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Isolation of *N*-2′-Hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-D-*erythro*-sphingadienine from Fruiting Bodies of Two *Basidiomycetes* Fungi

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Fruiting bodies of *Clitocybe geotropa* and *Clitocybe nebularis* were extracted with chloroform-methanol and the extracts were fractionated by chromatography. The title compound was isolated in pure form from both extracts. The structure was verified by NMR spectroscopy, sugar and fatty acid analysis, and FAB-MS.

Glycosphingolipids of animal tissue have been extensively studied during the last decades, and various biological functions for these cell surface-located compounds have been demonstrated [1-3]. However, the structure and function of glycosphingolipids of plant and fungal tissue is less well known. We have examined the neutral glycosphingolipid content in fruiting bodies of two *Basidiomycetes* species (*Clitocybe geotropa* and *Clitocybe nebularis*), using chromatographic methods, NMR spectroscopy and mass spectroscopy.

Results and Discussion

Ground and lyophilized fruiting bodies of *C. geotropa* or *C. nebularis* were extracted with chloroform-methanol. The crude extracts were divided by partitioning into polar and non-polar fractions. The non-polar fractions were further investigated. Column chromatography on silica gel in solvent systems used for resolving mono-, di- or trihexosyl ceramide mixtures gave several fractions containing pure compounds, among them nebularine [4] (from *C. nebularis*) and a compound with a chromatographic mobility corresponding to a monohexosyl ceramide. This latter compound was the

Abbreviations: TMS, trimethylsilane; DMSO, dimethylsulfoxide.

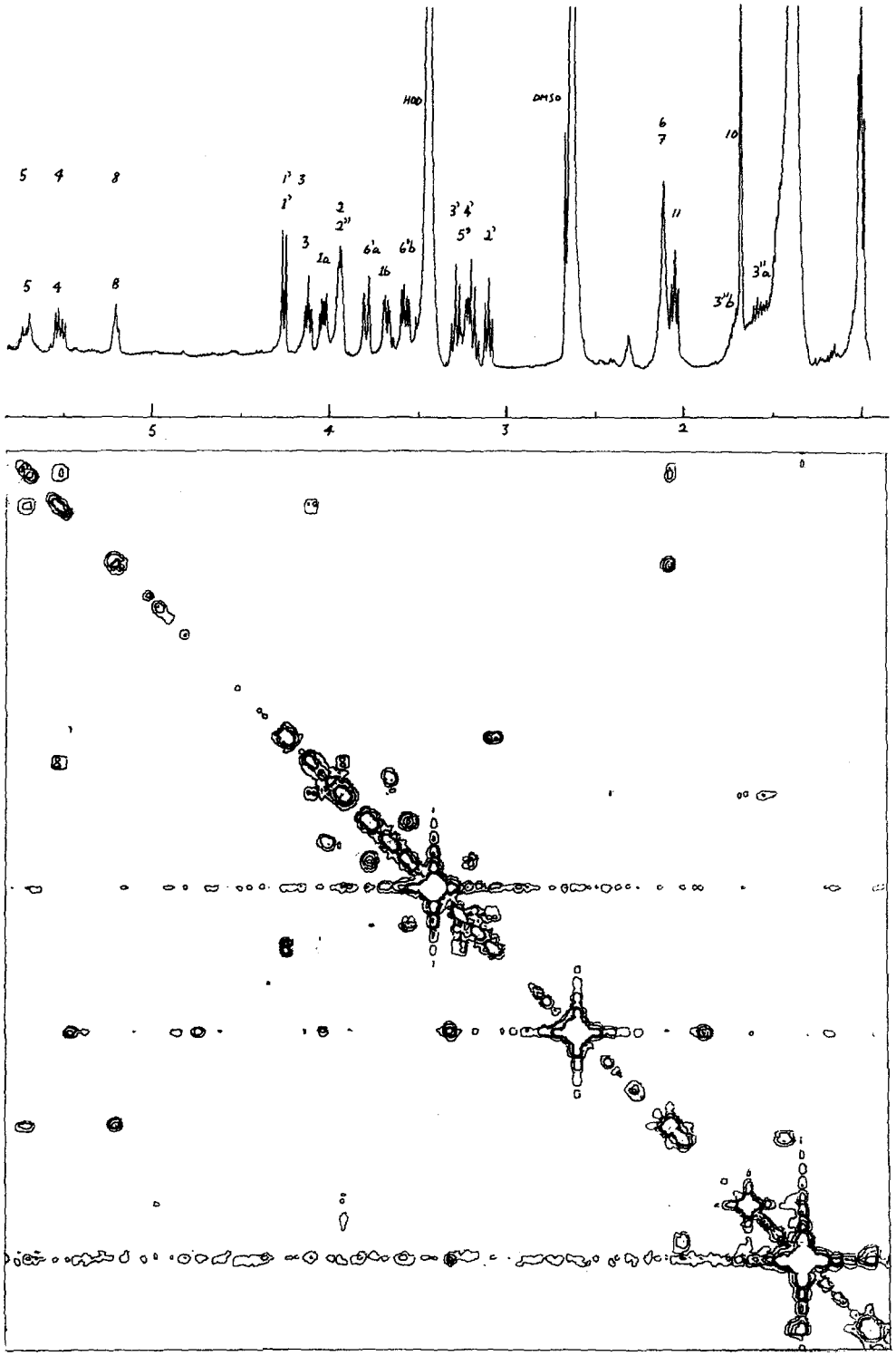

Figure 1. COSY-45-2D proton NMR spectrum of the glycosphingolidpid from *C. nebularis.*

