Isolation and Structure of a New Ceramide from the Basidiomycete 
*Hygrophorus eburnesus*

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A new ceramide, named hygrophamide (**1**), was isolated from the fruiting bodies of the basidiomycetes *Hygrophorus eburnesus* Fr. The structure of the compound was elucidated as (2S,3S,4R,2'R)-2-(2'-hydroxy-9'Z-ene-tetracosanoylamino)-octadecane-1,3,4-triol (**1**) by spectral and chemical methods.  

**Key words:** *Hygrophorus eburnesus*, Basidiomycete, (2S,3S,4R,2'R)-2-(2'-Hydroxy-9'Z-ene-tetracosanoylamino)-octadecane-1,3,4-triol, Hygrophamide

**Introduction**  
In recent years, renewed attention has been paid to constituents of higher, often edible mushrooms, Basidiomycetes, because of their possible medicinal usage [1]. Anti-viral, -biotic, -inflammatory, -hypoglycemic, -hypocholesterinemic and -hypotensive properties were ascribed to ingredients of such filamentous fungi [2]. It was assumed that, at least in part, observed therapeutic effects were due to a stimulation of the immune system [3 – 5]. On a chemical basis, the medical effects of basidiomycetes were attributed to glyproteins and proteoglycans [6]. Glycolipids of higher mushrooms have not yet been studied, except for an early, less detailed report [6, 7].

Sphingolipids, e.g., ceramides, cerebrosides, glycosphingolipids (GSL), sphingomyelin and sphingosine derivatives or analogs are important constituents of cell membranes and are assumed to play important roles as antigens and receptors there [8]. Some showed anti-uncerogenic, -hepatotoxic, -tumor, immunostimulatory activities [9 – 11].

The basidiomycete *Hygrophorus eburnesus* belongs to the family Hygrophoraceae. This edible mushroom grows in symbiosis with trees [12]. By literature search, there have been no reports on the chemical analysis of constituents of fungi for the genus *Hygrophorus*. In continuation of our studies on basidiomycete-derived bioactive secondary metabolites [13 – 17], the chemical constituents of the mushroom *Hygrophorus eburnesus* were investigated. This report describes the structure elucidation of a new cerebroside (2S,3S,4R,2'R)-2-(2'-hydroxy-9'Z-ene-tetracosanoylamino)-octadecane-1,3,4-triol (**1**).

**Results and Discussion**  
Hygrophamide (**1**), C_{42}H_{83}NO_{5} (high resolution FAB-MS showed [M-1]− m/z 680.6168 calcld. for C_{42}H_{82}NO_{5} 680.6189), was obtained as white powder. Its IR spectrum exhibited strong hydroxyl absorption bands at 3435 cm\(^{-1}\) and bands at 1651, 1540, 1261 cm\(^{-1}\) due to an amide group. EI-MS showed the characteristic fragments ion at m/z 682 [M+1]+, 664 [M+1-H_{2}O]+, 357 [M+1-H_{2}O-C_{22}H_{43}]+, 300 [C_{18}H_{36}O_{3}]+. In its \(^{13}\)C NMR (DEPT) spectrum, the signals at \(\delta\) 130.3 (CH × 2) showed the presence of one double bond group which was also revealed by the signals at \(\delta\) 5.49 (2H, bs) in its \(^{1}\)H NMR spectrum. The signals at \(\delta\) 14.3 (CH_{3} × 2), \(\delta\) 53.0 (6H, t, \(J = 6.7\) Hz) were assigned to be the two straight chain terminal methyl groups. At \(\delta\) 1.21 – 1.45 ppm in \(^{1}\)H NMR and \(\delta\) 23.0 – 35.8 ppm in \(^{13}\)C NMR the overlapped signals of methylenes suggested the existence of two long aliphatic chains. In addition, \(^{1}\)H NMR spectrum also presented a characteristic amide NH doublet at \(\delta\) 8.57 (1H, d, \(J = 8.8\) Hz; exchangeable with D_{2}O) and \(^{13}\)C NMR (DEPT) spectrum of **1** showed the presence of an amide functionality at \(\delta\) 175.3 (C-1') and \(\delta\) 53.0 (C-2). These evidences...
Scheme 1. Structure of 1 and related derivatives.

led to the conclusion that compound 1 was a ceramide.

