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Novel glycoinositolphosphosphingolipids, basidiolipids, from *Agaricus*

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From the edible mushroom, the basidiomycetes *Agaricus bisporus* and *Agaricus campestris*, a novel carbohydrate-homologous series of four glyco-inositol-phospho-sphingolipids, designated basidiolipids, was isolated and the constituents purified. The chemical structures of the basidiolipids were elucidated to be: Man*p*β1-2inositol1-phospho-ceramide, Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide, Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide and Gal*p*α-6Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide. All four glycolipids contained a ceramide which was composed of phytosphingosine and predominantly α-hydroxy-behenic and α-hydroxy-lignoceric acid.

Keywords: *Agaricus*; basidiolipids; glyco-inositol-phospho-ceramides; glycolipids; sphingolipids.

Two groups of glycosphingolipids (GSL) exist that are distinguished from one another by the relation of their carbohydrate to the ceramide moiety. Classical GSL have their sugar portion linked directly to the ceramide by a glycoside. In the second group, the glyco-inositol-phospho-sphingolipids (GIPSL), carbohydrate is coupled to the lipophilic portion of the molecule via an intermittent inositol phosphate. The occurrence of the latter group of sphingolipids has typically been detected in plants, fungi, yeast, protozoans and more recently in worms (Aschelminthes) [1] (for review, see references [2,3]). The occurrence of GIPSL could now also be recognized in higher fungi, the basidiomycetes. In search of agents that might display adjuvant immune activities, a crude aqueous extract of the edible mushroom, the basidiomycete *Agaricus bisporus*, as well as, *Agaricus campestris*, was observed to be strongly positive in animals for immune adjuvancy (data to be published elsewhere). In the process of identifying the immunologically relevant substances, four carbohydrate-containing components were isolated from the mushroom and purified. All four substances could be characterized as GIPSL and their chemical structures elucidated. The four compounds, designated as a group 'basidiolipids', lipids of basidiomycetes, belong to a homologous series with a ceramide composed of phytosphingosine and α-hydroxy-C_{22}- and C_{24}-fatty acids: Man*p*β-2inositol1-phospho-ceramide, Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide, Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide and Gal*p*α-6Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2inositol1-phospho-ceramide.

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Abbreviations: Bl, basidiolipids (basiolipids treated with exoglycosidases were designated, e.g. Bl-2/αGal, Bl-2 de-α-galactosylated with α-galactosidase, etc.; GIPSL, glyco-inositol-phospho-sphingolipids; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry

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MATERIALS AND METHODS

Cultivated mushroom, *A. bisporus*, was purchased from a local farm (Schlüchtern, Germany). Naturally grown mushroom, *A. campestris*, was collected from a regional lawn.

All solvents used were of analytical grade. DEAE-Sephadex A-25 was from Pharmacia, Uppsala, Sweden. Silicagel Si 60 and Dowex H^+/OH^- column material as well as TLC plates were from Merck, Darmstadt, Germany. Silicagel reversed-phase C-18 bulk material was purchased from Waters, Milford, MA, USA. All enzymes, taurodeoxycholate and d_6-dimethyl sulfoxide were from Sigma, Munich, Germany.

Isolation and purification of basidiolipids

For extraction of glycolipids with organic solvents, a prior lyophilization step was required, because of the high water content of the mushrooms. To save drying of the material, an extraction procedure using water was therefore established. For comparison, glycolipid extraction with organic solvents was also performed.

Water extraction procedure. Fresh mushrooms (2 kg) were mashed with a mixer after the stalks had been removed. To this slurry, 4 L of distilled water was added, and the mixture was stirred for 1 h at 4 °C. After centrifugation at 4000 r.p.m. at 4 °C for 20 min, the supernatants were collected, and the water removed by *in vacuo* rotary evaporation. The crude extract was then redissolved in 200 mL water and dialyzed for 3 days in a dialysis membrane (diameter = 5 cm) of 12 kDa MWCO against several changes of distilled water. The dialyzed material was again evaporated to dryness. Subsequently, the dry extract was suspended in 500 mL chloroform/methanol/water (30 : 60 : 8 by vol.) under vigorous shaking and sonifying at room temperature, and then centrifuged at 4000 r.p.m. for 10 min at room temperature. The extraction step was repeated twice with 500 mL chloroform/methanol/water 60 : 35 : 8. The supernatants were collected and evaporated to dryness by *in vacuo* rotary evaporation.

Anion-exchange chromatography. A glass column (vol. = 250 mL, diameter = 3.5 cm) was filled with DEAE-Sephadex A-25 (acetate form), and equilibrated with chloroform/methanol/water (60 : 35 : 8, by vol.). The mushroom extract was dissolved in the same solvent mixture and applied to the column. After a prewashing step with chloroform/methanol/water (60 : 35 : 8, by vol.) and methanol, 1 L each, the acidic components were eluted with 1 L of 0.08 M potassium acetate in methanol. The eluate was evaporated to dryness, redissolved in water and dialysed for 2 days against frequent changes of distilled water, as described above. The dialysate containing the basidiolipids was brought to dryness, and stored in a vacuum desiccator.

