

## Novel neogala-series glycosphingolipids with terminal mannose and glucose residues from *Hirsutella rhossiliensis*, an aureobasidin A-resistant ascomycete fungus

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Received on October 22, 2009; revised on December 2, 2009; accepted on December 3, 2009

*Hirsutella rhossiliensis*, a nematophagous fungus belonging to the Ascomycota, is resistant to aureobasidin A (AbA). In this fungus, the biosynthetic pathway leading to mannosylinositolphosphoceramide, which is inhibited by AbA, was not detected. Instead, this fungus contains neutral complex glycosphingolipids (GSLs) and monoglycosylceramides. Except for monoglycosylceramides, neutral GSLs share a neogala-series core structure, Gal $\beta$ 1–6Gal $\beta$ 1–Cer. Among the GSLs of *H. rhossiliensis*, three novel GSLs with terminal Man and Glc residues on the sugar chain were elucidated. We analyzed GSL structure using compositional sugar, fatty acid, and sphingoid analyses, methylation analysis, matrix-assisted laser desorption ionization time-of-flight/mass spectrometry (MALDI-TOF MS), and <sup>1</sup>H nuclear magnetic resonance spectroscopy (NMR). The following structures were determined: Man $\alpha$ 1–3Gal $\beta$ 1–6Gal $\beta$ 1–6Gal $\beta$ 1–Cer; Glc $\alpha$ 1–2Gal $\beta$ 1–6Gal $\beta$ 1–6Gal $\beta$ 1–Cer; and Man $\alpha$ 1–3Gal $\beta$ 1–6(Glc $\alpha$ 1–4)Gal $\beta$ 1–6Gal $\beta$ 1–Cer. In the ceramides, the fatty acids were predominantly saturated h24:0-acids and the sphingoids were predominately t18:0- or t18:1-sphingoids. In contrast, the ceramides of Glc $\beta$ 1-Cer contained d18:2- and d19:2-sphingoids. These findings indicate the presence of a novel biosynthetic pathway of neogala-series GSLs in fungi.

**Keywords:** Ascomycota/aureobasidin A/glycosphingolipid/*Hirsutella rhossiliensis*/neogalatriasylceramide

### Introduction

The major autotrophic fungi are classified into three phyla: the Ascomycota (*Aspergillus*, *Penicillium*, *Acremonium*, etc.), the Basidiomycota (*Cryptococcus*, etc.), and the Zygomycota (*Mucor*, *Rhizopus*, etc.). Glycosphingolipids (GSLs), which consist of a hydrophilic carbohydrate and a hydrophobic ceramide moiety, are essential membrane components of all eukaryotic cells, including fungi. To date, GSLs have been investigated mainly in pathogenic fungi belonging to the Ascomycota and

Basidiomycota. In these fungi, two different types of GSLs have been identified: one is composed of neutral monoglycosylceramides (ceramide monohexosides, CMHs), Gal $\beta$ 1-Cer and Glc $\beta$ 1-Cer (Warnecke and Heinz 2003), and the other is composed of glycosylinositolphosphoceramide (GIPCs), which are characterized by a common core structure of inositolphosphoceramide (IPC) with the addition of Man, GalN, Galp, Galf, and/or Xyl (Loureiro Penha et al. 2001; Heise et al. 2002; Simenel et al. 2008; Takahashi et al. 2009). These GIPCs are also present in other organisms such as protozoa, nematodes, and plants, but not in mammals. GIPCs containing Galf are found in many pathogenic fungi and are suggested to function in infection and pathogenesis (Suzuki et al. 2008). Inhibition of IPC synthase, which catalyzes the transfer of inositol phosphate from phosphatidylinositol to ceramide to give IPC, is lethal to most fungi. Therefore, GIPCs are also essential components for fungal growth (Cheng et al. 2001; Hu et al. 2007). The well-known IPC synthase inhibitor aureobasidin A (AbA) is a potentially broad-spectrum antifungal drug (Takesako et al. 1993; Heidler and Radding 2000; Zhong et al. 2000). Recently, we reported that fungal species belonging to the Zygomycota are entirely resistant to AbA (Aoki, Uchiyama, Yamauchi, et al. 2004). These fungi do not have acidic GIPCs, but instead have unusual neutral neogala-series GSLs with the following representative structure: Gal $\alpha$ 1–6Gal $\alpha$ 1–6Gal $\alpha$ 1–6Gal $\beta$ 1–6Gal $\beta$ 1–Cer. Therefore, we speculated that AbA-resistant fungi might have complex neutral GSLs instead of GIPCs.

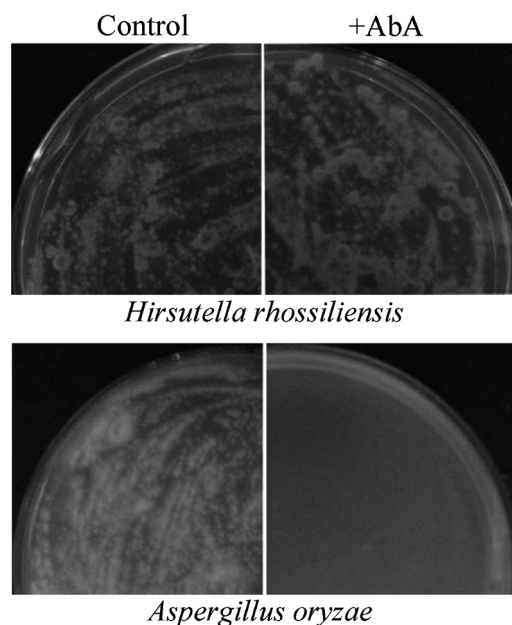
In this study, we examined the relationship between AbA resistance and the presence and composition of complex neutral GSLs. We screened the species group in Deuteromycota which is formerly known as the fungi imperfecti, now in the Ascomycota. These fungi are distinguished on the basis of their morphological characteristics, and their sexual reproductive body is unknown. Our results show that *Hirsutella rhossiliensis*, an AbA-resistant fungus that infects nematodes and insects, has five neutral neogala-series GSLs including three novel GSLs, but no acidic phosphoinositol-containing GSLs. This is the first report of an AbA-resistant fungus within the Ascomycota.

