	5.06	4.39	4.98	
Coupling constants (Hz)	**	3.7	3.7	**	6.1	**	

*, singlet; **, broad singlet.

(lane 8). AGL6 was also treated with mixed glycosidases (*Charonia lampas*) including α-galactosidase, but no hydrolyzed product was detected (data not shown).

Discussion

Mycoglycolipids were isolated from the Shimeji mushroom, *Hypsizygus marmoreus*, and their structures were characterized by chemical analysis. They have a common-core structure, Manα1-2Ins-[PO_4]-ceramide, which is a characteristic structure in basidiomycetes. The ceramide moiety of all but the simplest of these lipids, AGL0, consists of phytosphingosine and a long chain containing mostly 2-hydroxy fatty acid. Families of such glycosphingolipids containing inositol phosphate have been found in plants, fungi, yeast, and protozoa, and these compounds have been referred to as "Phytoglycolipids" and/or "Mycoglycolipids," indicating their origins (Weiss and Stiller 1972; Weete 1974; Costello et al. 1993; Dickson 1998; Levery et al. 1998; Toledo et al. 2001; Dickson and Lester 2002; Levery et al. 2002; Bennion et al. 2003). However, the distribution of these lipids extends into the animal kingdom as well. For example, they have been reported in nematoda (*Ascaris suum*, Galα1-2Ins-[PO_4]-ceramide) (Sugita et al. 1996) and annelida (*Tylorrhynchus heterochetus*, Manα1-2Ins-[PO_4]-ceramide, Fucα1-5Ins-[PO_4]-ceramide, and related compounds) (Sugita et al. 2000), although their ceramide components are different, consisting, instead, of dihydroxysphingoids (d17:0, d18:0, and/or d18:1).

The aliphatic components of AGLs typically consist of phytosphingosine as the sole sphingoid, and 2-hydroxy C22:0 and C24:0 acids as the main fatty acids. In some AGLs, 2-hydroxy C16:0, C18:0, C24:1 acids are also detected. The simplest AGL we studied, AGL0, contained both groups, those are both nonhydroxy and hydroxy C16 and C18 acids. The composition of phytoceramide found so far in fungal cells and higher mushrooms typically consists of phytosphingosine and 2-hydroxy fatty acids (Weete 1974; Jennemann, Bauer, Bertalanffy, Geyer, et al. 1999; Jennemann, Geyer, et al. 2001; Aoki et al. 2004; Arigi et al. 2007). The additional presence of nonhydroxy saturated fatty acids in AGLs appears to be a characteristic of these particular lipids. It was noted during the course of this work that the recovery of hydroxyl fatty acid is improved using methanolysis with aqueous methanolic HCl instead of using methanolic HCl.

Interestingly, mycoglycolipids from several species are composed of neutral glycosides, such as mannose, galactose, glucose, and fucose, but they lack amino sugars, such as *N*-acetylgalactosamine or *N*-acetylglucosamine. Most mushroom mycoglycolipids also have a core structure, Galβ1-6Manα1-2Ins-[PO_4]-ceramide, except for those from *Cantharellus cibarius* which has Manα1-3/6Manα1-2Ins-[PO_4]-ceramide (Jennemann, Geyer, et al. 2001). The sugar structure of AGL4, Galα1-3(Fucα1-2)Galβ1-6Manα, is the same as Av-1 from *Amanita virosa* (Jennemann, Geyer, et al. 2001). Furthermore, the α-galactose extension on AGL3 is similar to the one in basidiolipid Bl-4, namely Galα1-6Galα1-6Galα1-6(Fucα1-2)Galβ1-6Manα1-2Ins-[PO_4]-ceramide, from *Agaricus bisporus* and *campestris* (Jennemann, Bauer, Bertalanffy, Geyer, et al. 1999). Galα1-2 and its repeating sequence in AGL5 and AGL6 are novel structures on the nonreducing sugar end in mycoglycolipids. These two mycoglycolipids therefore distinguish *H. marmoreus* from other basidiomycetes.

Since many mushroom mycoglycolipids contain Gal and Fuc, it could be predicted that they might express human blood group type H and B determinants. Thus blood group type B polyclonal and monoclonal antibodies recognize the sugar structure in AGL4. Furthermore, AGL3 which has human blood group H determinant has been isolated as a minor component from *H. marmoreus*. It is well known that various lectins isolated from plants recognize blood groups and aggregate human red blood cells. Similarly, galectins and mannose-binding proteins interact with Gal, Fuc, and Man in mushroom mycoglycolipids (Cooper et al. 1997; Boulianne et al. 2000). It is interesting that mycoglycolipids from many mushrooms, which usually are plant parasites, are consistent with those determinant structures.

