Sterols from a Vietnamese Wood-Rotting *Phellinus* sp.

Dang Ngoc Quang[a,b], Dang Dinh Bach[a], and Yoshinori Asakawa[b]

[a] Faculty of Chemistry, Hanoi University of Education, 136-Xuan Thuy Road, Cau Giay district, Hanoi, Vietnam
[b] Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Yamashiro-cho, Tokushima 770-8514, Japan

Reprint requests to Dr. Dang Ngoc Quang. E-mail: quangdn@dhsphn.edu.vn

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Phytochemical examination of the methanolic extract from fruit bodies of an unidentified Vietnamese *Phellinus* species led to the isolation of four compounds, one of which is a new steroid, 25-hydroxy-ergosta-7,24(28)-dien-3β-ol, named phellinol, together with senexonol, trametenolic acid B and ergosta-4,6,8(14),22-tetraen-3-one. Their structures were determined by 2D NMR, MS, IR and UV spectroscopy. In addition, the absolute configuration of senexonol was established by X-ray crystallographic analysis of its *p*-bromobenzoate derivative as 22(*R*)-hydroxy-4(*S*),14(*S*)-dimethyl-cholesta-8,24-dien-3-one. All compounds moderately suppressed the lipopolysaccharide (LPS)-induced production of nitric oxide (NO) in RAW 264.7 cells.

Key words: Phellinus, Sterol, Phellinol, Nitric Oxide

Introduction

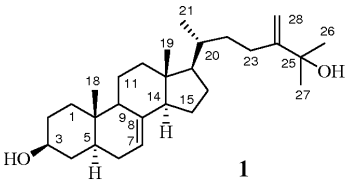
Wood-rotting fungi of the genus *Phellinus* are rich sources of aromatics, polysaccharides, sterols and triterpenes. Since 1969, when Efimenko [1] reported the isolation of ergosterol from *P. pini* var. *abietis*, there have been many reports on antitumor polysaccharides [2], odorous constituents produced by various species of *Phellinus* [3], phenolic pigments from sporocarps of *P. igniarius* and other species [4], triterpenoids from *P. gilvus* [5] and *P. pomaceus* [6], and antioxidants and free radical scavenging activity of *P. baumii* [7]. In the course of our phytochemical studies on Vietnamese fungi, we also reported antimicrobial aromatic compounds from the ascomycete *Xylaria intracolorata* [8] and a sterol from *Xylaria* sp. [9]. In continuation, the methanolic extract of an unidentified *Phellinus* sp. was studied and four compounds were isolated. Their inhibitory activities with regard to NO production in RAW 264.7 cells was examined.

Results and Discussion

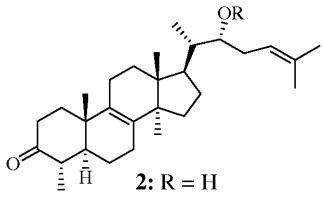
The fruit bodies of the *Phellinus* sp. were air-dried and extracted with methanol. The methanolic extract was concentrated and subjected to silica gel, Sephadex LH-20 and MPLC, to give four compounds, three of which were previously reported as senexonol (**2**) [10], trametenolic acid B (**3**) [11] and ergosta-4,6,8(14),22-tetraen-3-one (**4**) [9].

The EIMS of phellinol (**1**) exhibited a molecular peak at $m/z = 414.3508$, corresponding to the molecular formula of $C_{28}H_{46}O_2$ as determined by HREIMS. Its ^{1}H NMR spectrum showed the presence of an olefinic proton ($\delta_H = 5.16$), an exo-ethylene, together with protons of four methyl groups. The ^{13}C NMR spectrum (Table 1) contained 28 carbon signals, including two oxygenated and four olefinic carbon atoms. These spectral data of **1** suggested a typical ergostane skeleton [12 – 14]. In addition, **1** is similar to ergosta-7,24(28)-dien-3β-ol [12] with a notable difference of a hydroxyl group at C-25, which was confirmed by low field shifts of the signals of two methyl groups H-26 ($\delta_H = 1.35$) and H-27 ($\delta_H = 1.25$) as well as C-25 ($\delta_C = 73.6$). Consequently, phellinol (**1**) was deduced to be 25-hydroxyergosta-7,24(28)-dien-3β-ol as shown in Fig. 1.

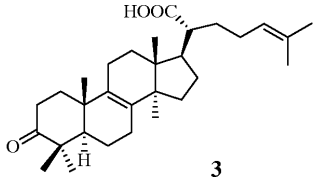
Senexonol (**2**) was previously purified from the Polyporous fungus *Fomes senex* [10] without any ^{1}H and ^{13}C NMR spectral data. In the present paper, full spectral data of **2** are reported. Furthermore, the absolute structure of **2** was determined by X-ray crystallographic analysis of its *p*-bromobenzoate (**5**, ORTEP drawing shown in Fig. 2) allowing us to determine its absolute structure as 22(*R*)-hydroxy-4(*S*),14(*S*)-



1



2: R = H
5: R = *p*-Br-C_6H_4-CO



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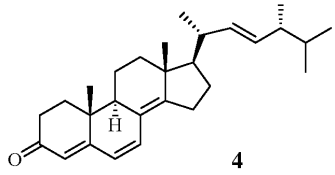

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Fig. 1. Structures of **1** – **5**.

