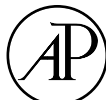
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Archives of Biochemistry and Biophysics 405 (2002) 205–213

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Glycosphingolipids from *Magnaporthe grisea* cells: expression of a ceramide dihexoside presenting phytosphingosine as the long-chain base

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Received 7 February 2002, and in revised form 1 July 2002

Abstract

Magnaporthe grisea is a fungal pathogen that infects rice leaves and causes rice blast, a devastating crop disease. *M. grisea* produces active elicitors of the hypersensitive response in rice that were previously identified as ceramide monohexosides (CMHs). Using several chromatographic approaches, mass spectrometry, and nuclear magnetic resonance, we identified ceramide mono- and dihexosides (CDH) in purified lipid extracts from *M. grisea* cells. As described by other authors, CMH consists of a ceramide moiety containing 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecenoic or 2-hydroxyhexadecenoic acids and a carbohydrate segment consisting of one residue of glucose. CDHs, however, contain β-galactose (1 → 4)-linked to β-glucose as sugar units and phytosphingosine as the long-chain base, bound to a C24 α-hydroxylated fatty acid. To our knowledge, this is the first report on the occurrence of CDH in a fungal species and illustrates the existence of an alternative path of ceramide glycosylation in fungal cells. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: *Magnaporthe grisea*; Glucosylceramide; Phytosphingosine; Ceramide dihexoside

The filamentous ascomycete *Magnaporthe grisea* is a fungal pathogen that infects rice leaves and causes rice blast, one of the most economically devastating crop diseases worldwide [1]. This disease is disseminated by water-splashed conidia, which adhere to the host leaf, germinate, and differentiate, followed by fungal penetration of plant surfaces [2]. This process results in the generation of fungal leaf spots, which are typical of rice blast [3].

M. grisea produces active elicitors of the hypersensitive response in rice [4,5] that were identified as ceramide monohexosides (CMHs,[3] cerebrosides), which are glycosphingolipids consisting of a sugar residue attached to a ceramide moiety. Treatment of rice leaves with *M. grisea* CMHs induced the accumulation of antimicrobial compounds, cell death, and expression of pathogenesis-related proteins in rice leaves and effectively protected rice plants against fungal infection [4,5]. In other models, fungal CMHs functioned as inductors of fruiting body formation in *Schizophyllum commune* [6] and, as recently demonstrated by our group, are crucial antigenic com-

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[3] *Abbreviations used:* CMH, ceramide monohexoside; GlcCer, glucosylceramide; GSC, glucosylceramide synthase; CHD, ceramide dihexoside; FAB, fast atom bombardment; IPC, inositolphosphorylceramide; ER, endoplasmic reticulum.

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PII: S0003-9861(02)00365-X

pounds involved with the growth of the human pathogen *Cryptococcus neoformans* [7] and differentiation of *Candida albicans* and *Pseudallescheria boydii* [8].

Although a few reports suggesting the function of glycosphingolipids in fungal cells are available, significant progress has been made on the field of their biosynthesis and structural elucidation. CMHs from the fungal species *M. grisea* [5], *C. neoformans* [7,9], *P. boydii* [8], *H. capsulatum* [10], *P. brasiliensis* [11], *Aspergillus fumigatus* [11,12], *Aspergillus niger* [9], *Fusarium* sp. [13], *Sporothrix schenckii* [14], *Fonsecaea pedrosoi* [15], *C. albicans* [16], *Pichia pastoris* [17], and *Rhynchosporium secalis* [17] were characterized in detail, all of them presenting a ceramide moiety containing 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acids and a carbohydrate segment consisting of one residue of glucose or galactose.

Fungal cells are believed to construct two different pools of ceramides to be used for the synthesis of different sphingolipids [18]. Ceramide backbones with C16 or C18 fatty acids linked to a 4,8-diene-9-methyl-sphingobase, which were widely identified in several fungal species [7–16], are exclusively precursors for glucosylceramide (GlcCer) synthesis. Ceramide backbones with relatively long-chain C24 and C26 fatty acids bound to phytosphingosine are thought to be restricted to the synthesis of the inositol-containing phosphosphingolipids [17–21]. In a recent investigation, however, Leipelt and colleagues [22] have identified and characterized novel glucosylceramide synthases (GCS) from plants, animals, and fungi, including *M. grisea*. Genetic studies revealed that the expression of the GCS from *M. grisea* in a *P. pastoris* GCS null mutant resulted in the biosynthesis of GlcCer with the usual ceramide moieties comprising C16 and C18 fatty acids in an amidic linkage with 9-methyl-4,8-sphingadienine, but also in the occurrence of GlcCer with phytosphingosine and mainly long-chain (C26) α-hydroxy fatty acids in amide linkage. These results indicated that GCS could accept both classes of ceramide as substrates to form GlcCer. These data also supported a previous report by Lester and coworkers [23], which described the occurrence of a ceramide tetrahexoside consisting of (Gal_3-Glc)-*N*-hydroxytetracosonyl-hydroxysphinganine in *Neurospora crassa*.

