Glycobiology and Extracellular Matrices:

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Newly Discovered Neutral Glycosphingolipids in Aureobasidin A-resistant *Zygomycetes*

IDENTIFICATION OF A NOVEL FAMILY OF GALA-SERIES GLYCOLIPIDS WITH CORE Galα1-6Galβ1-6Galβ SEQUENCES*

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Kazuhiro Aoki‡§, Ryosuke Uchiyama‡, Suguru Yamauchi‡, Takane Katayama‡, Saki Itonori¶, Mutsumi Sugita¶, Noriyasu Hada‖, Junko Yamada-Hada‖, Tadahiro Takeda‖, Hidehiko Kumagai‡, and Kenji Yamamoto‡

From the ‡Graduate School of Biostudies, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan, the ¶Faculty of Liberal Arts and Education, Shiga University, 2-5-1, Hiratsu, Otsu, Shiga 520-0862, Japan, and the ‖Kyoritsu University of Pharmacy, 1-5-30, Shibakoen, Minato-ku, Tokyo 105-8512, Japan

We found for the first time that *Zygomycetes* species showed resistance to Aureobasidin A, an antifungal agent. A novel family of neutral glycosphingolipids (GSLs) was found in these fungi and isolated from *Mucor hiemalis*, which is a typical *Zygomycetes* species. Their structures were completely determined by compositional sugar, fatty acid, and sphingoid analyses, methylation analysis, matrix-assisted laser desorption ionization time-of-flight/mass spectrometry, and ^{1}H NMR spectroscopy. They were as follows: Galβ1-6Galβ1-1Cer (CDS), Galα1-6Galβ1-6Galβ1-1Cer (CTS), Galα1-6Galα1-6Galβ1-6Galβ1-1Cer (CTeS), and Galα1-6Galα1-6Galα1-6Galβ1-6Galβ1-1Cer (CPS). The ceramide moieties of these GSLs consist of 24:0, 25:0, and 26:0 2-hydroxy acids as major fatty acids and 4-hydroxyoctadecasphinganine (phytosphingosine) as the sole sphingoid. However, the glycosylinositolphosphoceramide families that are the major GSLs components in fungi were not detected in *Zygomycetes* at all. This seems to be the reason that Aureobasidin A is not effective for *Zygomycetes* as an antifungal agent. Our results indicate that the biosynthetic pathway for GSLs in *Zygomycetes* is significantly different from those in other fungi and suggest that any inhibitor of this pathway may be effective for mucormycosis, which is a serious pathogenic disease for humans.

Sphingolipids are essential membrane components of both mammalian and fungal cells. The early steps in their biosynthetic pathways up to the formation of sphingosine are the same, but the subsequent pathways are very different in both cells (1). In mammalian cells, sphingosine is attached to fatty acids to yield ceramide. In fungi, phytoceramide is produced from a phytosphingosine having an additional hydroxylation on C-4 and 2-hydroxy fatty acid. The phytoceramide gives rise to inositol-containing sphingolipids such as inositol phosphorylceramide (IPC),[1] mannose-IPC (MIPC), and inositol phosphoryl-MIPC (2, 3). This step involves the transfer of the phosphoinositol group from phosphatidylinositol to the 1-hydroxy group of the phytoceramide to yield IPC, which is then mannosylated to yield MIPC (4). Because sphingolipid synthesis is essential for the growth and viability of fungi, it is likely that a blocking of the synthesis would efficiently inhibit cell growth. Therefore, the enzymes catalyzing the synthesis of inositol-containing sphingolipids that are present in fungi but absent in humans have been focused as targets for antifungal agents (5). One of these enzymes is IPC synthase, which catalyzes the transfer of the inositolphosphate from phosphatidylinositol to ceramide to give IPC (6).

Aureobasidin A is well known and widely used as an antifungal agent for *Eumycetes* including yeasts and fungi. It exhibits strong fungicidal activity against many pathogenic fungi, including *Candida albicans*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (7, 8). Recent studies (6) have shown that this antifungal agent inhibits IPC synthase in fungal cells. The inhibition of this enzyme causes the depletion of essential sphingolipids in the fungal cells. Because it is recognized that all fungi have this enzyme, Aureobasidin A is potentially a broad spectrum antifungal (9).

We found that all *Zygomycetes* species tested were resistant to the antifungal agent Aureobasidin A, a cyclic depsipeptide produced by *Aureobasidium pullulans* (10). The *Zygomycetes* species do not have inositol-containing sphingolipids but contain novel neutral glycosphingolipids (GSLs) consisting glucose or galactose as sugar constituents. This suggests a remarkable difference in GSLs between *Zygomycetes* species and other fungi. We supposed that the lack of a synthetic pathway for inositol-containing sphingolipids in their cells might be the cause of the resistance of *Zygomycetes* species to Aureobasidin A. We also suggested a new synthetic pathway for GSLs containing galactooligosaccharides with Galβ1-6Gal and Galα1-6Gal residues in *Zygomycetes* species such as *Mucor* and *Rhizopus* species. A novel family of neutral GSLs found from *Zygomycetes* species belongs to a homologous series with a phytoceramide consisting of phytosphingosine and 2-hydroxy C24–C26 fatty acids. This is the first report that *Zygomycetes* species are resistant to the antifungal agent Aureobasidin A

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[1] The abbreviations used are: IPC, inositol phosphorylceramide; AbA, Aureobasidin A; Cer, ceramide; CMS, ceramide monosaccharide; CDS, ceramide disaccharide; MS, mass spectroscopy; CTS, ceramide trisaccharide; CTeS, ceramide tetrasaccharide; CPS, ceramide pentasaccharide; Glc, glucose; Gal, galactose; GalCer, galactosylceramide; GSL, glycosphingolipid; MALDI-TOF/MS, matrix-assisted laser-desorption ionization-time-of-flight MS; MIPC, mannose-IPC; NGL, neutral GSL; GLC, gas-liquid chromatography.

and that they contain a novel family of galactose-containing GSLs but not phosphoinositol-containing sphingolipids.