Organic solvent extraction procedure. Two kilograms of fresh mushrooms were mashed with a mixer, after the stalks had been removed, and, after freezing at – 20 °C, lyophilized. The yield of dry mushroom powder was between 6 and 7% of the original wet weight. Twenty millilitres organic solvent, i.e. either of methanol, or propanol-2/*n*-hexane/water (55 : 35 : 10, by vol.), or chloroform/methanol/water (60 : 35 : 8, by vol.) was added to 1 g of dry powder. Each extraction mixture was allowed to stand for 1 h at 50 °C. Supernatants were collected after centrifugation at 4000 r.p.m. for 10 min at room temperature. The extraction steps with the organic solvents were repeated twice, as described before. Collected supernatants were evaporated to dryness and dialyzed. The basidiolipids were isolated by anion-exchange column chromatography, as described above.

Separation of basidiolipids

The four basidiolipid compounds that were detected on TLC as major carbohydrate-positive bands, were separated, using a glass column (vol. = 250 mL, diameter = 3.0 cm). Silicagel Si60 LiChroprep material was filled into the column and washed with 500 mL chloroform/methanol (8 : 2, by vol.). The dry acidic glycolipid fraction obtained from 2 kg mushrooms was dissolved in 100 mL of chloroform/methanol (8 : 2, by vol.) and applied to the column. The column was washed with the same solvent mixture followed by chloroform/methanol/water (65 : 25 : 4, by vol.), 1 L each. Single basidiolipids were eluted from the column with 3 L of chloroform/methanol/water (60 : 27 : 5, by vol.). Fractions of 100 mL were collected, and screened for their content by TLC

For a final purification, fractions showing single basidiolipid compounds on TLC were rechromatographed on a smaller silicagel column (vol. = 40 mL, diameter = 1.8 cm). The same solvent system as described before was used, except that the solvent volumes were adjusted to the smaller column size.

Quantitation of the basidiolipids

An aliquot of each extract was spotted on a TLC plate. The plate was developed in chloroform/methanol/0.3% aqueous $CaCl_2$ (50 : 40 : 10, by vol.). Carbohydrate-positive bands were detected with orcinol-sulfuric acid spray reagent at a temperature of 110 °C for 10 min. The amount of the single acidic glycolipid compounds from *A. bisporus* extracts was determined, using a Shimadzu CS-9000 TLC-scanner at a wave length of 440 nm.

HF-cleavage of phosphodiesters

A solution containing 200 μg of basidiolipid was brought to dryness in a screw cap polyethylene tube. One-hundred microlitres fluorohydrogenic acid, 48% in water, was added and the reaction mixture allowed to stand for 16 h at room temperature. Excess of fluorohydrogenic acid was removed under a flow of nitrogen.

Fatty acid and sphingoid base determination

Fatty acid was determined after acid hydrolysis of 200 μg of the respective basidiolipid using 1 mL HCl/H_2O/methanol (8.6 : 9.4 : 82, by vol.) for 20 h at 80 °C. The solvent was evaporated and the sample redissolved for a second hydrolysis for 1 h at 100 °C in 1 mL 1 M aqueous HCl. The solvent was evaporated again and fatty acids and sphingosin base were separated by extraction for three times with 1 mL chloroform under intensive sonication and following centrifugation at 4000 r.p.m. for 10 min. A small DEAE-Sephadex A-25 column (acetate form; 0.3 mL) was used to separate the lipophilic components. The sphingoid was eluted with chloroform/methanol/water (30 : 60 : 8, by vol.) and fatty acids with chloroform/methanol/1 M aqueous HCl (30 : 60 : 8, by vol.). The solvents were evaporated under a stream of nitrogen. Fatty acids were determined by GC/MS as described before [4]. The sphingoid base was identified by TLC with running solvent chloroform/methanol/water (60 : 35 : 8, by vol.) and colour formation using either iodine vapour, sulfuric acid, ninhydrin-spray or fluoram-spray reagent. Further characterization of the sphingoid was by GC/MS as previously described [4].

Carbohydrate constituent analysis

Carbohydrate constituents were released by acid hydrolysis, converted into corresponding alditol acetates and analysed by capillary GC as detailed elsewhere [5].

Methylation analysis

To determine the linkage positions of the monosaccharide constituents present, glycolipids were permethylated and hydrolyzed [6]. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by GC/MS using the instrumentation and microtechniques described earlier [7,8].

Colorimetrical phosphorus determination

Two hundred micrograms of each basidiolipid were cleaved with hydrofluoric acid as described above. After removal of HF, 200 μL distilled water were added to each of the samples. Ten and 20 μL from the aqueous solutions were diluted to a volume of 200 μL with water. As a standard, sodium dihydrogen phosphate at a concentration of 1 mg $PO_4^{(3-)}$ per 10 mL water was used. 0, 10, 20 and 30 μL, as double values were also diluted to 200 μL with water. For determination of phosphorus, 200 μL of a freshly made solution of 0.375% ammonium heptamolybdate, 2.04% sodium acetate and 2% L-ascorbinic acid was added to each sample. After incubation for 2 h at 37 °C, 400 μL water were added and the light blue color was measured in a spectrophotometer with a wavelength of 578 nm.

