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Glycoinositolphosphosphingolipids (basidiolipids) of higher mushrooms

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The basidiolipids of six mushroom species, i.e. the *basidiomycetes Amanita virosa* (engl., death cup), *Calvatia exipuliformis* (engl., puffball), *Cantharellus cibarius* (engl., chanterelle), *Leccinum scabrum* (engl., red birch boletus), *Lentinus edodes* (jap., Shiitake), and *Pleurotus ostreatus* (engl., oystermushroom), were isolated, and their chemical structures investigated. All glycolipids are structurally related to those of the *Agaricales* (engl., field mushroom). They are glycoinositolphosphosphingolipids, their ceramide moiety consisting of t18:0-trihydroxysphinganine and an α-hydroxy long-chain fatty acid. In contrast to a previous study [Jennemann, R., Bauer, B.L., Bertalanffy, H., Geyer, R., Gschwind, R.M., Selmer, T. & Wiegandt, H. (1999) *Eur. J. Biochem.* **259**, 331–338], the glycoside anomery of the hexose (mannose) connected to the inositol of all investigated basidiomycete glycolipids, including the basidiolipids of *Agaricus bisporus*, was determined unequivocally to be alpha. Therefore, the root structure of all basidiolipids consists of α-D-Man*p*-2Ins1-[*P*O_4]-Cer. In addition, for some mushroom species, the occurrence of an inositol substitution position variant, α-Man*p*-4Ins1-[*P*O_4]-Cer, is shown. The carbohydrate of chanterelle basidiolipids consists solely of mannose, i.e. *Cc1*, Manα-3 or -6Manα; *Cc2*, Manα-3(Manα-6)Manα-. All other species investigated show extension of the α-mannoside in the 6-position by β-galactoside, which, in some instances, is α-fucosylated in 2-position (Fucα-2)Galβ-6Manα-. Further sugar chain elongation at the β-galactoside may be in 3- and/or 6-position by α-galactoside, e.g. *Ce4, Po2*, Galα-3(Galα-6)(Fucα-2)Galβ-6Manα-, whereas *A. virosa*, Av-3, has a more complex, highly α-fucosylated terminus, Galα-3(Fucα-2)(Fucα-6)Galα-2(Galα-3)Galβ-6Manα-. *L. edodes* basidiolipids show further elongation by α-mannoside, e.g. *Le3*, Manα-2Manα-6Galα-3(Fucα-2)Galβ-6Manα-, *C. exipuliformis* glycolipid by α-glucoside, i.e. *Ce3*, Glcα-6Galβ-6Manα-. Basidiolipid *Ls1* from *L. scabrum,* notably, has a 3-α-mannosylated α-fucose, i.e. Galα-6(Manα-3Fucα-2)Galα-6Galβ-6Manα-. In conclusion, basidiolipids, though identical in their ceramide constitution, display wide and systematic mushroom species dependent variabilities of their chemical structures.

Keywords: basidiomycetes; fungi; glyco-inositol-phosphoceramides; glycolipids; glycosphingolipids

In recent years, renewed attention has been paid to constituents of higher, often edible mushrooms, *Basidiomycetes*, because of their possible medicinal usage (reviewed in [1]). Anti-viral, -biotic, -inflammatory, -hypoglycemic, -hypocholesterinemic and -hypotensive properties were ascribed to ingredients of such filamentous fungi (reviewed in [2]). It was assumed that, at least in part, observed therapeutic effects were due to a stimulation of the immune system [3–7]. On a chemical basis, the medicinal effects of basidiomycetes were attributed to glycoproteins and proteoglycans. Such glycoconjugates, however, with the exception of α(1–6)-Glc-branched (1–3)α-D-glucans, remained as structurally not well characterized [8–10]. Glycolipids of higher mushrooms have, apparently, not yet been studied, except for an early, less detailed report [11] and structural studies on the basidiolipids, i.e. glycosyl-inositol-phosphoceramides (G-Ins*P*Cer), of the field mushroom *Agaricus bisporus* [12,13]. Basidiolipids of this mushroom species are derived from a common root structure of a D-Man*p*-2Ins1-[*P*O_4]-Cer by extension of the carbohydrate moiety, whereby the ceramide consists of trihydroxysphinganine and a long chain α-hydroxy-fatty acid. As revealed in the present report, the anomeric linkage between mannose and inositol was found to be alpha. In contrast to higher

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Abbreviations: Av, *Amanita virosa*; Bl, basidiolipid (i.e. Bl-1, Man*p*α-2Ins1-*P*O_4-Cer; Bl-2, Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*α-2Ins1-*P*O_4-Cer; Bl-3, Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*α-2Ins1-*P*O_4-Cer; Bl-4, Gal*p*α-6Gal*p*α-6Gal*p*α-6[Fuc*p*α-2]Gal*p*β-6Man*p*α-2Ins1-*P*O_4-Cer); Cc, *Cantharellus cibarius*; Ce, *Calvatia exipuliformis*; Cer, ceramide (containing a *N*-α-hydroxy fatty acid and trihydroxysphinganine); ganglioside Gtet1, (GM1) II^{3}NeuAcGg$_4$Cer; G-Ins*P*Cer, glycosylinositolphosphoceramide; Ins*P*Cer, inositolphosphoceramide; Le, *Lentinus edodes*; Ls, *Leccinum scabrum*; Po, *Pleurotus ostretatus*; trihydroxysphinganine (phytosphingosine), (2*S*,3*S*,4*R*)-2-aminooctadecane-1,3,4-triol; PFG, pulsed field gradient.

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Table 1. Conditions chosen for ESI-MS/MS determinations. Before measurement, ceramides were liberated from G-Ins*P*Cer by mineral acid hydrolysis.

Substances measured:	Purified G-Ins*P*Cer	Fragments of G-Ins*P*Cer, glycoinositol-phosphate characterization	Specific detection of G-Ins*P*Cer by $[PO_3^-]$ fragment	Specific detection of Cer by fragment from sphingosine part	Fragments of Cer1: characterization		Specific detection of $[IO_4^-]$ treated G-Ins*P*Cer by $[PO_3^-]$ fragment
					Fatty acids	Sphingosine	
Scan modus (polarity)	[-] MS1 (total ions)	[-] product ion	[-] precursor ion *m/z* 79	[+] precursor ion *m/z* 264	[-] product ion	[+] product ion	[-] precursor ion *m/z* 79
Charge of detected ions	Negative	Negative	Negative	Positive	Negative	Positive	Negative
Cone (V)	40–50	40–50	40–50	23	55	23	55
Collision energy (eV)	–	50–90	100–140	44	35	35	100

mushrooms, glycolipids of lower fungi, e.g. *Aspergillus, Saccharomyces, Schizosaccharomyces, Candida, Histoplasma*, have been well investigated (reviewed in [14–16]). The latter glycolipids also are G-Ins*P*Cer, and are based on the same root structure of an α-D-Man*p*-2Ins1-[PO_4]-Cer, and a ceramide constituent composition similar to that of the basidiolipids. Still, in some cases reported, the anomery and the position of the glycosidic and phosphoric acid linkages to inositol remain uncertain.

To probe their structural distribution amongst basidiomycetes species, and in continuation of the previous studies on the basidiolipids of the *Agaricales* [12,13], the present investigation was devoted to the characterization of G-Ins*P*Cer of other higher mushrooms, *Amanita virosa* (engl., death cup), *Cantharellus cibarius* (chanterelle), *Lentinus edodes* (Shiitake), *Pleurotus ostreatus* (oyster-mushroom), *Calvatia exipuliformis* (engl., puffball), and *Leccinum scabrum* (engl., red birch boletus).

MATERIALS AND METHODS

The mushrooms *C. cibarius, L. edodes* and *P. ostreatus* were purchased from a local market, *C. exipuliformis, A. virosa* and *L. scabrum* were collected in regional meadows or forests. Isolation and purification of the glycolipids from freeze-dried mushrooms was according to a previously described method using chloroform/methanol/water [12].

Quantitation of the basidiolipids

An aliquot of each extract was spotted on TLC (HPTLC-Plate, Merck, Darmstadt, Germany). After development in chloroform/methanol/0.3% aqueous $CaCl_2$ (50 : 40 : 10, v/v) carbohydrate containing bands were detected with orcinol-sulfuric acid spray-reagent at 110 °C for 10 min. The amount of the acidic glycolipid compounds from the different mushroom extracts was determined by scanning at 440 nm (Shimadzu CS-9301 TLC-scanner).

Mass spectrometric analyses

All analyses were performed with a triple quadrupole instrument [VG micromass (Cheshire, UK) model Quattro II] equipped with a nanoelectrospray source operating at an estimated flow rate of 20–50 nL·min^{-1}. Gold-sputtered borosilicate glass capillaries Type D were purchased from Teer Coatings Ltd (Worcestershire, UK). Samples were dissolved in methanol. Ammonium acetate (5 mM) was added for measurements in the positive-ion modes. The electrospray capillary was filled with 5–10 μL sample and positioned at a distance of 1–3 mm in front of the cone. The source temperature was set to 30 °C and the spray was started by applying 800–1200 V to the capillary. For each spectrum 10–30 repetitive scans of 10–30 s duration were averaged. All tandem MS experiments were performed with argon as collision gas at a nominal pressure of 2×10^{-3} mbar. The parameters for the cone voltage and the collision energy for the different scan modes are listed in Table 1.

Phosphorus determination

Phosphorus was determined by colorimetry as described elsewere [12], or by ESI-MS/MS in the precursor ion scan mode of 79 (*m*/*z*), specific for phosphate.