EXPERIMENTAL PROCEDURES

Strains and Growth Conditions—Fungal strains *Aspergillus oryzae* (NBRC4075), *Aspergillus niger* (NBRC31125), *Mucor fragilis* (NBRC-6449), *Mucor racemosus* (NBRC4581), *Rhizopus oryzae* (NBRC9364), *Rhizopus microsporus* (NBRC30499), *Rhizopus stolonifer* (NBRC-32998), *Rhizomucor pusillus* (NBRC9745), *Penicillium oxalicum* (NBRC5748), and *Absidia corymbifera* (NBRC32279) were obtained from the National Institute of Technology and Evaluation Biological Resource Center. *Mucor hiemalis* number 314 and *Acremonium* sp. were isolated from soil and identified in our laboratory (11, 12). These strains were cultivated in YPG medium (0.5% yeast extract, 0.5% peptone, 0.5% NaCl,1% glucose, pH 6.5) at 28 °C for 48 h in 2-liter shaking flasks containing 700 ml of the medium on a reciprocal shaker at 120 rpm (12). For assaying of the growth inhibition by Aureobasidin A (AbA), fungal strains were grown on YPG plates containing 0.1–10 μg/ml AbA for 48 h at 28 °C (13).

Materials—QAE-Sephadex A-25, DEAE Sephadex A-25, and D-[U-^{14}C]glucose were purchased from Amersham Biosciences. Iatrobeads 6RS-8060 was obtained from Iatron Laboratories Inc. Silica gel 60 precoated plates were from Merck, magnesium silicate (Florisil) was from Nacalai Tesque, green coffee bean α-galactosidase was from Sigma, and jack bean β-galactosidase was from Seikagaku Co. Aureobasidin A was obtained from Takara Bio Inc. All other reagents used were of best grade available commercially.

Extraction and Purification of Sphingolipids—Sphingolipids were prepared from mycelia by consecutive extractions, as described elsewhere (14). Lipid extracts were saponified with 0.5 M KOH in methanol-water (95:5, v/v) at 37 °C for 6 h. The hydrolysate was acidified to pH 1.0 with concentrated HCl and then dialyzed against tap water for 2 days followed by concentration and precipitation with acetone. The sphingolipids were fractionated on a QAE-Sephadex A-25 column (20 × 300 mm, OH^- form). The neutral fraction was further purified by silica gel chromatography (column, 15 × 600 mm) with a linear gradient elution system of chloroform-methanol-water (400 ml of 90:10:0.5 by volume to 420 ml of 40:60:10 by volume). The polar fraction was then applied to a column of DEAE-Sephadex A-25 (20 × 200 mm, acetate form), as described elsewhere (14).

Carbohydrate and Fatty Acid Composition Analyses—For determination of the compositions of the fatty acids and sugars in GSLs, 100–200 μg of GSLs were methanolyzed in thick glass test tubes with 200 μl of freshly prepared 1 M anhydrous methanolic HCl using a microwave oven (14, 15). After methanolysis, the fatty acid methyl esters were extracted three times with 400 μl of n-hexane and then analyzed by capillary gas-liquid chromatography (GLC)/MS (14, 15). The remaining methanolic phase was evaporated to dryness for deacidification under a nitrogen stream. The residue containing methylglycosides was trimethylsilylated and then analyzed by GLC. Sphingoids prepared from GSLs by methanolysis with 1 M aqueous methanolic HCl at 70 °C for 18 h were converted to their *O*-trimethylsilyl (*N*-free) derivatives and then analyzed by GLC/MS (14, 15).

Methylation for Sugar Linkage Analysis—For determination of the sugar linkages of oligosaccharides in GSLs, 300 μg of a purified GSL was partially methylated with NaOH and CH_3I in Me_2SO (16). The permethylated GSL was acetolyzed and hydrolyzed with 300 μl of a mixture of HCl-water-acetic acid (0.5:1.5:8 by volume.) by exposure to the maximum power of the microwave oven for 1 min and then was reduced with $NaBH_4$ and acetylated with a mixture of acetic anhydride-pyridine (1:1, v/v) at 100 °C for 15 min. The partially methylated alditol acetates thus obtained were analyzed by GLC and GLC/MS (14, 15).

TLC—TLC was performed on silica gel 60 precoated plates with a neutral solvent system, chloroform-methanol-water (60:35:8 and 80:20:1, by volume). Detection was performed by spraying with orcinol-H_2SO_4 reagent for sugars, 5% H_2SO_4-ethanol reagent for organic substances, Dittmer-Lester reagent and Hanes-Isherwood reagent for phosphorus, and ninhydrin reagent for free amino groups.

Labeling Studies of GSLs—Fungal cells were grown on YPG liquid medium at 28 °C for 48 h, and then mycelia were collected and washed with distilled water. Mycelia were incubated with 20 μl of [^{14}C]glucose (7.4 MBq/ml) at 28 °C. Incubation was stopped at the appropriate times, and lipids were extracted from the mycelia with a solvent mixture of chloroform-methanol-water (30:30:10, by volume). They were separated by TLC and visualized with an imaging analyzer (Fujifilm, BAS2000).

Cleavage of Sugar Linkages by Exoglycosidases—α-Galactosidase from green coffee beans and β-galactosidase from jack beans were used for exoglycosidase cleavage of the sugar linkages of oligosaccharides in GSLs. Samples (10–30 μg) were suspended in 0.1 ml of 50 mM Tris-HCl buffer (pH 6.5) for α-galactosidase treatment and 50 mM citrate buffer (pH 3.5) for β-galactosidase treatment, respectively, in the presence of 0.1 mg of sodium taurodeoxycholate. Each reaction was carried out with 0.25 units of α-galactosidase and 0.5 units of β-galactosidase, respectively, at 37 °C for 12 h and was stopped by adding 0.5 ml of chloroform-methanol (2:1, v/v). The hydrolysate, after extraction into the lower phase, was dried under a nitrogen stream and then analyzed by TLC.

