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Investigation of the Constituents of *Inonotus mikadoi*

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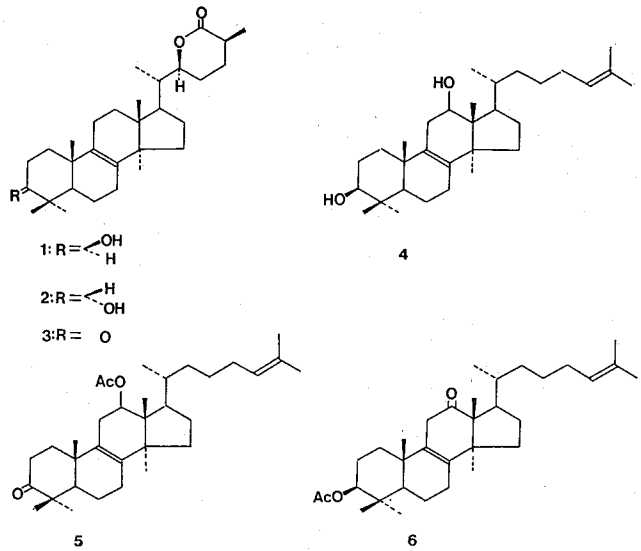
Investigation of the constituents of *Inonotus mikadoi* led to isolation and characterization of ergosterol peroxide (**7**), cerevisterol (**8**) and a cerebroside (**11**).

KEY WORDS: Basidiomycetes/ *Inonotus mikadoi*/ Steroid/ Cerebroside/

Basidiomycetes are the natural sources rich in biologically active substances. In our studies on the biologically active components of Basidiomycetes of *Gasteromycetes,* we have isolated three new lanostane derivatives **1**, **2** and **3** from *Astraeus hygrometricus*[1]. Antitumor lanostane-type triterpene, inotodiol (**4**), was isolated from *Inonotus obliquus* (*Mucronoporaceae*) along with **5** and **6**.[2,3] Several polysaccharides which have an antitumor activity were isolated from *I. cuticularis,*[4] *I. kanekirae*[5] and *I. sciurinus*.[6] We report here the investigation of the constituents of *I. mikadoi*.

The methanol extract from the fresh fruit bodies of *I. mikadoi* was partitioned with water and ethyl acetate. Ethyl acetate extract was separated by silica gel and Sephadex LH-20 column chromatography to give compounds **7**, **8** and **11**.

Compound **7**, mp 181–182°C, $[\alpha]_D^{21}+57.7°$ ($CHCl_3$), showed absorption at 3400(OH) cm^{-1} in the IR spectrum. Its ^1H NMR spectrum showed the presence of four secondary



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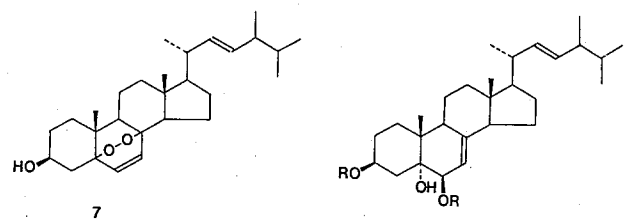
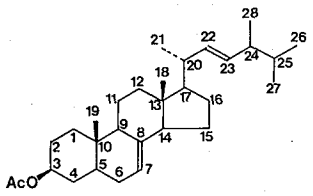


7

8: R=H
9: R=Ac



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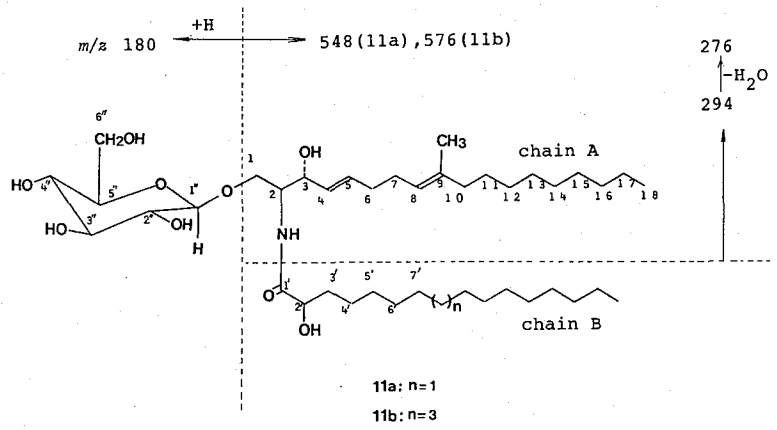