Ceramide isolation and characterization by ESI-MS/MS

The ceramide and fatty acid composition of the isolated basidiolipids was determined by ESI-MS/MS spectrometry. About 100 μg of the respective glycolipid were treated with 100 μL HF, 48% in H_2O for 20 h at room temperature. Under this condition, the ceramide-phosphoric acid ester linkage is cleaved. HF was removed under a stream of nitrogen and the ceramides were separated from the carbohydrate derivatives by silica gel reversed-phase chromatography (C-18 bulk material, 55–105 μm, Waters, Milford, MA), using small (200 μL volume) glass columns. Carbohydrates were eluted with distilled water, 2 mL, the ceramides using 2 mL each of methanol and chloroform/methanol 1 : 1, (v/v). Each ceramide fraction was dissolved in 1 mL chloroform/methanol 1 : 1, (v/v). A sample aliquot was further diluted 1 : 20 with methanol, containing 5 mM ammonium acetate, to enhance the signal in the mass spectrometer. The masses of the ceramides were determined by ESI-MS/MS in the precursor ion mode (parent scan) of 264 (*m*/*z*), which is a typical mass subunit for ceramides, according to a method of Liebisch *et al*. [17]. Fatty acid composition was determined in the negative- and sphingoid base composition was specified in the positive product ion mode [18].

Determination of the sphingoid base by TLC

The sphingoid composition was further specified chromatographically, after hydrolysis of the original glycolipid compound. Approximately 100 μg of the respective basidiolipid fraction was dried under nitrogen, and, subsequently, redissolved in 1 mL of aqueous HCl (37%)/H_2O/methanol 8.6 : 9.4 : 82.0, (v/v). The reaction mixtures were incubated for 20 h at 80 °C. After addition of 1 mL propan-1-ol, the solvent was removed by rotary evaporation. All samples were once more treated with 200 μL 1 M aqueous HCl for 1 h at 100 °C, and the mineral acid evaporated with the help of 1 mL propan-1-ol. The lipophilic constituents remaining after total hydrolysis were separated by the use of small reversed-phase columns of 200 μL volume, as described before. The sphingoid bases were identified on HPTLC by their chromatographical migration properties as compared to standard sphingoid bases using the solvent system of chloroform/methanol/2% aqueous sodium tetraborate 45 : 45 : 12, (v/v). A well saturated atmosphere in the TLC chamber was achieved by using a small propeller attached to the lid, inside of the TLC tank, for approximately 5 min before lowering the TLC plate into the running solvent.

Carbohydrate constituent analysis

Carbohydrate constituents were released by acid hydrolysis after HF treatment (see above), converted into their corresponding alditol acetates and analyzed by capillary GC/MS as detailed elsewhere [19].

Carbohydrate permethylation analysis

For determination of linkage positions of monosaccharide constituents, glycolipids were permethylated and hydrolyzed [20]. Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by GC/MS using the instrumentation and microtechniques described previously [21,22].

Exoglycosidase cleavage

The anomeric configuration and sequence of monosaccharides were determined by applying different amounts of single basidiolipid compounds according to their estimated carbohydrate chain lengths. The basidiolipids were dissolved in 200 μL citrate/citric acid buffer and after addition of the appropriate exoglycosidase, the reaction mixtures were incubated for 16 h to 72 h at 37 °C (for details, see [12]).

For each sample, a small silica gel reversed-phase C-18 column (0.2 mL volume, C-18 bulk material, 55–105 μm, Waters, Milford, MA, USA) was prepared. Each sample was applied to the column after adding 1 mL 0.1 M aqueous potassium chloride solution. Under these conditions, the glycolipids and the detergent taurodeoxycholate bound to the column material. Distilled water was used to remove the cleaved carbohydrate, buffer salt and enzyme. This eluate was kept for the determination of the sugar constituent released by the enzyme. With methanol, the glycolipids and the detergent were eluted. Subsequently, each sample was dried. After redissolution in 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 bound to the column material. The glycolipid already eluted with the water fraction could, in this way, be separated from taurodeoxycholate.

In order to confirm the specificity of the respective exoglycosidase, after removal of buffer salts by consecutively passing the sample over small Dowex-$H^{(+)}$- and Dowex-$OH^{(-)}$-columns (Roth, Karlsruhe, Germany), the released sugar was identified by TLC in a well saturated chromatography chamber, running solvent pyridine/ethylacetate/glacial acetic acid/water 5 : 5 : 1 : 2, v/v. Carbohydrate was visualized with orcinol spray reagent.

Total hydrolysis of the enzyme-treated basidiolipid 'end products'

The determination of the inositol-bound, glycosidase resistant sugar, was carried out after hydrolysis of the enzyme products with mineral acid, as described above. The released sugar was analyzed by comparison to standards by TLC, running solvent pyridine/ethylacetate/glacial acetic acid/water 5 : 5 : 1 : 2, v/v.

Periodate oxidation analysis

Determinations of inositol substitution positions were performed according to a method described previously [12,23,24].

Release of glycosyl-inositols by ammonolysis

The alkaline cleavage of basidiolipid Bl-1 was performed as described previously [25,26].

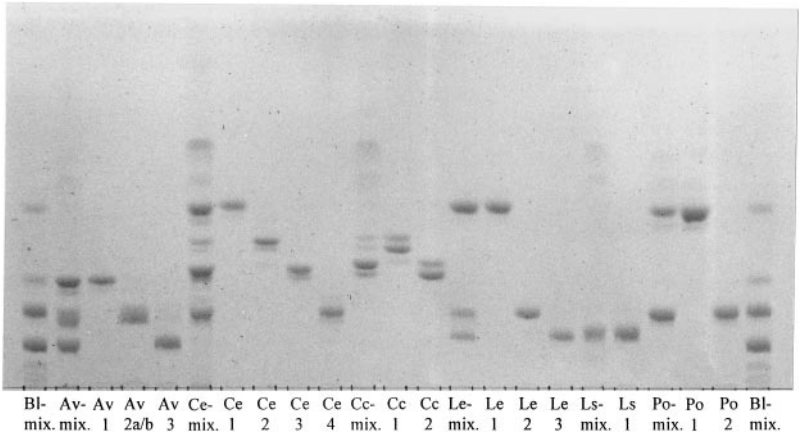


Fig. 1. TLC of purified and isolated glycoinositolphosphosphingolipids (basidiolipids) from different mushrooms. Bl, *Agaricus bisporus*; Av, *Amanita virosa*; Ce, *Calvatia exipuliformis*; Cc, *Cantharellus cibarius*; Le, *Lentinus edodes*; Ls, *Leccinum scabrum*; Po, *Pleurotus ostreatus*. The whole acidic extract of each mushroom is indicated by 'mix' and the isolated fractions in serial Arabic numerals. Running solvent was chloroform/methanol/0.3% aqueous $CaCl_2$, 45 : 45 : 10, (v/v). Visualization was performed at 110 °C for 15 min, using orcinol/H_2SO_4 spray reagent.

NMR spectroscopy

All NMR experiments were performed at a concentration of 5–8 mg per mL on a Bruker AMX500 spectrometer equipped with a pulsed field gradient (PFG) accessory and a triple resonance probe, under the conditions described previously [12]. The chemical shifts of the basidiolipids Ce-1, Le-1, Po-1, and Cc-1/–Man were identified by HMQC experiments. The assignment was performed by comparison of the very similar chemical shift pattern of the basidiolipids investigated here with that from B1-1 of *A. bisporus*.

The assignment of the released mannosyl-inositol (5 mg·mL^{-1}) after ammonolysis in D_2O at 298 K was performed according to the procedure described [12]. The proton chemical shift was referenced to internal D_2O (4.65 p.p.m.) and the carbon chemical shift in terms of the frequency ratios Ξ [27]. The ^{1}H-^{13}C coupling constants were determined from a coupled HMQC spectrum.

Additionally a 1D-HMQC spectrum for the determination of the ^{1}H-^{13}C coupling constant of mannosyl-Ins*P*Cer (Bl-1 from *A. bisporus*) was performed in d_6-dimethylsulfoxide/D_2O 98 : 2 v/v at 308 K.

RESULTS

Freeze dried mushrooms, collected from regional meadows and forests, or purchased on the market, were extracted with chloroform/methanol/water. The basidiolipids, present in the extracts, were separated by ion-exchange chromatography. The chromatographic properties of the newly isolated basidiolipids were comparable to those of the acidic basidiolipids previously isolated from the field mushroom, *A. bisporus* [12]. Separation into single compounds was achieved by purification and isolation using silica gel column chromatography. On TLC, the newly isolated basidiolipids of each of the mushroom species investigated, migrated with rates, grossly similar to the *A. bisporus* glycolipids (Fig. 1). Whereas, the TLC of G-Ins*P*Cer from *A. bisporus* showed four main components (designated Bl-1, Bl-2, Bl-3, Bl-4), the major glycolipid TLC bands of the mushrooms newly investigated numbered: *A. virosa* three bands, Av-1, Av-2, and Av-3, *C. exipuliformis* four bands, Ce-1, Ce-2, Ce-3, Ce-4, *C. cibarius* two double bands, Cc-1 and Cc-2, *L. edodes* three bands, Le-1, Le-2, Le-3, *L. testacio scabrum* one band, Ls-1, and *P. ostreatus* two bands, Po-1 and Po-2 (Fig. 1).

Based on their complete chemical constitution as shown below, not including the simplest mushroom glycolipid compound observed, i.e. Man-Ins-*P*O_4-Cer, the highest concentrations of basidiolipids were found in *A. virosa*, followed by *C. cibarius*. High levels, similar to those previously determined in *A. bisporus*, were observed in *C. exipuliformis*, *L. edodes*, *L. scabrum* and *P. ostreatus* (see later).

All mushroom glycolipids analyzed contained ceramide phosphate with t18:0-trihydroxysphinganine as the sole sphingoid base and a long chain α-hydroxy-fatty acid as lipophilic constituents.

Trihydroxysphinganine presence was determined by ESI-MS/MS in the respective isolated ceramide fractions, using the positive product ion mode. Fragments, which are typical for t18:0 trihydroxysphinganine with the masses of 318, 300, 282 and 264 (*m*/*z*) were detected, whereby, in contrast, a sphingenine containing ceramide standard led to the formation of the mass fragments of solely 264 and 282 (*m*/*z*) under the conditions choosen. Furthermore, in the sphingoid fraction of the total hydrolysate of any selected newly isolated basidiolipids, the mass of 318 (*m*/*z*), specific for trihydroxysphinganine could be detected by ESI-MS/MS in the positive ion mode, as well as in the positive precursor ion mode of 264 (*m*/*z*), characteristic for sphingolipids. Trihydroxysphinganine could also be analyzed chromatographically in a borate containing running solvent, whereby no other sphingoid base was detected (data not shown).

