Some Peroxysterols and Ceramides from “Phellinus ribis”, a Korean Wild Mushroom

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Abstract : Studies on the chemical constituents from a Korean wild mushroom, Phellinus ribis, were carried out. A triterpenoid, two peroxysterols, and a chlorobenzene compound were isolated from the hexane soluble fraction of the methanol extract of dried fruiting bodies of the basidiomycetes. Those compounds identified were 3-hydroxy-20(29)-lupen-28-oic acid (betulinic acid), 5,8-epidioxyergosta-6,22-dien-3-ol(ergosterol peroxide), 5,8-epidioxyergosta-6,9(11),22-trien-3-ol (dehydroperoxyergosterol), and 1,2,4,5-tetrachloro-3,6-dimethoxybenzene. Structural studies were carried out on molecular species of a ceramide and cerebroside isolated from the chloroform soluble fraction of the methanol extract. For ceramide, the major component fatty acids were α-hydroxy fatty acid isomers of C_{22:0} ~ C_{25:0}; the predominant long-chain bases were trihydroxy sphinganine of C_{17} ~ C_{18}. The structure of a cerebroside containing mono-sugar was assumed that the long-chain base was C_{19:2} sphingadienine; the major fatty acids were C_{16}~C_{15} α-hydroxy fatty acid isomers.

Keywords : Phellinus ribis, triterpenoids, peroxysterols, chlorobenzene, ceramides, cerebrosides

1. Introduction

A Korean wild mushroom, Phellinus ribis, has been used as a folk remedy for treating cancers and epilepsy. However, no biologically active principles from the mushroom have yet been reported. We found that extracts from the dried fruiting body of Phellinus ribis have significant cytotoxic, antiflammatory and antibiotic effects in vitro(unpublished results). During a preliminary survey of the lipids of the basidiomycete, we
found that comparatively large quantities of sphingolipids (ceramides) were present in this mushroom.

Dried fruiting bodies were extracted with methanol at 60°C and the extract was concentrated and successively partitioned with hexane, chloroform, ethylacetate, butanol, and water. The obtained fractions were concentrated and their chemical constituents were separated and purified by using silica gel column chromatography, TLC, and preparative liquid chromatography and so forth.

Four compounds were isolated from hexane-soluble fractions of the MeOH extract: 3-hydroxy-20(29)-lupen-28-oic-acid (betulinic acid), 5,8-epidioxyergosta-6,22-dien-3-ol (ergosterolperoxide), 5,8-epidioxyergosta-6,9(11),22-trien-3-ol (dehydroperoxysterol), and 1,2,4,5-tetrachloro-3,6-dimethoxybenzene. Molecular species of a ceramide and a cerebrosido were isolated from the chloroform-soluble fraction. For the structure of ceramide, the predominant long-chain bases were trihydroxy-sphinganines of C₁₇ - C₁₈; the major component fatty acids were C₂₃:₀ - C₂₅:₀. α-hydroxy fatty acid isomers. A cerebrosido containing mono-sugar was assumed that the long-chain base was C₁₀₂ sphingadicnine; the major fatty acids were C₁₆:₀-C₁₅ α-hydroxy fatty acid isomers.

2. Experimental

Materials and Reagents - The basidiomycetes were collected in the Chungbuk province in 1993 and validated as *Phellinus ribis*. The authentic galactocerebroside from bovine brain was purchased from Sigma Chemical Co., Ltd. USA. All the solvents used in these experiments were of analytical reagent grade.

Instruments - EI- and FAB-MS spectra were obtained with JMS-AX505H (Jeol Ltd.,Japan) and Trio 2A GC-MS systems (V.G. Ltd. Altrincham, England). ¹H- and ¹³C-NMR spectra were obtained on a Bruker ARX 300(300MHz) spectrometer. HPLC and MPLC instruments were Hitachi L-6200 and L-6000 series, respectively.

Methanalysis of ceramide and cerebrosido - An appropriate amount of cerebrosido/ or ceramide(0.1-0.5mg) was refluxed with 2N HCl in methanol at 75°C for 12h according to Gaver and Sweeley[1].

GLC and GC-MS analysis - After cooling, the resulting fatty acid methyl ester was extracted into hexane for GC-MS analysis. The remaining methanol solution was evaporated to dryness and made alkaline with 3N-NaOH, followed by extraction with Et₂O. The ethereal solution was evaporated to dryness and the residue was redissolved in anhydrous pyridine. The TMS ether derivative was formed with HMDS-TMCS (= 2:1, v/v) for GC-MS analysis. The TMS ether derivatives were analyzed with a gas-chromatograph (HP-5890 series II, Hewlett-Packard, Ltd., USA). The column used was a HP-5 fused silica capillary column (0.32mm, i.d. x 25m; split ratio 1/60, He carrier gas, flow rate 2ml/min). The column temperature was programed from 160°C to 320°C at 4°C/min. The GC eluates were MS analyzed at 70 eV.