We now report on the purification and structural elucidation of a ceramide dihexoside (CDH) from *M. grisea*. Remarkably, the ceramide moiety of this glycolipid presented phytosphingosine as the long-chain base bound to a C24 α-hydroxylated fatty acid. To our knowledge, this is the first report on the identification of a CDH from fungal cells. Additionally, the description of the natural occurrence of this class of ceramide in a non-inositol-containing fungal glycolipid demonstrates the existence of alternative steps of ceramide glycosylation in fungal cells.

Materials and methods

Microorganisms

M. grisea strain $P_2/86$ was obtained from Dr. Vanda Magalhães (Instituto Agronômico de Campinas, SP, Brazil). Mycelial cells were cultivated at room temperature in Sabouraud's modified liquid medium, containing (g/liter) 20 glucose, 10 peptone, and 5 of yeast extract. After 7 days of growth, fungal cells were obtained by filtration, washed three times with cold water, and stored at −20 °C.

Lipid extraction

M. grisea cells (160 g, wet weight) were successively extracted at 28 °C with chloroform/methanol 2:1 and 1:2 (v/v). Extracts were combined and dried and the crude lipid extract was partitioned according to Folch and coworkers [24]. The lipids recovered from the Folch et al. lower phase were fractionated on a silica gel column eluted with chloroform, acetone, and then methanol. The glycolipid fraction eluted with methanol was purified by further silica gel column chromatography. This column was eluted sequentially with the following mixtures: chloroform/methanol (95:5, 9:1, 8:2, 7:3, and 1:1, v/v) and finally methanol. Fractions of 5 mL were collected and analyzed by high-performance thin-layer chromatography (HPTLC), developed with chloroform/methanol/water 65:25:4 (v/v). The spots were visualized with iodine and by spraying with orcinol/H_2SO_4. A purified glycosphingolipid was obtained in the chloroform/methanol 9:1 (v/v) fraction, which was further chemically characterized. The chloroform/methanol 7:3 (v/v) fraction was further purified by chromatography on Iatrobeads RS 2060 (Macherey & Nagel, Düren, Germany), using the same elution system, yielding a partially purified glycosphingolipid fraction. Final purification was attained after passage through a second Iatrobeads column eluted with chloroform/methanol mixtures (9:1, 85:15, 8:2, 75:25, 7:3, and 6:4, v/v) and finally methanol. The 8:2 chloroform/methanol fraction contained the finally purified glycolipid, as visualized by HPTLC.

Sugar analysis

The purified glycosphingolipids were hydrolyzed with 3 M trifluoroacetic acid at 100 °C for 3 h, and the resulting monosaccharides were characterized by HPTLC and quantified by gas chromatography (GC) as alditol acetate derivatives [25] using an OV-225 fused silica capillary column (30 m × 0.25 mm i.d.), with temperatures programmed from 50 to 220 °C at 50 °C/min.

Structural determination

Fatty acid methyl esters were prepared by acid methanolysis using 1 mL of toluene:methanol (1:1 v/v) containing 2.5% concentrated sulfuric acid (overnight at 70 °C). Samples were diluted in 0.5 mL of deionized water and extracted twice with hexane:chloroform (4:1 v/v). The combined extracts were dried by vacuum centrifugation and trimethylsilylated by treatment with 100 μL of *bis*-(trimethylsilyl)trifluoroacetamide/pyridine(1:1 v/v; 30 min at 60 °C). The reagent was removed by vacuum centrifugation, and the samples were dissolved in hexane for gas chromatography–mass spectrometry (GC–MS). GC–MS was performed with a Kratos MS80 RFA spectrometer (Kratos, Manchester, UK) directly interfaced to a Carlo Erba 5160 chromatograph. Helium (0.7 mL/min) was used as the carrier gas, and samples were introduced by splitless injection (splitless time 30 s) into a BPX-5 fused silica column (25 m × 0.2 mm; SGE, Milton Keynes, UK). The injector and interface oven were maintained at 250 °C. One minute after injection, the column oven temperature was programmed from 60 to 200 °C at 40 °C/min, then at a rate of 3 °C/min to 230 °C, and a final 8 °C ramp to 265 °C with a hold for 10 min. Electron ionization spectra were recorded at an ionization energy of 70 eV, a trap current of 100 μA, and a source temperature of 220 °C. Chemical ionization spectra were obtained using isobutane as the reagent gas with an emission current of 250 mA. The magnet was scanned at 0.6 s/decade over the range 550–40. Final structural elucidation was obtained after fast atom bombardment (FAB) analysis, whose spectra were obtained with a Kratos MS80 spectrometer, fitted with an Ion Tech saddle-field atom gun supplied with high-purity xenon gas. Spectra of the underivatized glycolipids were recorded in both the positive and the negative ion modes using either 3-nitrobenzyl alcohol, or a 1:1 (v/v) mixture of glycerol and dithiothreitol–dithioerythritol (5:1,w/w) as liquid matrices. Approximately 10 μL of each sample was loaded onto the FAB probe. The instrument was operated at an accelerating voltage of 4 kV and a resolution of 1000 (10% valley), and the magnet was scanned at 10 s/decade of mass over the range 2000–200. Peracetyl derivatives (1–5 μg) were analyzed from 3-nitrobenzyl alcohol matrix. Sugar and ceramide-derived fragment ions are described using the nomenclature introduced by Domon and Costello [26,27].