Exoglycosidase treatment

For the determination of the anomeric configuration and sequence of monosaccharides, 20 μg of each basidiolipid was diluted in 200 μL citrate-citric acid buffer and after addition of

Table 1. Enzymes used for the cleavage of glycolipid terminal sugars

Enzyme	Source	Amount of enzyme used for cleavage (nkat)	Buffer	pH	Detergent
α-Galactosidase (EC 3.2.1.22)	*Coffea arabica* (green coffee beans)	8.35	0.05 M citrate	6.0	0.1% taurodeoxycholate
α-Fucosidase (EC 3.2.1.51)	bovine kidney	0.84	0.05 M citrate	5.5	0.1% taurodeoxycholate
β-Galactosidase (EC 3.2.1.23)	bovine testes	1.67	0.05 M citrate	4.3	0.1% taurodeoxycholate
β-Mannosidase (EC 3.2.1.25)	*Turbo cornutus* (snail)	2.17	0.05 M citrate	4.0	0.1% taurodeoxycholate
α-Mannosidase (EC 3.2.1.24)	*Canavalia ensiformis* (Jack beans)	15.00	0.05 M citrate	4.0	0.1% taurodeoxycholate

1.0 unit of enzyme activity is equivalent to 16.67 nkat.

the appropriate enzyme, the reaction mixtures were cleaved for 16 h at 37 °C (for details, see Table 1).

For each sample a small silicagel reversed-phase C-18 column (0.2 mL volume) was filled. The probe was applied to the column after adding 1 mL 0.1 M aqueous potassium chloride solution. Under this conditions, the glycolipids, as well as, the detergent taurodeoxycholate bound to the column material. Water was used to remove buffer salt and enzyme, with methanol, the glycolipids and the detergent were eluted. Subsequently, the sample was brought to dryness. After redissolution in pure water, the mixture of glycolipid and detergent was applied to a second reversed-phase column. Without addition of a salt containing aqueous solution, only the detergent did bind to the column material. The glycolipid was already in the water fraction and could be separated from taurodeoxycholate in this way.

Periodate oxidation analysis

For determination of inositol substitution positions, 5 mg Bl-1 and Bl-3 were treated with $NaIO_4$, and the oxidation products reduced with sodium borohydride according to the procedure of Sugita *et al*. [1]. The reaction mixture was passed through a silica gel reversed-phase C-18 column. For this, 2 mL silica gel RP-18 was prewashed with 10 mL methanol and water, respectively. The sample was diluted to a salt concentration of ≈ 0.2 M and applied to the column. Hydrophilic components were washed through with 10 mL of water. Lipophilic material was eluted with 10 mL methanol and chloroform/methanol/water (60 : 35 : 8, by vol.), respectively. The organic solvents of the eluate were evaporated. The following hydrolysis and ion exchange chromatography was carried out according to Sugita *et al*. [1]. For cleavage of inositol phosphate ester linkages, the Bl-1 and Bl-3 periodate oxidation products were treated with HF as described. Thereafter, free phosphoric acid was removed by passing the reaction mixture over a 1-mL Dowex 1 × 8, $OH^{(-)}$-form column. The inositol periodate oxidation fragment was eluted with 10 mL of water and chromatographed on TLC with the running solvent pyridine/ethyl acetate/glacial acetic acid/water (5 : 5 : 1 : 1, by vol.) on a silica gel plate. Visualization was performed with periodate/benzidine reagent.

As a second step, the inositol periodate oxidation fragment was acetylated and identified by GC/MS.

Chemical determination of inositol-1-phosphate

Two hundred μg of the single basidiolipid components were hydrolyzed according to Sugita *et al*. [1]. To remove lipophilic components, the hydrolysis products were passed through a small silica gel reversed-phase C-18 column (0.3 mL volume). The water fraction was evaporated to dryness and chromatographed on a TLC plate with inositol-1-phosphate as a standard. Running solvent was pyridine/ethyl acetate/glacial acetic acid/water (5 : 5 : 1 : 3, by vol.). Color formation was performed using a sodium periodate/benzidine reagent.

NMR spectroscopy

All NMR experiments were performed on a Bruker AMX500 spectrometer equipped with pulsed field gradient (PFG) accessory and a triple resonance probe. Owing to aggregation of the basidiolipid at lower temperatures producing severe line broadening, all experiments were recorded at 353 K in d_6-DMSO. Proton and carbon chemical shifts were referenced to internal DMSO (2.49/39.5 p.p.m.). For the phosphorus chemical shifts phosphoric acid (85%) was used as external standard (δ_p = 0.0 p.p.m., at 300 K). Pulsed field gradients were used to select the coherence pathways and to suppress spectral artefacts [9]. In all two-dimensional spectra the numbers of complex points and sweep widths were 4196 points/9.0 p.p.m. for ^1H and 512 points/100 p.p.m. or 40 p.p.m. for ^{13}C. Quadrature detection in the indirect dimension was achieved with the time-proportional-phase-incrementation (TPPI) method [10]. For the