11a: n=1
11b: n=3

methyls [δ 0.82 (d, J=6.8 Hz), 0.83 (d, J=6.8 Hz), 0.91 (d, J=7.0 Hz) and 1.00 (d, J=6.6 Hz)] and two tertiary methyls [δ 8.01 and 0.88 (each s)]. The signal at δ 3.96 (1H, m) was assigned to the proton on carbon bearing the hydroxy group. The signals at δ 5.17 (2H, m), 6.24 (1H, d, J=8.5 Hz) and 6.50 (1H, d, J=8.5 Hz) were assigned to the protons on the double bond. The ^{13}C NMR (Table 1) spectrum of **7** showed the presence of four signals [δ 119.6 (d), 130.7 (d), 132.2 (d) and 135.5 (d)] assigned to olefinic carbons, three signals [δ 66.3 (d), 82.1 (s) and 82.7 (s)] assigned to carbons bearing an oxygen atom, six methyls, seven methylenes, six methines and two quaternary carbons.

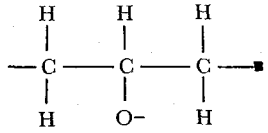
The EIMS of **7** exhibited a molecular ion at m/z 428. These spectral data agreed with a molecular formula for **7** of $C_{28}H_{44}O_3$, which was confirmed by HRMS. On the basis of above results, it was concluded that compound **7** was based on the ergosterol skeleton found in Basidiomycetes. In the EIMS, **7** gave a characteristic fragment ion peaks at m/z 410 [M–H_2O], 396 [M–O_2] and 303 [M-side chain (C_9H_{17})], among which the peak at m/z 396 was characteristic of ergosterol peroxide.[7] Compound **7** was identified from the spectral data and direct comparison with synthetic compound derived from ergosterol.[8]

Compound **8**, mp 253–255°C, $[\alpha]_D^{21}$ −74.0° ($CHCl_3$), showed absorption at 3400(OH)

Table 1. The ^{13}C NMR data of compounds 7, 8 and 10.

Carbons	7	8	10
1	30.1 t	33.8 t	36.8 t
2	32.6 t	32.5 t	27.4 t
3	66.3 d	67.6 d	73.4 d
4	41.2 t	41.8 t	33.8 t
5	82.7 s	76.1 s	40.0 d
6	130.7 d	74.2 d	29.5 t
7	119.6 d	120.4 d	117.2 d
8	82.1 s	141.6 s	139.4 s
9	44.6 d	43.8 d	49.3 d
10	38.0 s	38.0 s	34.1 s
11	23.4 t	22.4 t	21.4 t
12	28.6 t	40.0 t	39.3 t
13	43.6 s	43.8 s	43.2 s
14	55.9 d	55.2 d	55.0 d
15	23.4 t	23.4 t	22.9 t
16	29.4 t	28.4 t	28.3 t
17	56.2 d	56.2 d	55.9 d
18	12.8 q	12.5 q	12.0 q
19	18.1 q	18.7 q	12.8 q
20	39.9 d	40.8 d	40.5 d
21	19.9 q	19.8 q	21.1 q
22	135.5 d	136.2 d	135.5 d
23	132.2 d	132.1 d	131.9 d
24	42.8 d	43.1 d	43.0 d
25	33.1 d	33.3 d	33.2 d
26	20.6 q	20.1 q	19.6 q
27	20.9 q	20.1 q	19.6 q
28	17.6 q	17.8 q	17.9 q

cm^{-1} in the IR spectrum. Its ^1H NMR spectrum (in C_5D_5N) showed the presence of four secondary methyls [δ 0.87, 0.89, 0.97 and 1.07 (each 3H, d, J=6.8 Hz)] and two tertiary methyls [δ 0.67, 1.53 (each 3H, s)]. The signal at δ 4.83 (1H, ddt, J=11.5 and 5.5 Hz) was assigned to the proton on the carbon atom bearing the oxygen atom, which was coupled with the signal at δ 3.03 (1H, dd, J=13.2 and 11.5 Hz). Thus, the partial structure I for compound **8** was deduced. The presence of the partial structure II was indicated from the signal at δ 4.34 (1H, d, J=5.1 Hz) coupled with that at δ 5.74 (1H, d, J=5.1 Hz). Also, the ^1H NMR spectrum of **8** showed the presence of two olefinic protons [δ 5.32 (2H, m)]. The ^{13}C NMR