Fatty acids were determined by ESI-MS/MS in the negative product ion mode from the basidiolipid derived ceramides. In all newly isolated basidiolipids, only masses, which could be correlated with hydroxy-fatty acids were detected (Table 2). The majority of the fatty acids were C_{22}-OH:0, C_{24}-OH:0 and C_{24}-OH:1, whilst C_{24}-OH:0 was present in all basidiolipids investigated (Table 2). In addition, the basidiolipid ceramides of *C. cibarius* showed, besides the monohydroxy-fatty acids, the mass of a di-hydroxy fatty acid [C_{24}-$(OH)_2$:0].

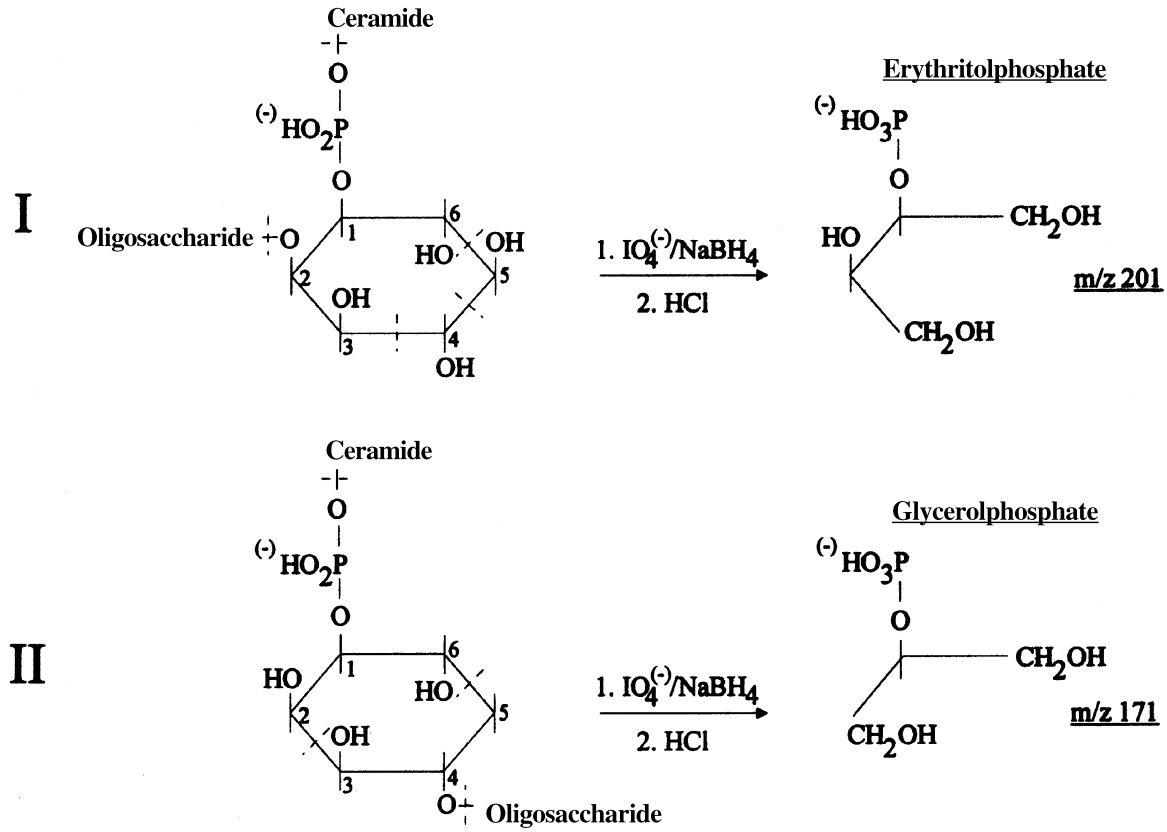
With the phosphate specific product ion *m*/*z* 79[PO_3^-] all G-Ins*P*Cer could be detected in the negative precursor ion mode of the ESI-MS/MS, demonstrating phosphate as a component of the G-Ins*P*Cer (Table 2). In confirmation of the mass spectrometry data, phosphorus was detected by

Table 2. Mass determination of ceramides and ceramide-fragments from basidiomycetes after HF cleavage [*m*/*z*] using ESI-MS/MS (top; Av-2a, Av-2b, Ce-1, Ce-2, Cc-1, Le-1, Le-3, Po-1, not determined) and mass determination of glyco-inositol-phospho-ceramides and their carbohydrate-fragments, isolated from basidiomycetes [*m*/*z*] using ESI-MS/MS (bottom).

Basidiomycetes components	Mass (a.m.u.) of the main ceramides[a] $[M + H^+]^+$	Mass (a.m.u.) of the fatty acid part[b] ($R\text{-}CO_2^-$) $[M - H^+]^-$	Mass correlation to fatty acids	Characteristic sphingosine fragments (% of basepeak) 264/282/300/318 (a.m.u.)[c]
Amanita virosa:				
AV-1	684	383	C_{24}OH:0	92/100/55/7
Av-3	684	383	C_{24}OH:0	61/100/39/4
Calvatia exipuliformis				
Ce-3	600/656/682/684	299/355/381/383	$C_{18/22/24}$OH:0, C_{24}OH:1	40/100/49/3
Ce-4	600/656/682/684	299/355/381/383	$C_{18/22/24}$OH:0, C_{24}OH:1	52/100/66/5
Cantharellus cibarius				
Cc-2	684/700	383/399	C_{24}OH:0, $C_{24}(OH)_2$:0	55/100/32/4
Lentinus edodes				
Le-2	684	383	C_{24}OH:0	50/100/49/<4
Leccinum scabrum				
Ls-1	656/682/684	355/381/383	$C_{22/24}$OH:0, C_{24}OH:1	68/100/42/4
Pleurotus ostreatus				
Po-2	656/682/684	355/381/383	$C_{22/24}$OH:0, C_{24}OH:1	52/100/57/3

Basidiomycetes components	Mass (a.m.u.) of the main G-Ins*P*Cer $[M - H^+]^-$	Mass (a.m.u.) of the corresponding glyco-inositol phosphate fragment[d] $[M - H^+]^-$	Precurson ion mode of 79 *m*/*z* for phosphate	Carbohydrate composition[e]
Amanita virosa				
AV-1	1556	891	1556	3H/D/I-P
Av-2a	1718	1053	1718	4H/D/I-P
Av-2b	1866	1199	1866	4H/2D/I-P
Av-3	2026	1361	2026	5H/2D/I-P
Calvatia exipuliformis				
Ce-1	1002/1058/1084/1086	421	1002/1058/1084/1086	H/I-P
Ce-2	1164/1220/1246/1248	583	1164/1220/1246/1248	2H/I-P
Ce-3	1326/1382/1408/1410	745	1326/1382/1408/1410	3H/I-P
Ce-4	1634/1690/1716/1718	1053	1634/1690/1716/1718	4H/D/I-P
Cantharellus cibarius				
Cc-1	1248/1264	583	1248/1264	2H/I-P
Cc-2	1410/1426	745	1410/1426	3H/I-P
Lentinus edodes				
Le-1	1086	421	1086	H/I-P
Le-2	1718	1053	1718	4H/D/I-P
Le-3	1880	1215	1880	5H/D/I-P
Leccinum scabrum				
Ls-1	1852/1878/1880	1215	1852/1878/1880	5H/D/I-P
Pleurotus ostreatus				
Po-1	1058/1084/1086	421	1058/1084/1086	H/I-P
Po-2	1690/1716/1718	1053	1690/1716/1718	4H/D/I-P

[a] Detection in the positive ion mode, as well as in the precursor ion mode of 264 (*m*/*z*), specific for sphingolipids (sphingoid-fragment). [b] Determination by negative product ion mode. [c] In contrast to the measured trihydroxysphinganines above, mass determination of characteristic sphingosine fragments (264/282/300/318) from a ceramide standard, containing sphingenine resulted in an intensity distribution of 100/6–13/0/0%, respectively. [d] Determination by collision-induced-dissociation (daughter scan) ESI-MS/MS, inositol and phosphate included. [e] H, Hexose; D, Deoxyhexose; I, Inositol; P, Phosphate.