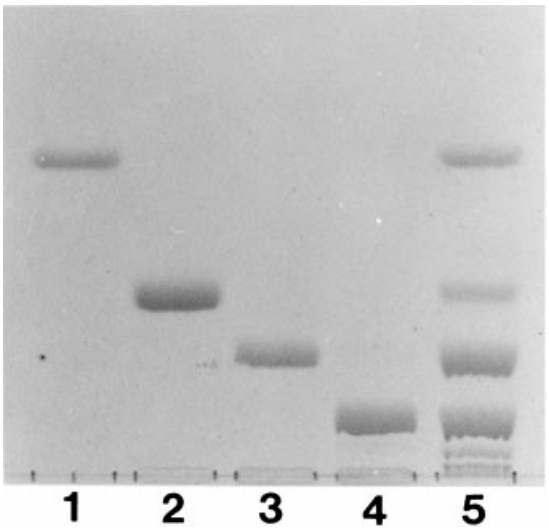

Fig. 1. TLC of acidic glycolipids from *A. bisporus*. 1–4, isolated glycolipid components Bl-1–4; 5, total acidic glycolipids. Running solvent: chloroform/methanol/0.3% aqueous $CaCl_2$ (45 : 45 : 10, by vol.); visualization with orcinol/sulfuric acid spray reagent at 110 °C for 10 min.

Table 2. Quantitation of glycolipids from *Agaricus bisporus*, as extracted with different solvents[a] (mg of glycolipid extracted from 1 g freeze-dried mushroom)

Extraction-solvent:	C/M/W[b] (60 : 35 : 8)	I/H/W[c] (55 : 35 : 10)	Methanol	Water
Glycolipid				
Bl-1	3.73	2.81	2.69	0.70
Bl-2	0.20	0.12	0.11	0.08
Bl-3	0.64	0.31	0.28	0.23
Bl-4	0.48	0.15	0.17	0.23

[a] Calculated values are correlated to the molecular weight of the single components
[b] C/M/W, chloroform/methanol/distilled water.
[c] I/H/W, propanol-2/*n*-hexane/distilled water.

assignment of the chemical shifts, the following experiments were recorded: DQF-COSY [11], z-TOCSY [12] with a MLEV-17 mixing sequence of 80 ms, HMQC [13] and HMBC [13]. The anomeric configuration was defined by a NOESY experiment with τ_m = 300 ms [14]. For the inositol substitution, two one-dimensional selective TOCSY experiments with and without ^{31}P-decoupling, respectively, were recorded [15].

The data were processed with the software package UXNMR (Bruker). After apodization with a 90° shifted sinebell, zero filling to 512 real points and baseline correction were applied for the indirect dimensions. For the direct dimensions zerofilling to 4196 real points, Lorentz-to-Gauss transformation, and baseline correction were used.

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)

Samples were dissolved in 0.1% aqueous trifluoroacetic acid, and 1 μL applied on a thin layer of indole-2-carboxylic acid created from a solution (50 mg·mL^{-1}) in acetone. The samples were air-dried and analysed using a VoyagerRP work station (Perseptive Biosystems, Wiesbaden, Germany). The spectra were collected in the reflector mode of the instrument at an acceleration voltage of 15 000 V, a grid voltage of 58.000% and a delay time of 80 ns. The *m*/*z*-values were calculated using the default-calibration of the instrument.

RESULTS

Preparation of basidiolipids

A mixture of glycolipids was extracted from mushrooms, *A. bisporus* or *A. campestris*, using water, methanol or aqueous organic solvent mixtures. Even though, the extraction with water offers the advantage that prior drying of the mushroom material could be avoided, the yield of basidiolipid components obtained, in particular of Bl-1, would favour extraction with organic solvents (Table 2). These glycolipids were stable under the usual alkaline saponification conditions, and could be separated by anion-exchange chromatography. Isolation and purification of single components was achieved by column chromatography. Thereby four compounds could be obtained that migrated on silica gel TLC with organic solvent systems and stained positive with iodine vapour for their lipid nature, and with oricinol–sulfuric acid spray reagent for carbohydrate (Fig. 1).

They were designated as basidiolipids, i.e. Bl-1, Bl-2, Bl-3 and Bl-4. Bl-1 was extracted from the mushroom in greatest quantity followed by Bl-3, Bl-4 and Bl-2 (Table 2). From the lamellae of the mushroom, the yield of all four extracted basidiolipid compounds, in identical ratio to one another, was approximately three times as much as from the cap, and 10 times as much as from the stalk (data not shown).

Only Bl-1 could be stained on TLC with Hanes–Isherwood's phosphate-detecting spray-reagent [16]. On TLC, none of the glycolipids reacted with chlorine-tolidine reagent for substances that can be converted to chloramines.

C, H, N elemental analysis

In order to gain preliminary information of the constitution of the basidiolipids, Bl-1 was analysed and the number of C and H atoms in the molecule calculated with the understanding of the existence of one atom of nitrogen.

Bl-1: found (%) C 54,80 H 8.44 N 1.17; found $C_{54}H_{101}$ N; calc. C_{53} H_{105} N.

MALDI-TOF-mass spectrometry

The molecular mass peak data of all four basidiolipids suggested a molecular heterogeneity with the presence of two components in comparable quantity in each lipid compound. These two components were always distinguished from one another by a mass difference of 28, indicating an equivalent of two CH_2 groups (Table 3). Furthermore, the molecular masses of Bl-2, Bl-3 and Bl-4, respectively, differed by 162, the equivalent of one hexose unit in each case (Table 3). The molecular mass of Bl-1 differed from Bl-2 by the mass of two hexoses and one desoxy-hexose.

