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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 basidomycetes. Those compounds identifed were 3-hydroxy-20(29)-lupen-28-oic acid (betulinic acid), 5,8-epidioxy ergosta-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 $C_{22:00}$ ~ $C_{25:00}$; 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dried fruiting body of *Phellinus ribis* have significant cytotoxic, antiflammatory and antibiotic effects *in vitro*(unpublished results).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 basidomycete, 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 (dehydroperoxyergosterol), and 1,2,4,5-tetrachloro-3,6-dimethoxybenzene. Molecular species of a ceramide and a cerebroside were isolated from the chloroform-soluble fraction. For the structure of ceramide, the predominant long-chain bases were trihydroxy-sphinganines of C_{17} ~ C_{18}; the major component fatty acids were $C_{22:00}$ ~ $C_{25:00}$ α-hydroxy fatty acid isomers. 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.

2. Experimental

Materials and Reagents - The basidomycetes 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). ^{1}H- and ^{13}C-NMR spectra were obtained on a Bruker ARX 300(300MHz) spectrometer. HPLC and MPLC instruments were Hitachi L-6200 and L-6000 series, respectively.

Methanolysis of ceramide and cerebroside - An appropriate amount of cerebroside/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 Et_2O. 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 cerebroside 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 H_2O and successively partitioned with hexane, $CHCl_3$, 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, $CHCl_3$: MeOH: H_2O from 100:0:0 to 6:4:1). Fraction 2 was rechromatographed over a SiO_2 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, C_{18} 5μm, Labomatic Inst. Ltd., USA; solvent: MeOH at 9 ml/min) to give compound **4** (26mg). Fraction 3 was rechromatographed on a SiO_2 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 SiO_2 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 SiO_2 column chromatography (70-230 mesh) with solvent gradient($CHCl_3$: MeOH: H_2O, from 100:0:0 to 6:4:1). Fraction 6, an eluate when the solvent was $CHCl_3$: MeOH = 100: 1, was concentrated to dryness, redissolved in $CHCl_3$ and MeOH(= 2:1), and purified by preparative TLC (adsorbent: silica gel, G.E.Merck; solvent: $CHCl_3$: MeOH: H_2O = 65:25:4, v/v) to give compound **5** (25mg; R_F 0.75) and a yet-unidentified material (5mg; R_F 0.72). Fraction 9 was further purifed by repeated SiO_2 column chromatography to give compound **6** (2 mg, R_F 0.62 at $CHCl_3$: MeOH: H_2O =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 λ_{max} (in MeOH) nm 205; EI-MS spectrum, m/z (rel. intensity) $[M]^+$ 456 (72, calculated for $C_{30}H_{48}O_3$; 456.71), $[M+1]^+$ 457 (23), $[M-H_2O]^+$ 438 (26), $[M-H_2O-CH_3]^+$ 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 : coloreless needles (from MeOH), UV λ_{max} (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) ; ^{1}H NMR (CD_3OD, 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, J_1=3Hz,J_2=2.1Hz, C-11), 2.50 and 2.27 (2H, dd, C-12)

Compound 3 : coloreless needles (from MeOH), UV λ_{max} (in MeOH) nm 206; EI-MS spectrum, m/z (rel. intensity) $[M]^+$ 428 (31, calculated for $C_{28}H_{44}O_3$ 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); ^{1}H NMR (CD_3OD, 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 λ_{max} (in MeOH) nm 293; EI-MS (%) m/z M^+ 274 (49, calculated for $C_8H_6O_2Cl_4$;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 υ_{max} (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)

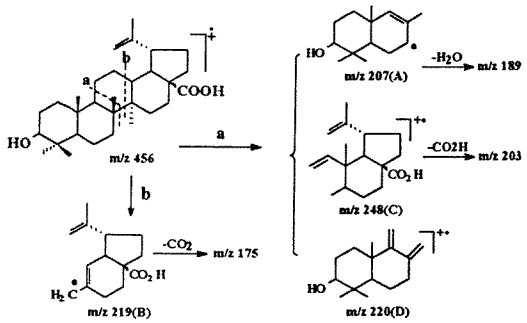
compound 6 : white powder; FAB-MS (composition ratio, %), m/z, $[M+Li]^+$ 720 (13), 558 (3)

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-H_2O), 423 (M- H_2O - CH_3), 410 (M-46), 248 (C), 220 (D), 219 (B), 207 (A), 203 (C-45), and 189 (A-18). This fragmentation pattern

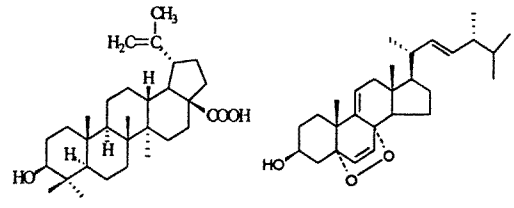
203 (C-45), and 189 (A-18). This fragmentation pattern strongly indicated that the compound was of the lup-20)29)-ene type and allowed allocation of the carboxyl group to C-17 and that of the hydroxyl group to rings A/B[2, 3]. The main fragment ions are shown below. Compound **1** was thus assumed to be a lupane type triterpenoid, betulinic acid, rather than ursane- or oleanane triperpenoids such as ursolic and boswellic acids.