When submitted to glycosidase treatment for determination of anomeric configuration, commercially available α-galactosidase (green coffee beans) did not cleave the nonreducing end galactose residue on AGL6. Under the same enzyme conditions, the nonreducing end galactose residue on AGL5 was readily cleaved. This enzyme seems to have no restriction for positional sugar attachment, such as for Galα1-2 in AGL5 or Galα1-3 in AGL4, or for Galα1-2Ins in Fagopyritol B1 (Horbowicz et al. 1998) or α-Gal residues from the surface of human blood group type B erythrocytes (Zhu et al. 1996). One explanation for the seemingly paradoxical resistance of the AGL6 sugar structure to coffee bean α-galactosidase could be that access of the enzyme's active site to the terminal Galα1-2Gal linkage is sterically hindered by some conformational effect within the repeated Galα1-2Gal motif. This seems worthy of further investigation.

It has been reported that mycoglycolipid itself has a good immuno adjuvant property and is recognized by human heterophile antibodies (Jennemann, Bauer, Bertalanffy, Selmer, et al. 1999; Jennemann, Sandhoff, et al. 2001). Immunologically, an important feature is that mycoglycolipids contain many galactose residues, especially α-Gal. Evidence is accumulating that endogenous α-galactosylceramide is a potent antigen recognized by natural killer T cells (NKT cells) (Kawano et al. 1997; Brossay et al. 1998; Spada et al. 1998). It will be reported elsewhere that AGLs can stimulate murine NKT cells in a CD1d-dependent manner.

Materials and methods

Isolation and purification of glycoinositolphospholipids

Cultivated Shimeji mushroom, *H. marmoreus* (Hokuto Co., Nagano, Japan), was purchased from a local market. Fresh mushrooms (1.3 kg) were dried in an incubator at 50°C and mashed with a blender. The dried powder (131.5 g) was extracted two times with 3 L of chloroform/methanol/water (60:35:8, v/v) at 50°C (Jennemann, Bauer, Bertalanffy, Geyer, et al. 1999). The extracts were evaporated and subjected to mild alkaline hydrolysis in 0.5 M KOH in methanol/water (95:5, v/v) at 37°C overnight to eliminate glycerolipids. The hydrolysate was acidified to pH 1.0 with 2 M HCl, kept for 1 h at room temperature, dialyzed against tap water for 2 days, concentrated, and precipitated with acetone. The alkaline-stable product (1.3 g) was taken up in chloroform/methanol/water (30:60:8, v/v) and applied to a DEAE-Sephadex A-25 column (bed volume 35 mL, acetate

form; Amersham Biosciences AB, Sweden). The column was eluted successively with the same solvent (five volumes) and methanol (one volume) as neutral solvents, and with 0.45 M ammonium acetate in methanol (five volumes) as a polar solvent; the contaminating acetate of the effluent was removed by dialysis (Sugita et al. 1996). The separation of neutral and acidic glycolipids was monitored by TLC as described below. The acidic lipid fraction (476 mg) was applied to a column of porous silica gel (2.0 × 100 cm, Iatrobeads 6RS-8060, Iatron Lab., Japan) and individual glycolipids were eluted with a linear gradient of chloroform/methanol/3 M NH_4OH (1100 mL of 70:30:3, v/v, to 1250 mL of 50:50:17). Final purification of the glycolipids was achieved by repeating the chromatography of this fraction two more times using Iatrobeads columns (1.2 × 60 cm). The columns were treated with solvent mixtures of 1-propanol/water/15 M NH_4OH (75:15:5 and 75:20:5, v/v).

Thin-layer chromatography (TLC)

Silica gel 60 pre-coated plates (E. Merck, Germany) were developed with a neutral solvent system of chloroform/methanol/water (60:40:10, v/v) and a basic solvent system of 1-propanol/water/15 M NH_4OH (75:30:5, v/v). Detection was performed with the orcinol–H_2SO_4 reagent for sugar, Dittmer–Lester reagent (Dittmer and Lester 1964) and Hanes–Isherwood reagent (Hanes and Isherwood 1949) for phosphorus, and the 5% H_2SO_4/ethanol reagent for organic substances.