### Results

#### *Growth of various fungi in the presence of AbA*

Previously, we found that all tested species within the Zygomycota (*Mucor hiemalis*, *Rhizopus microsporus*, *Rhizomucor pusillus*, etc.) were resistant to AbA, although the representative Ascomycota species were sensitive (Aoki, Uchiyama, Yamauchi, et al. 2004). Subsequently, we found that membranes of Zygomycota species contained unusual neutral GSLs, which are novel neogala-series GSLs. Thus, we speculated that AbA-resistant fungi, if they belong to phyla other than

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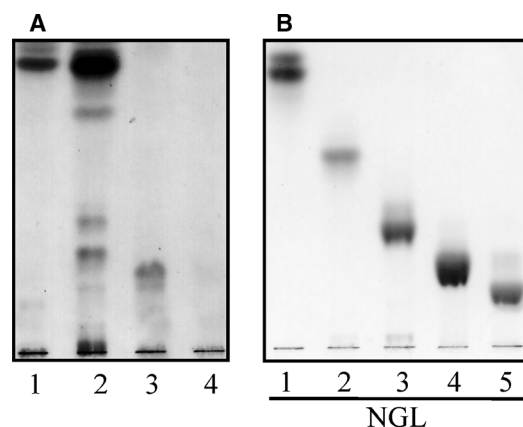


**Fig. 1.** Growth of two Ascomycota fungi on the medium containing AbA. *H. rhossiliensis* was grown on potato medium plates containing 5  $\mu\text{g/mL}$  AbA for 5 days at 20°C. *A. oryzae* was grown on YPG medium plates containing 5  $\mu\text{g/mL}$  AbA for 2 days at 28°C.

the Zygomycota, may contain unusual GSLs. In this study, we first screened AbA-resistant fungal species within the Ascomycota, including the so-called fungi imperfecti formerly classified into Deuteromycota. The fungal strains were cultured on agar plates containing 1–10  $\mu\text{g/mL}$  AbA for 2–5 days. As expected, AbA inhibited the growth of most species, including *Aspergillus oryzae*, *Emericellopsis glabra*, *Hemicarpenites ornatus*, *Rosellinia aquila*, *Hypocrea lutea*, *Epicoccum nigrum*, *Monocillium indicum*, etc. (data not shown). However, the growth of *Hirsutella rhossiliensis* was barely inhibited by AbA (Figure 1). This result suggested that the GSLs of *H. rhossiliensis* differed from those in other Ascomycota species.

#### Isolation of neutral GSLs from *H. rhossiliensis*

We extracted GSLs from the mycelia of *H. rhossiliensis* grown in the YPG liquid medium. GSLs were then separated on the basis of their polarities into neutral and acidic fractions using ion-exchange column chromatography. Each fraction was analyzed by TLC with a chloroform–methanol–water system. The GSLs of *H. rhossiliensis* were present only in neutral fractions (Figure 2A). Surprisingly, the neutral GSLs of *H. rhossiliensis* clearly differed from the GSLs of common Ascomycota fungi such as *A. oryzae*. They consisted of five components, which were identified as mono-, di-, tri-, tetra- and pentasaccharide-containing GSLs on the basis of their TLC mobilities. We tentatively designated these compounds as NGL1, NGL2, NGL3, NGL4, and NGL5, respectively. We checked no fatty acylation on the hydroxyl group in sugars and on hydroxy fatty acids (data not shown). The NGL1 band consisted of two spots, which we presumed were different aliphatic components. These neutral GSLs were then separated by silica-gel column chromatography. The purity of each GSL was confirmed by TLC (Figure 2B). The yields of GSLs from 300 g dried mycelia were



**Fig. 2.** TLC analyses of GSLs extracted from *H. rhossiliensis*. (A) TLC of polar GSLs and neutral GSLs. Lane 1, neutral GSLs from *Aspergillus oryzae*; lane 2, neutral GSLs from *H. rhossiliensis*; lane 3, polar GSLs from *Aspergillus oryzae*; lane 4, polar GSLs from *H. rhossiliensis*. (B) TLC of purified neutral GSLs from *H. rhossiliensis*. Lanes 1–5, purified NGL1, NGL2, NGL3, NGL4, and NGL5, respectively. Orcinol– $\text{H}_2\text{SO}_4$  reagent was used for detection.

as follows: 8.7 mg NGL1; <1 mg NGL2; 2.4 mg NGL3; 11.8 mg NGL4; and 3.0 mg NGL5.

#### Sugar and aliphatic components of GSLs from *H. rhossiliensis*

To determine the structures of neutral GSLs from *H. rhossiliensis*, we first analyzed the composition of sugars and aliphatic components. Each GSL (NGL1–NGL5) was methanolized and then analyzed by GC and GC/MS (Table I). We detected Glc and a trace amount of Gal in NGL1; only Gal in NGL2 and

**Table I.** Ceramide compositions of neutral GSLs from *H. rhossiliensis*. Results were calculated from the peak area of GC detected by FID, with reference to standards for response factors.

		NGL1	NGL2	NGL3	NGL4	NGL5
Sugar	Glc	1.0			1.6	1.0
	Gal	tr.	1.0	1.0	7.0	3.0
	Man				1.0	1.0
	Hex <sup>a</sup>	1	2	3	4	5
	Fatty acid (%)					
	14:0					0.7
	15:0					0.7
	16:0		7.6	1.6	1.0	7.6
	16:1			0.3		1.6
	18:0		3.9	1.0	0.7	4.4
	18:1		2.2	0.3		1.6
	20:0					4.6
	22:0		2.5	0.3		3.2
	24:0			1.3		
	h16:0		4.4	2.1	1.9	3.8
	h16:1	100				
	h18:0		2.2	2.1	2.0	5.4
	h20:0			0.6	0.6	1.0
	h22:0		4.0	5.1	5.2	5.2
	h23:0		2.7	4.4	2.8	
	h24:0		70.6	80.9	85.9	60.4
Sphingoid (%)	d18:2	32.2				
	d19:2	67.8				
	t18:0		47.1	42.0	38.2	37.3
	t18:1		52.9	58.0	61.8	62.7

h, 2-hydroxy fatty acid; t, trihydroxy sphingoid; d, dihydroxy sphingoid; tr, trace.

<sup>a</sup> Results calculated from MALDI-TOF MS analyses (PSD mode).

NGL3; Gal, Glc, and Man (7:1.6:1) in NGL4; and Gal, Glc, and Man (3:1:1) in NGL5. In terms of aliphatic components, NGL1 was quite different from NGL2–NGL5. The C16 hydroxy fatty acids (h16:1) were unique in NGL1, whereas an h24:0 long-chain fatty acid was predominant in NGL2–NGL5. Similarly, dihydroxy 18:2 (d18:2) and d19:2 sphingoids were unique in NGL1. Trihydroxy 18:0 (t18:0) and t18:1 sphingoids were common in all neutral GSLs. Since the aliphatic compositions of NGL2–NGL5 were exactly the same, these NGLs were assumed to be synthesized from a common precursor.

