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# Sphingolipids of Mushrooms†

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ABSTRACT: Ceramides and cerebrosides isolated from cultivated and wild mushrooms together comprise about 0.3–0.7% of the cell dry weight which is approximately 5% of the wet weight. The long-chain bases obtained from these sphingolipid fractions are (1) 4-hydroxyheptadecasphinganine, (2) 16-methyl-4-hydroxyheptadecasphinganine, (3) 4-hydroxyoctadecasphinganine, (4) 17-methyl-4-hydroxyoctadecasphinganine, (5) 18-methyl-4-hydroxynonadecasphinganine, (6) 19-methyl-4-hydroxyeicosasphinganine, (7) 20-methyl-4-hydroxyheneicosasphinganine, (8) 20-methyl-4-hydroxyheneicosa-$X_1$-sphingenine, (9) 20-methyl-4-hydroxyheneicosa-$X_2$-sphingenine, (10) 4-hydroxydocosasphinganine, (11) 4-hydroxydocosa-$X_1$-sphingenine, and (12) 4-hydroxydocosa-$X_2$-sphingenine. The occurrence in biological materials of bases 5, 7, 8, 9, 10, 11, and 12 has not been previously reported. The major normal fatty acids are palmitate, stearate, octadecenoate and octadecdienoate, whereas the predominant hydroxy fatty acids are hydroxytetradecanoate, hydroxypentadecanoate, hydroxypalmitate, and hydroxystearate. The hexose from the cerebrosides of the wild mushrooms is glucose.

The order Eumycophyta, true fungi, consists of three classes of increasing morphological complexity, Phycomycetes, Ascomycetes, and Basidiomycetes; included in the last class are mushrooms. Although considerable work has been done on the structural determination of the toxic peptides and central nervous system (CNS) active principles, which act at postganglionic parasympathetic effector sites, of poisonous mushrooms, genus *Amanita*, little is known of their lipid composition. Sphingolipids are considered to be ubiquitous constituents of cell membranes and it was therefore of interest to know of the presence or absence of these compounds in cells which elaborate substances active on the CNS. Since the CNS-active compounds are substituted tetrahydrofurans and isoxazole derivatives (Wieland, 1968; Simons, 1971) which would be soluble in lipids, it was thought that the knowledge obtained might assist in understanding the mechanism of transport of these substance across cell membranes as well as yielding information concerning the nature of receptor sites. It was also hoped that this study might disclose the presence of novel long-chain bases[1] which would serve as models in organic syntheses in future studies for the search of metabolic antagonists in the treatment of diseases of the CNS. Therefore, we investigated the sphingolipid content of several species of *Amanita* along with the cultivated edible mushroom, *Agaricus bisporus*, and attempted to characterize each component of these complex lipids.

## Methods and Materials

Infrared spectroscopy on KBr disks and column chromatography (cc) were performed as described previously (Weiss and Stiller, 1970a,b). All reagent grade solvents were distilled and the column packings, silicic acid (Merck or Mallinckrodt), and Florisil (100 mesh) were washed several times by suspension in methanol and centrifugation; after additional washing of the Florisil with water, the adsorbents were dried at 115°. Column fractions were analyzed for long-chain bases, serine, ethanolamine (Meltzer, unpublished results), hexose (Radin *et al.*, 1955), phosphate (Marinetti, 1962), and ester groups (Meltzer, 1958).

Analytical thin-layer chromatography (tlc) plates (20 × 20 cm) containing silica gel G (0.2 mm thick) were prepared (Weiss and Stiller, 1965) and preparative plates (2 mm thick) were purchased from Brinkmann Instrument Co. Ascending chromatography with solvent systems chloroform–methanol–water (100:42:6) (Carter and Hirschberg, 1968) and chloroform–methanol–concentrated $NH_4OH$ (65:35:5) (Rouser *et al.*, 1965) were used to examine the purity of fractions obtained by cc. Preparative tlc with the former solvent system was employed when further purification was necessary. Components were detected after separation by a variety of reagents (Mangold, 1969), preferably iodine vapor, and removed by successive suspension of the adsorbent-containing band in warm cholroform–methanol (2:1) and methanol with centrifugation.