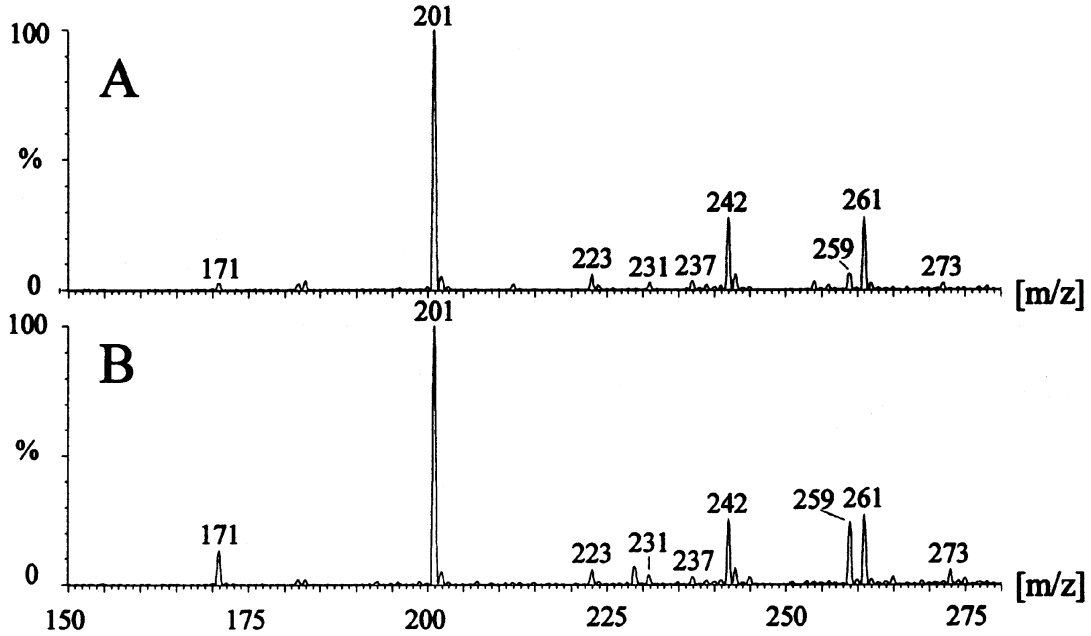


Fig. 2. Fragmentation scheme of sodium periodate treated G-Ins*P*Cer and mass determination of the isolated fractions. I, fragmentation scheme of oligosaccharide-2Ins1-P-Cer linked components; II, fragmentation scheme of oligosaccharide-4Ins1-P-Cer linked components. (A) mass determination of the inositol-phosphate fragments from component Po-2 after IO_4^- treatment, exemplary for a low amount glycerolphosphate (*m/z* 171) containing G-Ins*P*Cer. (B) mass determination of the inositol-phosphate fragments of component Ce-4 after IO_4^- treatment, exemplary for a moderate amount glycerolphosphate (*m/z* 171) containing G-Ins*P*Cer. (A and B were measured in precursor ion mode of 79, specific for phosphate); *m/z* 171 glycerolphosphate; *m/z* 201, erythritolphosphate; *m/z* 261, 259, 242, 231, peaks of non or incompletely periodate-cleaved inositolphosphate residues, i.e. *m/z* 259 noncleaved-, *m/z* 261 one time cleaved-, *m/z* 242, noncleaved [-H_2O]-, and *m/z* 231, twice cleaved (C_3-C_4-C_5- or C_4-C_5-C_6-cleaved) residues of inositolphosphate. For IO_4^- cleavage conditions, see [12].

Table 3. ESI-MS/MS determination of the phosphoinositol fragment of basidiolipids after sodium periodate cleavage, reduction and hydrolysis [12] (see also, Fig. 2). Detection in the precursor ion mode with *m/z* −79, specific for phosphate[a]. +, detectable amounts, no percentage could be given due to the relatively high background.

	Percentage of base peak	
Component	Erythritol-phosphate[b] corresponding to R-2Ins1-P (*m/z* 201)	Glycerol-phosphate[b] corresponding to R-4Ins1-P (*m/z* 171)
Av-1	100	+
Av-3	100	7
Ce-1	100	11
Ce-4	100	13
Cc-2	100	11
Le-1	100	7
Le-2	100	20
Ls-1	100	6
Po-1	100	< 4
Po-2	100	< 4

[a] The specific precursor ion mode for phosphate was chosen because it is more selective than the normal MS spectrum. The detection of nonspecific background noise, which usually appear in this low mass range thus can be avoided. [b] In addition, the appearance of erythritol and glycerol was confirmed chromatographically after HF-cleavage of the respective inositol-phosphate fragment as described in Materials and methods and Results.

colorimetry in all glycolipids investigated after cleavage of the phosphodiester using HF (data not shown).

As has been shown previously in the case of the *Agaricales* basidiolipids by NMR spectroscopy and periodate oxidation, linkage of phosphate and of the oligosaccharide reducing end β-mannoside to myo-inositol was in its 1- and 2-position, respectively [12]. After oxidation with periodate and reduction of the products with borohydride, the masses of the resulting inositol-phosphate fragments of compounds Av-1, Av-3, Ce-1, Ce-4, Cc-2, Le-1, Le-2, Ls-1, Po-1 and Po-2 were characterized by ESI-MS/MS in the precursor ion mode of 79 (*m/z*), specific for phosphate. In each sample the ion 201 (*m/z*) was detected as base peak. This ion was expected for a 4-carbon inositol fragment. In addition, a small peak at 171 (*m/z*), reflecting the presence of a 3-carbon inositol fragment linked to phosphorus, was obtained (Table 3). An example for a low-(Po-2) and moderate (Ce-4) content of fragment 171 (*m/z*), specific for a R-4Ins1-P-Cer linkage, as well as the periodate fragmentation schemes for R-2Ins1-P-Cer and R-4Ins1-P-Cer are shown in Fig. 2.

This result could be confirmed chromatographically, after hydrolysis of the phospho-inositol fragment by HF treatment. All samples investigated in this way, showed erythritol as the remaining inositol periodate-oxidation/borohydride reduction fragment. This is indicative for a divalent substitution of the inositol in 1,2-positions. However, in some of the newly isolated glycolipids, periodate oxidation/borohydride reduction of the substituted inositol yielded, in addition to a major portion of erythritol, substantial amounts of glycerol in agreement with the ESI-MS/MS fragment of −171 (*m/z*) (data not shown). This result shows the additional occurrence of inositol substitution in 1- and 4-position. Occurrence of this type of inositol substitution, besides compounds with linkage in 1- and 2-position, was observed for glycolipids of *A. virosa*, *C. exipuliformis*, *C. cibarius*, *L. edodes*, *L. scabrum* and *P. ostreatus* (data not shown). A tendency was also recognized that the proportion of 1-,4-substituted inositol as compared to the 1-,2-linkage in G-Ins*P*Cer of different mushrooms increased with the size of its carbohydrate portion (data not shown).

Determination of the anomeric configuration between mannose and inositol by NMR -

The proton and carbon chemical shifts of Ce-1, Cc-1/–Manα, Le-1 and Po-1 correlate well with that of Bl-1 (from *Agaricus bisporus*) (Table 4), respectively. This indicates the close structural relation among each other.

The previous investigation of the anomeric configuration of mannosyl-Ins*P*Cer was performed in dimethylsulfoxide-d_6/2% D_2O at 353 K [12]. In order to establish independent criteria for this assignment, the mannosyl-inositol fragment of Bl-1 released by ammonolysis was reinvestigated by NMR spectroscopy in D_2O at 298 K (Table 4).

The anomeric resonance of mannose was observed at 4.97 p.p.m. which is in the region expected for α-Man*p* residues under the conditions used. Furthermore, the $^3J_{1,2}$ coupling constant was found to be in the range of 1.7–2.1 Hz, which is clearly within the limits observed for the α-configuration of mannosyl residues. For β-Man*p* the $^3J_{1,2}$ coupling constants and the chemical shift values are typically in the range of 0.9 Hz and 4.7–4.8 p.p.m., respectively.

The magnitude of the $^1J_{H1,C1}$ coupling constant, 173 Hz, provided further evidence for the α-anomeric configuration (empirical results for β-hexopyranose ≈ 160–165 Hz, for α-hexopyranose 170–175 Hz [28]. A very similar value of 175 Hz was found for Man-Ins*P*Cer in dimethylsulfoxide/D_2O at 308 K.

The NOESY spectrum of the released mannosyl-inositol fragment in D_2O at 298K, only the dipolar correlations between mannose H-1/H-2 and mannose H-1 and inositol H-2 could be observed (Fig. 3). This result is consistent with a configurational assignment of α-, rather than β-, for the mannose residue, in agreement with the α-anomeric configuration obtained from the chemical shift values and coupling constants.

Determination of the carbohydrate constitution

The mushroom glycolipids revealed novel oligosaccharide structures that reflected specific species dependent variations (carbohydrate constituent analysis, methylation analysis (Table 5), proposed chemical structures (Table 6).

Basidiolipids of *Amanita virosa*

Compound Av-1, with a mass spectrometrically determined molecular mass of 1556 (Table 2) contains inositol, mannose, galactose and fucose in a molar ratio of 1 : 1 : 2 : 1, as obtained from carbohydrate constituent analysis.

Table 4. Chemical shifts of basidiolipids Ce-1, Le-1, Po-1 and Cc-1(after α-mannosidase treatment), compared to Bl-1 from *Agaricus bisporus* in Me_2SO-d_6 at 353 K (top) and ^1H, ^{13}C, chemical shifts of mannosyl-inositol of basidiolipid (bottom).

	Mushroom component									
	Bl-1		Ce-1		Le-1		Po-1		Cc-1/–Manα[a]	
Reference to carbon atoms	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
Ins 1	75.53	3.83	75.31	3.76	75.27	3.74	75.32	3.76		
Ins 2	77.95	4.03	78.01	3.97	78.10	3.97	77.98	3.97	78.10	3.97
Ins 3	70.44	3.26	70.49	3.21	70.46	3.22	70.60	3.22		
Ins 4	72.16	3.38	72.09	3.37	72.11	3.37	72.08	3.37	72.14	3.37
Ins 5	75.32	3.00	75.58	2.98	75.44	2.97	75.75	2.97	75.89	2.97
Ins 6	72.11	3.53	72.25	3.52	72.37	3.51	72.33	3.52		
Man 1	100.40	5.04	100.41	5.04	100.53	5.04	100.50	5.04	100.40	5.04
Man 2	70.50	3.73	70.10	3.73	70.21	3.73	70.24	3.73	70.23	3.73
Man 3	70.61	3.56	70.57	3.56	70.54	3.55	70.69	3.56	70.65	3.55
Man 4	66.83	3.48	66.88	3.48	67.02	3.48	66.85	3.48		
Man 5	72.54	3.86	72.48	3.85	72.53	3.83	72.46	3.84	72.50	3.83
Man 6	61.06	3.50/3.63	61.09	3.49/3.63	61.11	3.49/3.61	61.13	3.49/3.63		
Sphingosine 1	64.06	4.06/3.81	63.69	4.08/3.69	63.60	4.08/3.67	63.72	4.08/3.69		
Sphingosine 2	50.42	3.97	50.55	3.88	50.42	3.88	50.55	3.88		
Sphingosine 3	73.27	3.45	73.88	3.46	73.03	3.43	72.88	3.45		
Sphingosine 4	70.61	3.40	70.57	3.40	70.54	3.39	70.60	3.41	70.65	3.40

Reference to carbon atoms	^1H	^{13}C
Mannose		
1	4.97	102.1
2	3.94	70.8
3	3.70	70.97
4	3.53	67.2
5	3.86	73.3
6	3.69/3.62	61.4
Inositol		
1	3.96	80.6
2/6	3.41/3.47	70.9/72.3
3/5	3.46/3.50	73.2/73.2
4	3.11	75.43

[a] Chemical shifts of the mannosidase cleaved CC-1 product could only be partly determined, because of the limited amount of less than 1 mg.