#### MALDI-TOF MS analyses

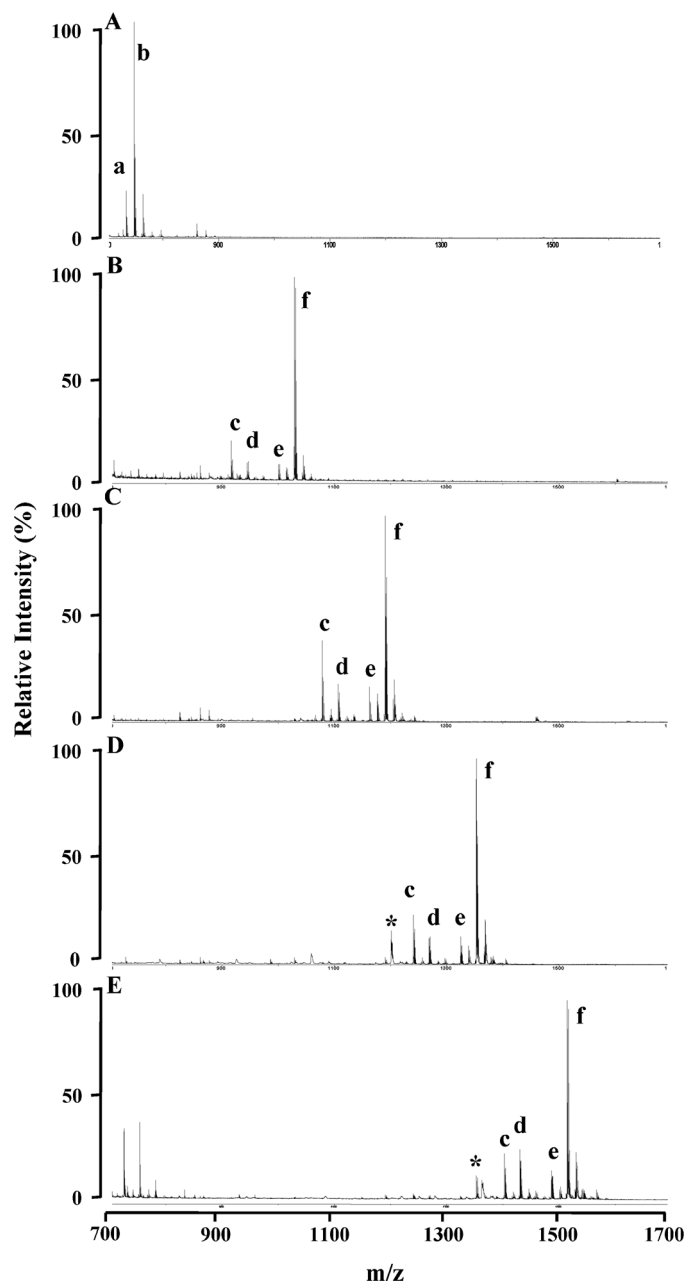
Next, we analyzed the purified NGLs by positive-ion mode MALDI-TOF MS. The presence of different fatty acid and sphingoid species resulted in mass spectra with several different pseudomolecular ions (Figure 3, and Tables I and II). The mass spectrum of NGL1 showed two ion peaks:  $[M+Na]^+$  ions at  $m/z$  734.5 and 748.5, which were consistent with the values calculated from their proposed structures (1 mole each of Hexose (Hex), fatty acid (h16:1), and sphingoid (d18:2 or d19:2)). Eight ion peaks of  $[M+Na]^+$  at  $m/z$  916.6, 918.6, 944.7, 946.7, 1000.7, 1002.7, 1028.8, and 1030.8 from the NGL2 spectrum were assigned to 2 moles of Hex and 1 mole each of fatty acid (h16:0, h18:0, h22:0, or h24:0) and sphingoid (t18:0 or t18:1). Similarly, in the spectra of NGL3–NGL5, eight ion peaks correspond to 3, 4, and 5 moles of Hex, respectively, and the same eight combinations of fatty acid and sphingoid as in NGL2. Other minor peaks contain the  $[M+K]^+$  ion.

#### Anomeric configuration analyses of sugar components of NGLs by $^1H$ NMR

To determine the anomeric configurations of the sugar residues, we subjected each of NGL3–NGL5 to 400 MHz  $^1H$  NMR spectroscopy (Figure 4 and Table III). From the anomeric region of the spectrum of each GSL, the following anomeric proton resonances of sugars were observed: 3 moles of  $\beta$ -Gal (I, II, and III in Figure 4A) from NGL3; 3 moles of  $\beta$ -Gal (I, II, III, and III' in Figure 4B), 0.4 moles of  $\alpha$ -Man (IV in Figure 4B), and 0.6 moles of  $\alpha$ -Glc (IV' in Figure 4B) from NGL4; 3 moles of  $\beta$ -Gal (I, II, and III in Figure 4C), 0.9 moles of  $\alpha$ -Man (IV in Figure 4C), and 1.0 moles of  $\alpha$ -Glc (V in Figure 4C) from NGL5. In the spectrum of NGL4, III' and IV' showed the same molar concentration and were absent from the spectrum of NGL5 (compare Figure 4B and C), suggesting that the  $\alpha$ -linkage between Glc and Gal in NGL4 differs from that in NGL5. Furthermore, the  $\alpha$ -linkage between Man and Gal in NGL4 is the same as that in NGL5 because the position of  $\alpha$ -Man is the same in NGL4 and NGL5 (IV in Figure 4B and C). These results indicate that NGL3 contains three  $\beta$ -Gal residues; NGL4 contains three  $\beta$ -Gal residues and either an  $\alpha$ -Man or an  $\alpha$ -Glc; and NGL5 contains three  $\beta$ -Gal residues, an  $\alpha$ -Man (with the same linkage as in NGL4) and an  $\alpha$ -Glc (with a different linkage from  $\alpha$ -Glc in NGL4).

#### Exoglycosidase digestion of GSLs

We used specific exoglycosidases to analyze the anomeric configuration and linkage of terminal Man and Glc residues. Each of NGL3, NGL4, and NGL5 was treated either singly or with a combination of  $\beta$ -galactosidase,  $\alpha$ -glucosidase, and  $\alpha$ -mannosidase, and then products were analyzed by TLC



**Fig. 3.** MALDI-TOF MS spectra of purified GSLs from *H. rhossiliensis*. Analyses were performed in the positive-ion linear mode. Pseudomolecular ions are given in average masses. Panels A–E show spectra of NGL1, NGL2, NGL3, NGL4, and NGL5, respectively. Multiple peaks in the same panel contain different ceramide moieties. Peaks a–f correspond to those in Table II. Asterisks indicate probable peaks of contaminated triaacylceramide in panel D and tetraacylceramide in panel E.

(Figure 5). The results show that NGL3 was completely hydrolyzed by  $\beta$ -galactosidase (lane 2), whereas NGL4 and NGL5 were resistant to  $\beta$ -galactosidase (lanes 4 and 10). When NGL4 was treated with either  $\alpha$ -glucosidase or  $\alpha$ -mannosidase, residual NGL4 and a new spot corresponding to the NGL3 position were detected (lanes 5 and 7). The spots at the NGL3 position that appeared after each enzyme treatment were completely hydrolyzed by the addition of  $\beta$ -galactosidase (lanes 6 and 8).