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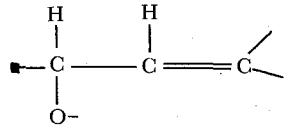


II

spectrum (Table 1) of **8** indicated the presence of four signals [δ 120.4 (d), 132.1 (d), 136.2 (d) and 141.6 (s)] assigned to olefinic carbons, three signals [δ 67.6 (d), 74.2 (d) and 76.1 (s)] assigned to carbons bearing oxygen atom, seven methyls, six methines and two quaternary carbons. The molecular formula. $C_{28}H_{46}O_3$, of compound **8** was determined by the high resolution mass spectrum on the peaks at m/z 412 (M–H_2O), 394 (M–$2H_2O$), and 376 (M–$3H_2O$). Acetylation of **8** gave diacetate **9** indicating the presence of two secondary hydroxy groups and a tertiary hydroxy group in **8** in the molecule. Thus, the molecular formula of compound **8** was assigned to $C_{28}H_{46}O_3$. The peaks at m/z 412, 394 and 376 were shown to M–H_2O, M–$2H_2O$ and M–$3H_2O$, respectively, in which assignment were confirmed by HRMS. These fact indicated that compound **8** was based on the ergosterol skeleton.

The MS of compound **8** showed a peak at m/z 251 [M-side chain (C_9H_{17})–$3H_2O$], which indicated the presence of one double bond on side chain. The partial structures I and II were placed in the ring portion of ergosterol skeleton.

The comparison of ^{13}C NMR spectra of compound **8** and 5,6-dihydro ergosterol acetate (**10**)[9] indicated almost same chemical shifts on the ring C, D and side chain. The partial structure I and II could be placed on the ring A and B. The ^1H NMR signal at δ 4.83 was appeared down field than usual proton on carbon bearing a hydroxy group in the ergosterol skeleton. This phenomenon was explained the effect of neighboring oxygen function. From this reason, the secondary hydroxy group in the partial structure I and tertiary hydroxy group were placed on C–3 and C–5, respectively. Remaining partial structure II was put on the ring B from the ^{13}C NMR data. Thus the structure of **8** was represented as ergosta 7,22-diene-3,5,6-triol, which was known compound named as cerevisterol.[10] The spectral data of compound **8** was good agreement with literature data of cerevisterol.

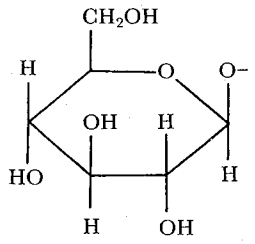
Compound **11**, mp 180–182°C, $[\alpha]_D^{21}$+18.7° (EtOH), showed absorption at 3360(OH), 1640 and 1530(CONH) cm^{-1} in the IR spectrum. Its ^1H NMR spectrum (in C_5D_5N) showed the presence of three methyls [δ 0.88 (3H×2, brt), 1.63 (3H, s)] and many methylenes [δ 1.27 (ca. 42H), 2.20 (2H, brt) and 2.17 (4H, brt)]. The signal at δ 1.63 was attributed to a methyl on double bond from the chemical shift. The signals at δ 5.99 (2H, m) and δ 8.37 (1H, d, J= 7.8 Hz) were assigned to the proton on double bond and amide proton, respectively. The

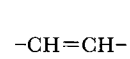
Table 2. The ^{13}C NMR data of compound 11.

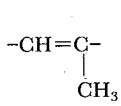
CH_3	CH_2	CH	C
14.2×2	22.8×2	54.3	135.5
16.0	25.7	71.2	175.5
	28.1	72.1	
	28.2	72.3	
	29.5×3	74.6	
	30.0×10	77.9×2	
	32.1×2	105.0	
	32.9	124.0	
	35.4	131.5	
	39.9	132.2	
	62.4		
	69.7		

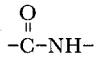
complex signals between δ 3.8–5.0 were estimated to protons of carbohydrate. These ^{1}H NMR data suggested that **11** was a sphingolipid. The ^{13}C NMR spectrum (Table 2) showed the presence of three methyls, many methylenes, eleven methines and two quaternary carbons. The signals at δ 105.6 (s), 74.6 (d), 77.9 (d), 71.2 (d), 77.9 (d) and 62.4 (t) were assigned to glucopyranose carbons (C″–1–C″–6) from the comparison of reported data.[11] The signal at δ 175.5 was assigned to amide carbon. The signals at δ 124.0 (d), 131.5 (d), 132.2 (d) and 135.5 (s) were assigned to olefinic carbons. The remaining low field signals at δ 69.7 (t), 72.1 (t) and 72.3 (d) were attributed to carbons bearing oxygen atom.