^{1}H-nuclear magnetic resonance spectroscopy (^{1}H NMR)

A sample of CDH was dissolved in 0.5 mL $CDCL_3$–CD_3OD (2:1 v/v) (99.9%; Merck, Darmstadt, Germany). The ^{1}H-NMR spectrum was acquired at 400 MHz using a DRX-400 Bruker Avance spectrometer, at a probe temperature of 303 K and a sweep width of 20 ppm. Suppression of the residual HOD resonance was obtained by presaturation pulse during the preparatory delay period.

Results and discussion

Extraction, purification, and structural elucidation of glycosphingolipids from M. grisea

Analysis of lipid extracts from *M. grisea* revealed the occurrence of orcinol-reactive bands with R_fs corresponding to mono- (CMH) and dihexosylceramides (CDH) (Fig. 1). These molecules were purified and their structures elucidated.

FAB-MS analysis of the *M. grisea* CMH indicated the existence of two components that differed in their fatty acid compositions. In the negative ion spectrum of native CMH (not shown) deprotonated molecules were observed at m/z 752 and 724. Elimination of hexose results in the doublet of Yo ions at m/z 590 and 562. The corresponding positive ion spectrum revealed $[M + Na^+]$ signals at m/z 776 and 748 (Fig. 2, inset) and Y'o fragments at m/z 574 and 546 (not shown). These findings were confirmed by FAB-MS of the peracetylated glycolipid shown in Fig. 2. $[M + Na^+]$ signals were observed at m/z 1028 and 1000, indicating addition of six acetyl groups to the mass of the underivatized glycolipids, which is consistent with hydroxy acid-containing monohexosylceramides. Additionally, $[M + H^+]$-60 fragments at m/z 946 and 918 were present. The ceramide moiety was represented by peaks at m/z 658 and 630 (Yo^+Na^+), 598 and 570 ($Yo–HOAc^+Na^+$), and 538 and 510 ($Yo–2HOAc^+Na^+$). The B1 carbenium ion at m/z 331 is indicative of a terminal hexose, and this assignment is supported by the presence of the expected secondary fragments at m/z 229, 169, and 109 (fragments at m/z 169 and 109 not shown in Fig. 2). The low mass end of the spectrum was dominated by an abundant W'' ion at m/z 276 which is diagnostic of a C19 sphingadienine [13] originated from the long-chain base. The hexose was identified as glucose by GC–MS (data not shown). Taken together, these data and previous reports by other authors [4,5] lead to the conclusion that CMHs consisting of *N*-2′-hydroxyoctadecenoyl-1-*O*-β-D-glucopyranosyl-9-methyl -4,8-sphingadienine and *N*-2′-hydroxyhexadecenoyl -1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine are components of *M. grisea* cells.