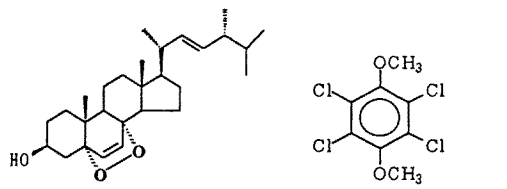
This is the first time that **1** has been isolated from *Phellinus ribis*. The 3-α-*O*-(3,4-dihydroxy cinnamoyl) derivative is known to have an antiinflammatory effect.

Compound **2** showed a molecular ion peak at m/z 426, and frgamentation ion peaks at m/z 408 $[M-H_2O]^+$ and 394 $[M-O_2]^+$ in the EI-MS spectrum. This suggested that **2** was the homologue of **3** having one additional double bond. The ^{1}H-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 $[M-H_2O]^+$, 396 $[M-O_2]^+$, 378 $[M-O_2-H_2O]^+$ and 303 [M-side chain $(C_9H_{17})]^+$, among which the peak at m/z 396 was characteristic of ergosterol peroxide[8]. The ^{1}H-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 ^{1}H NMR data are in good agreement with those of ergosterol peroxide. The assignment of carbon signals in the ^{13}C NMR spectrum of **3** was confirmed by using 2D-NMR.



1 **2**



3 **4**

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 populimum* in humans [12,13]. Recently, antitumor activity in certain tumor cell was also reported for the peroxide compounds isolated from basidomycetes [14,15].

Compound **4**, a white crystal, was identified as

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 R_F, 0.72 on TLC (silica gel plate, solvents, $CHCl_3$: MeOH: H_2O = 65: 25: 4, v/v), showing a ceramide position compared with the R_F values of cerebrosides (compound **6:** R_F 0.62; galactocerebroside: R_F 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 characterisitc 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 $[M-COOCH_3]$;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 duc to 1,2 cleavage with loss of the methoxycarbonyl group.

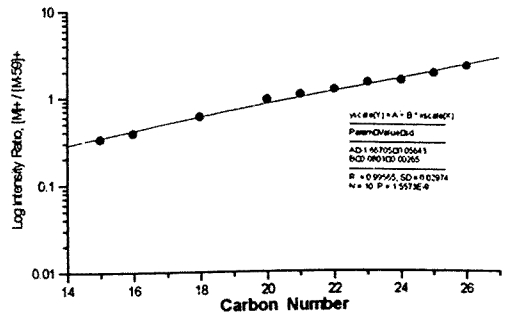


Fig. 1 Linear relationship of the relative intensity ratio, $log[M]^+/[M-59]^+$, with C numbers of α-hydroxy fatty acid methyl esters.

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 $C_{24:0}$, $C_{23:0}$, $C_{22:0}$ and $C_{25:0}$ 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 $C_{16:0}$ and $C_{15:0}$ 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.

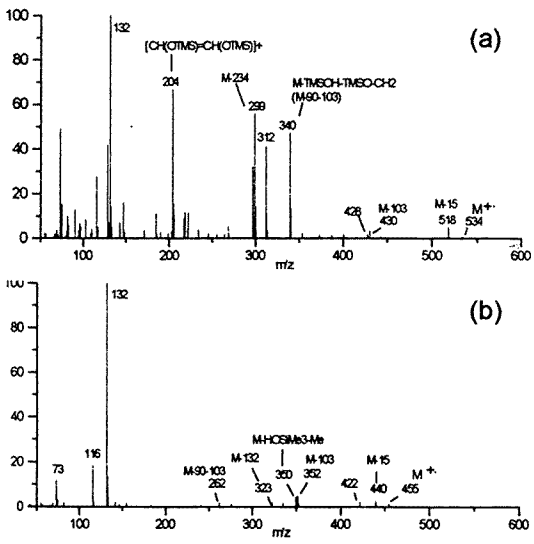
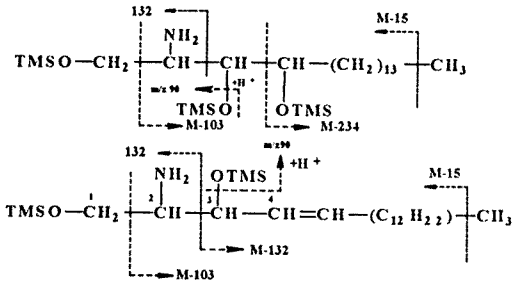
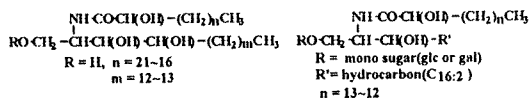


Fig. 2 The EI-MS spectra of TMS ether derivatives of the corresponding sphingoid base of compound 5(a) and 6(b)

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 C_{18} 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-hydroxysphinganine of $C_{18:0}$. The other one was 4-hydroxysphinganine of $C_{17:0}$.



5 **6**

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 vicinary diol. Consequently, it was concluded that the TMS ether of the long chain base of **6** is 1,3-bis-*O*-trimethylsilyl-nonadecasphingadienine (freebase, MW 311).

Acknowledgment

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