Chemical composition of four basidiolipids

All four basidiolipids could be hydrolyzed with hydrogen fluoride. In the respective hydrolysates, free phosphate was detected by reaction with molybdate reagent, and ceramide by TLC. The ceramide was further cleaved by acid hydrolysis yielding its constituent sphingoid base and fatty acids, which were characterized by GC/MS. The sphingoid present consisted solely of hydroxy-sphinganine (C_{18}). The majority of the fatty acids were α-hydroxy-behenic (C_{22}) and α-hydroxy-lignoceric

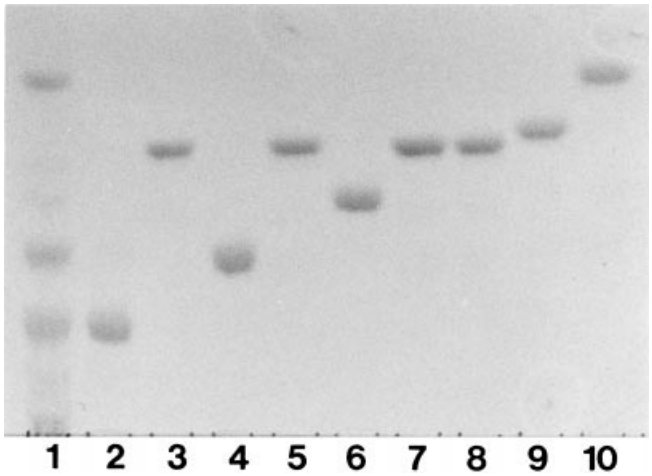

Fig. 2. TLC of isolated, exoglycosidase treated acidic glycolipids from *A. bisporus*. 1, total acidic glycolipids; 2, isolated component Bl-4; 3, Bl-4, treated with α-galactosidase; 4, isolated component Bl-3; 5, Bl-3, treated with α-galactosidase; 6, isolated component Bl-2; 7, Bl-2, treated with α-galactosidase; 8, Bl-2/-αGal, treated with β-galactosidase; 9, product of 7 (Bl-2/-αGal), treated with α-fucosidase; 10, product of 9 (Bl-2/-αGal/-αFuc) treated with β-galactosidase. Running solvent chloroform/methanol/0.3% aqueous $CaCl_2$ (45 : 45 : 10, by vol.); visualization with orcinol/sulfuric acid spray reagent at 110 °C for 10 min.

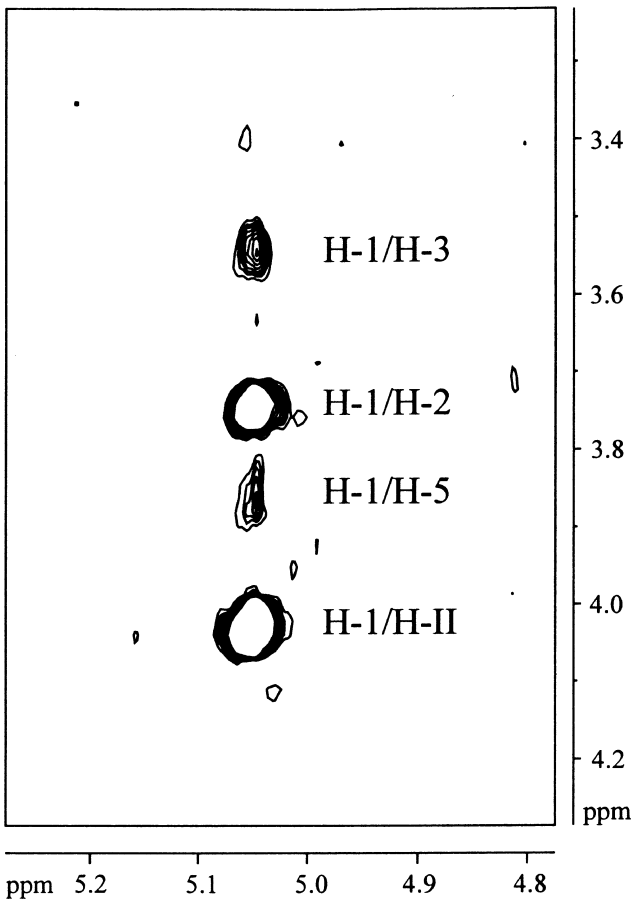

Fig. 3. Part of a 2D 500-MHz NOESY spectrum of the basidiolipid Bl-1. Shown are the NOE crosspeaks of the anomeric proton. The protons of the mannose are labelled with Arabic characters, those of the inositol with Roman. The two NOE contacts between the H-1/H-3 and the H-1/H-5 protons of the mannose are diagnostic for the β anomer. The interresidue linkage position between H-1^{Man} and H-2^{Ino} elucidated by the HMBC spectrum is additionally confirmed by the NOE H-1/HII.

(C_{24}) acid in approximately comparable percentages. Besides of these two fatty acids, only a minor contribution of α-hydroxy-C_{21}- and C_{23}-fatty acids could also be detected (data not shown).

Free inositol, as well as, monosaccharide constituents from the hydrolysate were measured as their acetate and alditol acetates, respectively, by gas-liquid chromatography. All four basidiolipids contained 1 M of inositol and mannose, as well as varying ratios of galaxies. The basidiolipids, except Bl-1, contained 1 M of fucose (Table 4).

