Sterols from a Vietnamese Wood-Rotting Phellinus sp.

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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 C28H46O2 as determined by HREIMS. Its 1H NMR spectrum showed the presence of an olefinic proton (δH = 5.16), an exo-ethylene, together with protons of four methyl groups. The 13C 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 (δH = 1.35) and H-27 (δH = 1.25) as well as C-25 (δC = 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 1H and 13C 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)-

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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 IC₅₀ value of 28.96 µM [9]. Compounds 1–3 were also 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 IC₅₀ 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 CHCl₃ as a solvent. IR spectra were measured on a JASCO FT/IR-5300 spectrophotometer. The ¹H and ¹³C NMR spectra were recorded on a Varian Unity 600 NMR spectrometer (600 MHz for ¹H and 150 MHz for ¹³C), using CDCl₃ as a solvent. Chemical shifts are given relative to TMS (δ = 0.00 as internal standard (¹H) and δ = 77.0 ppm (ppm) from CDCl₃ (¹³C)). 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, CHCl₃-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 with MeOH and CHCl₃ (1:1, 400 mL) to give 3 fractions. Fraction 2 (2.34 g) was purified by MPLC, on a RP-18 Lobar column (Merck). Column chromatography was carried out on Sephadex LH-20 (Amersham Pharmacia Biotech, λ = 0.15 mm, eluent MeOH (200 mL), flow rate 1 mL/min to give 2 (150.8 mg) was purified by MPLC, on a RP-18 Lobar column (Merck). Column chromatography was carried out on Sephadex LH-20 (35 × 1000 mm), using MeOH-CHCl₃ (1:1, 200 mL), followed by MPLC under the same conditions as sub-fraction 3 to give 2 (15.4 mg) and 3 (32.7 mg).

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

\[\alpha_D^{20} = +6.8^\circ (c = 0.10, \text{CHCl}_3), \quad \text{IR (KBr): } \nu = 3322 \quad \text{(OH)}, 1467, 1378, 1259, 1156, 1042 \text{ cm}^{-1}. \quad \text{- 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). \quad \text{- HREIMS: } m/z = 414.3508 [M^+] \text{ calcd. for C}_{36}H_{49}O_{3}BrNa 631.2763. \quad - \text{IR (KBr): } \nu_{\text{max}}: 1711 (C=O), 1590 (C=C), 1433, 1376, 1275, 1115, 1012, 766 \text{ cm}^{-1}

\[\text{- FABMS: } m/z = 631 [M+Na]^+. \quad \text{- HRFABMS: } m/z = 631.2747 [M+ Na] \text{ calcd. for C}_{36}H_{49}O_{3}BrNa 631.2763. \quad - \text{1H NMR (600 MHz, CDCl}_3\text{): } \delta = 0.74 \text{ (s, 3H, 18-H)}, 0.90 \text{ (s, 3H, 29-H)}, 1.03 \text{ (d, J = 6.3 Hz, 3H, 28-H)}, 1.04 \text{ (d, J = 6.0 Hz, 3H, 21-H)}, 1.20 \text{ (s, 3H, 19-H)}, 1.63 \text{ (d, J = 1.1 Hz, 6H, 26-H, 27-H)}, 5.12 \text{ (tt, J = 1.4, 7.1 Hz, 1H, 24-H)}, 5.16 \text{ (dd, J = 3.3, 9.9 Hz, 1H, 22-H)}, 7.58 \text{ and 7.89 (d, J = 8.8 Hz, benzene)}, \quad \text{- }^{13}\text{C NMR (CDCl}_3\text{) data see Table 1.}

**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].

**Acknowledgements**

Financial support from the Japan Society for the Promotion of Science in the form of a postdoctoral fellowship to D. N. Quang (No. P04162) is gratefully acknowledged. Our special thanks go to Ms. M. Nukada (Kurashiki Sakuyo University, Japan) for her identification of the fungal sample. We thank Dr. Masami Tanaka, Mr. Shigeru Takaoka and Ms. Yasuko Okamoto (TBU, Japan) for recording NMR, X-ray and mass spectra, respectively. Our special thanks are due to Dr. Takashi Nishizawa and Dr. Gen-Ichiro Soma (TBU, Japan) for their help in examination of NO production.

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

To a solution of 2 (14.9 mg) in pyridine (1.5 mL) was added p-Br-C₆H₅COCl (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^\circ (c = 0.10, \text{CHCl}_3). \quad \text{- IR (KBr) } \nu_{\text{max}}: 1711 (C=O), 1590 (C=C), 1433, 1376, 1275, 1115, 1012, 766 \text{ cm}^{-1}

\[\text{- FABMS: } m/z = 631 [M+Na]^+. \quad \text{- HRFABMS: } m/z = 631.2747 [M+ Na] \text{ calcd. for C}_{36}H_{49}O_{3}BrNa 631.2763. \quad - \text{1H NMR (600 MHz, CDCl}_3\text{): } \delta = 0.74 \text{ (s, 3H, 18-H)}, 0.90 \text{ (s, 3H, 29-H)}, 1.03 \text{ (d, J = 6.3 Hz, 3H, 28-H)}, 1.04 \text{ (d, J = 6.0 Hz, 3H, 21-H)}, 1.20 \text{ (s, 3H, 19-H)}, 1.63 \text{ (d, J = 1.1 Hz, 6H, 26-H, 27-H)}, 5.12 \text{ (tt, J = 1.4, 7.1 Hz, 1H, 24-H)}, 5.16 \text{ (td, J = 3.3, 9.9 Hz, 1H, 22-H)}, 7.58 \text{ and 7.89 (d, J = 8.8 Hz, benzene)}, \quad \text{- }^{13}\text{C NMR (CDCl}_3\text{) 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 F² full matrix least-squares. Diffractometer: DIP Image plate. A colorless crystal of C₃₆H₄₉BrO₃ having approximate dimensions 0.8 × 0.1 × 0.01 mm, f. w. 608.29 monoclinic, \[a = 16.550 (14) \AA, b = 7.551 (6) \AA, c = 27.126 (4) \AA, \beta = 101.748 (4)^\circ, \quad V = 3318.9 (6) \AA^3, Z = 4, \quad \text{MoK}_\alpha \text{ radiation, } \lambda = 0.71073 \AA, \mu = 1.271 mm^-1, 9458 \text{ reflections, 722 parameters}; \quad \text{only coordinates of H atoms refined, } R = 0.0732, R_w = 0.1722, \quad S = 1.006. \text{ Crystallographic data for 5 (deposition number CCDC 615653) have been deposited at the Cambridge Crystallographic Data Centre. Copies of the data can be obtained, free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.}

**Senexonol (2)**

\[\alpha_D^{20} = +39^\circ (c = 0.1, \text{CHCl}_3), \quad \text{- 1H NMR (600 MHz, CDCl}_3\text{): } \delta = 0.77 \text{ (s, 3H, 18-H)}, 0.88 \text{ (s, 3H, 29-H)}, 0.96 \text{ (d, J = 6.6 Hz, 3H, 21-H)}, 1.03 \text{ (d, J = 6.7 Hz, 3H, 28-H)}, 1.20 \text{ (s, 3H, 19-H)}, 1.66 \text{ (s, 3H, 27-H)}, 1.75 \text{ (s, 3H, 26-H)}, 3.67 \text{ (m, 1H, 22-H)}, 5.18 \text{ (dt, J = 1.4, 6.6 Hz, 1H, 24-H)}, \quad \text{- }^{13}\text{C NMR (150 MHz, CDCl}_3\text{) data see Table 1.}
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