Av-1 was sensitive to the sequential treatment with exoglycosidases in the order of α-galactosidase, α-fucosidase and β-galactosidase (data not shown). The final product of the enzymatic degradation contained mannose linked to inositol as determined after total hydrolysis with mineral acid (data not shown). These results establish the monosaccharide sequence in the oligosaccharide chain of Av-1 as Galα-(Fucα-)Galβ-Man-Ins.

Methylation analysis of the product of the Av-1 degradation by α-galactosidase, as compared to the original compound, showed the appearance of galactose, mono-substituted in position 2, whereby, the di-substituted galactose, observed in methylation analysis of intact Av-1, had disappeared (Table 5). The fucose residue of Av-1 must therefore be linked in position 2, and the terminal galactose in position 3 of the subterminal galactose. In addition, the subterminal β-galactosidase-sensitive galactose residue must be linked to the inositol-bound mannose in position 6 (Table 5). This suggests for Av-1 the structure of a Galpα-3(Fucpα-2)Galpβ-6Manp(α)-2/(4)Ins1-phospho-ceramide (Table 6).

Compound Av-3, with a mass spectrometrically determined molecular mass of 2026 (Table 2), contains inositol, mannose, galactose and fucose in a mol proportion of 1 : 1 : 4 : 2, as shown by carbohydrate constituent analysis. Av-3 was cleaved by α-galactosidase with the concomitant loss of two galactose residues. This was assumed from the similarly fast migration rate of the Av-3/α-galactosidase product (data not shown). Treatment of this product with α-fucosidase resulted in the appearance of a TLC double band with a migration rate that was for both bands slightly faster as compared to that of the precursor

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

	Peak ratios[b] of component											
Alditol acetate[a]	Av-1	Av-3	Ce-2	Ce-3	Ce-4	Cc-1	Cc-2	Le-2	Le-3	Ls-1	Po-2	Linkage
2,3,4,6-ManOH	–	–	–	–	–	1.00[c]	1.14	0.86	0.94	0.89	–	Man(1-
2,3,4-ManOH	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]	0.59	–	1.00[c]	1.00[c]	1.00[c]	1.00[c]	−6)Man(1-
2,4,6-ManOH	–	–	–	–	–	0.76	–	–	–	–	–	−3)Man(1-
3,4,6-ManOH	–	–	–	–	–	–	–	–	1.02	–	–	−2)Man(1-
2,4-ManOH	–	–	–	–	–	–	1.00[c]	–	–	–	–	−3,-6)Man(1-
2,3,4,6-GalOH	0.88	1.71	0.84	(0.32)[d]	2.30	–	–	–	–	1.04	1.42	Gal(1-
2,3,4-GalOH	–	–	–	1.01	(1.12)[d]	–	–	1.15	1.06	0.83	–	−6)Gal(1-
3,4-GalOH	–	–	–	–	–	–	–	–	–	1.32	–	−2,-6)Gal(1-
4,6-GalOH	0.97	0.94	–	–	–	–	–	1.00	0.91	–	–	−2,-3-)Gal(1-
4-GalOH	–	1.07	–	–	0.66	–	–	–	–	–	0.92	−2,-3,-6)Gal(1-
2,3,4,6-Glc	–	–	–	0.74	–	–	–	–	–	–	–	Glc(1-
2,3,4-FucOH	0.63	1.59	–	–	0.91	–	–	0.56	0.83	(< 0.06)	0.69	Fuc(1-
2,4-FucOH	–	–	–	–	–	–	–	–	–	0.49	–	−3)Fuc(1-

	Peak ratios[b] of component								
Alditol acetate[a]	Av-1 (-Galα)	Av-3 (-2Galα)	Ce-4 (-2Galα)	Le-2 (–Manα)	Le-2 (–Manα/-Galα)	Ls-1 (-Galα)	Ls-1 (–Manα)	Po-2 (-2Galα)	Linkage
2,3,4-ManOH	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]	1.00[c]	−6)Man(1–
2,3,4,6-ManOH	–	–	–	–	–	0.54	–	–	Man(1–
2,3,4,6-GalOH	–	–	–	0.37	–	–	0.65	–	Gal(1–
2,3,4-GalOH	–	–	–	–	–	1.02	0.90	–	−6)Gal(1–
3,4,6-GalOH	0.83	1.04	0.99	–	0.95	0.93	–	0.80	−2)Gal(1–
4,6-GalOH	–	–	–	0.92	–	–	–	–	−2,-3)Gal(1–
3,4-GalOH	–	1.31	–	–	–	–	1.39	–	−6,-2)Gal(1–
2,3,4-FucOH	1.39	1.82	+	0.15	0.77	< 0.02	0.54	0.42	Fuc(1–
2,4-FucOH	–	–	–	–	–	0.81	–	–	−3)Fuc(1–

[a] 2,3,4-FucOH, etc. = 2,3,4-tri-*O*-methyl-L-fucitol, etc. [b] Values obtained by combined GC/MS. [c] set to 1.0. [d] Contaminant.

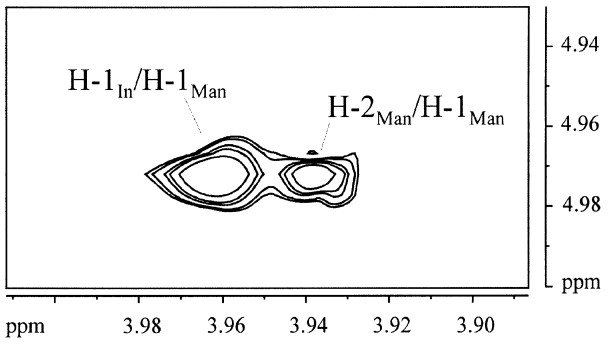


Fig. 3. Part of a 2D 500-MHz NOESY spectrum of the released mannosyl-inositol of basidiolipid Bl-1 after ammonolysis in D_2O at 298 K. Shown are all detectable NOE cross-peaks of the anomeric proton. The two cross-peaks between H-2_{Man}/H-1_{Man} and H-1_{In}/H-1_{Man} are in agreement with an α-anomeric configuration of the mannosyl residue. Cross-peaks between H-3_{Man}/H-1_{Man} and H-5_{Man}/H-1_{Man} could not be observed.

molecule (data not shown). After further treatment with α-galactosidase, only the upper band of the TLC double band disappeared. The product of the enymatic cleavage, migrating as the remaining lower band, when treated again, first with a larger amount of α-fucosidase followed by α-galactosidase, resulted in the formation of the same product, as that obtained from the upper band described before. This observation is taken as evidence that Av-3 contains of two fucose residues, a fact that is consistent with the carbohydrate constituent analysis, and the fact that a third α-linked galactose of Av-3 can only enzymatically be cleaved from the core molecule after the previous removal of both fucose residues. The Av-3/-2Galα/-2Fucα/-Galα product was further hydrolyzed with β-galactosidase, and, after total hydrolysis of the ensuing product, the remaining inositol-linked sugar, determined as mannose.

The linkage positions of the sugar chain monosaccharides and the ramified fucoses could be specified by methylation analysis of intact Av-3, as well as, the Av-3/-2Galα product (Table 5). In this analysis, a monomethylated galactitol derivative was regognized amongst the permethylation products. After methylation of the

Table 6. Structures of glycoinositolphosphosphingolipids, basidiolipids, from different mushrooms. For ceramide constitution, see Table 2.

Mushroom Component	Dried mushroom ($mg \cdot g^{-1}$)	Structure
Amanita virosa		
Av-1	1.67	Gal*p*α-3(Fuc*p*α-2)Gal*p*β-6Man*p*(α)-2/(4)Ins1-*P*O_4-Cer
Av-2a/b Mix:		
Av-2a		Av-1/+Galα
Av-2b	1.31	Av-3/-Galα
Av-3	1.10	Gal*p*α-3(Fuc*p*α-2)(Fuc*p*α-6)Gal*p*α-2(Gal*p*α-3)Gal*p*β-6Man*p*(α)-2/(4)Ins1-*P*O_4-Cer
Calvatia exipuliformis		
Ce-1	1.21	Man*p*α-2/(4)Ins1-*P*O_4-Cer
Ce-2	0.27	Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Ce-3	0.44	Glc*p*α-6Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Ce-4, main component	0.32	Gal*p*α-3(Gal*p*α-6)(Fuc*p*α-2)Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Cantharellus cibarius		
Cc-1	1.03	Man*p*α-3 or − 6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Cc-2	1.97	Man*p*α-3(Man*p*α-6)Man*p*α-2/(4)Ins1-*P*O_4-Cer
Lentinus edodes		
Le-1	1.35	Man*p*α-2/(4)Ins1-*P*O_4-Cer
Le-2	0.37	Man*p*α-6Gal*p*α-3(Fuc*p*α-2)Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Le-3	0.22	Man*p*α-2Man*p*α-6Gal*p*α-3(Fuc*p*α-2)Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer
Leccinum scabrum		
Ls-1	1.41	Man*p*α-3Fuc*p*α-2(Gal*p*α-6)Gal*p*α-6Gal*p*β-6Man*p*(α)-2/(4)Ins1-*P*O_4-Cer
Pleurotus ostreatus		
Po-1	3.33	Man*p*α-2/(4)Ins1-*P*O_4-Cer
Po-2	1.41	Gal*p*α-3(Gal*p*α-6)(Fuc*p*α-2)Gal*p*β-6Man*p*α-2/(4)Ins1-*P*O_4-Cer

original compound, hydrolysis and reduction using $NaBD_4$, the triple-substituted galactose residue was substantiated by mass spectrometry, to represent a 2,3,6-substituted galactose. From these results, the chemical constitution of Av-3 is deduced as Galpα-3(Fucpα-2)(Fucpα-6)Galpα-2(Galpα-3)Galpβ-6Manp(α)-2/(4)Ins1-phospho-ceramide (Table 6).