FAB-MS - FAB-MS spectra of ceramide/ or cerebrosido samples were obtained on 3-NOBA (3-nitrobenzylalcohol) or LiCl saturated NOBA matrix using a xenon primary beam with acceleration voltage of 3kV, filament current of 5mA, gun voltage of 1 kV, scanning from 50 - 2000 amu at a velocity of 30 sec. over the mass range.

Isolation - The air-dried fruiting body material (4kg) was finely ground and extracted with MeOH at 60°C. The obtained extracts were concentrated in vacuo to afford a residue (206 g) which was suspended in H₂O and successively partitioned with hexane, CHCl₃, EtOAc, and BuOH. The hexane fraction (24.5g) was first separated by silicic acid column chromatography (Merck Art 7734; 70-230 mesh) into 8 fractions (solvent, CHCl₃ : MeOH: H₂O from 100:0:0 to 6:4:1). Fraction 2 was rechromatographed over a SiO₂ column (Merck Art. 9385;230-400mesh) eluting with hex.:EtOAc:MeOH, from 100:0:0 to
0:0:100), followed by further separation with preparative LC (column: Nucleosil, 20 x 250mm, C18 5μm, Labomatic Inst. Ltd., USA; solvent: MeOH at 9 ml/min) to give compound 4 (26mg). Fraction 3 was rechromatographed on a SiO2 column (230–400mesh) with a solvent gradient (Hexane:EtOAc:MeOH = 100:0:0 to 0:0:100) to give 12 subfractions. Subfraction 4 was purified by preparative LC to give compound 1 (2mg). Repeated SiO2 column chromatography of subfraction 6 gave a mixture of compounds 1, 2, and 3, which were further purified by preparative LC eluting with MeOH to give compound 1 (5mg, RT., 2.6min.), compd. 2 (8mg R.T., 3.2min.) and compd 3 (20mg, R.T., 3.7 min.).

The chloroform fraction (22.4g) was separated by SiO2 column chromatography (70–230 mesh) with solvent gradient(CHCl3: MeOH: H2O, from 100:0:0 to 6:4:1). Fraction 6, an eluate when the solvent was CHCl3: MeOH = 100: 1, was concentrated to dryness, redissolved in CHCl3 and MeOH(=2:1), and purified by preparative TLC (adsorbent: silica gel, G.E. Merck; solvent: CHCl3: MeOH: H2O = 65:25:4, v/v) to give compound 5 (25mg; Rf 0.75) and a yet-unidentified material (5mg; Rf 0.72). Fraction 9 was further purified by repeated SiO2 column chromatography to give compound 6 (2 mg, Rf 0.62 at CHCl3: MeOH: H2O = 65:25:4, v/v). Compound 5 was assumed to be a ceramide from the corresponding IR spectral data.

**Compound 1:** Crystal (from MeOH); UV λmax (in MeOH) nm 205; EI-MS spectrum, m/z (rel. intensity) [M]+ 456 (72, calculated for C20H14O3, 456.71), [M+1]+ 457 (23), [M+2H]+ 438 (26), [M+2H2O]+ 423 (16), 410 (12), 395 (10), 248 (64), 220 (31), 219 (26), 207 (68), 203 (39), 189 (100), 175 (32), 147 (18), 135 (34), 121 (23), 119 (22), 107 (18), 109 (16), 106 (15).

**Compound 2:** colorless needles (from MeOH); UV λmax (in MeOH) nm 206; EI-MS spectrum, m/z (rel. intensity) [M]+ 426 (74), [M+1]+ 427 (22), 408 (66), 394 (74), 376 (62), 365 (34), 328 (38), 315 (72), 299(88), 251 (100), 152 (43); 1H NMR (CD3OD, 300MHz) δ 1.30 (3H, s, C-19), 1.22 (3H, d), 1.14 (3H, d), 1.03-1.12 (6H, Me-2), 0.97 (3H, s, C-18), 4.0 (1H, m, C-3), 5.43 (2H, m, C-22 and C-23), 6.84 (1H, d, J=8.4Hz, C-7), 6.50 (1H, d, J=8.4Hz, C-6), 5.67 (1H, dd, J1=3Hz, J2=2.1Hz, C-11), 2.50 and 2.27 (2H, dd, C-12).