In the negative ion FAB spectrum of the native CDH, a molecular ion $[M - H]^-$ was observed at m/z 1006 (Fig. 3, inset A). The positive ion FAB spectrum of the peracetylated sample (Fig. 3) shows $[M + Na^+]$ signals at m/z 1450, indicating addition of 10 acetyl groups to the mass of the underivatized glycolipid. Ions at m/z 331 and 619, the latter derived from the hexose–hexose residue, were detected. In the deuteroacetylated spectrum

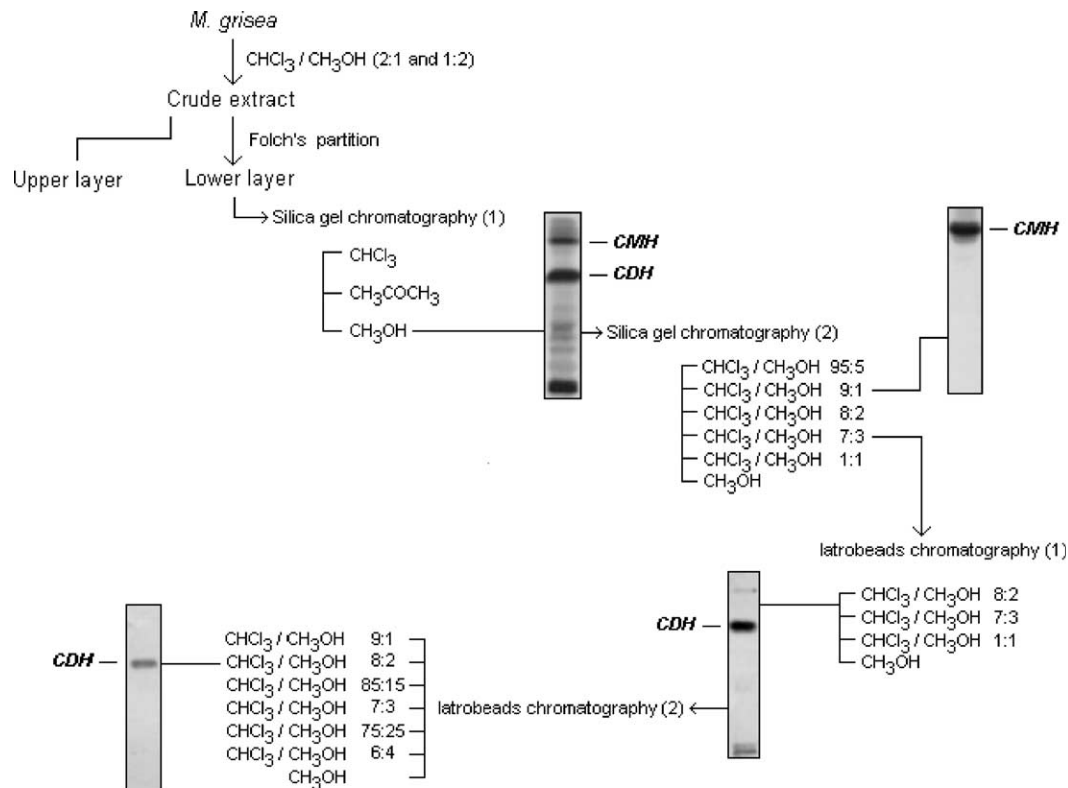

Fig. 1. Overview of the strategy used for purification of CMH and CDH from *M. grisea* mycelia. Purified or partially purified extracts were resolved by HPTLC and visualized by reaction with orcinol–H_2SO_4.