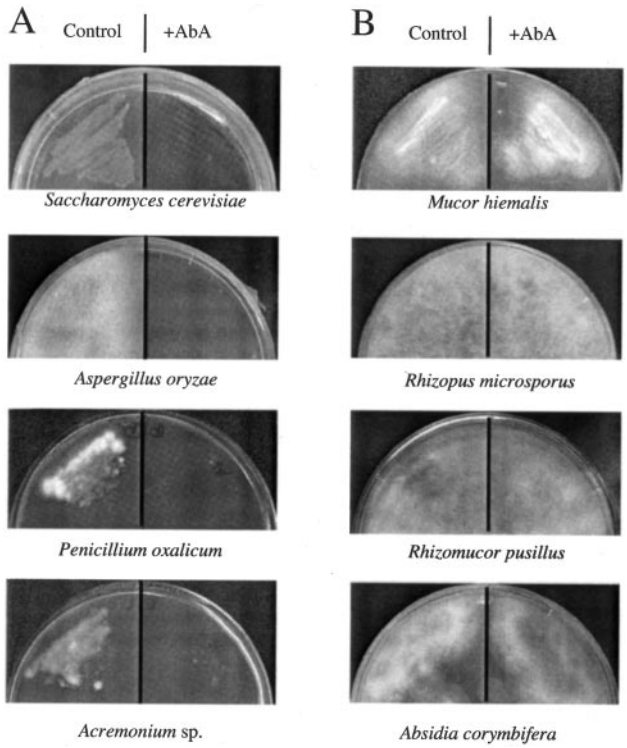


FIG. 1. **Growth of various fungi on medium containing Aureobasidin A.** Various fungal strains were grown on YPG plates containing 5 μg/ml AbA for 48 h at 28 °C. Yeast was grown for 48 h at 37 °C. *A*, yeast *S. cerevisiae* (W303-1A), *A. oryzae* (NBRC4075), *P. oxalicum* (NBRC5748), and *Acremonium* sp. *B*, *Zygomycetes* species of *M. hiemalis* number 314, *R. microsporus* (NBRC30499), *R. pusillus* (NBRC9745), and *A. corymbifera* (NBRC32279).

GLC and GLC/MS—A Shimadzu GC-18A gas chromatograph with a capillary column (0.22 mm × 25 m) of Shimadzu HiCap-CBP 5 was used for determination of sugars, aliphatic compositions, and sugar linkages. The following temperature programs were used, 2 °C/min from 140 to 230 °C for sugars, 2 °C/min from 170 to 230 °C for fatty acids, and 2 °C/min from 210 to 230 °C for sphingoids. The partially methylated alditol acetates were analyzed with GLC and GLC/MS equipped with a HiCap-CBP 5 capillary column, as described above. Electron impact and chemical ionization mass spectra were obtained with a Shimadzu GCMS-QP 5050 GLC/MS under the following conditions: oven temperature, 80 °C (2 min) → 180 °C (20 °C/min) → 240 °C (4 °C/min); interface temperature, 250 °C; injection port temperature, 240 °C; helium gas pressure, 100 kilopascal; ionizing voltage, 70 eV (electron impact) and 100 eV (chemical ionization); ionizing current, 60 μA (electron impact) and 200 μA (chemical ionization); and reaction gas (chemical ionization), isobutane.

^{1}H NMR Spectroscopy—NMR spectra of the purified neutral GSLs were obtained with a JEOL A-500 500 MHz ^{1}H NMR spectrometer at 60 °C as the operating temperature. Each purified GSL was dissolved in 0.6 ml of dimethyl sulfoxide-d_6 containing 2% D_2O with the chemical shift being referenced to the solvent signals (δ_H = 2.49 ppm) in Me_2SO-d_6 as the internal standard.

Matrix-assisted Laser-desorption Ionization Time-of-Flight MS (MALDI-TOF/MS)—MALDI-TOF/MS analyses of the purified neutral GSLs were performed with a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer equipped with a Work station SPARC station, operating in the positive-ion linear mode (14). Ions were formed by a pulsed ultraviolet laser beam (N_2 laser, 337 nm; 3-ns wide pulses/s). The matrix used was 7-amino-4-methylcoumarin (Sigma) (17).