Table 1. ^{13}C NMR chemical shift data for **1** – **2** and **5** (δ values, 150 MHz, $CDCl_3$).

H	1	2	5
1	37.1	37.1	37.1
2	31.5	38.0	38.0
3	71.1	213.5	213.5
4	38.0	45.0	45.0
5	40.2	49.7	49.7
6	29.6	22.0	22.0
7	117.5	25.5	25.5
8	139.5	135.5	135.4
9	49.4	132.6	132.5
10	34.2	36.6	36.6
11	21.5	21.9	21.8
12	39.5	30.9	31.0
13	43.4	44.8	44.9
14	55.0	49.4	49.5
15	22.9	29.7	30.9
16	27.9	27.2	27.0
17	55.9	47.2	47.3
18	11.8	15.7	15.7
19	13.0	17.5	17.5
20	36.3	41.6	40.0
21	18.9	12.6	13.4
22	35.4	73.3	78.0
23	27.6	29.1	26.7
24	156.8	121.3	120.4
25	73.6	135.2	133.7
26	29.3	26.0	25.7
27	29.7	18.0	17.9
28	106.7	11.4	11.4
29		24.4	24.4
Benzoate			165.4, 131.6, 131.0, 129.9, 127.7

dimethylcholesta-8,24-dien-3-one. This is the second report of the very rare carbon skeleton which has only one methyl group at C-4.

Previously, **4** was shown to inhibit the nitric oxide (NO) production in RAW 264.7 cells with an IC_{50} value of 28.96 μM [9]. Compounds **1** – **3** were also

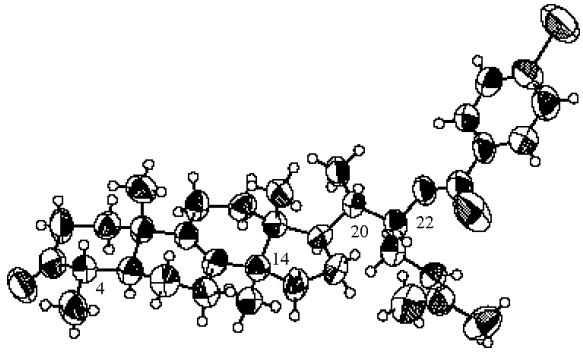

Fig. 2. ORTEP drawing of **5**.

evaluated regarding their inhibitory activities against NO production stimulated by LPS in RAW 264.7 cells. They moderately suppressed the LPS-induced production of NO with IC_{50} values of 22.6, 31.3 and 85.4 μM, respectively.

Experimental Section

General

Melting points were determined on a Yanagimoto micromelting point apparatus and are uncorrected. Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as a solvent. IR spectra were measured on a JASCO FT/IR-5300 spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C), using $CDCl_3$ as a solvent. Chemical shifts are given relative to TMS ($\delta = 0.00$ as internal standard (^{1}H) and $\delta = 77.0$ (ppm) from $CDCl_3$ (^{13}C)). Mass spectra were recorded on a JEOL JMS AX-500 spectrometer. Preparative medium-pressure liquid chromatography (MPLC) was performed with a Work-21 pump (Lab-Quatec Co., Ltd., Japan) and a

Lobar column (Merck). Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech, $CHCl_3$-MeOH, 1 : 1).

Fungal material

Fruit bodies of *Phellinus* sp. were collected in Nho Quan district, Ninh Binh province, Vietnam in February, 2002 and identified by Ms. Makiko Nukada (Kurashiki Sakuyo University, Kurashiki, Japan). A voucher specimen (VN02-2) has been deposited at the Faculty of Pharmaceutical Sciences, Tokushima Bunri University, Tokushima, Japan.

Extraction and isolation

Fresh fruit bodies of the *Phellinus* sp. (75.3 g) were extracted with MeOH, and the MeOH extract was concentrated to give a residue (4.2 g), which was chromatographed on a Sephadex LH-20 column (35 × 1000 mm), using MeOH-$CHCl_3$ (1 : 1, 400 mL) to give 3 fractions. Fraction 2 (2.34 g) was further separated on a silica gel column, MeOH-$CHCl_3$ (1 : 20, 600 mL) to afford 11 sub-fractions. Sub-fraction 3 (150.8 mg) was purified by MPLC, on a RP-18 Lobar column, eluent MeOH (200 mL), flow rate 1 mL/min to give **2** (47.4 mg) and **4** (6.3 mg). Sub-fraction 7 (126.7 mg) was rechromatographed on a Sephadex LH-20 column using MeOH-$CHCl_3$ (1 : 1, 200 mL), followed by MPLC under the same conditions as sub-fraction 3 to give **1** (15.4 mg) and **3** (32.7 mg).