To determine the length of fatty acid (FA) and long-chain base (LCB), the compound 1 was treated with methanolic hydrochloric acid to yield a mixture of fatty acid methyl ester (FAME, 1a) and long-chain base (LCB). The EI-MS of 1a displayed the molecular ion at $m/z$ 396 [M]+ and prominent fragment ion 337 [M-COOCH₃]+. The optical rotation of 1a ($[\alpha]_{20}^{20}$−3.3°, c 0.02, CHCl₃), which was very close to those of methyl esters of 2-(R)-hydroxy fatty acids reported earlier [18], identified fatty acid methyl as the R-isomer.

1a (5 mg) was treated in 0.5 ml of CS₂ by addition of 0.5 ml DMDS and 2 mg I₂. The reaction was carried out in a 10 ml flask closed with a Teflon-lined cap and kept 40 h at 60°. Samples were treated with Na₂S₂O₃ solution (5% in distilled water) and twice extracted with petroleum ether. The organic extract was conducted to EI-MS analysis immediately. The MS spectrum gave remarkable fragment-ion peaks at $m/z$ 257 for A and 201 for C due to cleavage of the bond between the carbons bearing a methylthio group. These data indicated that the double bond in the fatty acid is located at C-9' [19]. Combined the above facts it revealed 1a as methyl 2-hydroxy-9-ene-tetracosanoate.

The geometry of the C-9'/C10' double bond was determined to be cis Z on the basis of the 13C NMR chemical shift of the methylene carbon adjacent to the olefinic carbon, which is observed at $\delta$ 26.8 (C-8'), 26.8 (C-11') in (Z) isomer (at $\delta$ ≈ 32 if in E isomers) [19, 20], as also evidenced by the triplet-like signals of olefinic protons (H-9' and H-10') that appear at $\delta$ 5.49 in the 1H NMR of 1 [21].

On the other hand, the LCB component was reacted with acetic anhydride/pyridine (1:1, v/v) to afford peracetate of the LCB (1b). EI-MS of peracetyl LCB (1b) displayed the molecular ion at $m/z$ 485 [M]+ and prominent fragment ions at $m/z$ 443 [M-Ac]+, 425 [M-CH₃COOH]+, 365 [M-2CH₃COOH]+. It suggested the LCB part of 1 to be 2-amino-1, 3, 4-trihydroxyl-octadecane.

Comparing the chemical shifts and coupling constants of C-1, C-2, C-3, C-4 with those of

Table 1. 1H and 13C NMR (pyridine-D₅) data of hygrophamide (1).