The linkage positions of the monosaccharide constituents were established by permethylation, hydrolysis and GC/MS of the partially methylated monosaccharides as alditol acetates (Table 5).

Exoglycosidase analysis

Bl-1 proved refractory to both enzymes, α-mannosidase and β-mannosidase, even after incubation with 15 nkat α-enzyme or

Table 3. Molecular mass of basidiolipids as measured by MALDI-TOF-MS

Basidiolipid	Bl-1	Bl-2	Bl-3	Bl-4
$g \cdot mol^{-1}$	1060	1529	1691	1853
$g \cdot mol^{-1}$	1088	1557	1719	1881

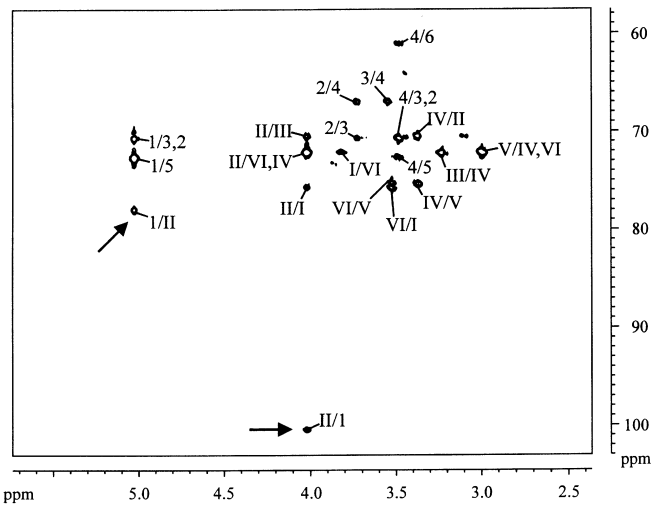

Fig. 4. Relevant region of a 2D 500-MHz HMBC spectrum of the basidiolipid Bl-1. The protons of the mannose are labelled with Arabic characters, those of the inositol with Roman, respectively. The linkage position between H-1^{Man} and H-2^{Ino} can be determined by the corresponding HMBC crosspeaks. Both possible long range $^{1}H/^{13}C$ couplings across the glycosidic bond (H-1^{Man}/C-2^{Ino} and H-2^{Ino}/C-1^{Man}) can be seen (marked with the arrows).

2.17 nkat β-enzyme for 16 h at 37 °C (data not shown). By high resolution 500-MHz ^{1}H-NMR, the hexopyranose was shown to be β-linked to the inositol moiety. Bl-2 was hydrolysed with α-galactosidase yielding a new glycolipid which still contained one residue of galactose, besides fucose and mannose. It migrated on TLC between the uncleaved compound and Bl-1 (Fig. 2, lane 7). The product of α-galactosidase treatment, furthermore, was also cleaved readily by α-fucosidase yielding still another glycolipid which, while migrating on TLC differently from the product Bl-2/-αGal, also chromatographed between the uncleaved compound and Bl-1 (Fig. 2, lane 9). The product of the Bl-2 α-fucosidase cleavage contained only one galactose and one mannose as hexose constituents (data not shown).

The latter intermediate, Bl-2/-αGal/-αFuc, could be further hydrolysed by β-galactosidase with the concomitant formation of a glycolipid that migrated on TLC in several running solvents identically to Bl-1 (Fig. 2, lane 10). This final exoglycosidase cleavage product showed, upon permethylation carbohydrate constituent analysis, only the presence of one terminal mannose residue (data not shown). Bl-3, as well as, Bl-4 were hydrolyzed in presence of α-galactosidase resulting in the formation of one and the same glycolipid that had TLC properties identical to Bl-2/-αGal (Fig. 2, lanes 3, 5 and 7). The latter product could equally well as Bl-2/-αGal be further disintegrated by the sequential application of the two glycosidases, α-fucosidase and β-galactosidase. The final products, Bl-3/-αGal/-αFuc/-βGal or Bl-4/-αGal/-αFuc/-βGal were, as in the case of the Bl-2 degradation, both indistinguishable from Bl-1, and yielded,

Table 4. Carbohydrate constituent analysis of four basidiolipids. Results are mean values of capillary gas chromatography and capillary gas chromatography/chemical ionization (ammonia)/mass fragmentography

	Inositol	ManOH	GalOH	FucOH
Bl-1	1.2	1	–	–
Bl-2	1.3	1	1.95	0.9
Bl-3	1.3	1	2.9	0.9
Bl-4	1.2	1	3.7	0.9

ManOH = D-mannitol, etc. Mannitol set to 1.0.