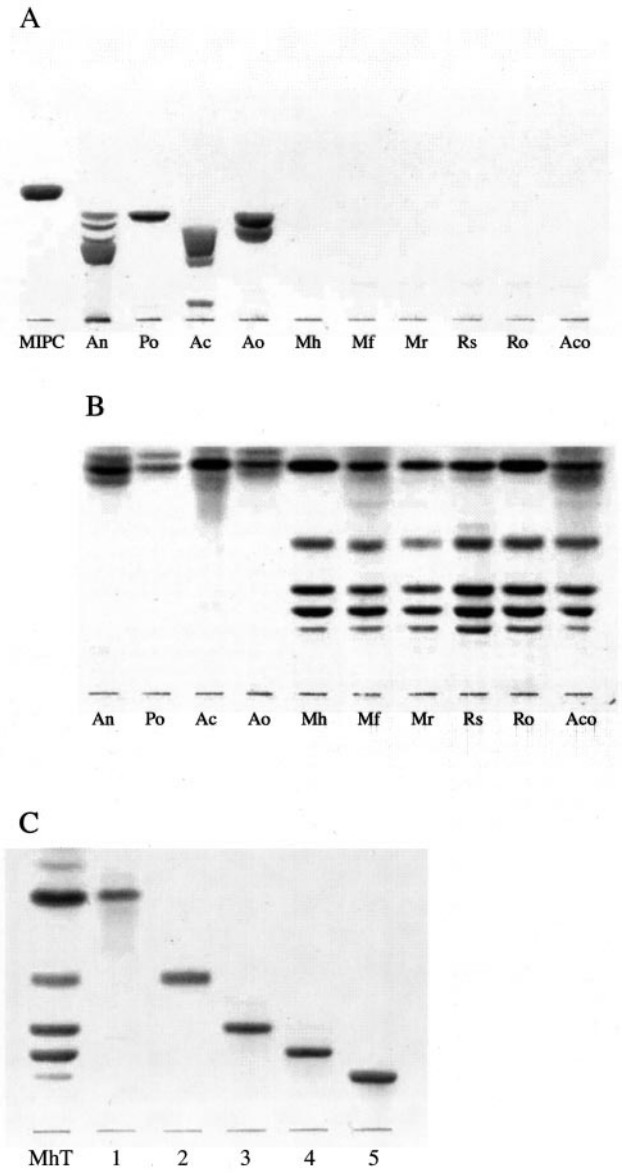


FIG. 2. **TLC analyses of glycolipids from various fungi.** TLC was performed with chloroform-methanol-water (60:35:8, by volume, neutral solvent), with visualization with orcinol-H_2SO_4 reagent. *A*, TLC of polar GSLs from various fungi. *B*, TLC of NGLs from various fungi. MIPC was purified from *S. cerevisiae* (W303-1A). *Lanes An*, *Po*, *Ac*, *Ao*, *Mh*, *Mf*, *Mr*, *Rs*, *Ro*, and *Aco* are polar GSLs from *A. niger* (NBRC31125), *P. oxalicum* (NBRC5748), *Acremonium* sp., *A. oryzae* (NBRC4075), *M. hiemalis* number 314, *M. fragilis* (NBRC6449), *M. racemosus* (NBRC4581), *R. stolonifer* (NBRC32998), *R. oryzae* (NBRC9364), and *A. corymbifera* (NBRC32279), respectively. *C*, TLC of purified NGLs from *M. hiemalis* number 314. *Lane MhT*, total NGL fraction of *M. hiemalis* number 314; *lanes 1–5*, purified GSLs isolated from NGLs: CMS, CDS, CTS, CTeS, and CPS, respectively.

RESULTS

Growth of Fungi Resistant to AbA—During studies on GSLs of fungi, we found that all *Zygomycetes* species examined were resistant to AbA and grew in the medium containing AbA (0.1–10 μg/ml). As shown in Fig. 1*A*, AbA strongly inhibits the growth of filamentous fungi such as *A. oryzae*, *P. oxalicum*, and *Acremonium* sp., previously reported for the yeast *Saccharomyces cerevisiae* (W303-1A) and other fungi (7–10, 13). However, growth inhibition by AbA was not observed for any *Zygomycetes* species such as *M. hiemalis*, *R. microsporus*, *R. pusillus*, and *A. corymbifera* (Fig. 1*B*). These results suggested that the GSLs in *Zygomycetes* species were very different from those in other fungi.

TABLE I
Chemical compositions of the purified GSLs from M. hiemalis
Plus (+) and minus (−) signs indicate the presence and the absence, respectively.

	CMS	CDS	CTS	CTeS	CPS
Sugar					
Glc	+				
Gal		+	+	+	+
Fatty acid (%)					
h14:0[a]	10.5				
h16:0	89.5				
h24:0		65.2	63.2	63.8	67.9
h25:0		10.8	17.3	17.2	15.4
h26:0		24.0	19.5	19.0	16.7
Sphingoid (%)					
d19:2	53.6				
d20:1	27.5				
Unknown	18.9				
t18:0	−	>99	>99	>99	>99

[a] h, 2-hydroxy fatty acid; d, dihydroxy; t, trihydroxy sphingoid.

Therefore, we analyzed the GSLs in *Zygomycetes* species to investigate the AbA resistance mechanism.

GSLs of Various Fungi Zygomycetes Species—GSLs of fungi were separated into neutral, acidic, and zwitterionic fractions by ion-exchange column chromatography based on their polarities. Each fraction was analyzed by TLC with a chloroform-methanol-water system. The GSLs of all *Zygomycetes* species examined were recovered in the neutral fraction but not in the acidic and zwitterionic fractions. In general, acidic GSLs are found in all fungal cells and have been reported to be inositolphosphate-containing sphingolipids such as glycosylinositolphosphoceramides (1–3). Surprisingly, they were not found in *Zygomycetes* species (Fig. 2*A*), and only neutral GSL (NGLs) being present. A TLC of the NGLs of all *Zygomycetes* species gave the same pattern but they were apparently different from those of other fungi (Fig. 2*B*). The NGLs of all *Zygomycetes* species comprised five components, which were identified to be ceramide mono-, di-, tri-, tetra-, and pentasaccharides (tentatively named as CMS, CDS, CTS, CTeS, and CPS, respectively). On the other hand, for the other fungi, only ceramide monosaccharide was found in the neutral fraction. Then these NSLs of *M. hiemalis* number 314 were further purified by silica gel column chromatography and confirmed by TLC, as shown in Fig. 2*C*. The yields were CMS, 20.8 mg; CDS, 6.3 mg; CTS, 16.1 mg; CTeS, 14.0 mg; and CPS, 2.3 mg, respectively. The purified NGLs were then analyzed by MALDI-TOF/MS and quantified by carbohydrate constituent analysis.