<table>
<thead>
<tr>
<th>δC</th>
<th>δH</th>
<th>H-1/HCOSY selected</th>
<th>HMBC selected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1(CH₂)</td>
<td>62.1</td>
<td>4.51(dd, 10.5, 4.1)</td>
<td>H-2</td>
</tr>
<tr>
<td>2(CH)</td>
<td>53.0</td>
<td>5.11(m)</td>
<td>NH, H-1, 3</td>
</tr>
<tr>
<td>3(CH)</td>
<td>76.8</td>
<td>4.36(dd, 6.5, 4.0)</td>
<td>H-2, 4</td>
</tr>
<tr>
<td>4(CH)</td>
<td>73.0</td>
<td>4.28(m)</td>
<td>H-3, 5</td>
</tr>
<tr>
<td>5(CH₂)</td>
<td>34.2</td>
<td>1.91(m)</td>
<td>H-2, 5</td>
</tr>
<tr>
<td>6(CH₂)</td>
<td>25.9</td>
<td>1.71(m)</td>
<td>H-3</td>
</tr>
<tr>
<td>7~</td>
<td>29.6</td>
<td>1.21 ~</td>
<td>H-2</td>
</tr>
<tr>
<td>17(CH₂)</td>
<td>32.4</td>
<td>1.45</td>
<td></td>
</tr>
<tr>
<td>18(CH₂)</td>
<td>14.3</td>
<td>0.86(t, 6.7)</td>
<td></td>
</tr>
<tr>
<td>18'</td>
<td>175.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2'</td>
<td>72.5</td>
<td>4.62(dd, 7.7, 3.8)</td>
<td>H-3'</td>
</tr>
<tr>
<td>3'(CH₂)</td>
<td>35.8</td>
<td>2.03(m)</td>
<td>H-2', 4'</td>
</tr>
<tr>
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<td>34.6</td>
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<td>1.96(m)</td>
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<tr>
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<td>23.0</td>
<td>1.75(m)</td>
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<tr>
<td>8'(CH₂)</td>
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<tr>
<td>9'(CH)</td>
<td>130.3</td>
<td>5.49(bs)</td>
<td>H-8', 11'</td>
</tr>
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<td>12~</td>
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<td>24'(CH₃)</td>
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<tr>
<td>NH</td>
<td>8.57</td>
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phytosphingosine-type LCB possessing (2S, 3S, 4R) configuration \([14, 22 – 24]\), the relative configurations of C-2, C-3, C-4 were predicted to be S, S, R, respectively. The \(^1\)H NMR signals of the basic structure of 1 (Table 1) are in good agreement with those of the known ceramides, which is composed of (2S, 3S, 4R)-phytosphingosine and 2‘R-fatty acid. These results led us to establish the structure of compound 1 as (2S, 3S, 4R, 2’R)-2-(2’-hydroxy-9’Z-ene-tetracosanoylamino)-octadecane-1,3,4-triol.

**Experimental Section**

**General**

Melting point was obtained on an XRC-1 apparatus (Sichuan, P.R. China) and is uncorrected. Optical rotation was taken on a Horiba Sepa-300 polarimeter (Horiba, Tokyo, Japan). \(^1\)H, \(^{13}\)C NMR and two-dimensional NMR spectra were recorded on Bruker DRX-500 (Karlsruhe, Germany) at 500 MHz for \(^1\)H and 125 MHz for \(^{13}\)C NMR, chemical shifts \(\delta\) in ppm to TMS as internal standard and coupling constants in Hz. Mass spectra were measured with a VG Autospec 3000 mass spectrometer (VG, England). Infrared (IR) spectra were obtained on a Bio-Rad FTS-135 infrared spectrophotometer (Bio-Rad, Richmond, CA, USA) employing the EI mode (ionizing potential 70eV) and a capillary column GC-MS spectrometer (San Jose, CA, USA) employing the HP-5 (Hewlett-Packard, Palo Alto, CA, USA) silicon column temperature was varied from 160 to 240 \(^\circ\)C/min.

**Material**

Column chromatography was carried out on silica gel (200 – 300 mesh) and TLC was carried out on plates precoated with silical gel F254 (Qingdao Marine Chemical Ltd., Qingdao, People’s Republic of China). RP-8 (LiChroprep, 40 – 63 \(\mu\)m, Merck, Germany).

**Fungal material**

The fresh fruiting bodies of *Hygrophorus eburnesus* were collected at Lijiang of Yunnan Province in July 2002 and identified by Ms X. H. Wang at Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan, People’s Republic of China. A voucher specimen has been deposited at the Herbarium of Kunming Institute of Botany, Chinese Academy of Sciences, People’s Republic of China (HKAS 39214).