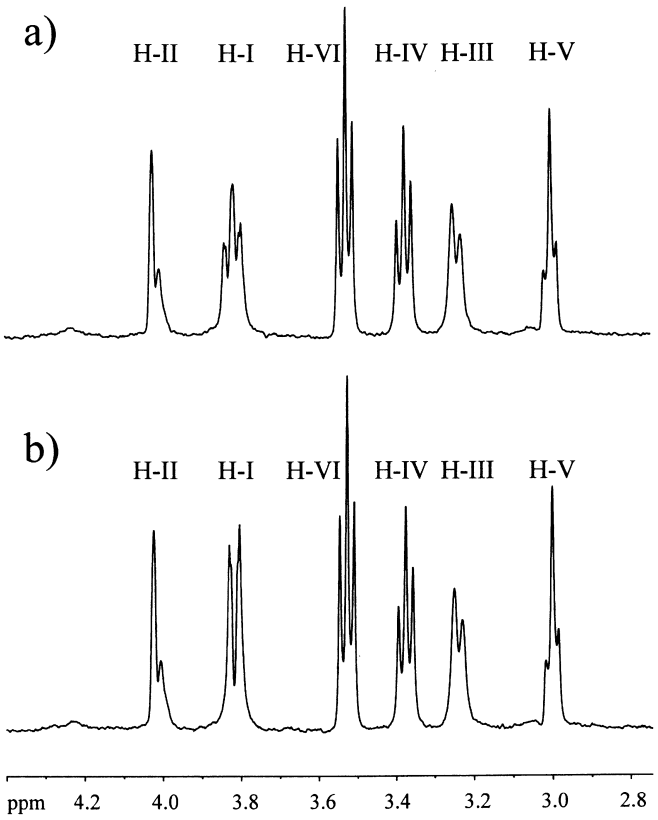

Fig. 5. 1D 500 MHz selective TOCSY-spectra of the basidiolipid Bl-1 (a) without and (b) with ^{31}P-decoupling. Additionally to the typical homonuclear coupling pattern of myo-inositol (b) a $^{3}J_{31P1H}$ coupling can be seen in the pattern of the H-I signal (a). A corresponding $^{3}J_{31P1H}$ coupling was observed for the H-1/H-1′ protons of sphingosin (data not shown). Thus the position of the phosphorous could be determined as inositol-1-phosphate.

before and after permethylation, the same mass spectra (data not shown).

Identification of inositol1-phosphate

The linkage of inositol to phosphate could be confirmed by the isolation of inositol1-phosphate from the HCl-hydrolysate of all basidiolipids, and TLC comparison with an authentic standard.

Establishment of inositol substitution positions by periodate oxidation

The basidiolipids were oxidized with periodate, the reaction products reduced with borohydride, and subsequently hydrolyzed with mineral acid. Among the periodate oxidation products, erythritol could be identified by TLC and, as its trimethylsilyl-derivative, by GC (data not shown). The formation of erythritol was indicative for the divalent substitution of the myo-inositol in 1 and 2 positions.

NMR spectroscopy

The assignment of the different spin systems was achieved by a combination of COSY, TOCSY, HMQC and HMBC spectra. Owing to the well resolved chemical shifts of the anomeric proton of mannose and the amide proton of *N*-acyl-sphingosine, these spin systems could be easily identified by the TOCSY spectrum. The remaining cyclic spin system corresponding to an inositol residue could also be detected in the COSY and TOCSY spectra. The severe overlap of the proton resonances was resolved by their carbon chemical shifts. Therefore, the resonance assignment was mainly done by a combination of HMQC and HMBC spectra (for full assignments, see Table 6)

Assignment of the C-1 anomeric proton of mannose in Bl-1

Usually, the anomeric configuration of monosaccharide residues can be identified by $^{3}J_{HH}$ coupling constants, which are large for antiperiplanar orientation of the vicinal ring protons and small for their gauche orientation. As the gauche orientation may refer either to an equatorial/axial or a diequatorial combination, the anomeric configuration of mannose cannot be distinguished by this criterion. In such a case, NOE signals between synaxial H1/H3 and H1/H5 protons are diagnostic for the β-anomer [17]. For the basidiolipid, exactly these two intraresidue NOE crosspeaks were observed, together with strong NOE crosspeaks between the H1/H2 protons of mannose and the H1 proton of mannose and the H2 proton of inositol (see Fig. 3).

This indicates a β-linked mannose, and it confirms the substitution Man*p*β-2inositol (see below).

Assignment of the inositol substitution

For the inositol spin system, the pattern of intraring coupling constants was consistent with *myo*-inositol. Owing to the characteristic proton coupling constants of the myo-inositol ($^{3}J_{1,2}$ and $^{3}J_{2,3}$ are small [ax/eq and eq/ax], the remaining ^{3}J values are large [ax/ax]) the one equatorial ring proton H-2 could be identified as a starting point for the assignment. The interresidue linkage position Man*p*1–2inositol could be determined by the HMBC spectrum (see Fig. 4).