Analytical procedure

Compositional analysis of sugars of the isolated acidic glycolipid, AGL, employing alditol acetate derivatives was carried out by GC. Each purified glycolipid (300 μg) was hydrolyzed with 300 μL of 2 M HCl at 100°C for 20 h. After removing free fatty acid with *n*-hexane, the remaining phase was evaporated, reduced with 2% $NaBH_4$ in 0.01 M NaOH, and acetylated with acetic anhydride/pyridine (1:1, v/v) at 100°C for 15 min. For determination of sugar linkages, permethylation of the samples was performed, using 300 μg each of the purified glycolipids, with NaOH and CH_3I in DMSO by the method of Ciucanu and Kerek (1984). The permethylated glycolipids were acetolyzed and hydrolyzed in thick glass test tubes (16 × 125 mm with Teflon-lined screw caps; Pyrex, Iwaki Glass Co., Tokyo, Japan) with 300 μL of a mixture of HCl/water/acetic acid (0.5:1.5:8, v/v) by exposing to the maximum power (500 W) of the microwave oven (Sharp RE-Z3W6, 100 V, 60 Hz) for 1 min (Itonori et al. 2004), and then reduced and acetylated as described above. For determination of the branching sugar structure, 1.5 mg of sample was hydrolyzed by 1 mL of 0.01 M HCl at 100°C for 1 h. De-fucosylated glycolipids were dialyzed and lyophilized. For further purification, the hydrolysates were purified by an Iatrobeads column chromatography using a single elution with 1-propanol/methanol/water (75:15:5, by volume).

Preparation of inositol phosphate from the acidic glycolipid

To confirm the inositol phosphate content of AGLs, a purified AGL was methanolyzed in a thick glass tube with 200 μL of freshly prepared 1 M anhydrous methanolic HCl, using a microwave. The sample was exposed to the maximum power of the microwave oven for 2 min. After removing fatty acid methyl esters with *n*-hexane, the dried residue was subjected to Folch partitioning, and the upper phase containing the water-soluble component was lyophilized and then analyzed by GC and GC-MS after trimethylsilylation with bis (trimethylsilyl)acetamide/trimethlylchlorosilane/pyridine (10:2:5, by volume) at 60°C for 1 h (Kishimoto and Hoshi 1972).

Determination of linkage between sugar and inositol by periodate oxidation

Modifications of earlier procedures were used to determine the linkage of sugar with inositol (Hsieh et al. 1978; Sugita et al. 1996; Aoki et al. 2004). A 3-mL portion of 80 mM $NaIO_4$ in a 0.2 M sodium acetate buffer (pH 4.0) was added to 0.5 mL of an ethanol solution containing approximately 5 mg of purified AGL. The oxidative reaction was allowed to proceed in the dark at 4°C for 144 h. The reaction was stopped by adding ethylene glycol, and then made alkaline with a 3 M ammonia solution. To reduce the oxidized product, 1 mL of 5% $NaBH_4$ was added in a 1 M ammonia solution, and the mixture was allowed to react at room temperature for 12 h. After the addition of acetic acid, the reaction mixture was dialyzed against tap water and lyophilized. The periodate oxidation products were subjected to methanolysis. After removing fatty acid methyl esters, the phosphoalcohol in the hydrolysate was dried under a nitrogen stream. The phosphate group was removed by incubating the product with 2 U of alkaline phosphatase (*Escherichia coli*; Sigma Chemical Co., Saint Louis, MO) in a 0.2 M ammonium acetate buffer (pH 8.5) at 37°C for 24 h. After incubation, the reaction mixture was dried under a nitrogen stream, acetylated, and analyzed by GC and GC-MS.

Aliphatic constituents and HF treatment

Sphingoids were prepared from AGLs by methanolysis with 1 M aqueous methanolic HCl at 70°C for 18 h (Gaver and Sweeley 1965), and the fatty acid methyl esters produced were extracted three times with 400 μL of *n*-hexane. Sphingoids were converted into their *O*-trimethylsilyl derivatives and fatty acid methyl esters were analyzed by GC and GC-MS. For selective cleavage of phosphate bonds, 3 mg of sample in a polyethylene tube was treated with 4.5 mL of 47% HF (w/v) and allowed to stand at room temperature for 48 h. The reaction mixture was dialyzed against tap water to remove HF, and the tube contents were lyophilized to prepare ceramide from AGL.

GC and GC-MS

Compositional analyses of sugars, fatty acids, and sphingoids of AGLs were carried out by using a Shimadzu GC-18A gas chromatograph equipped with a flame ionization detector (FID) and a capillary column of Shimadzu HiCap-CBP 5 (0.22 mm × 25 m, Shimadzu Co., Kyoto, Japan). Electron impact mass spectra were taken using a Shimadzu GCMS-QP 5050 gas chromatograph-mass spectrometer with the same capillary column under the following conditions: ionizing voltage, 70 eV; ionizing current, 60 μA; interface temperature, 250°C; injection port temperature, 240°C; helium gas pressure, 100 kPa. Oven temperatures were programmed as follows: alditol acetate derivatives, 170°C to 230°C (2°C/min) for GC; partially methylated alditol acetate derivatives, 140°C to 230°C (4°C/min) for GC, 80°C (2 min) to 160°C (20°C/min) to 240°C (4°C/min) for GC-MS; inositol phosphate TMS derivatives, 140°C to 240°C (5°C/min) for GC, 80°C (2 min) to 170°C