**Compound 3:** colorless needles (from MeOH); UV λmax (in MeOH) nm 206; EI-MS spectrum, m/z (rel. intensity) [M]+ 428 (31, calculated for C26H34O2, MW 428.3291), [M+1]+ 429 (10), 410 (44), 396 (100), 377 (34), 363 (54), 327 (30), 328 (38), 303 (32), 301 (30), 285 (42), 267 (36), 251 (28), 239 (24), 213 (22), 161 (32), 152 (48); 1H NMR (CD3OD, 300MHz) δ 1.14-1.02 (15H, Me), 1.21 (3H, d), 3.97 (1H, m, C-3), 5.41 (2H, m, C-22 and C-23), 6.45 (1H, d, J=8.4Hz, C-7), 6.72 (1H, d, J=8.4Hz, C-6).

**Compound 4:** White crystal, mp, 164°C; UV λmax (in MeOH) nm 293; EI-MS (%) m/z M+ 274 (49, calculated for C14H10OCL, 275.945), [M+2]+ 276 (64), [M+4]+ 278 (30), 261 (100), 259 (79), 263 (49), 265 (12), 211 (37), 209 (38).

**Compound 5:** White powder, IR (KBr) cm−1: 3340, 3210, 2920, 2840, 1620, 1535, 1460, 1350, 1270, 1100, 1060, 1020, 720; FAB-MS (composition ratio, %), m/z, [M+Li]+ 690 (30.8), 676 (25.1), 662 (27.5), 704 (7.1), 648 (9.5).

**Compound 6:** white powder; FAB-MS (composition ratio, %), m/z, [M+Li]+ 720 (13), 558 (3).

### 3. Results and Discussion

Column chromatography of the hexane soluble fraction of the MeOH extract afforded four compounds: two peroxide compounds, a triterpenoid, and a chlorobenzene derivative, which were identified by comparison of spectral data with those reported in literature.

The MS of 1 exhibited diagnostically important peaks at m/z 456 (M), 438 (M-H2O), 423 (M-H2O - CH3), 410 (M-46), 248 (C), 220 (D), 219 (B), 207 (A), 203 (C-45), and 189 (A-18). This fragmentation pattern
203 (C-45), and 189 (A-18). This fragmentation pattern strongly indicated that the compound was of the lup-
(20)29)-ene type and allowed allocation of the carboxyl
group to C-17 and that of the hydroxyl group to rings
A/B[2, 3]. The main fragment ions are shown below.
Compound 1 was thus assumed to be a lupane type
triterpenoid, betulinic acid, rather than ursane- or
oleanane triterpenoids such as ursolic and boswellic
acids.

This is the first time that 1 has been isolated from
Phellinus ribis. The 3α-O-(3,4-dihydroxy cinnamoyl)
derivative is known to have an antinflammatory effect.

Compound 2 showed a molecular ion peak at m/z
426, and fragmentation ion peaks at m/z 408 [M-H2O]⁺
and 394 [M-O₂]⁺ in the EI-MS spectrum. This
suggested that 2 was the homologue of 3 having one
additional double bond. The ¹H-NMR spectrum of 2
displayed four methyl doublet peaks at δ 1.22, 1.14,
1.03, 1.05, and two methyl singlet peaks at δ 1.30, 0.97
in the high field region showing two singlet peaks
characteristic of two angular methyl protons at δ 0.97
(C-18) and 1.30 (C-19). In comparison of the
chemical shift of 2 with those of 3, the C-18 proton
shift of 2 is upfield, while the C-19 proton shift is
downfield. These chemical shift differences are very
similar to the relationship between androstane and its
9(11)-ene homologue[4]. Consequently, 2 was defined
as 9,11-dehydroergosterol peroxide[5,6,7]. This is the
first report of the isolation of compound 2 from
Phellinus ribis.

Compound 3 showed very similar spectral data to
those of 2. The MS spectrum of 3 showed a molecular
ion peak at m/z 428 and fragment ion peaks at m/z 410
[M-H₂O]⁺, 396 [M-O₂]⁺, 378 [M-O₂·H₂O]⁺ and 303
[M-side chain (C₃H₇)]⁺, among which the peak at m/z
396 was characteristic of ergosterol peroxide[8]. The
¹H-NMR spectrum of 3 showed signals for C-6 and C-7
protons at δ 6.72 and 6.45 (each 1H, d, J=8.4Hz).
The high field region displayed at least four methyl
doublet peaks and two, methyl singlet peaks between δ
1.02 and 1.22. A broad multiplet at δ 3.97 is due to
the C-3 proton, and the multiplet at δ 5.41 (2H, dd) was
assigned as two protons at C-22 and C-23 in the side
chain [9,10]. The ¹H NMR data are in good agreement
with those of ergosterol peroxide. The assignment of
carbon signals in the ¹³C NMR spectrum of 3 was
confirmed by using 2D-NMR.