Both possible long range $^{1}H/^{13}C$ couplings across the glycosidic bond (H-1^{Man}/C-2^{Ino} and H-2^{Ino}/C-1^{Man}) could be observed. The position of the phosphorylation could be identified as inositol1-phospho-sphingosine. With a selective

Table 5. Methylation analysis of basidiolipids. Results are mean values of capillary gas chromatography and capillary gas chromatography/chemical ionization (ammonia)/mass fragmentography

	Peak ratios[c] of component					
Alditol acetate[b]	Bl-1	Bl-2	Bl-3	Bl-4	Bl-2/-αGal[a]	Linkage
2,3,4-ManOH	–	1.00[c]	1.00[c]	1.00[c]	1.00[d]	– 6)Man(1-
2,3,4,6-ManOH	1.00	–	–	–	–	Man(1-
3,4-GalOH	–	1.3	1.1	1.15	–	– 6,-2)Gal(1-
2,3,4-GalOH	–	–	1.15	2.4	–	– 6)Gal(1-
3,4,6-GalOH	–	–	–	–	0.95	– 2)Gal(1-
2,3,4,6-GalOH	–	0.75	0.55	0.7	–	Gal(1-
2,3,4-FucOH	–	0.1	0.1	0.15	0.65	Fuc(1-

[a] Bl-2/-αGal, designates Bl-2 de-α-galactosylated with α-galactosidase [b] 2,3,4-FucOH, etc. = 2,3,4-tri-*O*-methyl-L-fucitol, etc.[c] values obtained by combined GC/MS.[d] 2,3,4-ManOH set to 1.0.

Table 6. ^{1}H, ^{13}C and ^{31}P chemical shifts[a] of basidiolipid Bl-1 in Me_2SO-d_6 at 353 K.

	Mannose		Inositol		Sphingosine		
Reference to carbon atoms	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{31}P
1	5.05	101.5	3.82	75.6	4.06/3.80	64.0	2.73
2	3.73	70.1	4.02	77.9	3.95	50.3	
3	3.53	70.6	3.24	70.4	3.43	73.2	
4	3.48	66.9	3.38	72.1	3.39	70.5	
5	3.85	72.6	3.00	75.3	1.28/1.50		
6	3.62/3.50	61.1	3.53	72.0			
NH					7.29		

[a] The chemical shifts were referenced to internal Me_2SO (2.49/39.5 p.p.m.) and to external H_3PO_4 (85%. 300 K).

TOCSY spectrum starting at H-5^{Ino}, the inositol spin system could be selected. Additional to the homonuclear coupling pattern (see Fig. 5b), the $^{3}J_{HP}$ coupling due to the phosphate substitution can be seen in the coupling pattern of H-1^{Ino} (see Fig. 5a).

DISCUSSION

In recent years, glycoconjugates that could be isolated from higher mushrooms, the *Basidiomycetes*, have met with increasing interest. Such complexes were glycoproteins or proteoglycans that possessed immunoenhancing and antitumor activities [18–21]. In search of substances that would display immune adjuvant properties, we have initiated a study of the glycolipids of *Basidiomycetes*, in particular, the readily available edible cultured mushroom *A. bisporus,* and naturally grown *A. campestris*. Both of these agaricales gave the same results, and indeed contain the same glycolipids that are highly immune adjuvant (data to be published elsewhere). A homologous series of four glycolipids could be isolated from these mushrooms, and purified. They were designated basidiolipid components Bl-1, Bl-2, Bl-3 and Bl-4. The chemical structures of these GIPSL have been elucidated on the basis of elemental analysis, MALDI-TOF-MS and NMR-spectroscopy of the whole molecules, constituent composition, exoglycosidase cleavage and linkage analysis of the oligosaccharide chain, fatty acid and sphingoid determination of the ceramide moiety, hydrochloric acid hydrolysis and isolation of inositol phosphate, hydrofluoric acid hydrolysis and cleavage of the hexose–inositol-phosphate linkages, as well as, determination of the periodate oxidation products. From the data obtained, the following structures for the four basidiolipids can be proposed:

Man*p*β-2Ins1-$PO_4^{(-)}$-Cer

Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2Ins1-$PO_4^{(-)}$-Cer

Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2Ins1-$PO_4^{(-)}$-Cer

Gal*p*α-6Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*β-2Ins1-$PO_4^{(-)}$-Cer

The basidiolipids belong to the GIPSL, a class of the sphingolipids that has previously been reported from higher plants [2], yeasts and lower fungi, such as *Aspergillus niger* [22], *Histoplasma capsulatum* [23], *Saccharomyces cerevisiae* [24,25], *Neurospora crassa* [26], *Paracoccidioides brasiliensis* [27], as well as, from protozoa (for review, see reference [3]). However, GIPSL also occur in the animal kingdom, where such a glycolipid was recently found in the worm, the nematode *Ascaris suum* [1]. With the identification of the basidiolipids, it could be shown that higher mushrooms also contain this class of glycoconjugates. They typically have the C_{18}-sphingoid phytosphingosine and an α-hydroxy long-chain fatty acid as ceramide moiety that is linked into the 1-position of myo-inositol via a phosphodiester bridge. The C-2 position of the inositol is substituted by a β-D-mannopyranoside. A GIPSL with this structural element has recently been described from the pathogenic fungus *P. brasiliensis* [27]. However, unlike the further elongation of the oligosaccharide chain in the case of the *P. brasiliensis* GIPSL with a β-galactofuranosyl residue in the 6-position of the inositol-linked mannose, no galactofuranose could be detected in the basidiolipids. Similar to numerous other glycoconjugate oligosaccharide chains, the basidiolipids also show the conspicuous branching substitution on the second, equatorially linked, hexapyranosyl unit, in this case, by fucose [28]. Apart from this, the oligosaccharide chain of the basidiolipids is characterized by the mutual substitution of hexopyranosyl-residues in their 6-positions. It may be expected that this type of linkage could result in special conformations of the carbohydrate region of the basidiolipids.

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