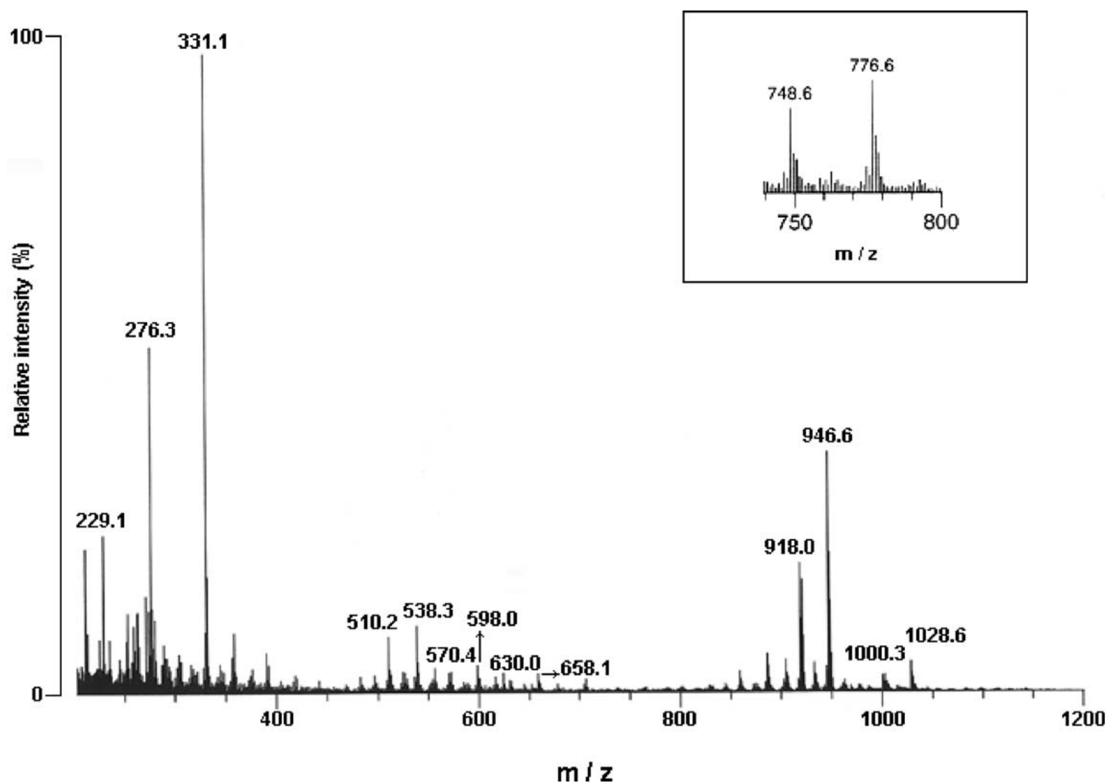

Fig. 2. FAB-MS analysis of peracetylated CMH from *M. grisea*. Inset: positive ion spectrum (molecular ion region) of the underivatized CMH. See Results and discussion for interpretation of fragments.

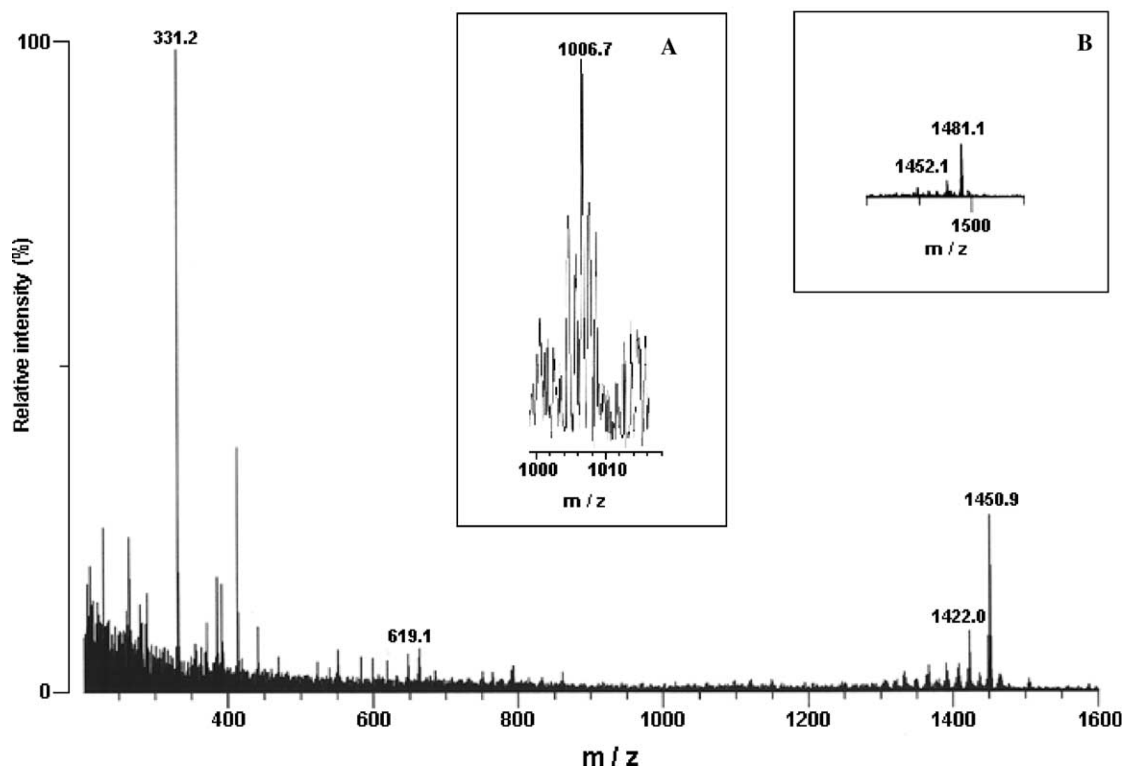

Fig. 3. Positive ion FAB spectrum of peracetylated CDH from *M. grisea*. Insets show the molecular ion regions of FAB spectra of underivatized (A) and deuteroacetylated (B) CDH. See Results and discussion for interpretation of fragments.