Ergosterol peroxide (5α,8α-epidioxyergosta-6,22-
dien-3β-ol) is also frequently obtained from fungi and it
has been suggested that it is an important metabolite in
the biosynthesis of ergosterol [11]. Other reports tell
it has immunosuppressive, antiallergic and antiviral
activity and is also responsible for the antiallergic
effects of a mushroom, Tricholoma populatum in
humans [12,13]. Recently, antitumor activity in certain
tumor cell was also reported for the peroxide
compounds isolated from basidiomycetes [14,15].

Compound 4, a white crystal, was identified as

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1,2,4,5-tetrachloro-3,6-dimethoxybenzene by GC-MS. It is known to be found usually in the wood-rotting fungi, and has antibiotic properties.

Compound 5 gave IR peaks at diagnostic values (cm⁻¹) of 3300-3400, 1620, 1535 (aliphatic secondary amide), and 1000-1100 (alcoholic CO), absorption bands from ceramides. 5 gave a single spot of Rf, 0.72 on TLC (silica gel plate, solvents, CHCl₃; MeOH: H₂O = 65: 25: 4, v/v), showing a ceramide position compared with the Rf values of cerebrosides (compound 6: Rf 0.62; galactocerebroside: Rf 0.64). The FAB-MS spectrum of 5 gave four peaks of [M+Li⁺]⁺ at m/z 690 (1.13), 676 (0.92), 662 (1.01), 704 (0.26) and 648 (0.35) indicating that it is a molecular species of ceramide. The FAB-MS spectrum of 6 showed two main peaks of [M+Li⁺]⁺ at m/z 720 (cerebroside MW = 713) and 558 (the related ceramide, MW=551).

Trimethylsilyl ether derivatives of the fatty acid methyl ester fraction of compound 5 yielded four main peaks with a composition ratio of 1: 0.9: 0.7: 0.2 each, respectively. The mass spectra of these peaks showed characteristic fragmentation patterns of an α-hydroxy fatty acid methylester. One of the main components at RT 20.7 min., exhibited ions at m/z 398 [M⁺], 339 [M-COOCH₃;M-59], 366 [M-32], 353 [M-45], 320 [M-78], 294 [M-104], and 103 [16]. The large peak at m/z M-59 may be due to 1,2 cleavage with loss of the methoxy carbonyl group.

![Graph](image)

**Fig. 1** Linear relationship of the relative intensity ratio, log[M⁺]/[M-59⁺], with C numbers of α-hydroxy fatty acid methyl esters.

We found that the relative intensity ratio, log [M⁺]/[M-59⁺], increased linearly with carbon number as shown in Fig. 1.

From the above results, it was concluded that the fatty acid fraction of a molecular species of the ceramide 5 was composed of four α-hydroxy fatty acid analogues of C₂₄₀, C₂₅₀, C₂₂₀, and C₂₅₀ with a composition ratio of 10:6:9:1.

Similarly, it was concluded that the main fatty acid composition of the cerebroside, 6 were α-hydroxy fatty acid analogues of C₁₆₀ and C₁₅₀ with a composition ratio of 2:1.

GC-MS of trimethylsilyl ether derivatives of the corresponding long-chain base of 5 showed the presence of at least four peaks, which were analyzed by GC-MS. Fig. 2 shows the EI-MS spectra of the TMS ether derivatives of the predominant sphingoid bases contained in 5 and 6, respectively.

![Graph](image)

**Fig. 2** The EI-MS spectra of TMS ether derivatives of the corresponding sphingoid base of compound 5(a) and 6(b)

The mass spectrum of the main peak(a) exhibited ions at m/z 518 (M-15), at m/z 132, 204, and 299 (M-234), indicating the presence of trihydroxy- but not dihydroxy- groups in the polar part [17]. The fragmentation rule was figured out as shown below.
The hydrocarbon part was easily analogized to be saturated C₁₈ from the ions at m/z 430 (M-103), 401 (M-132) and 340 (M-90-103). Accordingly, One of the main long chain base of the ceramide 6 was thus identified as 4-hydroxyysphinganine of C₁₈₀. The other one was 4-hydroxyysphinganine of C₁₇₀.

\[
\begin{align*}
\text{ROCH₂C₆H₄CH₃ - CH₂(CH₃)₇CH₃} & \quad \text{ROCH₂C₆H₄CH₃ - CH₂(CH₃)₇CH₃} \\
R = \text{Monosugar(aldose or galactosyl),} & \quad n = 6-20 \quad (1) \\
R' = \text{hydrocarbon(C₃₆-38),} & \quad n = 26-100 \quad (2)
\end{align*}
\]

It is obvious that a long chain base of compound 6 contains a double bond at the C-4 position based on the ions at m/z 221 (M-234) and 204 fragment, which would be, due to a vicinal diol. Consequently, it was concluded that the TMS ether of the long chain base of 6 is 1,3-bis-0-trimethylsilyl-nonadecasphingadienine (freebase, MW 311).

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