Gas chromatography (gc) was done on a Perkin-Elmer 881 equipped with flame ionization detector. A 6.0 ft × 0.25 in. i.d. glass column packed with 3% SE-30 on gas chrom Q was used for the determination of long-chain bases, long-chain alcohols, fatty acids, and simple sugars. The nitrogen flow rate was 15 ml/sec and the injector and detector temperatures were 270°. Column temperatures of 195 and 240° were employed for determining bases of chain length up to C-18 and

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[1] The nomenclature used is that recommended by IUPAC-IUB Commission on Biochemical Nomenclature. In all cases, the configurations at carbon atoms 2, 3, and 4 are unknown.

beyond C-18, respectively. The lower and upper range of the series of normal and iso alcohols of chain length C-14 to C-21 was analyzed at 150 and 165°, respectively. Fatty acids and sugars were analyzed at column temperatures of 165° and programming from 100 to 210° at 2°/min (Dijong *et al.*, 1971), respectively. Bases, alcohols, and methyl glycosides were determined as trimethylsilyl derivatives which were prepared with either the reagent of Gaver and Sweeley (1965) or Carter and Gaver (1967). Fatty acids were converted to methyl esters with diazomethane prepared from Diazald (Aldrich Chemical) and the hydroxy fatty acid methyl esters were further converted to $Me_3Si$ ether derivatives.

The logarithms of the retention times of standard long-chain bases *vs.* their carbon numbers were plotted to obtain equivalent chain lengths. This plot was used to calculate the equivalent chain lengths of unknown long-chain bases. In addition, due to the lack of several long-chain base standards, the exceedingly helpful retention data presented by Carter and Gaver (1967) was adapted for our use.

Reference compounds were obtained as follows. (1) Cerebrosides were prepared according to the procedure of Carter *et al.* (1947) and also purchased from Supelco. (2) Sphingosine was isolated as the sulfate salt after acid hydrolysis of cerebrosides. (3) Dihydrosphingosine was obtained by reduction of sphingosine free base in ethanol over platinum. (4) *O*-Methyl ether stereoisomers of sphingosine were isolated as described previously (Carter *et al.*, 1951; Weiss, 1964). (5) The ceramides *N*-myristoylsphingosine and *N*-palmitoyldihydrosphingosine were synthesized (Weiss and Raizman, 1958). (6) Phytosphingosine was obtained from the yeast *Hansenula ciferri* (Weiss and Stiller, 1970b). (7) Psychosine and fatty acid esters were commercial products. (8) Some $\alpha$- and $\beta$-methyl glycosides were purchased and others were prepared by refluxing 1.0 mg of the appropriate sugar in 2 ml of dry 1 N HCl–methanol for 18 hr in a heating block at 78° in Teflon-lined screw-cap centrifugation tubes; after removal of solvent under a stream of nitrogen, the residues were stored over phosphorus pentoxide until preparation of their $Me_3Si$ derivatives. (9) Similarly, normal alcohols were purchased and the branched chain iso and anteiso alcohols were prepared from the corresponding fatty acid mixtures BC-1 and BC-L (Applied Science) (Carter and Hirschberg, 1968) by dissolving 10 mg of dried fatty acids in 2 ml of dry diethyl ether containing 5 mg of $LiAlH_4$; after standing several hrs at room temperature, 2 ml each of methanol and 2.5 N NaOH were successively added and the alcohols were removed by two extractions with 4-ml portions of ethyl acetate–diethyl ether (1:1) with centrifugation; the combined extracts were concentrated under nitrogen and the alcohols stored under anhydrous conditions. (10) Cultivated edible mushrooms, *Agaricus bisporus*, grown in Pennsylvania, were purchased locally and *Amanita muscaria* and *Amanita rubescens* were harvested during Autum in the New Jersey, New York, and Massachusetts countryside; the mushrooms were refrigerated no more than several days before use.