(Fig. 3, inset B) an m/z value of 1481 is observed, which confirms that at least 10 acetyl groups had been added. All this is consistent with a CDH presenting phytosphingosine and a C24 α-hydroxylated fatty acid, which was confirmed by GC–MS analysis. Analysis of the methanolyzed and trimethylsilylated CDH from *M. grisea* revealed two peaks (Fig. 4). Its EI mass spectrum showed weak molecular ions at m/z 470 and 442, others at 455 (M-15) and 427 (M-15), and base peaks at 411 (M-59) and 383 (M-59). The base peak fragments, originating from facile cleavage between the carboxyl group and the carbon 2, are characteristic of 2-hydroxy fatty acid methyl esters [28], thus identifying the compounds as 2-hydroxy tetracosanoic and 2-hydroxy docosanoic acids. The latter accounts for the minor peak at m/z 1422 in the acetylated (Fig. 3) and m/z 1452 in the perdeuterocetylated spectra, respectively (Fig. 3, inset B).

GC–MS analysis of alditol acetate derivatives revealed the presence of galactose and glucose in a 1:1 ratio (not shown). The exact linkage between these sugars was revealed by ^{1}H NMR analysis. H-1 signals at 4.29 ppm ($J = 7.5$ Hz) and 4.36 ppm ($J = 7.5$ Hz) (Fig. 5) were consistent with the occurrence of a βGal*p*-(1 → 4)-β-Glc*p*-group as the glycan sequence [29]. This observation was compatible with that obtained by enzymatic degradations of the CDH. Treatment of CDH with a bovine β-galactosidase resulted in its total conversion to products comigrating with CMH in HPTLC analysis (data not shown), while an extensive digestion with α-galactosidase did not yield bands corresponding to CMH.

Glycosylation of ceramides in M. grisea

The structural identification of a CDH with phytosphingosine and a C24 α-hydroxy fatty acid in an amide linkage suggests that biosynthesis of glycosphingolipids in *M. grisea* varies from that described for other species. In *Saccharomyces cerevisiae*, sphingolipid synthesis begins in the endoplasmic reticulum, where phytosphingosine-containing ceramide is converted to inositolphosphorylceramide (IPC) before transport to the Golgi apparatus for further glycosylation [18]. On the contrary, in mammalian cells, unmodified ceramide is transported from the endoplasmic reticulum to the Golgi apparatus, where it is converted to complex glycosphingolipids [30]. Our results indicate that, in *M. grisea*, two independent groups of ceramides are similarly glycosylated by its enzymes, since fungal cells synthesized both mono- and dihexosyl ceramides containing clearly different ceramide moieties.

In another model, the expression of GCS in a *P. pastoris* GCS null mutant resulted in the synthesis of the