(20°C/min) to 240°C (4°C/min) for GC-MS; periodate oxidized products, 140°C to 230°C (2°C/min) for GC; sphingoid TMS derivatives, 210°C to 230°C (2°C/min) for GC, 80°C (2 min) to 210°C (20°C/min) to 230°C (4°C/min) for GC-MS; fatty acid methyl esters, 170°C to 240°C (4°C/min) for GC, 80°C (2 min) to 170°C (20°C/min) to 240°C (4°C/min) for GC-MS.

MALDI-TOF MS

MALDI-TOF MS analysis of purified AGLs was performed using an Applied Biosystems/Voyager-DE STR™ Biospectrometer with a nitrogen laser (337 nm) and an acceleration voltage of 20 kV, operating in the reflector negative-ion mode. The matrix used was 7-amino-4-methylcoumarin (Sigma Chemical Co.). External mass calibration was provided by the $[M-H]^-$ ions of angiotensin II (1046.2 mass units; Sigma Chemical Co.) and neurotensin (1446.6 mass units; Sigma Chemical Co.).

^{1}H-NMR spectroscopy

The NMR spectra of purified AGLs were obtained using a JEOL-α500 500 MHz ^{1}H-NMR spectrometer at an operating temperature of 60°C. The purified glycolipid was dissolved in 0.65 mL of $[^2H_6]$DMSO containing 2% ^2H_2O, and the chemical shift was referenced to the solvent signals (δH 2.49 ppm) in $[^2H_6]$DMSO as the internal standard.

Cleavage of sugar linkages by exoglycosidases

α-Galactosidase [EC 3.2.1.22] from green coffee beans (Sigma Chemical Co.), β-galactosidase [EC 3.2.1.23] from jack beans (Seikagaku Co., Tokyo, Japan), or mixed glycosidases from *Charonia lampas* (Seikagaku Co.) were used. Samples of 10–20 μg were suspended in 0.1 mL of 0.15 M citrate-phosphate buffer (pH 6.5 for α-galactosidase, pH 3.5 for β-galactosidase, and pH 4.0 for mixed glycosidase), containing 0.1 mg of sodium taurodeoxycholate and 0.1 mg of galactonic acid γ-lactone for α-galactosidase. The reaction was carried out with 0.2 U of α-galactosidase at 25°C for 20 h, and 0.2 U of β-galactosidase and glycosidase mixed at 37°C for 18 h. Each incubation was stopped by adding 0.1 mL of methanol and applied to a reversed-phase tC18 cartridge (Sep-Pak cartridge, Waters Co., Milford, MA). After washing the resin with 20 mL of water, the hydrolysates were eluted with 9 mL of methanol and then analyzed by TLC.

TLC immunostaining

TLC immunostaining was performed as described (Itonori et al. 1992). The acidic glycolipid fraction of the Shimeji mushroom, *H. marmoreus*, and the neutral glycolipid fraction extracted from blood group type B red blood cells were spotted on a plastic TLC plate (Polygram SIL G; Macherey-Nagel, Postfach, Germany) and resolved using chloroform–methanol–water (65:35:10, v/v). The plate was dried and blocked with phosphate-buffered saline containing 1% bovine serum albumin. The plate was incubated, with shaking, for 2 h with a monoclonal mouse anti-blood group type B antibody, immunized by a turtle red blood cell surface antigen (Wako Chemical Co., Tokyo, Japan). The plate was then washed, incubated for 1 h with horseradish peroxidase-conjugated goat anti-mouse IgG and IgM (heavy and light chain; Jackson Immunoresearch Laboratories Inc., West Grove, PA), washed again, and developed with 4-chloro-1-naphthol and H_2O_2.

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Conflict of interest statement

None declared.

Abbreviations

2-Gal, 2-linked Gal; 2,3-Gal, 2,3-linked Gal; 3-Gal, 3-linked Gal; 6-Man, 6-linked Man; AGL, acidic glycosphingolipid; FID, flame ionization detector; GC-MS, gas chromatography mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MIPC, Manα1-2Ins-[PO_4]-ceramide; NMR, ^{1}H-nuclear magnetic resonance spectroscopy; *t*-Fuc, terminal Fuc; *t*-Gal, terminal Gal; TLC, thin-layer chromatography; *t*-Man, terminal Man; TMS, trimethylsilyl.

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