**Table II.** Summary of MALDI-TOF MS spectra.  $[M+Na]^+$  ions calculated using monoisotopic mass

		NGL1		NGL2		NGL3		NGL4		NGL5	
		Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated
a	h16:1, d18:2	734.5	734.5								
b	h16:1, d19:2	748.5	748.5								
c	h16:0, t18:1			916.6	916.6	1078.5	1078.7	1240.7	1240.7	1402.6	1402.8
	h16:0, t18:0			918.6	918.6	1080.5	1080.7	1242.7	1242.7	1404.6	1404.8
d	h18:0, t18:1			944.7	946.7	1106.5	1106.7	1268.7	1268.8	1430.6	1430.8
	h18:0, t18:0			946.7	946.7	1108.5	1108.7	1270.8	1270.8	1432.6	1432.8
e	h22:0, t18:1			1000.7	1000.7	1162.5	1162.8	1324.8	1324.8	1486.6	1486.9
	h22:0, t18:0			1002.7	1002.7	1164.6	1164.8	1326.8	1326.8	1488.7	1488.9
f	h24:0, t18:1	866.6	866.7	1028.8	1028.7	1190.6	1190.8	1352.9	1352.9	1514.7	1514.9
	h24:0, t18:0	868.6	868.7	1030.8	1030.7	1192.6	1192.8	1354.9	1354.9	1516.7	1516.9

a–f correspond to those in Figure 3.

**Table III.** Chemical shifts (ppm) and  $J_{1,2}$  coupling constants (Hz) of anomeric protons of NGL3, NGL4, and NGL5

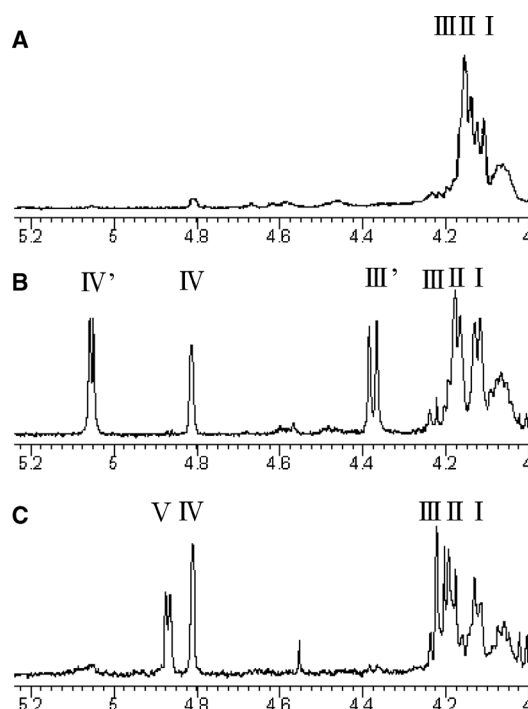
A	NGL3		Gal-III	6Gal-II	6Gal-I
			4.15 (6.34)	4.15 (6.34)	4.12 (6.34)
B	NGL4-a	Man-IV	3Gal-III	6Gal-II	6Gal-I
		4.81 (—)	4.17 (5.44)	4.17 (5.44)	4.12 (5.44)
		NGL4-b	Glc-IV'	2Gal-III'	
		5.05 (3.62)	4.37 (7.25)		
C	NGL5	Man-IV	3Gal-III	(Glc1-4)-V	6Gal-II
		4.81 (—)	4.19 (7.25)	4.87 (3.62)	4.19 (6.80)
					6Gal-I
					4.12 (6.80)

A–C correspond to those in Figure 4.

These results suggest that NGL4 contains two GSLs: an  $\alpha$ -Man added to NGL3 and an  $\alpha$ -Glc added to NGL3 (named NGL4-a and NGL4-b, respectively). Although previous analyses showed that NGL5 contains both Glc and Man residues, it was resistant to *Rhizopus*  $\alpha$ -glucosidase (lane 11). NGL5 was also resistant to other commercially available  $\alpha$ -glucosidases from *Saccharomyces cerevisiae* and *Bacillus stearothermophilus* (data not shown). In contrast, NGL5 was completely hydrolyzed by  $\alpha$ -mannosidase, forming a new spot corresponding to the NGL4 position (lane 12).  $\beta$ -Galactosidase treatment produced a new product which migrated a little faster than NGL3 (lane 13) and almost overlapped with the spot of detergent. Since this new product migrated apparently slower than NGL2, we supposed that this is a triaosylceramide. The results suggest that  $\alpha$ -Glc is attached at the Gal residue which is situated at the second residue from non-reducing end of NGL5. The resistance of such branched  $\alpha$ -Glc to  $\alpha$ -glucosidase treatment may result from steric hindrance.

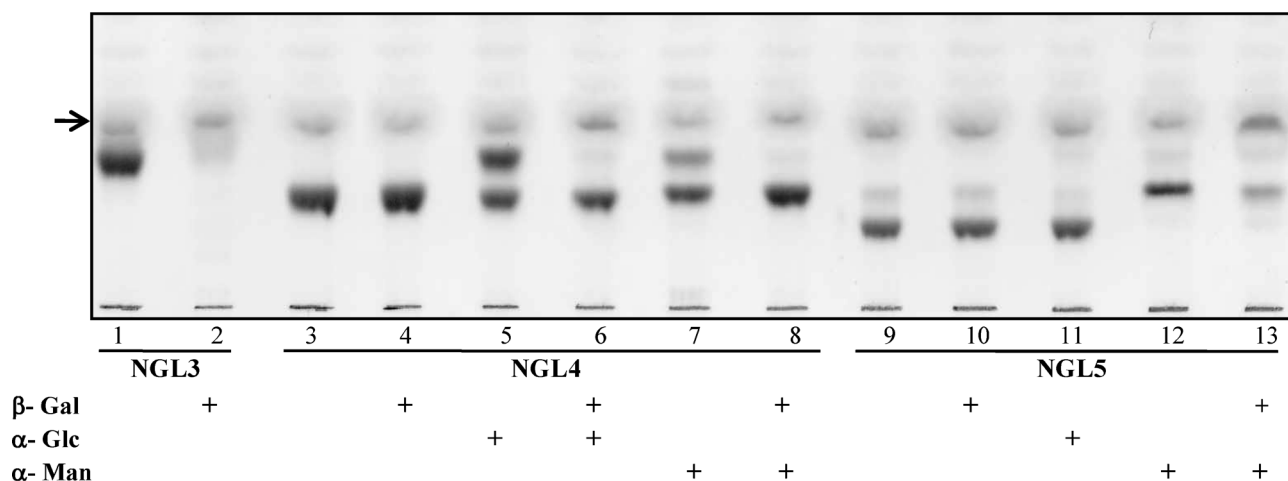
#### Methylation analyses for glycosidic linkages