**Extraction and isolation**

The fresh fruiting bodies of *H. eburnesus* were extracted with 95\% ethanol at room temperature (\(\times 3\), total 15 l), followed with chloroform/methanol (1:1, \(v/v\), \(\times 3\)) and methanol (\(\times 3\)) at 20 \(^\circ\)C. The combined extracts were concentrated in vacuo to give a crude extract, which was partitioned between H\(_2\)O and chloroform to provide chloroform extract (26 g). The mushroom residue after extraction was dried and weighed (135 g). The chloroform soluble fraction was subjected to column chromatography eluting with mixture solvent of chloroform/methanol from 100:0 (\(v/v\)) to 90:10 (\(v/v\)) to give 20 fractions. The fractions eluted by chloroform/methanol (98:2 and 95:5, \(v/v\)) were concentrated to small volume and then a white solid was precipitated from the solution. Then the solid was washed with acetone repeatedly, and 1 (190 mg) was obtained as white powders.

**Methanalysis of 1.** Compound 1 (15 mg) was refluxed with 3 ml 5\% hydrochloride-methanol at 60 \(^\circ\)C for 6 h. 10 ml cold water was poured into the reaction mixture, which was extracted with n-hexane for three times (5 ml \(\times 3\)). The combined organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo. The residue of organic phase was subjected to column chromatography employing the following steps: 1. Petroleum ether/ethyl acetate, 2. Petroleum ether/n-hexane, 3. Petroleum ether/ethyl acetate. The residue of organic phase was subjected to silica gel column chromatography using petroleum ether/ethyl acetate (9:1, \(v/v\)) as eluent to produce a tetraacetate of LCB (15 mg), which was characterized by EI-MS analysis and confirmed to be compound 1.

**Derivatization of 1a.** 1a (5 mg) was treated in 0.5 ml CS\(_2\) by addition of 0.5 ml DMDS and 2 mg I\(_2\). The reaction was carried out in a 10 ml flask closed with a Teflon-lined cap and kept 40 h at 60 \(^\circ\)C. The organic layer was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo to give a crude extract, which was partitioned between H\(_2\)O and chloroform to provide chloroform extract (12 mg). The combined extracts were concentrated in vacuo. Immediate EI-MS analysis gave fragments at \(m/z\) 257, \(C^+\) (\(m/z\) 201) in the MS spectrum.

2-Acetoamino-1,3,4-triacetoxy-octadecane (1b). The aqueous methanol layer was neutralized with saturated Na\(_2\)CO\(_3\) solution and concentrated in vacuo. The ether phase was dried over anhydrous Na\(_2\)SO\(_4\) and concentrated in vacuo to give a crude extract, which was partitioned between H\(_2\)O and chloroform to provide chloroform extract (12 mg). The combined extracts were concentrated in vacuo. Immediate EI-MS analysis gave fragments at \(m/z\) 257, \(C^+\) (\(m/z\) 201) in the MS spectrum.

**Hygrophamide (1).** M.p. 121 ~ 123 \(^\circ\)C, [\(\alpha\)]\(_D\) \(22^\circ\) + 7.65 \(c\) 0.3, pyridine. – UV (MeOH) \(\lambda_{max}\) (log \(\varepsilon\)) = 205 nm (3.43), 194 nm (3.09). – IR (KBr): \(\nu\) = 3435 (OH), 2921, 2849, 1651, 1540, 1468, 1261, 1077 cm\(^{-1}\). – HR-FABMS: \(m/z\) 680.6168 [(M-H)\(^+\)]\(^{-}\), calcd. for C\(_{46}\)H\(_{82}\)NO\(_3\) 680.6189. MS (EI, 70 eV): \(m/z\) (\%) = 682 (35) [M+1]\(^+\), 664 (55) [M+1-
H$_2$O$^+$, 455 (20), 437 (18), 424 (25), 382 (28), 357 (50), 325 (15), 300 (28), 272 (12), 225 (8), 97 (38). $^1$H and $^{13}$C NMR (pyridine-D$_5$) see Table 1.

Acknowledgement

We wish to acknowledge the financial support from the National Natural Science Foundation of China (30225048).