Ceramide Compositions of NGLs of M. hiemalis—The aliphatic components of the NGLs of *M. hiemalis* were determined by GLC and GLC/MS and summarized in Table I. The fatty acid composition of CMS comprised of C14h:0 (10.5%) and C16h:0 (89.5%) fatty acids, but CDS–CPS were composed of long chain fatty acids such as C24h:0, C25h:0, and C26h:0. The sphingoid components of CMS were 9-methyl-octadeca-4,8-sphingadienine (d19:2), eicosasphingenine (d20:1), and the unknown (18), whereas those of CDS–CPS were entirely 4-hydroxyoctadecasphinganine (phytosphingosine, t18:0). Because the ceramide compositions of CDS–CPS were entirely the same, these NGLs were supposed to be a series of intermediates of GSL biosynthesis. Such phytoceramides consisting of a 2-hydroxy fatty acid and phytosphingosine are generally found in fungal cells as the major aliphatic component of glycosylinositolphosphoceramides (1–3).

Structural Characterization of Oligosaccharides of NGLs—The sugar constituents of the purified NGLs were identified by GLC/MS, which revealed the presence of Glc in CMS and only Gal in all the other NGLs. MALDI-TOF/MS spectra of the NGLs showed the presence of pseudomolecular ions $[M+H]^+$ in

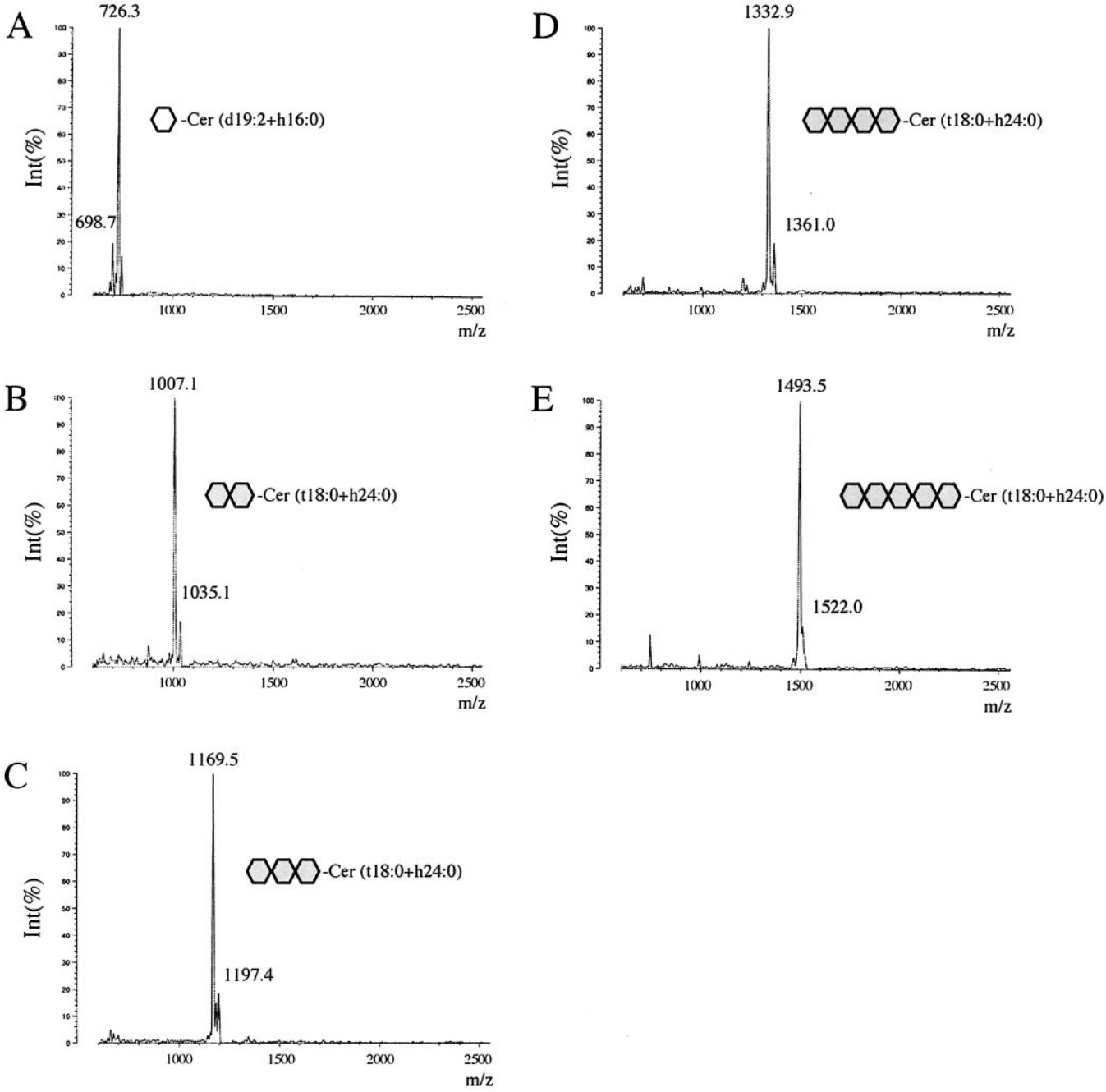


FIG. 3. **MALDI-TOF/MS spectra of purified GSLs.** Analyses were performed in the positive-ion linear mode. Pseudomolecular ions are given in average masses. The details are given in the text. The *open hexagon* is glucose and the *gray hexagons* are galactose. *A*, CMS; *B*, CDS; *C*, CTS; *D*, CTeS; and *E*, CPS.

the positive ion mode, as shown in Fig. 3. They had different pseudomolecular ions because of different ceramide species, which were in agreement with the mass values calculated from the proposed structures; the major $[M+H]^+$ ions of CMS at *m/z* 698.7 and 726.3 (Fig. 3*A*) coincided with the mass value of 1 mol each of Glc, fatty acid (2-hydroxy C14:0 or C16:0), and sphingoid (d19:2 or d20:1) (see Table I), those of CDS at *m/z* 1007.1 and 1035.1 (Fig. 3*B*) coincided with the mass value of 2 mol of Gal and phytoceramide (t18:0, and 2-hydroxy C24:0 and C26:0), those of CTS at *m/z* 1169.5 and 1197.4 (Fig. 3*C*) coincided with the mass value of 3 mol of Gal and phytoceramide (t18:0, and 2-hydroxy C24:0 and C26:0), those of CTeS at *m/z* 1332.9 and 1361.0 (Fig. 3*D*) coincided with the mass value of 4 mol of Gal and phytoceramide (t18:0, and 2-hydroxy C24:0 and C26:0), and those of CPS at *m/z* 1493.5 and 1522.0 (Fig. 3*E*) coincided with the mass value of 5 mol of Gal and phytoceramide (t18:0, and 2-hydroxy C24:0 and C26:0), respectively.