To completely determine the glycosidic linkages, we analyzed the partially methylated alditol acetates derived from NGLs and glycosidase-treated NGLs by GC and GC/MS. With reference to the data in the previous sections, the peaks in GC analyses (Figure 6) were assigned as follows: a large amount of terminal Glc (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol, t-Glc) and a trace amount of terminal Gal (1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylgalactitol, t-Gal) in NGL1; t-Gal and 6-substituted Gal (1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylgalactitol, 6-Gal) in NGL2; t-Gal and 6-Gal in NGL3; t-Glc, terminal Man (1,5-di-



**Fig. 4.**  $^1\text{H}$  NMR spectra of purified GSLs from *H. rhossiliensis*. Anomeric proton regions of the spectra are shown. (A) NGL3; (B) NGL4; (C) NGL5. I–V correspond to those in Table III.

*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, t-Man), 2-substituted Gal (1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylgalactitol, 2-Gal), 3-substituted Gal (1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylgalactitol, 3-Gal), and 6-Gal in NGL4; and t-Glc, t-Man, 3-Gal, 6-Gal, and 4,6-substituted Gal (1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylgalactitol, 4,6-Gal) in NGL5. The peak area of 6-Gal in NGL3 was significantly larger than that in NGL2, indicating that NGL3 contains additional 1–6-linked Gal. We also analyzed NGL4 and NGL5 after treatment with exoglycosidases (Table IV). NGL4 treated with both  $\alpha$ -glucosidase and  $\beta$ -galactosidase yielded t-Man, 3-Gal, and 6-Gal; NGL4 treated with both  $\alpha$ -mannosidase and  $\beta$ -galactosidase yielded terminal Glc, 2-Gal, and 6-Gal; NGL5 treated with  $\alpha$ -mannosidase and  $\beta$ -galactosidase yielded t-Glc, 4-substituted Gal (1,5,4-tri-*O*-acetyl-2,3,6-tri-*O*-methylgalactitol, 4-Gal), and 6-Gal. These



**Fig. 5.** Exoglycosidase digestion of neutral GSLs from *H. rhossiliensis*. NGL3, NGL4, and NGL5 were treated singly or with a combination of  $\alpha$ -mannosidase,  $\alpha$ -glucosidase, and  $\beta$ -galactosidase, and then analyzed by TLC. Lane 1, NGL3; lane 2, NGL3 incubated with  $\beta$ -galactosidase; lane 3, NGL4; lane 4, NGL4 incubated with  $\beta$ -galactosidase; lane 5, NGL4 incubated with  $\alpha$ -glucosidase; lane 6, NGL4 incubated with  $\alpha$ -glucosidase and then  $\beta$ -galactosidase (NGL4-a); lane 7, NGL4 incubated with  $\alpha$ -mannosidase; lane 8, NGL4 incubated with  $\alpha$ -mannosidase and then  $\beta$ -galactosidase (NGL4-b); lane 9, NGL5; lane 10, NGL5 incubated with  $\beta$ -galactosidase; lane 11, NGL5 incubated with  $\alpha$ -glucosidase; lane 12, NGL5 incubated with  $\alpha$ -mannosidase; and lane 13, NGL5 incubated with  $\alpha$ -mannosidase and then  $\beta$ -galactosidase. Arrow indicates detergent in reaction mixture. Orcinol- $H_2SO_4$  reagent was used for detection.

results indicate the following sequences: Man1-3Gal1-6Gal1-6Gal in NGL4-a; Glc1-2Gal1-6Gal1-6Gal in NGL4-b; and Glc1-4Gal1-6Gal in NGL5 treated with  $\alpha$ -mannosidase and  $\beta$ -galactosidase.

Taken together, our results suggest that the structures of NGL1, NGL2, NGL3, NGL4-a, NGL4-b, and NGL5 are Glc $\beta$ 1-Cer contaminated with a trace amount of Gal $\beta$ 1-Cer, Gal $\beta$ 1-6Gal $\beta$ 1-Cer, Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer, Man $\alpha$ 1-3Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer, Glc $\alpha$ 1-2Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer, and Man $\alpha$ 1-3Gal $\beta$ 1-6(Glc $\alpha$ 1-4)Gal $\beta$ 1-6Gal $\beta$ 1-Cer, respectively.

## Discussion

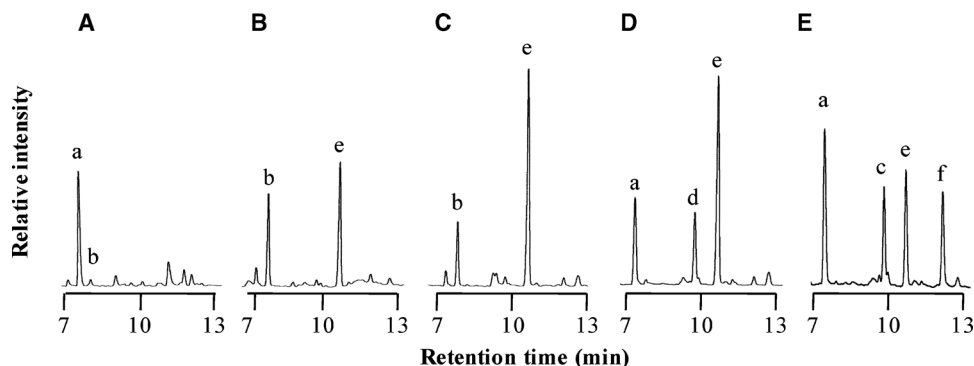
In this study, we structurally analyzed a series of GSLs from an AbA-resistant Ascomycota species, *H. rhossiliensis*. GIPC was not detected in this fungus, confirming its AbA resistance. Instead, we detected neutral complex GSLs of the neogala-series, which were designated as NGL1-NGL5. The major component of the NGL1 fraction was Glc $\beta$ 1-Cer, and the

**Table IV.** Methylated alditol acetate analyses of NGL4 and NGL5 from *H. rhossiliensis*. Results were calculated from the peak area of GC detected by FID, with reference to standards for response factors

Linkage	Relative amount				
	NGL4	NGL4-a	NGL4-b	NGL5	NGL5'
t-Glc			+		+
t-Man	+	+		++	
2-Gal	+		+		
3-Gal		+		+	
4-Gal					+
6-Gal	++	++	++	+	+
4,6-Gal				+	

A plus symbol (+) indicates approximately 1 mole. NGL5' indicates  $\alpha$ -mannosidase- and  $\beta$ -galactosidase-treated NGL5.