*Isolation of Sphingolipid Fractions.* Mushrooms were homogenized in a Waring blender with an equal volume of acetone (w/v) and filtered with suction. After rehomogenizing the precipitate two additional times with one-half the original volume of acetone and three times with the same volume of chloroform–methanol (2:1), the combined acetone and chloroform–methanol filtrates were concentrated to dryness separately under reduced pressure; methanol was added at intervals during concentration of the acetone solution. The residue from each extract was combined and a Folch dialysis (Folch *et al.*, 1951) was done after the addition of 160 ml of chloroform, 80 ml of methanol, and 60 ml of water; the upper phase was discarded and the same amount of fresh upper phase was added. After mixing and centrifugation, the lower phase was concentrated to dryness. The residue was loaded from chloroform on a 60-g silicic acid column and the neutral lipids were eluted with 1500 ml of chloroform and the polar lipids with 1 l. of methanol. In some instances, the mushrooms were homogenized in an equal volume of chloroform–methanol (2:1, w/v) and twice with one-half the original volume with filtration each time. Sephadex G-25, equal in weight to the water content of the combined chloroform–methanol filtrates, was added and the reaction mixture was processed according to the method of Williams and Merrilees (1970). The lipids were removed from the Sephadex by suspension three times in 800 ml of chloroform with centrifugation. After concentration of the chloroform supernatants to dryness, the residue was fractionated into neutral and polar lipids on a silicic acid column as described above.

After removal of methanol, the polar lipids were dissolved in chloroform–methanol (2:1) to a final concentration of 40–60 mg/ml (Carter and Hirschberg, 1968) followed by the addition of an equal volume of 1 N NaOH–methanol. The reaction mixture after 60–90 min at room temperature was neutralized with 1 N HCl and sufficient chloroform, methanol, and water were added to accomplish a ratio of 8:4:3 for a Folch partition. The lower phase was concentrated to dryness and the residue was reapplied from chloroform to a 30-g silicic acid column. After passage of 1 l. of chloroform which was discarded, the mild alkali-stable lipids were eluted with 1 l. of methanol. The methanol was removed and the residue was applied to a 20-g silicic acid column which was developed successively with 750 ml each of chloroform–methanol (96:4 and 80:20) to remove ceramides and cerebrosides, respectively. The ceramide and cerebroside fractions, after removal of solvent, were loaded on separate 10-g Florisil columns (Wells and Dittmer, 1965; Kishimoto and Radin, 1959). The ceramide-containing column was treated successively with 100 ml of chloroform (discarded) and 175 ml each of chloroform–methanol (96:4 and 42:4) to give ceramide I and ceramide II fractions, respectively. Similarly, the column containing cerebrosides was developed successively with 100 ml of chloroform–methanol (96:4) (discarded) and 200 ml each of chloroform–methanol (42:4 and 2:1) to yield cerebroside I and cerebroside II fractions, respectively.

*Isolation of Sphingolipid Components.* Fractions (2–5 mg), in Teflon-lined screw-cap centrifuge tubes, were hydrolyzed in 2 ml of concentrated HCl–methanol–water (3:29:4) in a heating block at 78° for 18 hr (Carter and Hirschberg, 1968). The fatty acids and their methyl esters were removed with three 2-ml portions of Skellysolve B (bp 68–74°) and the combined extracts were concentrated to dryness under nitrogen. The dried residue was esterified with diazomethane for gc analysis; a portion of the fatty acid methyl esters was treated with $Me_3Si$ reagent for determination of hydroxy fatty acids. The lower aqueous methanol phase was made alkaline to about pH 8.0 and the bases were removed with three 2-ml portions of chloroform. The long-chain bases, obtained from the combined chloroform extracts after concentration under nitrogen and drying over phosphorus pentoxide, were converted to $Me_3Si$ derivatives just before use; unused free bases were stored in the original solvent extract in the cold.