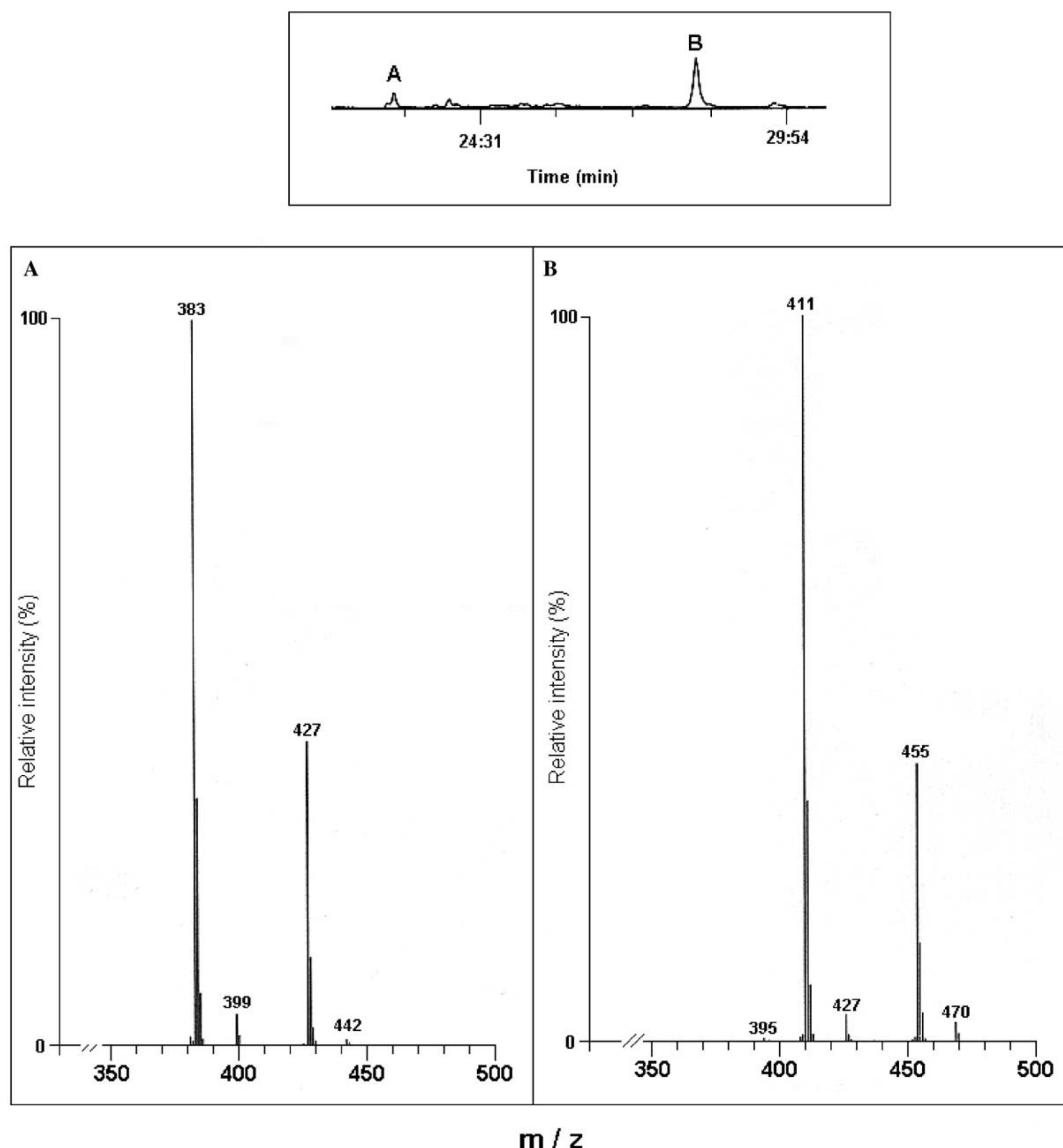

Fig. 4. GC–MS analysis of the methanolyzed and trimethylsilylated CDH from *M. grisea*. Chromatographic separation of fatty acids is shown at the top. Mass spectrometric analyses of peaks A and B, with retention times corresponding to C_{22} and C_{24} fatty acids, respectively, are shown at the bottom. See Results and discussion for interpretation of fragments.

usual GlcCer ceramide moieties comprising C16 and C18 fatty acids in an amidic linkage with 9-methyl-4,8-sphingadienine [22]. Remarkably, GlcCer with phytosphingosine and mainly long-chain α-hydroxy fatty acids was also detected in transformed cells [22]. This observation was possibly attributed to a disturbance of the protein targeting machinery by flooding the system with GCS molecules, resulting in overloading of the Golgi/ER retrieval capacity. In summary, these results indicate that GCSs are able to accept very long chain ceramides as substrates under manipulated conditions, in a modified cellular system. However, although this class of ceramides is naturally found in fungal cells, it is normally used for synthesis of inositol-containing sphingolipids, but not for GlcCer [17–21]. Therefore, as concluded by Leipelt and co-workers [22], the differences described for the ceramide backbones of inositol-containing sphingolipids and GlcCer in several fungal species may originate from compartmentalization of both kinds of ceramide, GCS or IPC synthases, rather than represent the occurrence of diverse levels of enzyme specificity.

We now demonstrate by structural determinations that the *M. grisea* enzymatic apparatus is able to glycosylate both phytosphingosine- and 9-methyl-