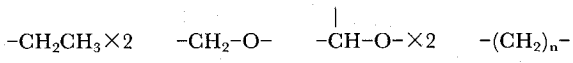
From the above evidence, the partial structure of compound **11** was shown below.











Recently, Kawai et al. isolated a cerebroside [(4E, 8E)-N-D-2′-hydroxypalmitoyl-1-O-β-D-glucopyranosyl-9-methyl-4,8-sphingodienine] from *Schizophyllum commune*, which has a fruiting-inducing activety on the same fungus.[12] ^{1}H NMR data of compound **11** was almost same with literature data. The FABMS of compound **11** showed peaks at *m/z* 750 $[M_I(727)+Na]^+$, 778 $[M_{II}(755)+Na]^+$, 576 $[M_{II}$-glucose$]^+$, 548 $[M_I$-glucose$]^+$, 294, 276 and 180 [glucose]$^+$. These results indicated that compound **11** was mixture of [**11a** (M_I=727), **11b** (M_{II}=755)] due to chain B (**11a**=palmitoyl, **11b**=stearoyl). Further purification of compound **11** is progress.

The isolation of this type cerebroside from Basidiomycetes is the second example, the compound **11** may be related to fruiting-inducing activity for *I. mikadoi*. The study on this point is underway.

EXPERIMENTAL

M.p.s. were taken on Yanagimoto melting point apparatus and are uncorrected. IR spectra were recorded on Hitachi type 215 spectrometer for KBr disks. ^{1}H and ^{13}C NMR spectra were taken with JEOL JMN FX 200 spectrometers for solution in deuterio-chloroform or $d_5$pyridine. Tetramethylsilane was used as internal standard and chemical shifts were given in δ (p.p.m.) value. Mass spectra were determined with JEOL JMS D-300 spectrometer. Optical rotations were measured with Union PM-201 polarimeter. Kiesel gel 60 (70–230 mesh or 230–400 mesh, Merck) and Sephadex LH-20 (Pharmacia) were used for chromatography, and precoated silica gel plates F_{254} (0.25 mm and 0.5 mm in thickness) were used for TLC.

Material. The fresh fruitbody of *I. mikadoi* were collected from the Kainan, Tokushima, Japan in July 1984.

Isolation of compounds 7, 8 and 11. Fresh fruit bodies (1.8 kg) were cut and extracted with hot MeOH (20 l) at three times. The MeOH solution was evaporated to dryness (136 g), dissolved in H_2O and extracted with EtOAc. The EtOAc extract was evaporated under red. pres. to give a residue (24.5 g). The residue was chromatographed on silica gel (600 g) column and eluted succesively with hexane-$CHCl_3$ and $CHCl_3$–MeOH to afford 15 fractions. Fr. 5 (3.2 g) containing compound **7** was chromatographed on silica gel column and eluted with hexane-EtOAc (2:1) to afford fr. 5–4 (1.10 g), which was crystallized from MeOH to give compound **7** (481 mg). Fr. 9 (0.25 g) containing compound **8** was filtrated, the filtrate was concentrated to give a residue (48 mg), which was purified by using preparative TLC ($CHCl_3$–MeOH=9:1) and crystallized from MeOH to give compound **8** (26 mg). Fr. 11 (1.65 g) containing compound **11** was chromatographed on silica gel column and eluted with $CHCl_3$–MeOH–H_2O (88:12:1) to give Fr. 11–2 (510 mg), which was crystallized from MeOH to give compound **11** (346 mg).

Compound **7**, colorless needles, mp 181–182°C, $[\alpha]_D^{21}$+57.7° (c=0.1, $CHCl_3$), IR ν_{max}^{KBr} cm^{-1}: 3350(OH), 1650, 1610, 1450, 1380, 1360, 1060, 1030, 960; EI-MS m/z (rel. int.): 428 $[M]^+$ (2), 410 $[M-H_2O]^+$ (6), 396 $[M-O_2]^+$ (19), 251 (25), 69 (100); HR-MS m/z: 428.3305 $[M]^+$ for $C_{28}H_{44}O_3$, required 428.3291; ^1H NMR δ ($CDCl_3$): 0.81 (3H, s), 0.82 (3H, d, J=6.8 Hz), 0.83 (3H, d, J=6.8 Hz), 0.88 (3H, s), 0.91 (3H, d, J=7.0 Hz), 1.00 (3H, d, J=6.6 Hz), 3.96 (1H, m, 3–H), 5.17 (2H, m, 26–H and 27–H), 6.24 and 6.50 (each 1H, d, J=8.5 Hz, 6–H and 7–H); ^{13}C NMR ($CDCl_3$): Table 1.