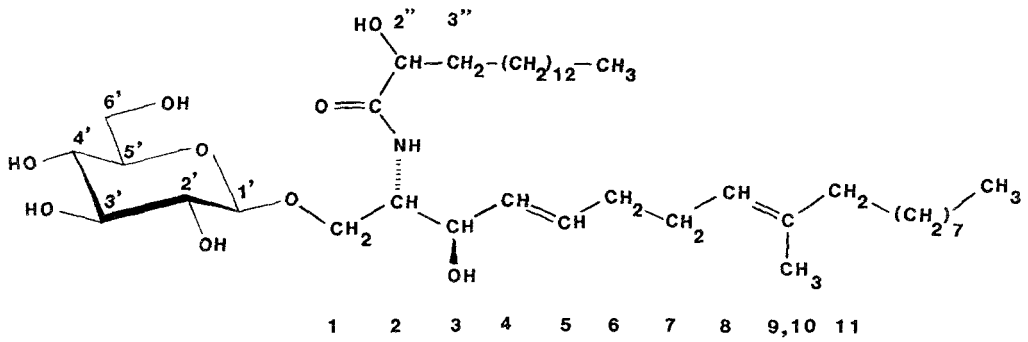

Figure 2. Proposed structure for the glycosphingolipid from *C. geotropa* and *C. nebularis*.

main neutral glycosphingolipid found (both species). The molecular weight, as determined by negative ion FAB-MS, was 728 ±0.4. Sugar analysis showed the presence of only glucose.

Treatment of the glycosphingolipid with methanolic hydrochloric acid gave the methyl ester of 2-hydroxyhexadecanoic acid as the major (80% in both species) fatty acid ester, as shown by GLC-MS. Treatment of the glycosphingolipid with methanolic potassium hydroxide followed by phase-transfer alkylation with methyl iodide gave the methyl ester of 2-methoxyhexadecanoic acid as the major (80% in both species) compound. Thus, the major acyl residue of the glycosphingolipid was 2-hydroxyhexadecanoyl. The former derivatization procedure methylated only the carboxyl function, whereas the latter conditions methylated both the carboxyl and the 2-hydroxyl functions.

The structure of the sphingosine moiety was deduced from the ^{13}C- and ^{1}H-NMR spectra. Thus, the ^{13}C-NMR spectrum of the intact glycosphingolipid corresponded well with that published [5] for *N*-octadecanoyl-1-*O*-β-D-glucopyranosyl-4-D-*erythro*-sphingenine. It corresponded less well with that for the L-*threo* compound. Two additional signals in the double-bonded carbon region were present (136.3 and 123.6 p.p.m.); one of them (136.3 p.p.m.), as judged by its low intensity, was from a tertiary carbon. There was also an additional signal at 72.3 p.p.m. and a different signal pattern in the region 15-40 p.p.m..

In the ^{1}H-NMR spectrum (Fig. 1), the glucosyl spin system (H-1' through H-6') could be fully assigned. The β configuration was evident from the coupling constant of H-1' (7.8 Hz). The sphingosine protons were divided into two independent spin systems, of which the H-1 through H-8 system could be fully assigned. On the basis of the data presented, the structure shown in Fig. 2 was proposed for the glycosphingolipid. The fatty acid C-2 configuration was not determined, nor were the absolute configurations of the glucose and sphingosine moieties. However, in this case it is assumed that the glucose has the D-configuration. The D-*erythro* configuration of the sphingosine is then indicated [5] by the ^{13}C-NMR spectrum (see above).

In conclusion, *C. geotropa* and *C. mebularis* both contain 2′-hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-D-*erythro*-sphingadienine as the major neutral glycosphingolipid. This compound has also been found [6] in the sea anemone *Metridium senile* and in the mycelia of *Penicillum* species [7]. It has been shown that this [8] and similar [9] compounds have fruiting-inducing activity when applied in low concentration to *Scizophyllum commune* mycelia. The present investigation indicates that this simple glycosphingolipid may have a more widespread occurrence in fungi.

Experimental

General Methods

All solvents used in the extraction and purification procedures were of analytical quality. TLC was performed on silica gel 60 HPTLC plates (Merck, Darmstadt, W. Germany) and spots were detected with the anisaldehyde spray reagent. For column chromatography, silica gel 60 (0.040-0.063 mm, Grace GmBH, Worms, W. Germany) was used. NMR spectra were recorded with a JEOL GX 400 instrument. The FAB-MS spectra were recorded with a VG ZAB-SE mass spectrometer. The primary beam consisted of xenon atoms with a maximum energy of 8 keV. The samples were dissolved in triethanolamine and the negative ions were extracted and accelerated over a potential of 10 keV. For GC-MS, a Hewlett-Packard 5790/5970 system was used, equipped with a 10 m capillary column, inner diameter 0.33 mm (a 60 % cyanopropyl stationary phase was used, phase thickness 0.15 μm). A temperature program (100-200°C, 5°/min) was run after each GC injection.