*25-Hydroxy-ergosta-7,24(28)-dien-3β-ol (**1**)*

$[\alpha]_D^{20} = +6.8°$ (c = 0.10, $CHCl_3$). – IR (KBr): $\nu = 3322$ (OH), 1467, 1378, 1259, 1156, 1042 cm^{-1}. – EIMS *m/z* (%) = 414 (27) [M^+], 396 (81), 381 (73), 314 (52), 271 (100), 231 (37), 161 (27), 147 (35), 119 (40), 95 (44), 55 (39). – HREIMS: $m/z = 414.3508$ [M^+] calcd. for $C_{28}H_{46}O_2$ 414.3498. – ^{1}H NMR (600 MHz, $CDCl_3$): $\delta =$ 0.80 (s, 3H, 19-H), 0.55 (s, 3H, 18-H), 0.98 (d, $J = 6.6$ Hz, 3H, 21-H), 1.25 (s, 3H, 27-H), 1.35 (s, 3H, 26-H), 3.60 (m, 1H, 3-H), 4.77 (d, $J = 1.1$ Hz, 1H, 28-H), 5.10 (d, $J = 0.6$ Hz, 1H, 28-H), 5.16 (t, $J = 2.2$ Hz, 1H, 7-H). – ^{13}C NMR (150 MHz, $CDCl_3$) data see Table 1.

*Senexonol (**2**)*

$[\alpha]_D^{20} = +39°$ (c = 0.1, $CHCl_3$). – ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.77$ (s, 3H, 18-H), 0.88 (s, 3H, 29-H), 0.96 (d, $J = 6.6$ Hz, 3H, 21-H), 1.03 (d, $J = 6.7$ Hz, 3H, 28-H), 1.20 (s, 3H, 19-H), 1.66 (s, 3H, 27-H), 1.75 (s, 3H, 26-H), 3.67 (m, 1H, 22-H), 5.18 (dt, $J = 1.4$, 6.6 Hz, 1H, 24-H). – ^{13}C NMR (150 MHz, $CDCl_3$) data see Table 1.

*Preparation of the p-bromobenzoate of **2***

To a solution of **2** (14.9 mg) in pyridine (1.5 mL) was added *p*-Br-C_6H_4COCl (55 mg). The reaction mixture was stirred at r. t. for 24 h. Work up as usual gave a residue (34.6 mg), which was purified by silica gel column chromatography using hexane-EtOAc (1 : 1) to yield **5** (12.7 mg) as white crystals from MeOH. M. p.: 154 – 158 °C; $[\alpha]_D^{20} = +30°$ (c = 0.10, $CHCl_3$). – IR (KBr) ν_{max}: 1711 (C=O), 1590 (C=C), 1433, 1376, 1275, 1115, 1012, 766 cm^{-1}. – FABMS: $m/z = 631$ [M+Na]$^+$. – HRFABMS: $m/z =$ 631.2747 [M+ Na] calcd. for $C_{36}H_{49}O_3BrNa$ 631.2763. – ^{1}H NMR (600 MHz, $CDCl_3$): $\delta = 0.74$ (s, 3H, 18-H), 0.90 (s, 3H, 29-H), 1.03 (d, $J = 6.3$ Hz, 3H, 28-H), 1.04 (d, $J =$ 6.0 Hz, 3H, 21-H), 1.20 (s, 3H, 19-H), 1.63 (d, $J = 1.1$ Hz, 6H, 26-H, 27-H), 5.12 (tt, $J = 1.4$, 7.1 Hz, 1H, 24-H), 5.16 (td, $J = 3.3$, 9.9 Hz, 1H, 22-H), 7.58 and 7.89 (d, $J = 8.8$ Hz, benzoate). – ^{13}C NMR ($CDCl_3$) data see Table 1.

*Crystal data for **5***

Data collection: Cell refinement: Scalepack (HKL). Data reduction: maXus [15]. Program used to solve structure: SHELXL-97 [16]. Refinement on F^2 full matrix least-squares. Diffractometer: DIP Image plate. A colorless crystal of $C_{36}H_{49}BrO_3$ having approximate dimensions 0.8 × 0.1 × 0.01 mm, f. w. 608.29 monoclinic, $a = 16.550$ (14) Å, $b = 7.551$ (6) Å, $c = 27.126$ (4) Å, $\beta = 101.748$ (4)°, $V = 3318.9$ (6) Å^3, $Z = 4$, MoK_α radiation, $\lambda = 0.71073$ Å, $\mu = 1.271$ mm^{-1}, 9458 reflections, 722 parameters; only coordinates of H atoms refined, $R = 0.0732$, $R_w = 0.1722$, $S = 1.006$. Crystallographic data for **5** (deposition number CCDC 611563) have been deposited at the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge from The Cambridge Crystallogrphic Data Centre *via* www.ccdc.cam.ac.uk/data_request/cif.

Bioassay

Inhibition of NO production in RAW 264.7 cells stimulated by LPS of **1** – **4** was tested by the same method as reported previously [9].

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