Compound Av-2comp. consists of two different molecular species, designated Av-2a and Av-2b, that were not separated during the isolation procedure. The corresponding masses were for Av-2a and Av-2b, 1718 and 1866, respectively (Table 2). The mass difference of 146 (*m*/*z*) between these two compounds must originate from differences in the carbohydrate moiety, and may correspond to one deoxyhexose. After α-galactosidase treatment, both glycolipids were degraded (data not shown). One of the two resulting products, designated Av-2b/-Galα, migrated on TLC comparable to Av-3/-2Galα, i.e. Fucα-2(Fucα-6)Galα-2Galβ-6Manα- (see above). The other α-galactosidase cleavage product, designated Av-2a/-2Galα, with a probable loss of two α-galactose residues from the native compound, migrated on TLC, faster than Av-2b/-Galα, and at a migration rate similar to the α-galactosidase product from Av-1, i.e. Fucα-2Galβ-6Manα-.

For further characterization, each of the two Av-2a/b α-galactosidase cleavage products was isolated by silica gel column chromatography, and the isolated compounds were treated further with exoglycosidases.

Component Av-2a/-2Galα could be degraded using the same enzymes as for the cleavage of Av-1/-Galα, i.e. consecutively by α-fucosidase followed by β-galactosidase. Based on these enzymatic cleavage results, the structure of Av-2a, most likely corresponds to Av-1 plus one additional α-galactose residue.

Compound Av-2b/-Galα could be degraded with exoglycosidases in an analogous way to Av-3/-2Galα. The structure of component Av-2b therefore is assumed to correspond to an Av-3/-Galα. Carbohydrate constituent and permethylation analyses were not performed with the Av-2a/b compound mixture, because of their inseparability with the methods available.

Basidiolipids of *Calvatia exipuliformis*

Compound Ce-1 showed a migration rate on TLC comparable to Bl-1 (Fig. 1). ESI-MS/MS daughter scan resulted in a mass fragment of 421 (*m*/*z*), which is typical for Man-Ins-*P*O_4 in the negative ion mode (Table 2). After hydrolysis with mineral acid, only mannose was analyzed as inositol bound sugar (data not shown). For Ce-1, Manα-Ins-Phos-Cer, the linkage of mannose to the 2-position of inositol was verified by NMR, showing similar chemical shifts for their ^1H and ^{13}C atoms as those of compound Bl-1 from *A. bisporus* (Table 4). Glycerol formation, besides erythritol, after periodate oxidation of Ce-1, suggests the presence of mannose-inositol-phosphate linkages as Man-2Ins1-Phos, and, to a minor extent, Man-4Ins1-Phos. The structure of Ce-1, therefore is that of a Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Ce-2, with a mass spectrometrically determined molecular mass of its glyco-inositol-phosphate fragment of 583 (Table 2), contains inositol, mannose, and galactose in a molecular ratio of 1 : 1 : 1, as shown by

carbohydrate constituent analysis. Ce-2 therefore is distinguished from Ce-1 by one additional hexose residue, i.e. galactose. The linkage of this galactose in 6-position of a subterminal mannose could be deduced from methylation analysis (Table 5). The anomery of the linkage between galactose and mannose was specified as β, by using the appropriate enzyme. In the total hydrolysate of the resulting enzyme-product, mannose could be analyzed as the sole sugar after total hydrolysis (data not shown). The structure of Ce-2 therefore is given as Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6)

Compound Ce-3, with a mass spectrometrically determined molecular mass of its glyco-inositol-phosphate fragment of 745 (Table 2), contains inositol, mannose, galactose, and glucose, in a molecular ratio of 1 : 1 : 1 : 1, as shown by carbohydrate constituent analysis. Ce-3 therefore is distinguished from Ce-2 by one additional hexose residue, i.e. glucose. The latter hexose was determined as α-anomerically linked in 6-position to the mannose-bound galactose of Ce-2 (data not shown). Therefore, the Ce-3 oligosaccharide has the structure Glcpα-6Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Ce-4, by using ESI-MS/MS in the negative-ion collision-induced-dissociation mode, showed a unique mass of 1053 (*m*/*z*) for its glyco-inositol-phosphate fragment (Table 2). This value exceeds the corresponding mass of Ce-3 by the equivalent of one hexose and deoxyhexose (Table 2). Carbohydrate constituent analysis revealed the presence of inositol, mannose, galactose, and fucose, in the mol ratio of 1 : 1 : 3 : 1.

The TLC-fraction Ce-4 could not be completely cleaved by α-galactosidase treatment, thereby showing its composite nature as a mixture of compounds. However, one major α-galactosidase hydrolysis product was obtained. This product had a TLC migration rate, as compared to the parent compound, that corresponded to a loss of two galactose residues. This fragment could be cleaved further by sequential treatment with α-fucosidase and β-galactosidase. The remaining inositol-bound carbohydrate was identified as mannose (data not shown). The sequence and anomeric glycosides are thus described to be $(Gal\alpha\text{-})_2$-(Fucα-)Galβ-Man-.

The sugar linkages of Ce-4 compound could not unequivocally be established by methylation analysis, because of the presence of glycolipid with a second different carbohydrate headgroup. Nevertheless, the linkage of the branched-chain fucose of the main α-galactosidase product, Ce-4/-2Galα, was shown by methylation analysis, to be in the 2-position of the terminal galactose, Fucα-2Galβ- (Table 5). The methylation analysis data for Ce-4 are in agreement with a proposed structure of the major compound as Galpα3(Galpα6)(Fucpα-2)Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6). The unexplained additional occurrence of 2,3,4-Me_3-galactitol amongst the permethylation products from Ce-4 may suggest an admixture of a Fucα-6Galα-6Galα-6Galβ-6Manα-2(4)Ins1-phospho-ceramide in this G-Ins*P*Cer fraction.

Basidiolipids of *Cantharellus cibarius*

Compound Cc-1 with a TLC migration rate much slower than *A. bisporus* Bl-1 [Man-Ins-Phos-Cer], revealed solely the presence of mannose as its carbohydrate constituent. Whereas after acid hydrolysis of Cc-1, a ratio of 1 : 3 for inositol and mannose was obtained by GC-analysis, the determination of the glyco-inositol-phosphate-residue by ESI-MS/MS resulted in a mass fragment 583 (*m*/*z*), characteristic for only two hexoses (Table 2). After enzyme treatment of Cc-1 with α-mannosidase, the product migrated on TLC in a comparable position to Bl-1, again showing mannose as its sole sugar (data not shown). Methylation analysis was suggestive of the existence of two different linkages for the terminal mannose, i.e. Manα1–3Man-, as well as, Manα1–6Man- (Table 5). NMR-analysis of the α-mannosidase cleavage product, Cc-1/–Manα, showed the same chemical shifts of decisive ^{1}H and ^{13}C atoms of mannose and inositol, as component Bl-1 from *A. bisporus* (Table 4). Accordingly, the mannose of Cc-1/–Manα will be linked to the inositol by a α-glycoside. The carbohydrate structures of the two G-Ins*P*Cer in Cc-1 therefore may be identified with Manpα-3/6Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Cc-2, as compared to Cc-1, consists of one additional mannose residue. Methylation analysis showed the presence only of terminal mannose and 3-,6-disubstituted mannose in Cc-2 glycolipid (Table 5). However, differently from Cc-1, we may assume, the inositol-linked mannose to be substituted by two mannose residues, one in 3-, the other in 6-position. Thus, the resulting structure of Cc-2 is (Manpα-3)(Manpα-6)Manpα-2(4)Ins1-phospho-ceramide (Table 6). Glycerol formation, besides erythritol, after periodate oxidation of Cc-2, in conjunction with the NMR data, similar to Ce-1, suggests the presence of mannose-inositol-phosphate linkages as Man-2Ins1-Phos, and, to a minor extent, Man-4Ins1-Phos.

Basidiolipids of *Lentinus edodes*

Compound Le-1, as shown by TLC (Fig. 1) ESI-MS/MS (Table 2), acid hydrolysis, NMR (Table 4), as well as, periodate cleavage is identical to Ce-1 (Table 3), i.e. Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Le-2 oligosaccharide constists of inositol, mannose, galactose, and fucose in the ratio of 1 : 2 : 2 : 1. By ESI-MS/MS, using the negative collision-induced-dissociation mode, for the glyco-inositol-phosphate-fragment, a mass of 1053 (*m*/*z*) was measured. This value corresponds to a mass of four hexoses, one deoxyhexose, inositol and phosphate (Table 2). A mass spectrum of component Le-2, as well as, its fragment ion- and precursor ion spectra, exemplary for all other investigated G-Ins*P*Cers are shown in Fig. 4. Le-2 could be sequentially cleaved with α-mannosidase, α-galactosidase, α-fucosidase and β-galactosidase. The enzyme resistant, inositol-bound carbohydrate was identified as mannose after total hydrolysis of the enzyme resistant glycolipid end product with mineral acid and TLC (data not shown). The linkages of the carbohydrate constituents, including the ramified fucose were determined by methylation analysis of Le-2, as well as, of its α-mannosidase and α-mannosidase/α-galactosidase products (Table 5). The structure of Le-2 oligosaccharide may thus be suggested as Manpα-6Galpα-3(Fucpα-2)Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Le-3 is distinguished from Le-2 by the presence of one additional α-mannose residue linked in the

2-position to the terminal mannose of the parent compound for comparison, (see Table 5). All the other steps of carbohydrate analysis, including mass determinations (Table 2), carbohydrate constituent analysis, exoglycosidase cleavage, and identification of the inositol-bound carbohydrate as mannose, confirmed the structural relation of Le-2 and Le-3. The latter compound therefore has the structure of Manpα-2Manpα-6Galpα-3(Fucpα-2)Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Basidiolipids of *Leccinum scabrum*