Subsequently, to determine the sugar linkages, the partially methylated alditol acetate derivatives of NGLs were analyzed by GLC and GLC/MS, and the results are shown in Fig. 4. The methylation analysis demonstrated the presence of one substituted Glc (1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl)glucitol, 1Glc) for CMS; on the other hand, one substituted Gal (1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl)galactitol, 1Gal) and 1,6 substituted Gal (1,5,6-tri-(*O*-acetyl)-2,3,4-tri-(*O*-methyl)galactitol, 1,6Gal) were detected from CDS–CPS. The peak area of 1,6Gal increased depending on the number of Gal residues; the ratio of 1,6Gal to each 1Gal ($n = 1.00$) in NGLs were 0.92 for

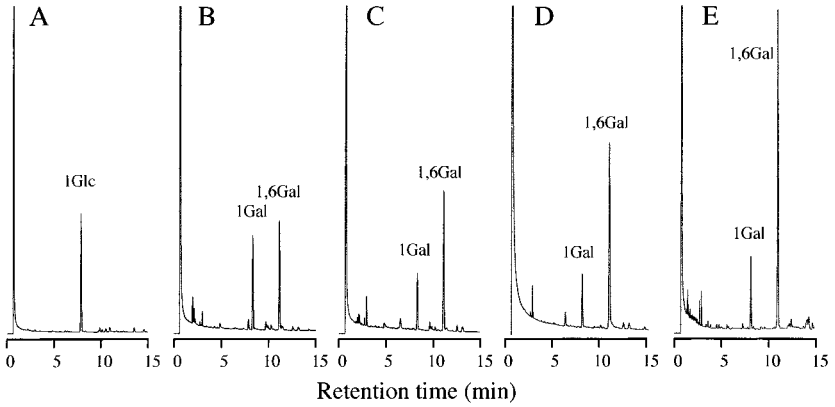


FIG. 4. **Gas chromatograms of partially methylated alditol acetates derived from purified GSLs for determination of glycosidic linkages.** Analyses were performed by GC under the conditions given under "Results." *A*, CMS; *B*, CDS; *C*, CTS; *D*, CTeS; *E*, CPS; *1Glc*, 1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl)glucitol; *1Gal*, 1,5-di-(*O*-acetyl)-2,3,4,6-tetra-(*O*-methyl)galactitol; and *1,6Gal*, 1,5,6-tri-(*O*-acetyl)-2,3,4,-tri-(*O*-methyl)galactitol.

CDS, 1.98 for CTS, 3.15 for CTeS, and 4.31 for CPS, respectively. This revealed that the Gal residues of CDS–CPS were linked to the C-6 position of their glycans.

Anomeric Configuration Analyses of Sugar Components of NGLs—For determination of the anomeric configurations of the sugar residues, CDS–CPS were subjected to proton NMR spectroscopy (Fig. 5). In the anomeric region of the spectrum of each glycolipid, the following anomeric proton resonances were observed: at 4.12 (J = 7.3 Hz) and 4.15 ppm (J = 7.3 Hz) demonstrating 2 mol of β-galactose for CDS (Fig. 5*A*), at 4.11 (J = 7.3 Hz), 4.16 (J = 7.3 Hz), and 4.69 ppm (J = 3.1 Hz) demonstrating 2 mol of β-galactose and 1 mol of α-galactose for CTS (Fig. 5*B*), at 4.12 (J = 7.3 Hz), 4.17 (J = 7.3 Hz), 4.70 (J = 3.0 Hz), and 4.72 ppm (J = 3.0 Hz) demonstrating 2 mol of β-galactose and 2 mol of α-galactose for CTeS (Fig. 3*C*), and at 4.12 (J = 7.3 Hz), 4.18 (J = 7.3 Hz), 4.71 (J = 3.1 Hz, 2H), and 4.72 ppm (J = 3.7 Hz) demonstrating 2 mol of β-galactose and 3 mol of α-galactose for CPS (Fig. 5*D*), respectively. Enzymatic hydrolysis of the above NGLs with α- and β-galactosidase also revealed the presence of α- and β-galactose residues. As a result, CDS was degraded to ceramide monosaccharide (galactosylceramide (GalCer)) by β-galactosidase, and CTS, CTeS, and CPS were also hydrolyzed to GalCer through the sequential actions of α- and β-galactosidase (data not shown).

From these findings, the following structures for NGLs were suggested, Glcβ1-1Cer for CMS, Galβ1-6Galβ1-1Cer for CDS, Galα1-6Galβ1-6Galβ1-1Cer for CTS, Galα1-6Galα1-6Galβ1-6Galβ1-1Cer for CTeS, and Galα1-6Galα1-6Galα1-6Galβ1-6Galβ1-1Cer for CPS, respectively. This is the first finding of novel glycan chains with Galβ1-6Gal and/or Galα1-6Gal in glycolipids from fungi.

Analyses of GSL Synthesis—To investigate the biosynthetic pathway for GSLs, fungal cells were incubated with [^{14}C]glucose, and then lipids extracted from mycelia were analyzed by TLC (Fig. 6). Our preliminary results showed that CMS–CPS were found to be labeled by ^{14}C on incubation within 1 h (Fig. 6*A*). However, GalCer was not detected on TLC even after incubation for 12 h (Fig. 6*B*). We supposed that the metabolic process yielding a digalactosylceramide from a phytoceramide might be rapid.