Similarly, for the analysis of cerebroside sugars, a separate sample, 2–4 mg in 2 ml of anhydrous 1 N HCl–methanol, was

TABLE I: Isolation Procedure and Sphingolipid Yields from Cultivated and Wild Mushrooms.

| | *Amanita muscaria* (g) | *Amanita rubescens* (g) | *Agaricus bisporus* (g) |
|---|---|---|---|
| 1. Cell wet weight[a] | 1107.0 | 341.0 | 1816.0 |
| 2. Cell dry weight | 69.0 | 16.2 | 87.7 |
| 3. Acetone-soluble material | 37.8 | 10.5 | |
| 4. Chloroform–methanol (2:1)-soluble material | 10.3 | 1.4 | 20.9 |
| 5. Lipids after Folch dialysis of steps 3 and 4 are combined | 17.4 | 3.7 | |
| 6. Silicic acid chromatography | | | |
| Neutral lipids | 13.4 | 2.1 | 10.2 |
| Polar lipids | 3.4 | 0.9 | 4.4 |
| Polar lipids after NaOH | 2.6 | | 2.8 |
| 7. Silicic acid chromatography of polar lipids | | | |
| Ceramides | 2.15 | 0.050 | 0.380 |
| Cerebrosides | 0.12 | 0.100 | 0.290 |
| 8. Florisil chromatography | | | |
| Ceramide I | 0.3202 | 0.0437 | 0.1344 |
| Ceramide II | 0.1346 | 0.0033 | 0.1264 |
| Cerebroside I | 0.0095 | 0.0253 | 0 |
| Cerebroside II | 0.0426 | 0.0306 | 0.0238 |

[a] See text for details.

TABLE II: Long-Chain Bases and Alcohols Derived from Sphingolipid Fractions of *A. muscaria*.

| Fraction | P[a,b] | % | ROH[a,c] | % |
|---|---|---|---|---|
| Ceramide | n17:0[a] | 10.2 | n14:0 | 5.6 |
| | i17:0[a] | 6.2 | i14:0 | 3.3 |
| | n18:0 | 22.6 | n15:0 | 11.4 |
| | i18:0 | 2.9 | i15:0 | 3.5 |
| | i19:0 | 7.8 | i16:0 | 7.2 |
| | i20:0 | 4.3 | i17:0 | 3.6 |
| | i21:0 | 33.5 | i18:$X_1$ | 36.8 |
| | | | i18:$X_2$ | 6.5 |
| | | | i18:0 | 2.6 |
| | n22:0 | 12.5 | n19:$X_1$ | 14.8 |
| | | | n19:$X_2$ | 3.7 |
| | | | n19:0 | 1.0 |
| Cerebroside | n17:0 | 2.4 | n14:0 | 0.3 |
| | i17:0 | 0.5 | i14:0 | 1.4 |
| | n18:0 | 6.8 | n15:0 | 1.5 |
| | i18:0 | 9.1 | i15:0 | 3.2 |
| | i19:0 | 8.7 | i16:0 | 9.5 |
| | i20:0 | 14.6 | i17:0 | 6.6 |
| | i21:0 | 36.2 | i18:$X_1$ | 25.2 |
| | | | i18:$X_2$ | 8.7 |
| | | | i18:0 | 5.2 |
| | n22:0 | 21.7 | n19:$X_1$ | 28.2 |
| | | | n19:$X_2$ | 8.3 |
| | | | n19:0 | 1.9 |

[a] Abbreviations used are: P, phytosphingosine; ROH, long-chain alcohol; n, normal; i, iso; T, trace. Ceramides I and II and cerebrosides I and II obtained from Florisil chromatography (Table I) were analyzed for long-chain bases, derived long-chain alcohols and fatty acids. The values obtained for each component from each of the sphingolipid pairs were averaged and presented in the appropriate tables. [b] Bases were obtained from sphingolipids hydrogenated over platinum by acid hydrolysis and analyzed by gc as trimethylsilyl derivatives. [c] Intact sphingolipids were degraded with periodic acid and the isolated aldehydes were reduced with sodium borohydride to alcohols which were determined by gc as trimethylsilyl derivatives. The subscripts represent different alcohols of unknown unsaturation. See text for details.

methanolyzed under the same conditions used for the isolation of the fatty acids and long-chain bases. After removal of the fatty acid methyl esters with Skellysolve B, the solution was concentrated to dryness followed by the addition of 0.5 ml of $H_2O$ and several drops of 1 N $NH_4OH$ to mild alkalinity. The long-chain bases were removed with three 2-ml portions of diethyl ether and the lower aqueous phase containing the methyl glycosides was concentrated to dryness under nitrogen by repeated additions of methanol–benzene (6:1). After drying over phosphorus pentoxide, the samples were derivatized with $Me_3Si$ reagent or stored under anhydrous conditions until used.