Extraction and Purification Procedure

Fruiting bodies of *C. geotropa* (A) and *C. nebularis* (B) were collected and frozen (-20°C) within 24 h. The frozen material was mixed with dry ice and ground in a Waring blender. The powder was suspended in distilled water, and the suspension was then lyophilized. The yields of dry powder from 400 g of frozen material was 23 g (A) and 32 g (B). The powder was extracted at room temperature with chloroform/methanol, 2/1 by vol. The yields of crude extracts after concentration were 6 g (A) and 4 g (B). The extracts were partitioned between chloroform/methanol/water, 8/4/3 by vol. The lower layers were collected and concentrated, yielding 3.0 g (A) and 1.2 g (B). Silica gel column chromatography of the residues, with ethyl acetate/acetic acid/methanol/water, 40/3/3/2 by vol, as eluant, first gave several fractions containing unidentified anisaldehyde-positive material (blue color), then fractions containing glycosphingolipid with a TLC mobility corresponding to monoglycosyl ceramide. In the case of *C. nebularis*, slower fractions containing nebularine [4] were also collected. Rechromatography of the glycosphingolipid fractions using ethyl acetate/acetic acid/methanol/water, 100/3/3/2 by vol, as eluant, gave chromatographically homogeneous material (A: 60 mg, B: 27 mg). Both preparations gave a strong $(M\text{-}H)^-$ ion = 727 in FAB-MS.

The ^{13}C-NMR spectra of the two preparations in $C^2HCl_3/C^2H_3O^2H$, 2/1 by vol, at 25°C were identical. Chemical shifts (TMS = 0.00 p.p.m.): 176.2 (C-1″), 136.3 (C-9), 134.3 (C-4), 129.4 (C-5), 123.6 (C-8), 103.5 (C-1′), 76.9 (C-3′), 76.6 (C-5′), 73.9 (C-2′), 72.4 (C-3), 72.3 (C-2″), 70.5

(C-4'), 68.8 (C-1), 61.9 (C-6'), 53.7 (C-2), and 40.0, 34.9, 32.9, 32.2, 30.0-29.6, 28.4, 28.0, 25.5, 23.0, 16.0, 14.2 (unassigned signals).

^{1}H-NMR chemical shifts (DMSO-$^2H_6$/^2H_2O, 98/2 by vol, 50°C, DMSO = 2.62 p.p.m.): 7.43 (d, 1H, NH), 5.72 (dt, 1H, *J* 15.2, 6.0 Hz, H-5), 5.53 (dd, 1H, *J* 6.8, 15.2 Hz, H-4), 5.22 (t, 1H, *J* 6.4 Hz, H-8), 4.26 (d, 1H, *J* 7.8 Hz, H-1'), 4.13 (t, 1H, *J* 6.8 Hz, H-3), 4.04 (dd, 1H, *J* 10.2, 5.8 Hz, H-1a), 3.94 (m, 2H, H-2, H-2''), 3.80 (dd, 1H, *J* 11.2, 1.6 Hz, H-6'a), 3.68 (dd, 1H, *J* 10.2, 3.4 Hz, H-1b), 3.58 (dd, 1H, *J* 11.2, 5.2 Hz, H-6'b), 3.30-3.16 (m, 3H, H-3', H-4', H-5'), 3.10 (t, 1H, *J* 7.8 Hz, H-2'), 2.12 (m, 4H, H-6, H-7), 2.06 (t, 2H, *J* 6.8 Hz, H-11), 1.75-1.68 (m, 1H, H-3''a), 1.68 (m, 1H, H-3''b), 1.50-1.30 (m, 20 H), 0.99 (t, 6H, H-19, H-16'').

Analytical Procedures

Sugar analysis was performed as described [10].

Fatty acid analysis was performed using two different methods: 1) Glycosphingolipid (1.0 mg) and methanolic hydrochloric acid (0.5 M, 0.5 ml) were heated to 80°C in a sealed ampoule for 18 h. The solution was extracted with hexane and the extract analyzed by GLC-MS. 2) Glycosphingolipid (1.0 mg) and methanolic potassium hydroxide (2 M, 0.5 ml) were heated to 100°C in a sealed ampoule for 18 h. The mixture was then partitioned between diethyl ether and water, and the aqueous layer was adjusted to pH 7 with hydrochloric acid. Aqueous tetrabutylammonium hydroxide (1.5 M, 5 ml) was added, and the mixture was extracted with dichloromethane. The extract was refluxed with methyl iodide (1 ml) for 30 min, then concentrated. The residue was triturated with diethyl ether and the supernatant was analyzed by GLC-MS.

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