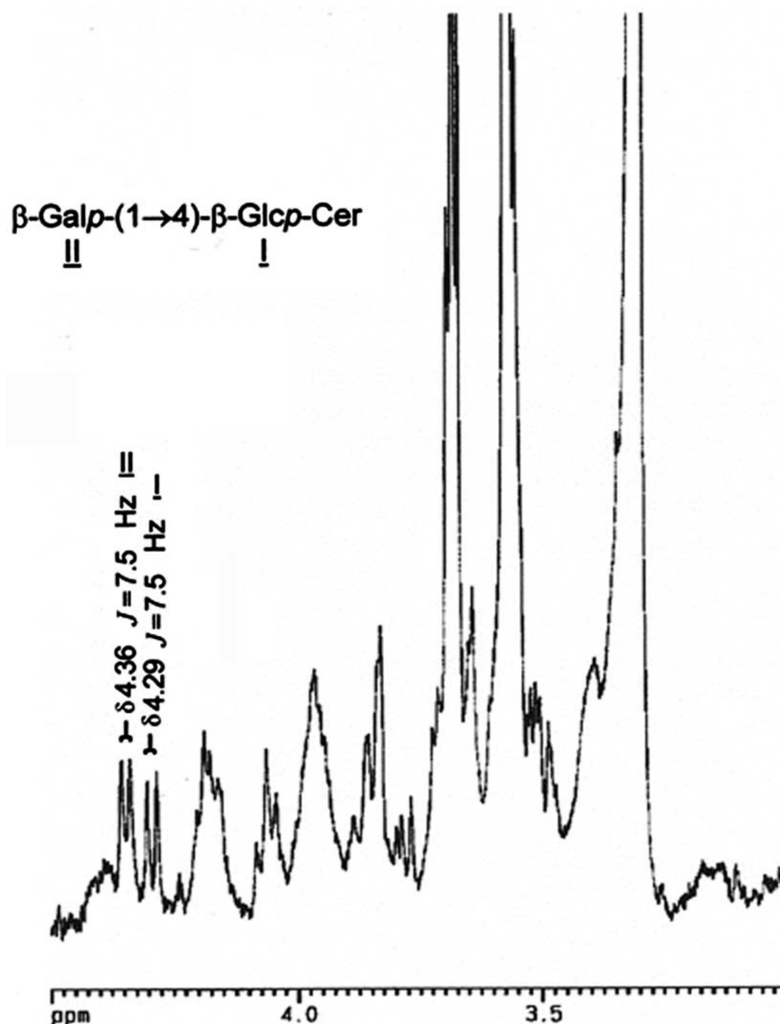

Fig. 5. Carbohydrate region of ^{1}H NMR spectrum, obtained from the CDH of *M. grisea*, including its H-1 region.

sphingadienine-containing ceramides under normal growth conditions, which is in accordance with the results of Leipelt and co-workers [22], with regard to GCS specificity. This observation therefore suggests that, in *M. grisea,* long-chain ceramides should also serve as substrates for the synthesis of GlcCer, which would be further glycosylated by galactosyl transferases to finally form more complex glycosphingolipids. These possibilities are supported by the results of Lester and co-workers [23], which described the occurrence of a ceramide tetrahexoside consisting of (Gal_3Glc)-*N*-hydroxytetracosonyl-hydroxysphinganine in *N. crassa*. Taken together, these observations raise the possibility that, contrarily to what has been proposed for several species of fungi [22], separation of ceramide pools for glycosphingolipid biosynthesis may not occur in fungal cells such as *M. grisea*. Our results and previous ones [23] therefore suggest the occurrence of an alternative path of ceramide glycosylation in fungal cells. This hypothesis is summarized in Fig. 6.

Although CMHs seem to have an important role in the interaction of *M. grisea* with the plant host [4,5], the functional relevance of CDHs remains to be established. However, complex sphingolipids and their biosynthesis are currently considered potential targets for new antifungal drugs. For instance, a gene encoding an enzyme involved in sphingolipid biosynthesis determines the sensitivity of *S. cerevisiae* to an antifungal plant defensin [31]. Inhibition of sphingolipid synthesis can also affect pathogenesis, as described for the human pathogen *C. neoformans*. In this fungus, down-regulation of *IPC1*, the gene encoding inositolphosphorylceramide synthase, significantly lowers the expression of virulence factors, impairing *C. neoformans* pathogenicity in an animal model, decreasing intracellular growth, and conferring a defect on in vitro growth at low pH [32]. Accordingly, an inhibitor of inositolphosphorylceramide synthase

Fig. 6. Glycosylation of ceramide-containing fungal lipids, modified from [22]. Steps of hydroxylation, desaturation, or methylation (not shown) would follow biosynthesis of ceramide backbones for further addition of sugar units. In *M. grisea*, we propose that the two classes of ceramides would be used by GCS, under standard cellular conditions, as substrates to form GlcCer. Phytosphingosine-containing GlcCer would be sequentially glycosylated with the addition of a galactose residue, catalyzed by a still uncharacterized galactosyl transferase.

(rustimicin) has a potent fungicidal activity against clinically important human pathogens that is correlated with sphingolipid inhibition [33]. The structural determination of novel fungal sphingolipids and consequent understanding of steps of lipid biosynthesis can therefore reveal the existence of new and efficient targets for the inhibition of fungal growth.

Acknowledgments

We thank Dr. Lothar Bergter for help with NMR analysis and Maria de Fatima F. Soares for technical assistance. This work was supported by Financiadora de Estudos e Projetos (FINEP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq),

Fundação de Amparo a Pesquisa no Estado do Rio de Janeiro (FAPERJ), and Programa de Apoio a Núcleos de Excelência (PRONEX-Carboidratos).

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