DISCUSSION

Sphingolipids have been established to be essential components of eukaryotic cells, where they are predominantly found on the plasma membrane. Inhibitors of specific steps of sphingolipid biosynthesis have proven useful for understanding sphingolipid functions, and some of them have been used as fungicidal agents. One of the more useful agents for blocking sphingolipid synthesis in fungi is AbA, which inhibits IPC synthase in fungi cells. As all fungi and plants seem to synthesize IPC as an intermediate of the biosynthetic pathway for GSLs (Scheme 1), AbA exhibits strong fungicidal activity

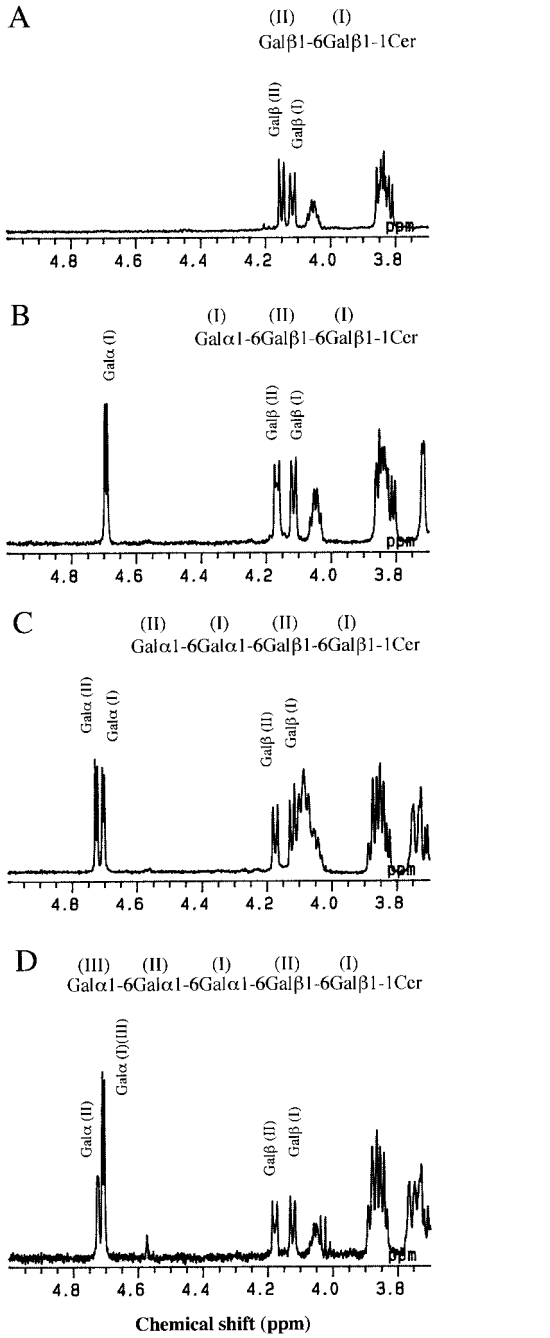


FIG. 5. **^{1}H NMR spectra of purified GSLs.** Anomeric proton regions of NGLs are shown, *A*, CDS; *B*, CTS; *C*, CTeS and *D*, CPS. The details are given under "Results."

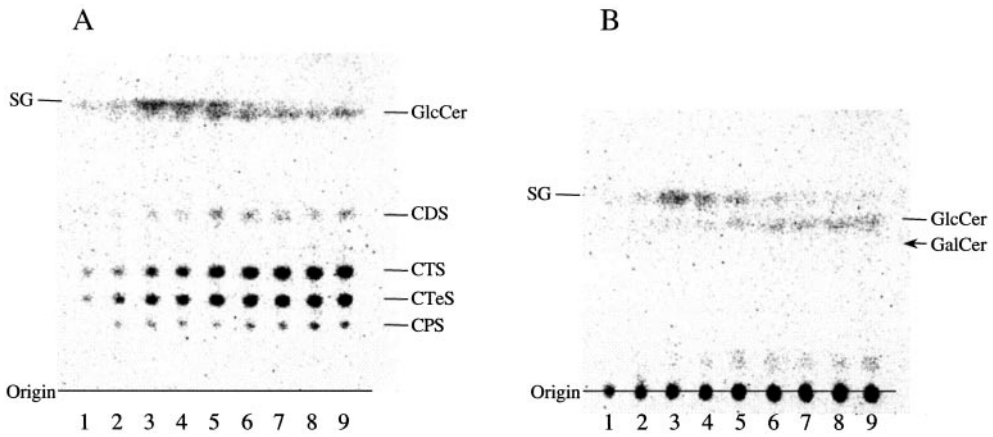
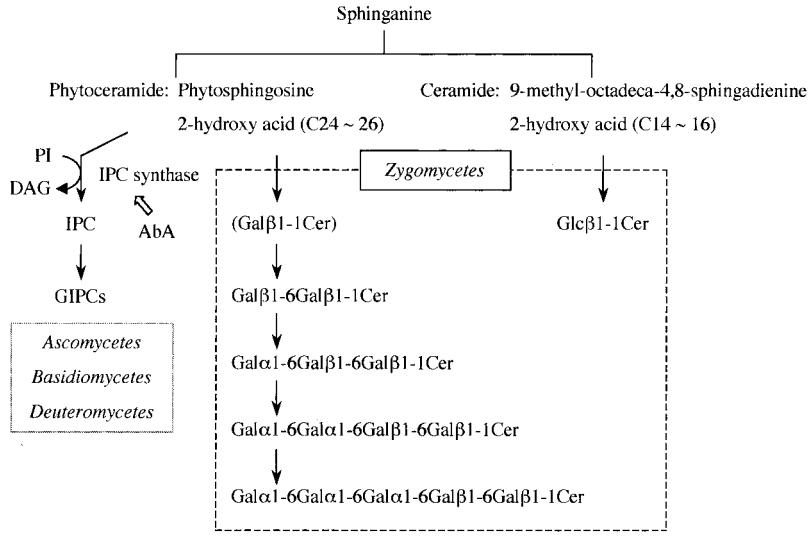