Compound Ls-1 carbohydrate constituent analysis revealed a molar ratio for inositol, mannose, galactose, and fucose of 1 : 2 : 3 : 1. These values are consistent with the measured mass of the glycosyl-inositol-phosphate fragment of 1215 (Table 2). A first hexose residue of Ls-1 could be released either by α-mannosidase or α-galactosidase, an indication of their terminal positions (data not shown). The methylation analysis of the α-galactosidase-treated compound, Ls-1/-Galα, revealed, that the 2-,6-double-substituted galactose residue of the original component Ls-1, had disappeared (Table 5). Instead, a new, in 2-position single-substituted galactose could be found (Table 5). This observation indicates, that the terminal α-galactose of Ls-1 must be linked to the 6-position, and the fucose residue in position 2 of subterminal galactose. In addition, methylation analysis of Ls-1 yielded a product with pseudomolecular masses ($[M + H]^+$, $[M + NH_4]^+$) of 321/338 (*m*/*z*), further characterized by electron impact mass spectrometry as fucose substituted in 3-position. Furthermore, the methylation analysis of the glycolipid product of Ls-1 treated with α-mannosidase, Ls-1/–Manα, indicated the existence of fucose now in terminal position, whereas the previously terminal mannose of Ls-1 had disappeared (Table 5). It is therefore suggested that the terminal mannose residue of Ls-1 is linked in 3-position to subterminal fucose, which itself is linked in 2-position of the di-substituted galactose. The glycolipid obtained after α-galactosidase and α-mannosidase treatment of Ls-1, i.e. Ls-1/-Galα/–Manα, could only further enzymatically be hydrolyzed by α-fucosidase (data not shown). The resulting component was cleaved sequentially using α-galactosidase and β-galactosidase (data not shown). The inositol linked sugar was determined chromatographically after hydrolysis of the final glycolipid product of the glycosidase treatment by mineral acid as mannose (data not shown).

The results, taken together, are suggestive of a structure of Ls-1 as Manpα-3Fucpα-2(Galpα-6)Galpα-6Galpβ-6Manp(α)-2(4)Ins1-phospho-ceramide (Table 6).

Basidiolipids of *Pleurotus ostreatus*

Compound Po-1, similar to Le-1 from *Lentinus edodes*, Po-1 was characterized by TLC (Fig. 1), ESI-MS/MS, hydrolysis under acidic conditions, NMR, and the products of its periodate degradation to be identical to Ce-1, i.e. Manpα-2(4)Ins1-phospho-ceramide (Table 6).

Compound Po-2 inositol-oligosaccharide consisted of inositol, mannose, galactose, and fucose in the mol ratio of 1 : 1 : 3 : 1. By ESI-MS/MS, using the negative collision-induced-dissociation mode, for the glyco-inositol-phosphate-fragment of Po-2, a mass of 1053 (*m*/*z*) was measured. This value corresponds to a mass of four hexoses, one deoxyhexose, inositol and phosphate (Table 2). Po-2 was cleaved by α-galactosidase with the appearance of a glycolipid product. As compared to the parent Po-2, the newly formed glycolipid migrated on TLC with a rate corresponding to the loss of two hexose residues. The enzyme degradation of Po-2/-2Galα could be continued with α-fucosidase and, furthermore, β-galactosidase. Finally, the inositol bound carbohydrate was determined as mannose (all data not shown). Methylation analysis of Po-2 revealed one mono-methylated hexose unit, which was attributed to a triple-substituted hexose residue, and, in addition, three terminal monosaccharides, two galactoses and one fucose (Table 5). After α-galactosidase treatment of the parent Po-2, methylation analysis of the product showed merely a mono-substituted galactose, indicating, that the two terminal galactose residues must have been linked to the former triple-substituted mono-saccharide. The latter must have consisted of galactose, as result of the carbohydrate constituent analysis. The linkage of the fucose was determined by methylation analysis of the product to be in 2-position of the triple-substituted galactose residue (Table 5). Furthermore, the existence of the 2,3,6-triple-substituted galactose residue was further substantiated by characterization of the peracetylated 4-mono-O-Me-galactitol, obtained from Po-2/-2Galα after permethylation, hydrolysis and reductamination with $NaBD_4$, by electron impact mass spectrometry. The data, taken together, suggest for Po-2 an oligosaccharide structure that corresponds to the main component of Ce-4, i.e. Galpα-3(Galpα-6)(Fucpα-2)Galpβ-6Manpα-2(4)Ins1-phospho-ceramide (Table 6).

DISCUSSION

Several recent studies have described mushrooms as a source of biological response modifiers. Polysaccharide preparations, obtained from higher mushrooms, effectively augmented alloreactive T-lymphocytes and stimulated cytokine gene expression accompanied by proliferation of human T-lymphocytes [7,29]. In addition, fungal glycolipids also may effect immune responses. It was demonstrated for lower fungi G-Ins*P*Cer that they could stimulate the thymus-dependent formation of IgG antibodies, a result of their CD1-dependent presentation by antigen-presenting cells [30] (reviewed in [31,32]). Basidiolipids, i.e. G-Ins*P*Cer components of a filamentous fungus, the basidiomycete *A. bisporus*, were shown to display immune adjuvant properties in mice [13]. In order to elucidate the prerequisites of chemical structures with regard to the capacity to stimulate immune mechanisms, as well as, to observe taxonomical variabilities, the G-Ins*P*Cer of six randomly chosen mushroom species were investigated.

As verified by the above described analysis techniques, all basidiolipids are based on a common mannose-inositol-phospho-ceramide root structure. With regard to their ceramide constitution, the sole occurrence of trihydroxy-sphinganine as sphingoid base, and the preponderance of hydroxylated fatty acids may reflect a requirement for basiolipid-containing membrane stabilization under physico-chemical stress situations. The notable presence of a dihydroxy-fatty acid in ceramides of chanterelle basidiolipids probably explains the pronounced TLC double band

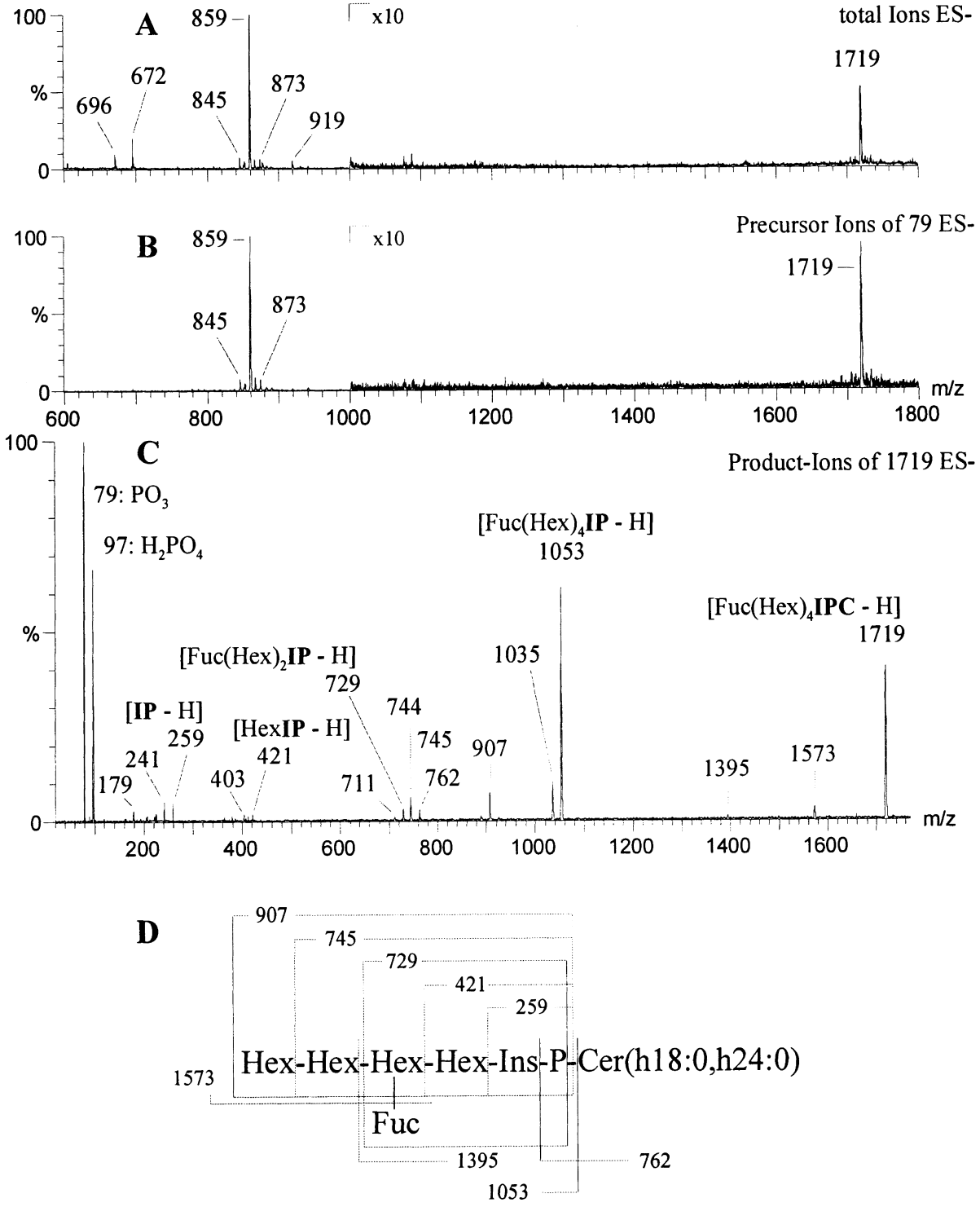


Fig. 4. ESI-MS/MS-spectra of Le-2. (A) Negative total ions; (B) negative precursor-ions of *m/z* 79, a fragment specific for phosphate-containing substances; *m/z* 1719 [Le-2(h18:0,h24:0)-H^+]$^-$, *m/z* 859 [Le-2(h18:0,h24:0)-$2H^+$]$^{2-}$. (C) Negative product-ions of *m/z* 1719: *m/z* 79 [PO_3]$^-$, *m/z* 97 [H_2PO_4]$^-$, *m/z* 179 [Hex or I–H^+]$^-$, *m/z* 241[259 − H_2O^+]$^-$, *m/z* 259 [IP − H^+]$^-$, *m/z* 403[421 − H_2O]$^-$, *m/z* 421 [HexIP − H^+]$^-$, *m/z* 711[729 − H_2O]$^-$, *m/z* 729 [Fuc(Hex)$_2$IP − H^+]$^-$, *m/z* 744[762 − H_2O]$^-$, *m/z* 745 [(Hex)$_3$IP − H +]$^-$, *m/z* 762 [Ins*P*Cer − H +]$^-$, *m/z* 907 [(Hex)$_4$IP − H^+]$^-$, *m/z* 1035 [Fuc(Hex)$_4$IP − H_2O]$^-$, *m/z* 1053 [Fuc(Hex)$_4$IP − H^+]$^-$, *m/z* 1395 [Fuc(Hex)$_2$Ins*P*Cer − H^+]$^-$, *m/z* 1573 [(Hex)$_4$Ins*P*Cer − H^+]$^-$; I : Inositol, P : Phosphate, C: Ceramide (h18:0,h24:0). (D) Fragmentation scheme.

formation, typical for glycolipids of this mushroom species (Fig. 1, Table 2). In contrast, TLC double band formation of those mushroom fractions containing only mono-hydroxy- or unsaturated fatty acids was much less pronounced.