*Reduction of Lipids.* Intact lipids (3–5 mg) were dissolved in 2 ml of ethanol containing 3 mg of platinum oxide in a 12-ml centrifuge tube which was connected to an absorption train and manifold with eight similar tubes having samples and standards to be reduced. Hydrogen was bubbled for 2 hr through each solution by means of capillary tubes connected to the manifold. Fresh ethanol was added at intervals to the solution kept at 35° to maintain a constant volume. The reaction mixture was centrifuged and the catalyst was washed several times by centrifugation with 1.0-ml portions of hot ethanol. The combined ethanol supernatants were concentrated to dryness and the product was hydrolyzed in the same manner as that described for the unreduced lipids to yield the fully saturated long-chain bases and fatty acids.

*Periodate Oxidation–Sodium Borohydride Reduction.* Intact sphingolipids, 1–3 mg of unreduced and reduced, were suspended in 2 ml of methanol–$H_2O$ (9:1) followed by the addition of 0.15 ml of 0.2 N aqueous periodic acid. After warming the reaction mixture at 40° for 15 min and standing at room temperature for 2 hr, the long-chain aldehydes were removed with three 2-ml portions of Skellysolve B. The combined extracts were concentrated to dryness and the residues, dissolved in 0.5 ml of methanol, were treated with 0.7 ml of 10% sodium borohydride in 0.1 N aqueous sodium hydroxide. After standing 2 or more hr at room temperature followed by the successive addition of 1 N HCl to approximately pH 6.0, 0.5 ml of $H_2O$, 0.3 ml of methanol, and 1.7 ml of chloroform were added (Carter and Hirschberg, 1968); the alcohols were recovered in the lower chloroform layer after centrifugation. The chloroform was removed under nitrogen and, after the addition of 1.0 ml of water and 0.3 ml of methanol, the alcohols were removed with three 2-ml portions of ethyl acetate. The combined extracts were either stored in the cold until ready for use or concentrated and the dried residues were analyzed by gc as $Me_3Si$ derivatives.

TABLE III: Long-Chain Bases and Alcohols Derived from Sphingolipid Fractions of *A. rubescens*.

| Fraction | P[a] | % | ROH | % |
|---|---|---|---|---|
| Ceramide | i17:0 | 4.8 | i14:0 | 6.4 |
| | n18:0 | 44.6 | n15:0 | 31.8 |
| | i18:0 | 0.3 | i15:0 | 1.6 |
| | i19:0 | 5.5 | i16:0 | 6.9 |
| | i20:0 | 2.7 | i17:0 | 1.7 |
| | i21:0 | 27.7 | i18:$X_1$ | 19.5 |
| | | | i18:$X_2$ | 9.1 |
| | | | i18:0 | 2.4 |
| | n22:0 | 14.4 | n22:$X_1$ | 17.5 |
| | | | n22:$X_2$ | 2.4 |
| | | | n22:0 | 0.7 |
| Cerebroside | i17:0 | 6.4 | i14:0 | 3.5 |
| | n18:0 | 13.1 | n15:0 | 6.9 |
| | i18:0 | 5.5 | i15:0 | 3.1 |
| | i19:0 | 22.0 | i16:0 | 20.9 |
| | i20:0 | 28.1 | i17:0 | 15.2 |
| | i21:0 | 15.2 | i18:$X_1$ | 14.4 |
| | | | i18:$X_2$ | 7.8 |
| | | | i18:0 | 4.8 |
| | n22:0 | 9.7 | n19:$X_1$ | 10.9 |
| | | | n19:$X_2$ | 7.4 |
| | | | n19:0 | 5.1 |

[a] See legend to Table II.