minor component was Gal $\beta$ 1-Cer. All other neutral GSLs contain a neogala-series core structure, Gal $\beta$ 1-6Gal $\beta$ 1-Cer (NGL2), which is common with GSLs in species in the Zygomycota. However, the linkage of the third Gal is quite different:  $\alpha$ 1-6 in



**Fig. 6.** Gas chromatograms of partially methylated alditol acetates derived from purified GSLs for determination of glycoside linkages. (A) NGL1; (B) NGL2; (C) NGL3; (D) NGL4; (E) NGL5; a, t-Glc and/or t-Man; b, t-Gal; c, 3-Gal; d, 2-Gal and/or 3-Gal; e, 6-Gal; f, 4,6-Gal.

zygomycetes and  $\beta$ 1-6 in *H. rhossiliensis*. In the zygomycete *Mucor hiemalis*, further elongation of  $\alpha$ 1-6-linked Gal residues occurs, and Gal $\alpha$ 1-6Gal $\alpha$ 1-6Gal $\alpha$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer appears to be the longest product (Aoki, Uchiyama, Yamauchi, et al. 2004). On the other hand, in *H. rhossiliensis*, NGL3 (Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer) is modified by the addition of Glc and/or Man residues. The results of chemical analysis and glycosidase digestion identified two structures in the NGL4 fraction, either  $\alpha$ 1-2-linked Glc or  $\alpha$ 1-3-linked Man attached to the non-reducing terminal Gal of NGL3. NGL4 containing  $\alpha$ 1-3-linked Man could be further modified to form NGL5, via branching of the  $\alpha$ 1-4-linked Glc at the second Gal residue from the reducing end of the sugar chain. Moreover, although the fine structure was not obtained, a PSD mode MALDI-TOF MS analysis suggested the presence of a hexasaccharide-containing GSL (data not shown). Among fungi other than those in the Zygomycota, the ascomycete *Neurospora crassa* is the only fungus known to have neutral oligosaccharide-containing GSLs ((Gal)<sub>3</sub>Glc-Cer, etc.) (Lester et al. 1974). Thus, NGL3, NGL4, and NGL5 from *H. rhossiliensis* are the first GSLs to be identified in fungi. Similar GSLs have been reported from lower animals: Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer from shellfish (Matsubara and Hayashi 1981, 1986); Gal $\alpha$ 1-6Gal $\alpha$ 1-6Gal $\beta$ 1-Cer and Gal $\alpha$ 1-6Gal $\alpha$ 1-6Gal $\alpha$ 1-6Gal $\beta$ 1-Cer from Japanese leech *Hirudo nipponia* (Noda et al. 1996; Sugita et al. 1996); Man $\alpha$ 1-4Gal $\beta$ 1-6Gal $\beta$ 1-Cer, Glc $\alpha$ 1-4Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer, Gal $\alpha$ 1-6(Man $\alpha$ 1-4)Gal $\beta$ 1-6Gal $\beta$ 1-Cer, and Glc $\alpha$ 1-4Gal $\beta$ 1-6(Glc $\alpha$ 1-4)Gal $\beta$ 1-6Gal $\beta$ 1-Cer from an earthworm, *Pheretima* sp. (Sugita et al. 1994, 1997); Fuc $\alpha$ 1-3Gal $\beta$ 1-6Gal $\beta$ 1-Cer from the parasitic cestode *Echinococcus multilocularis* (Persat et al. 1992); Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-6Gal $\beta$ 1-Cer from *E. multilocularis* (Persat et al. 1992), *Taenia crassiceps* (Dennis et al. 1992), and *Metroliasthes coturnix* (Nishimura et al. 1991); Gal $\alpha$ 1-4Gal $\beta$ 1-

6Gal $\beta$ 1-6Gal $\beta$ 1-Cer from *T. crassiceps* (Persat et al. 1992). Therefore, these GSLs from *H. rhossiliensis* containing Man $\alpha$ 1-3Gal $\beta$ -, Glc $\alpha$ 1-2Gal $\beta$ -, and/or Glc $\alpha$ 1-4Gal $\beta$ - structures may be the first discovery of these compounds in any organism.

Except for NGL1, all neutral GSLs from *H. rhossiliensis* share the same ceramide moiety composed of h24:0 linked to a t18:0- or t18:1 sphingoid. This composition is common among GIPCs from most fungal species. Since the ceramide moieties are uniform in NGL2-NGL5, they might be synthesized from a common precursor (Gal $\beta$ 1-Cer) by the sequential addition of monosaccharides. However, we detected very little Gal $\beta$ 1-Cer and Gal $\beta$ 1-6Gal $\beta$ 1-Cer in our analyses. It seemed that those enzymatic reactions of galactose addition to Gal $\beta$ 1-Cer or Gal $\beta$ 1-6Gal $\beta$ 1-Cer might proceed rapidly. In mammalian cells, Gal $\beta$ 1-Cer is synthesized by ceramide galactosyltransferase on the luminal side of the endoplasmic reticulum (ER), whereas Glc $\beta$ 1-Cer is synthesized in the Golgi (Sprong et al. 2003). Since the fungal ceramide galactosyltransferase is likely to be located in the ER as in mammalian cells, Gal $\beta$ 1-Cer may exist only as a transient intermediate in the ER. On the other hand, Glc $\beta$ 1-Cer, a major component of the NGL1 fraction, has a different ceramide composition; h16:1 linked to a d18:2- or d19:2-sphingoid. This suggests a different specificity or localization of its ceramide glucosyltransferase. The ceramide composition closely resembles that of CMH from other filamentous fungi (Warnecke and Heinz 2003). A different ceramide composition between Glc $\beta$ 1-Cer and neogala-series GSLs was also observed in *M. hiemalis* (Aoki, Uchiyama, Yamauchi, et al. 2004). The proposed biosynthetic pathways of GSLs in *H. rhossiliensis* are shown in Figure 7.