FIG. 6. **TLC analysis of sphingolipid biosynthesis of *M. hiemalis*.** Extracted lipids of *M. hiemalis* mycelia were analyzed on TLC with different solvent systems of chloroform-methanol-water (*A*, 60:35:8, by volume; *B*, 80:20:1, by volume) and visualized with an imaging analyzer. *Lanes 1–9*, 5-min incubation, 10-min incubation, 15-min incubation, 30-min incubation, 1-h incubation, 2-h incubation, 3-h incubation, 6-h incubation, and 12-h incubation, respectively. *Arrow* indicates of the corresponding position of GalCer with phytoceramide.



SCHEME 1. **Putative biosynthetic pathway for GSLs in *Zygomycetes* and other fungi.**

against many pathogenic fungi. However, we found that this agent was not entirely effective for the *Zygomycetes* species examined. We also found that *Zygomycetes* species did not have IPC but did have a novel family of neutral GSLs that are not found in other fungi. These GSLs isolated from *M. hiemalis*, which is a typical *Zygomycetes* species, were determined as Galβ1-6Galβ1-1Cer (CDS), Galα1-6Galβ1-6Galβ1-1Cer (CTS), Galα1-6Galα1-6Galβ1-6Galβ1-1Cer (CTeS), and Galα1-6Galα1-6Galα1-6Galβ1-6Galβ1-1Cer (CPS). Their aliphatic components were the same phytoceramides consisting of phytosphingosine and C24–C26 2-hydroxy fatty acids, which were bound through amide linkages. These ceramide moieties substantially differ from that of glucosylceramide (CMS) (Table I). The only glucosylceramide detected was ceramide monosaccharide, *i.e.* we did not identify a galactosylceramide with phytoceramide, which is supposed to be the precursor of a series of galactose-containing glycosphingolipids (CDS–CPS). It seemed that the enzymatic reaction to form CDS from galactosylceramide might proceed rapidly. In fact, there are some preliminary data about the existence of a very little amount of GalCer, which was found by means of sensible method using borated thin layer plate (data not shown). However, we could not know whether this GalCer is an intermediate of metabolic process of these novel NGLs or a degraded product from digalactosylceramide produced. Moreover, it could be speculated that digalactosylceramide is directly formed from phytoceramide by the addition of disaccharide from nucleotide diphosphate sugars. Such investigation is carrying out at present.

Galactooligosaccharides with similar structures in novel GSLs have been reported for the leech, *Hirudo nipponia* (*i.e.* Galα1-6Galα1-6Galβ1-1Cer) (19), the earthworm, *Pheretima* sp. (*i.e.* Galβ1-6Galβ1-6Galβ1-6Galβ1-1Cer) (20), and a parasitic cestode, *Echinococcus multilocularis* (*i.e.* Fucα1-3Galβ1-6Gal-Cer) (21). But this is the first finding of GSLs with the Galα1-6Galβ1-6Galβ sequence in any organism. Furthermore, there have been reports that humans and closely related mammals possess natural anti-α galactosyl (Galα1-3Gal) antibodies (22), which also strongly react with the epitopes of melibiose (Galα1-6Glc) and Galα1-6Gal (23). It has also been reported that sera of alveolar hydatid disease patients recognized the epitope of Galβ1-6Gal residues in GSLs of *E. multilocularis* (21), and the carbohydrate residues of its GSLs with Galβ1-6Gal sequences were inhibitors of human peripheral blood mononuclear cell proliferation (24). These findings suggest that the GSLs of *Zygomycetes* also might be immunogenic in humans.

The biosynthesis of GSLs in *Zygomycetes* species seemed to be different from that described for other fungal species. In most fungi, sphingolipid synthesis begins in the endoplasmic reticulum, where phytoceramide is converted to IPC before transport to the Golgi apparatus for further glycosylation (1–3). Our results indicated that two independent ceramide groups

existed in the *Zygomycetes* species, and the fungal cells synthesized neutral GSLs of both glucosylceramide and galactose-containing glycosphingolipids from different ceramide pools, because the ceramide structures of the two types of GSLs were significantly different. Although glycosylinositolphosphoceramides have been detected in many fungi as important constituents of cells, we could not obtain evidence of their presence in *Zygomycetes* species, nor could we detect inositolphosphate-containing sphingolipids. Surprisingly, *Zygomycetes* species showed strong resistance to AbA, and the above fact seems to be the reason why *Zygomycetes* species are resistant to AbA.

The roles of fungi in infections have been considered to be of lesser important, because only 5% of fungi have been found to be infectious. It has already been reported that aspergillosis (55%) is the most common invasive fungal disease, followed by mucormycosis (zygomycosis) (15%), fusariosis (15%), and acremoniosis (10%) (25). The pathogenic fungi responsible for these disease were not considered previously to be important human pathogens but are widely present in soil, plants, and elsewhere in the environment. *Aspergillu*s spp. and *Mucor* spp. have been shown recently to be human pathogens (26). In particular, *Mucor* spp. cause many diseases, and other members of the Mucorales family act as opportunistic human pathogens (27). Mucorales infections are observed in a variety of disease states that cause immunosuppression associated with leukemia (28), aplastic anemia (29), organ or bone marrow transplantation (28), renal disease (30), and asthma (31). Therefore, new effective drugs for mucormycosis are required immediately. In this point, an inhibitor of the synthesis of galactose-containing GSLs might be useful. Although the functional roles of these GSLs have not been elucidated, our finding may facilitate the development of new antifungal agents for Mucorales.

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