Compound **8**, colorless needles, mp 253–255°C, $[\alpha]_D^{21}$−74.0° (c=0.2, $CHCl_3$), IR ν_{max}^{KBr} cm^{-1}: 3400(OH), 1610, 1430, 1360, 1240, 1000, 940, 920; EI-MS m/z (rel. int.): 412 $[M-H_2O]^+$ (43), 394 $[M-2H_2O]^+$ (52), 376 $[M-3H_2O]^+$ (53), 251 $[C_{19}H_{23}]^+$ (100); HR-MS m/z: 412.3340 for $C_{28}H_{44}O_2$, required 412.3341, 394.3215 for $C_{28}H_{42}O$, required 394.3236, 376.3124 for $C_{28}H_{40}$, required 376.3130; ^1H NMR δ (C_5D_5N): 0.67 (3H, s), 0.87, 0.97, 1.07 and 1.26 (each 3H, d, J=6.8 Hz), 1.53 (3H, s), 3.03 (1H, dd, J=13.2 and 11.5 Hz, 4–H), 4.34 (1H, d, J=5.1 Hz, 6–H), 4.83 (1H, m, 3–H), 5.74 (1H, d, J=5.1 Hz, 7–H); ^{13}C NMR (C_5D_5N): Table 1.

Compound **11**, colorless needles, mp 180–182°C, $[\alpha]_D^{21}$+18.7° (c=0.1, EtOH), IR ν_{max}^{KBr} cm^{-1}: 3360(OH), 1640 and 1530(CONH), 1460, 1080; EI-MS m/z (rel. int.): 276 $[C_{18}H_{46}N]^+$ (63), 258 (32), 180 $[glu]^+$ (9), FAB-MS m/z (rel. int.): 778 $[M_{II}+Na]^+$ (15), 750 $[M_I+Na]^+$ (20), 576 $[M_{II}\text{-glu}+H]^+$ (12), 548 $[M_I\text{-glu}+H]^+$ (21), 294 $[C_{18}H_{49}NO]^+$ (8), 276 $[C_{18}H_{47}N]^+$ (15); ^1H NMR δ (C_5D_5N): 0.88 (3H×2, brt, 18–H_3 and 16′–H_3), 1.27 (ca. 42H, 11–H_2~17–H_2, 3′–H_2~15′–H_2), 1.63 (3H, s, 19–H_3), 2.02 (2H, brt, 6–H_2), 2.17 (2H×2, brt, 7–H_2, 10–H_2), 4.92 (1H, d, J=7.6 Hz, anomeric H), 5.99 (2H, m, 5–H, 8–H), 8.37 (1H, d, J=7.8 Hz, CONH); ^{13}C NMR (C_5D_5N): Table 2.

Ergosterol peroxide from ergosterol. A solution of ergosterol (50 mg) in $CHCl_3$ (50 ml) was stirred at 25°C under sun light for 5 hr, the reaction mixture was evaporated to give a residue, which was chromatographed on silica gel column and eluated with hexane-EtOAc (4:1) to give ergosterol peroxide, mp 182–183°C, EI-MS m/z: 428 $[M]^+$. This compound was identifide with compound **7** by direct comparison (TLC, IR, EI-MS and ^1H NMR).

Acetylation of compound 8. A solution of **8** (10 mg) in pyridine (0.5 ml) and Ac_2O (0.5 ml) was kept at room temperature for 12 hr., the reaction mixture was work up in usual way to give a residue, which was crystallized from MeOH to give a needles (6.4 mg), compound **9**, ^1H

NMR δ ($CDCl_3$): 0.82 (3H, d, J=7.0 Hz, 26-H_3), 0.83 (3H, d, J=7.0 Hz, 27-H_3), 0.92 (3H, d, J=7.0 Hz, 28-H_3), 1.04 (3H, d, J=7.0 Hz, 21-H_3), 1.08 (3H, s, 19-H_3), 1.58 (3H, s, 18-H_3), 4.91 (1H, d, J=3.5 Hz, 6-H), 5.12 (1H, m, 3-H), 5.26 (1H, m, 7-H), 2.02 and 2.06 (each 3H, s, $COCH_3$).

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