Generally, fungi that contain GIPC in their cell membranes are sensitive to AbA, which inhibits IPC synthase, the enzyme that catalyzes the first step of GIPC biosynthesis. The AbA-resistant fungi identified to date do not contain GIPC, or contain

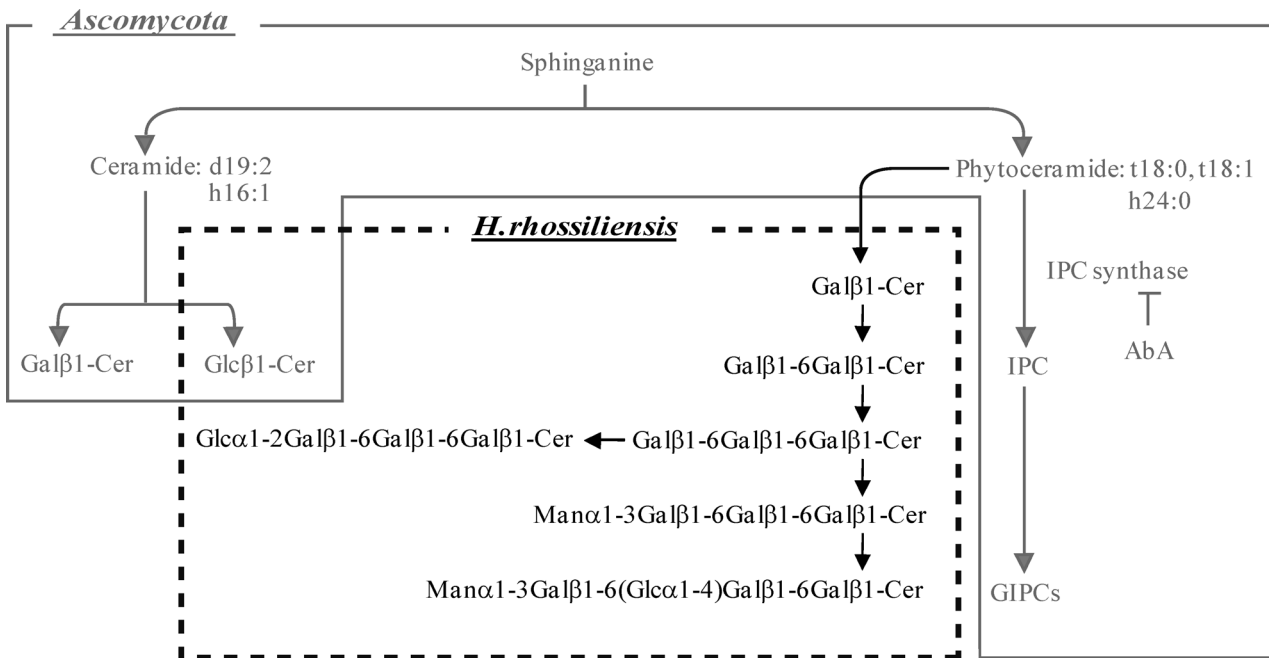


Fig. 7. Putative biosynthetic pathway for GSLs in *H. rhossiliensis*.

only trace amounts. It is worth noting that all AbA-resistant fungi have oligosaccharide-containing neutral GSLs. Thus, we can speculate that either neutral GSLs with long sugar-chains or GIPCs are essential for fungal growth. This study is the first report of an AbA-resistant fungus from the Ascomycota. There may be other AbA-resistant fungi within the Ascomycota and other fungal phyla. Therefore, inhibitors of fungal neutral GSL biosynthesis may have potential as antifungal drugs that are effective against AbA-resistant fungi.

*Hirsutella* species infect insects, mites, and nematodes. *H. rhossiliensis* is one of the most studied biological control agents for several plant-parasitic nematodes (Chen and Reese 1999). Another species within this genus, *Hirsutella thompsonii*, secretes the insecticidal protein Hirsutellin A (Maimala et al. 2002). However, the infectious and pathogenic mechanisms of *H. rhossiliensis* are poorly understood. Usually, carbohydrate epitopes on the cell surface of parasitic microbes are recognized by the hosts' immune systems. In invertebrates, pattern recognition receptors such as Toll-receptors are well known (Chamilos et al. 2008; Lamaris et al. 2009). The cell surface glycans of some parasites mimic those of the host, which enables the parasite to evade the host's immune systems. The unique GSLs in *H. rhossiliensis* may have a role in protecting the pathogen against the immune system of insects and nematodes. In fact, the nematode *Caenorhabditis elegans* contains carbohydrates with  $\beta$ 1–6-linked Gal and  $\alpha$ 1–3-Man at the non-reducing end (Natsuka et al. 2002; Griffiths et al. 2005). Another possibility is that GSLs themselves act as specific toxins for insects and nematodes. In humans, neogala-series GSLs from the pathogenic cestode *Echinococcus multilocularis* stimulate an immune response (Persat et al. 1992; Yamano et al. 2009) and inhibit the proliferation of peripheral blood mononuclear cells with a concomitant decrease in interleukin-2. This suggests that GSLs may play an immunologically important role in *Echinococcosis* (Persat et al. 1996). Previously, we demonstrated that phosphocholine-containing GIPCs from the plant pathogenic fungi *Acremonium* and *Trichoderma* induce apoptosis in cultured rice cells (Aoki, Uchiyama, Itonori, et al. 2004; Uchiyama et al. 2009). Furthermore, fungal-specific sphingolipids containing a d19:2 sphingoid were reported to interact with some plant defensins to enable infection (Thevissen et al. 2003; Park et al. 2005). The biological function of GSLs in *H. rhossiliensis* is expected to be elucidated in the near future.

## Material and methods

### Culture of fungal strains

The following Ascomycota fungal strains were obtained from the Japan Collection of Microorganisms (JCM), RIKEN BIOResource Center, Japan, and the National Research Institute of Brewing (RIB), Japan: *Hirsutella rhossiliensis* JCM 23109, *Emericellopsis glabra* JCM 10470, *Hemicarpenateles ornatus* JCM 12733, *Rosellinia aquila* JCM 22697, *Hypocrea lutea* JCM 23023, *Epicoccum nigrum* JCM 6029, *Monocillium indicum* JCM 10146, and *Aspergillus oryzae* RIB40. These strains were cultivated in the YPG medium (0.5% yeast extract, 0.5% peptone, 1% glucose, 0.5% NaCl) at 20–25°C for 10 days in a 2 L shaking flask containing a 700 mL medium at 200 rpm. To assay growth inhibition by AbA (Takara, Japan), fungal strains

were grown on potato medium plates or YPG plates containing 1–10  $\mu$ g/mL AbA for 2–5 days at 20–28°C (Aoki, Uchiyama, Yamauchi, et al. 2004).

### Extraction and purification of GSLs

The cultivated mycelia were harvested and freeze dried. The dried mycelia were extracted twice with chloroform–methanol–water (30:60:8, by volume). The extract was dried and subjected to mild alkaline hydrolysis with 0.5 M KOH in methanol–water (95:5, v/v) at 37°C for 12 h. The hydrolysate was acidified to pH 1.0 with concentrated HCl and then dialyzed against tap water for 2 days. Glycolipids in the dialysate were precipitated with acetone and then the dissolved solution was applied to a DEAE-Sephadex A-25 column (10 mL, OH<sup>−</sup> form, GE Healthcare Co., for small-scale preparation) or a QAE-Sephadex A-25 column (50 mL, OH<sup>−</sup> form, GE Healthcare Co., for large-scale preparation). Elution was carried out with five volumes of the above solution (chloroform–methanol–water, 30:60:8, by volume) to obtain the neutral GSLs fraction. Polar compounds were recovered with 0.05–0.45 M ammonium acetate in methanol. The neutral GSL fraction was acetylated with acetic anhydride–pyridine (3:2, by volume) at 20°C for 18 h, purified on a Florisil column (1.0  $\times$  35 cm, magnesium silicate, Nacalai Tesque, Japan), deacetylated with 0.5 M KOH in methanol at 37°C for 6 h, and dialyzed. Then, the purified neutral GSL fraction was applied to a column of porous silica gel (1.5  $\times$  55 cm, Iatrobeads 6RS-8060, Iatron Bab., Japan) and GSLs were eluted with chloroform–methanol–water (70:30:3, by volume).