TABLE IV: Long-Chain Bases and Alcohols Derived from Sphingolipids of *A. bisporus*.

| Fraction | P[a] | % | ROH | % |
|---|---|---|---|---|
| Ceramide | n17:0 | 3.8 | n14:0 | 1.0 |
| | i17:0 | 14.6 | i14:0 | 11.9 |
| | n18:0 | 66.9 | n15:0 | 75.3 |
| | i18:0 | 3.3 | i15:0 | 1.6 |
| | i19:0 | 2.5 | i16:0 | 1.9 |
| | i20:0 | 2.9 | i17:0 | 1.2 |
| | i21:0 | 5.0 | i18:$X_1$ | 5.0 |
| | | | i18:0 | 1.1 |
| | n22:0 | 1.0 | n19:$X_1$ | 1.0 |
| Cerebroside | i17:0 | | i14:0 | 0.9 |
| | n18:0 | 61.1 | n15:0 | 40.2 |
| | i19:0 | 5.3 | i16:0 | 2.2 |
| | i20:0 | | i17:0 | 0.5 |
| | i21:0 | 33.6 | i18:$X_1$ | 43.6 |
| | | | i18:$X_2$ | 11.4 |
| | n22:0 | | n19:$X_1$ | 1.2 |

[a] See legend to Table II.

## Results

Approximately 95% of the mushroom is water (Table I). The neutral and polar fractions represent 12–19 and 5%, respectively, of the cell dry weight. After treatment with alkali followed by Folch dialysis and successive passage through silicic acid and Florisil columns, the polar lipids, ceramides and cerebrosides combined, ranged from 0.3 to 0.7% of the dry weight. In *A. muscaria* and *A. bisporus* about 90% and *A. rubescens* 50% of the sphingolipids are ceramides.

The bases, all phytosphingosine homologs in each mushroom species, obtained after hydrolysis of the hydrogenated ceramides and cerebrosides of *A. muscaria*, range in length from 17 to 22 carbon atoms (Table II). The major bases are n18:0, i21:0, and n22:0. Except for the presence of the normal homologs 17:0, 18:0, and 22:0, all of the bases are of the iso series. The alcohols obtained from the intact lipids after periodate oxidation and sodium borohydride reduction disclose two unknown unsaturated components each in the i18 and n22 series (Table II); these disappear into their corresponding saturated analogs after similar treatment of oxidation and reduction of the hydrogenated intact lipids. The most unsaturated alcohols (i18:$X_1$[2] and n22:$X_1$) comprise the greatest, and the saturated (i18:0 and n22:0) the least, proportion of the i18 and n19 series, respectively.

In *A. rubescens* (Table III), the patterns of bases, except for the absence of n17:0, and alcohols obtained from the reduced and unreduced intact lipids, respectively, resemble those derived from *A. muscaria*. In *A. bisporus* (Table IV), the major base in the ceramides is n18:0, whereas in the cerebrosides n18:0 and i21:0 are predominant. In each sphingolipid fraction of *A. bisporus*, one or more of the three components comprising the i18 and n19 series of alcohols in genus *Amanita* are absent.

[2] The position, geometry, and nature of unsaturation are unknown. The subscripts indicate the presence of two different unsaturated bases.

TABLE V: Fatty Acids of *A. muscaria*.