In the case of the major portion of the basidiolipids, substitution of inositol by phosphate and by mannose was in 1- and 2-position, respectively. The same sequence of constituents was described previously for G-Ins*P*Cer from lower fungi (reviewed in [14–16,25]).

In contrast to the previous assumption that the anomeric configuration of the mannopyranosyl residue in Man-Ins*P*Cer was β, new data obtained for this study indicated instead an α configuration. The basis for the former assumption was a NOESY spectrum of native Bl-1 in dimethylsulfoxide, in which cross peaks were observed correlating Man H-1 not only with H-2 of the same residue, but with H-3 and H-5 as well. This was interpreted as consistent with an axial orientation for Man H-1 (as found in the β-configuration), which would allow sufficient proximity to the axial H-3 and H-5 for a substantial NOE to be detected [12]. A similar conclusion was drawn previously with respect to the configuration of the Man residue linked to inositol in G-Ins*P*Cers of the mycopathogen *Paracoccidioides brasiliensis* [33]. However, in a NOESY spectrum acquired in the present study on the released Man-Ins fragment in D_2O, the only intraresidue correlation observed was that between H-1 and H-2, while neither H-1/H-3 nor H-1/H-5 correlations were detected at a mixing time of 300 ms; this result is consistent with an equatorial orientation for H-1, and therefore indicates an α-anomeric configuration for the Man residue. The $^3J_{1,2}$ coupling constant and the chemical shift value of Man H-1 in the released mannosyl-inositol fragment are also consistent with an α-anomeric configuration, in agreement with results of a similar analysis of glycosyl-inositol fragments released from G-Ins*P*Cers of *P. brasiliensis* [25]. The α-anomery was confirmed by measurement of the $^1J_{H1,C1}$ coupling constant for the Man residue in both the released Man-Ins fragment and in the native Man-Ins*P*Cer; the values obtained (173 and 175 Hz, respectively), are typical for α-hexopyranoses and their derivatives, ≈ 10 Hz larger than values typical for the corresponding β-hexopyranoses [28,34]. Essentially identical values of $^1J_{H1,C1}$ were obtained with a Man-Ins*P*Cer extracted from the mushroom *Agaricus blazei*, as well with the mannosyl-inositol released from it by ammonolysis (S. B. Levery, M. S. Toledo, A. H. Straus, & H. K. Takahashi, unpublished results).

The new conclusion regarding the anomeric configuration of the inner Man residue in basidiolipids is consistent with the revised structures proposed for G-Ins*P*Cers of *P. brasiliensis* [25] in which the inositol is also substituted at O-2 by an α-mannopyronosyl residue. In that paper, it was suggested that the Man H-1/H-3 and H-1/H-5 correlations observed in NOESY spectra of G-Ins*P*Cers are a result of 'spin diffusion', that is, propogation of NOE by a three spin effect correlating two distant nuclei (H-1 and H-3) via a mutual cross-relaxation partner proximate to both (H-2). Further correlation could then proceed from H-3 to H-5. Although it was suggested that this effect might be promoted by an increase in the local correlation time of the Man residue from the attachment of additional glycosyl residues, it is apparent already in the results for Man-Ins*P*Cer. Note that although Man-Ins*P*Cer is not a very large molecule, it is in the regime producing negative NOEs at 500–600 MHz; furthermore, it must behave approximately as a bent rod in solution, and reorient (tumble) anisotropically. In such a case, the local correlation time experienced by a pair of interacting spins is expected to be dependent on both location within the molecule and the orientation of the internuclear vector with respect to the long axis [35]. Superimposed on this will be the effects of local conformational dynamics, determined by the relative flexibility and conformational space allowed for the Man-Ins and Ins-P-Cer linkages. It is thus conceivable that the propogation of NOEs from H-1 to H-3, and thence to H-5, could be anomalously enhanced by a unique geometrical arrangement of H-1, H-2, and H-3 in this α-Man residue relative to the long axis comprising the lipid part of the molecule. In contrast, the released mannosyl-inositol should behave as a disaccharide, a small molecule decidedly in the positive NOE regime, for which spin-diffusion is generally not detected under these conditions. The higher viscosity of dimethylsulfoxide relative to D_2O may be an additional factor increasing the correlation time for the native Man-Ins*P*Cer.

A spin diffusion model for development of the anomalous correlations is supported by a preliminary study of the time-dependence of growth of NOE cross peaks arizing from H-1 of Man in NOESY spectra of Man-Ins*P*Cer with increasing mixing times in the range of 50–750 ms (S. B. Levery, unpublished results). These show that the NOEs with both Man H-2 and Ins H-2 increase sharply in magnitude at very short mixing times (50–100 ms), while the magnitudes of those with Man H-3 and H-5 are only a small fraction in comparison. In the range from 250 to 750 ms, when the growth of NOEs with Man H-2 and Ins H-2 is beginning to flatten, the buildup rate of the NOE with Man H-3 begins to increase relative to that with Man H-2. These results are consistent with an indirect transfer of magnetism to Man H-3 via H-2. The significance of an alpha-glycosidic linkage positioned at the reducing end monosaccharide of glycosphingolipids, in contrast to the β glycoside, may be of immunological consequence. Thus, the CD1d-restricted, TCR-mediated activation of Vα14 NKT-cells by α but not β glycosylceramides has been shown [31,32,36,37].

Further was shown in the present paper that a minor portion of basidiolipids displayed corresponding linkages of phosphate and α-mannoside in 1- and 4-position, respectively. The observation that the proportion of 1-,4-disubstituted inositol, as compared to the 1-,2-linked hexitol increases with basidiolipids of higher carbohydrate chain length may perhaps reflect a more favourable accessibility for elongating glycosyl-transferases.

The common Man*p*α-2(4)Ins1-phospho-ceramide root structure was observed to be more or less extended by additional monosaccharides. Thereby, certain structural systematics of constitution are apparent. The mushroom species dependency of the carbohydrate structures is typically demonstrated by the absence of hexose types other than mannose in the basidiolipids of the chanterelle. All the other species investigated show extension of the α-mannoside in 6-position by β-galactoside, which in some instances is α-fucosylated in 2-position (Fucα-2)Galβ-6Manα-. This ramification, in this case by fucose, may

reflect a structural phenomenon more frequently observed with complex conjugated oligosaccharide chains in showing a 'second sugar substitution' at their reducing end (for examples, see [38]). Further basidiolipid sugar chain elongation at the β-galactoside root structure may be in 3- and/or 6-position by α-galactoside, e.g. Ce-4, Po-2, Galα-3(Galα-6)(Fucα-2)Galβ-6Manα-, whereas *A. virosa*, Av-3, with similar extension, reaches a more complex, highly α-fucosylated terminus, Galα-3(Fucα-2)(Fucα-6)Galα-2(Galα-3)Galβ-6Man(α)-. *L. edodes* basidiolipids show further elongation of the tetrahexosyl-core by an α-mannoside, e.g. *Le-3*, Manα-2Manα-6Galα-3(Fucα-2)Galβ-6Manα-. In contrast, *C. exipuliformis* glycolipid is derived from the nonfucosylated dihexosyl-core structure Galβ-6Manα- by extension with an α-glucoside, i.e. *Ce-3*, Glcα-6Galβ-6Manα-. However, basidiolipid Ls-1 from *L. scabrum,* also related to the same nonfucosylated dihexosyl-core structure, notably, has a 3-α-mannosylated α-fucose, i.e. Manα-3Fucα-2(Galα-6)Galα-6Galβ-6Man(α)-.

The root structure, Man-Ins-P-Cer, of the basidiolipids is well known from previous investigations including *Saccharomyces cerevisiae* [39–42], *Candida albicans* [43], *Cryptococcus neoformans* [44] and *Candida utilis* [40–42]. However, in the latter cases, the anomery of the mannose glycoside, as well as, it's linkage position to the inositol residue have not been specified. The structural characteristics of compound Ce-2 (Gal-Man-Ins-P-Cer) and Ce-3 (Glc-Gal-Man-Ins-P-Cer) were similarly described from Aspergillus niger [15,23,45]. But again, the anomery of the mannose glycoside and the inositol substitution positions had not been shown. In contrast, the chemical constitution of Cc-1 (Manα-3Manα-2(4)Ins1-P-Cer) has been reported from *Histoplasma capsulatum* [26,46] and *Paracoccioides brasiliensis* [25]. Apart from these afore mentioned glycoinositolphosphoceramides, all other structures presented from the present investigation show structurally novel oligosaccharide residues. In general, the higher oligosaccharides of the mushroom glycolipids, appear as quite alien when compared to those known from mammalian tissues. It is therefore of little surprise that in normal sera of humans and animals (e.g. cattle, rabbits), antibodies are present that recognize certain basidiolipids. These findings, including interactions of basidiolipids with the vertebrate immune system, are presently studied in greater detail.

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