### Aliphatic and sugar composition analyses

To determine the compositions of the aliphatic and sugar components of neutral GSLs, 50–100  $\mu$ g samples were methanolized in thick glass test tubes with 200  $\mu$ L of freshly prepared 1 M anhydrous methanolic HCl at 100°C for 3 h. After methanolysis, the fatty acid methyl esters were extracted five times with 200  $\mu$ L *n*-hexane and then analyzed by capillary GC/MS. The remaining methanolic phase was evaporated to dryness for deacidification under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated and then analyzed by GC.

### Methylation analysis to determine sugar linkages

To determine the sugar linkages of oligosaccharides in GSLs, 100  $\mu$ g purified GSL was partially methylated with NaOH and CH<sub>3</sub>I in DMSO (Noda et al. 1996). The permethylated GSL was hydrolyzed with 300  $\mu$ L HCl–water–acetic acid (0.5:1.5:8, by volume) at 80°C for 18 h, and then reduced with NaBH<sub>4</sub> and acetylated with acetic anhydride–pyridine (1:1, v/v) at 100°C for 15 min. The partially methylated alditol acetates obtained were analyzed by GC and GC/MS.

### Thin-layer chromatography

Silica gel 60 TLC plates (Merck, Germany) were developed for 5 cm using a neutral solvent system, chloroform–methanol–water (60:35:8, by volume). We sprayed the plates with orcinol–H<sub>2</sub>SO<sub>4</sub> reagent to detect sugars, 5% H<sub>2</sub>SO<sub>4</sub>–ethanol reagent to detect organic substances, Dittmer–Lester reagent to detect phosphorus (Dittmer and Lester 1964), and ninhydrin reagent to detect free amino groups.

### GC and GC/MS

We used a Shimadzu GC-18A gas chromatograph with a capillary column (Shimadzu HiCap-CBP 5; 0.22 mm × 25 m) to determine composition of sugars and aliphatic components, and sugar linkages. The following temperature programs were used: 2°C/min from 140°C to 230°C for sugar trimethylsilyl (TMS) derivatives; 2°C/min from 170°C to 230°C for fatty acid methyl esters; 4°C/min from 140°C to 230°C for partially methylated alditol acetate derivatives; and 2°C/min from 210°C to 230°C for sphingoid TMS derivatives. Electron impact (EI) mass spectra were obtained with a Shimadzu GCMS-QP 5050 GC/MS under the following conditions: oven temperature, 80°C (2 min) to 170°C (20°C/min) to 240°C (4°C/min) for sugar TMS derivatives; 80°C (2 min) to 170°C (20°C/min) to 240°C (4°C/min) for fatty acid methyl esters; 80°C (2 min) to 160°C (20°C/min) to 240°C (4°C/min) for partially methylated alditol acetate derivatives; 80°C (2 min) to 210°C (20°C/min) to 230°C (4°C/min) for sphingoid TMS derivatives; interface temperature, 250°C; injection port temperature, 240°C; helium gas pressure, 100 kPa; ionizing voltage, 70 eV; and ionizing current, 60 μA.

### MALDI-TOF MS

MALDI-TOF MS analyses of neutral GSLs were performed with an Applied Biosystems/Voyager-DE STR™ Biospectrometer with a nitrogen laser (337 nm) and an acceleration voltage of 20 kV, operating in the reflector and post source decay (PSD) positive-ion mode. The matrix used was α-cyano-4-hydroxycinnamic acid (proteomics Grade, Wako Chemical Co.). External mass calibration was provided by the [M+Na]<sup>+</sup> ions of angiotensin 1 (1296.69 mass units; proteomics Grade, Wako Chemical Co.) and bradykinin fragments 1–5 (573.31 mass units; Sigma Chemical Co., USA).

### <sup>1</sup>H NMR spectroscopy

NMR spectra of neutral GSLs were obtained with a JEOL JNM-ECS 400 MHz <sup>1</sup>H NMR spectrometer at an operating temperature of 60°C. The purified neutral GSL was dissolved in 0.6 mL dimethylsulfoxide-*d*<sub>6</sub> containing 2% D<sub>2</sub>O, and the chemical shift was referenced to the solvent signals (*d*<sub>H</sub> = 2.49 ppm) in DMSO-*d*<sub>6</sub> as the internal standard.

### Exoglycosidase digestion

α-Mannosidase and β-galactosidase from jack beans, and three α-glucosidases from *Rhizopus* sp. *Saccharomyces cerevisiae*, and *Bacillus stearothermophilus* (all purchased from Seikagaku Co., Japan) were used for exoglycosidase digestion to analyze sugar linkages in GSLs. Samples were suspended in the 100 μL 50 mM citrate buffer (pH 4.5) containing 0.1 mg sodium taurodeoxycholate and were incubated with either 0.2 units α-mannosidase, 2 units α-glucosidase, or 0.2 units β-galactosidase at 37°C for 16 h. The reaction was terminated by the addition of chloroform–methanol (2:1, v/v). The hydrolysates recovered from the lower phase were dried under a nitrogen stream and analyzed by TLC.

### Acknowledgements

We thank Dr. N. Hada (Keio University) for help with NMR analyses, and Messrs. M. Nishio and H. Kojima (Ritsumeikan University) for technical assistance with MALDI-TOF MS and NMR analyses.

### Supplementary data

Supplementary data for this article is available online at <http://glycob.oxfordjournals.org/>.

### Conflict of interest statement

The authors have no conflicts of interest to declare.

### Funding

The Japan Society for the Promotion of Science (B 16380063) to K.Y., and the 21st Century Center of Excellence (COE) program of the Ministry of Education, Culture, Sports, Science, and Technology, Japan, to the Graduate School of Biostudies and Institute for Virus Research, Kyoto University.

### Abbreviations

AbA, aureobasidin A; Cer, ceramide; CMH, ceramide monohexoside; FID, flame ionization detector; GC/MS, gas chromatography/mass spectrometry; GIPC, glycosylinositolphosphoceramide; GSL, glycosphingolipid; Hex, hexose; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PSD, post source decay; TLC, thin-layer chromatography; TMS, trimethylsilyl.

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