| Fatty Acid[a] | Ceramide | Cerebroside |
|---|---|---|
| n12:0 | 0.6 | 0.6 |
| n13:0 | 0.6 | 0.7 |
| n14:0 | 1.2 | 0.7 |
| i14:0 | 0.8 | 0.6 |
| HO-n14:0 | 2.7 | 8.1 |
| n15:0 | 3.5 | 2.5 |
| i15:0 | 0.2 | T |
| HO-n15:0 | 11.1 | 15.9 |
| n16:0 | 11.1 | 9.1 |
| n16:1 | 1.1 | 1.3 |
| HO-n16:0 | 18.4 | 21.3 |
| n17:0 | 0.8 | 0.9 |
| i17:0 | 0.4 | 0.3 |
| n18:0 | 7.1 | 8.7 |
| n18:1 | 21.1 | 15.7 |
| n18:2 | 16.6 | 11.5 |
| HO-n18:0 | 0.4 | 0.4 |
| n19:0 | 0.2 | 0.3 |
| n19:1 | 0.7 | |
| n20:0 | 0.9 | 0.9 |
| i20:0 | 0.2 | 0.4 |
| i20:1 | 0.4 | 0.1 |

[a] Fatty acids were obtained by acid hydrolysis of the sphingolipid fractions and analyzed by gc of their methyl esters or methyl esters–trimethylsilyl ethers derivatives. See Table II, footnote *a*. T = trace.

TABLE VI: Fatty Acids of *A. rubescens*.

| Fatty Acid[a] | Ceramide | Cerebroside |
|---|---|---|
| n13:0 | 0.5 | 0.4 |
| n14:0 | 1.1 | 0.5 |
| i14:0 | 0.6 | 0.5 |
| HO-n14:0 | | 3.4 |
| n15:0 | 2.8 | 1.5 |
| HO-n15:0 | 0.5 | 3.6 |
| n16:0 | 17.6 | 10.7 |
| n16:1 | 1.4 | 1.1 |
| HO-n16:0 | 3.4 | 32.7 |
| n17:0 | 2.8 | 1.4 |
| i17:0 | 0.8 | 0.6 |
| n18:0 | 9.2 | 5.7 |
| n18:1 | 30.0 | 11.7 |
| n18:2 | 24.4 | 14.8 |
| HO-n18:0 | 1.6 | 6.2 |
| n19:0 | 0.4 | 2.1 |
| n19:1 | 0.4 | |
| n20:0 | 1.3 | 1.0 |
| i20:0 | 1.3 | 1.0 |
| i20:1 | 0.4 | 1.1 |

[a] See legend to Table V.

TABLE VII: Fatty Acids of *A. bisporus*.

| Fatty Acids[a] | Ceramide | Cerebroside |
|---|---|---|
| n12:0 | 2.5 | 1.7 |
| n13:0 | 2.5 | 1.7 |
| n14:0 | 5.0 | 3.4 |
| HO-n14:0 | 7.4 | 3.4 |
| n15:0 | 2.5 | 1.7 |
| HO-n15:0 | 13.6 | 8.4 |
| n16:0 | 10.1 | 5.0 |
| n16:1 | T | 2.1 |
| HO-n16:0 | 21.2 | 42.7 |
| n17:0 | 2.3 | 2.8 |
| HO-n17:0 | 2.2 | 1.7 |
| n18:0 | 2.5 | 1.7 |
| n18:1 | 7.3 | 6.4 |
| n18:2 | 13.6 | 5.9 |
| HO-n18:0 | 7.3 | 13.4 |

[a] See legend to Table V.

The fatty acids of the ceramides and cerebrosides of each mushroom species range in length generally from 12 to 20 carbon atoms (Tables V–VII). The major normal fatty acids in each species are 16:0, 18:0, 18:1, and 18:2; the dominant hydroxy fatty acids are HO-n15:0 and HO-n16:0 in *A. muscaria*, HO-n16:0 in the cerebrosides, with little present in the ceramides, of *A. rubescens*, and HO-n16:0 in the ceramides and cerebrosides of *A. bisporus*.

In the cerebrosides of each *Amanita* species, the only sugar found was glucose. The molar ratios of 1:1 for hexose to base were determined by gc. Sugar analyses on the cerebrosides of *A. bisporus* were not done because of insufficient material.

## Discussion

Long-chain bases of varying diversity have been found as components of complex lipids in a wide array of biological materials ranging from plants (Carter and Koob, 1969; Sastry and Kates, 1964), bacteria (White *et al.*, 1969), protozoa (Carter and Gaver, 1968; Carter *et al.*, 1966), arthropods (O'Connor *et al.*, 1970), and mollusks (Hayashi and Matsubara, 1971) to mammals (Carter and Hirschberg, 1968). More than half a century ago, Zellner (1911a,b) reported the presence of phytosphingosine in *A. muscaria*. Recently, in a study of the total lipids of *A. bisporus* (Holtz and Schisler, 1971) no mention was made of the presence of sphingolipids. Similarly in an investigation of the lipids of the thermophilic fungus, *Humicola grisea* (Mumma *et al.*, 1971), no sphingolipids were reported in their polar lipid fraction which consisted of about 40% of the total lipids. It may be that the failure to find these compounds may have been due to the processing of insufficient amounts of material because these lipids comprise about 0.005% of the total mushroom weight.

The overall pattern of base composition, which appears to have no obvious symmetry, in both species of genus *Amanita* are similar and resemble qualitatively the distribution of bases in *A. bisporus*. No cerebroside I fraction was obtained from the cultivated mushroom (Table I). The lack of agreement, in some cases, between the percentages of long-chain bases and their corresponding alcohols is due to the differential destruction of bases, particularly the unsaturated compounds of the i18 and n19 series, during hydrolysis of the sphingolipids. Because of this considerable degradation, the long-chain bases are presented in the tables as reduced bases. Similarly, periodate oxidation of the free bases and sodium borohydride reduction of the isolated aldehydes does not yield the full pattern of alcohols which is obtained from treatment of the intact lipid with these reagents. The most reliable and reproducible data regarding the composition of phytosphingosines in the mushroom sphingolipids are those derived from the alcohols after appropriate treatment of the intact lipids. No dihydroxy bases were found after hydrolysis of either the unreduced or reduced lipids; sphingomyelin and phytoglycolipid (Carter and Koob, 1969), also, were not observed in these lipids.

The general structure firmly suggested for the phytosphingosines in the various sphingolipid fraction of *Amanita* and *Agaricus* is 1,3,4-trihydroxy-2-amino normal alkane or isoalkane; the unsaturated members may contain single or multiple olefinic and/or acetylenic bonds. The following arguments in support of this conclusion are: (1) the fatty acids in both ceramides and cerebrosides must be in amide linkage to survive the alkaline hydrolysis during purification; (2) the amino group in the ceramides must be on carbon atoms 1 or 2 and the carbohydrate and fatty acid moieties in the cerebrosides must occupy positions 1 and 2 to provide vicinal functional groups for periodate oxidation and thus yield long-chain aldehydes; and (3) identical thin-layer, column, and gas chromatographic behavior of the unknown bases and their derivatives with authentic n18:0 phytosphingosine and its derivatives, along with similar infrared spectra, strongly indicates that carbon atoms 1 and 2 carry the hydroxyl and amino functional groups, respectively (Carter and Hendrickson, 1963).

The phytosphingosine bases found in this study and al-

ready reported present in other biological materials (Karlsson, 1970) are (1) 4-hydroxyheptadecasphinganine, (2) 16-methyl-4-hydroxyheptadecasphinganine, (3) 4-hydroxyoctadecasphinganine, (4) 17-methyl-4-hydroxyoctadecasphinganine, and (5) 19-methyl-4-hydroxyeicosasphinganine. The new bases which have not been reported previously are (6) 18-methyl-4-hydroxynonadecasphinganine, (7) 20-methyl-4-hydroxyheneicosasphinganine, (8) 20-methyl-4-hydroxyheneicosa-$X_1$-sphingenine, (9) 20-methyl-4-hydroxyheneicosa-$X_2$-sphingenine, (10) 4-hydroxydocosasphinganine, (11) 4-hydroxydocosa-$X_1$-sphingenine, and (12) 4-hydroxydocosa-$X_2$-sphingenine.

Problems to be completed regarding this investigation are determination of the (1) configuration of carbon atoms 2, 3, and 4; (2) position, geometry, and type of unsaturation in the bases of the i21 and n22 series; and (3) anomeric configuration of the glycosidic linkage in the cerebrosides.

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