Pregnane-Type Steroids from the Inedible Mushroom *Thelephora terrestris*

Niko RADULOVIĆ,[a,b] Dang Ngoc QUANG,[a] Toshihiro HASHIMOTO,[a] Makiko NUKADA,[c] Masami TANAKA,[a] and Yoshinori ASAKAWA*,[a]

[a] *Faculty of Pharmaceutical Sciences, Tokushima Bunri University; Yamashiro-cho, Tokushima 770–8514, Japan:* [b] *Department of Chemistry, Faculty of Sciences and Mathematics, University of Niš; Višegradska 33, 18000 Niš, Serbia and Montenegro: and* [c] *Faculty of Food Culture, Kurashiki Sakuyo University; Kurashiki 710–0290, Japan.*

Received October 18, 2004; accepted November 22, 2004

Chromatographic fractionation of the methanol extract of fruiting bodies of the inedible Japanese mushroom *Thelephora terrestris* (Thelephoraceae) has led to the isolation and characterization of two unusual new pregnane-type steroids, 2β,3α-dihydroxypregna-4,7,16-trien-12,20-dione (2) and 1α,2β,3α-trihydroxypregna-4,7,16-trien-12,20-dione (3) named terresterones A and B (2, 3), as well as the previously known compound stizophyllin, now assigned as 2β,3α,12β-trihydroxypregna-4,7,16-trien-20-one (1). Elucidation of their structures and the revision of the stereochemical assignment of stizophyllin were achieved by means of extensive 1D and 2D NMR, UV, CD, IR, MS and molecular modeling experiments. This paper presents the first report on the isolation of true pregnane-type steroids from the Fungi kingdom.

Key words fungi; *Thelephora terrestris*; terresterone; stizophyllin; pregnane steroid

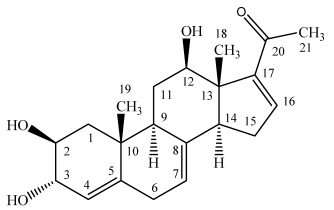
An apparent generality of the distinction[1)] in the biosynthesis of isoprenoid-derived membrane components between mammals (cholesterol), fungi (ergosterol), plants (sitosterol), eubacteria (bacteriohopanepolyols), and archaebacteria (archaebacterial core lipids) has led some authors (Ourisson,[2)] for example) to certain considerations on the evolution of steroid and triterpene metabolism. Until now the capability of fungi to produce true pregnane derivatives (C_{21}) as metabolites that do not originate from the triterpenoid branch of the mentioned biosynthetic pathway has not been reported. 8α,9α-Epoxy-4,4,14α-trimethyl-3,7,11,15,20-pentaoxo-5α-pregnane isolated from *Ganoderma concinna*[3)] has undergone the side chain cleavage of a lanostane precursor, however, it lacks the result of numerous enzymatic steps leading to demethylations which occur in the usual pathway from lanosterol to ergosterol in fungi, indicating that its properties are triterpenoid in nature.

Mushrooms belonging to the Thelephoraceae family are well known for being an abundant source of *p*-terphenyl derivatives.[4—6)] These derivatives have also been isolated from *Thelephora terrestris* in the course of this phytochemical investigation, but the results will be published elsewhere. Herein we report on the isolation and structural elucidation of two new pregnane-type steroids, named terresterones A, B (**2**, **3**), as well as on the revision of the stereochemical assignment of a previously known pregnane derivative stizophyllin (**1**), which was isolated from a higher plant (Fig. 1).

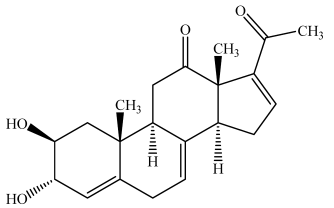
Results and Discussion

The methanol extract of dried fruiting bodies of the Japanese mushroom *Thelephora terrestris* was subjected to normal and reverse-phase (C-18) column chromatography, followed by reverse-phase preparative HPLC to afford pure samples of three pregnane-type steroids as described in the Experimental section.

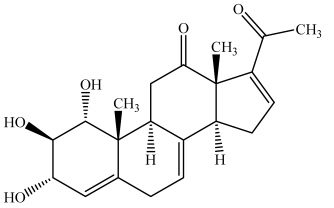
Compound **1**, isolated as an amorphous solid, showed a *quasi*-molecular ion (HR-FAB-MS) corresponding to the molecular formula $C_{21}H_{28}O_4Na$ and two typical C-18 and C-19 methyl groups in the high-field ^{1}H- and ^{13}C-NMR that suggested a pregnane skeleton possessing a keto group which can be placed at C-20 from the characteristic δ_C for an α,β-unsaturated ketone[7)] (Tables 1, 2). It was soon recognized from the perfect fit with the previously published spectral data that the identity of the compound was that of stizophyllin (**1**),[8)] a pregnane derivative isolated from a shrub native to continental tropical America. The long-range ^{1}H–^{13}C interactions observed in the HMBC spectrum of stizophyllin (**1**) corroborated the already established C–C connectivity. However, the proposed 2α,3β stereochemistry of the OH groups could not be substantiated by the cross peaks observed in the nuclear Overhauser effect spectroscopy (NOESY) spectrum. In order to investigate the stereochem-



1



2



3

Fig. 1. Structures of **1**—**3**

* To whom correspondence should be addressed. e-mail: asakawa@ph.bunri-u.ac.jp

Table 1. ^{1}H-NMR Spectral Data for Compounds **1**—**3** ($CDCl_3$, 600 MHz)

Position	**1**	**2**	**3**
1α	2.07 dd (13, 4)	1.93 dd (13.7, 3.8)	
1β	1.36 dd (12, 13)	1.39 dd (12.6, 13.7)	3.45 d (10.4)
2α	3.59 ddd (12, 8, 4)	3.56 ddd (3.8, 8.0, 12.6)	3.58 dd (7.0, 10.4)
3β	4.06 m	4.07 ddd (1.6, 3.6, 8.0)	4.10 m
4	5.24 br s	5.30 t (1.6)	5.26 br s
6α	2.47 m	2.61 br dd (6, 18)	2.54 br dd (4.1, 18.8)
6β	2.99 br d (18)	3.1 br d (18)	3.0 br d (18.8)
7	5.35 m	5.51 m	5.53 m
9α	2.31 m	2.71 m	2.82 m
11α	2.03 m	2.43 dd (11.5, 5.2)	2.98 dd (13.7, 5.2)
11β	1.59 ddd (13, 12, 10)	2.75 t (11.5)	2.75 t (13.7)
12α	3.89 dd (10, 5)		
14α	2.20 m	2.50 br dd (11.5, 1.6)	2.40—2.48 m (overlapped)
15α	2.37 m	2.47 m	2.40—2.48 m (overlapped)
15β	2.52 m		
16	7.00 br s	6.64 br s	6.64 br s
18	0.79 s	1.24 s	1.22 s
19	1.08 s	1.19 s	1.32 s
21	2.38 s	2.34 s	2.32 s

Table 2. ^{13}C-NMR Spectral Data for Compounds **1**—**3** ($CDCl_3$, 150 MHz)

Position	**1**	**2**	**3**
1	38.9	38.7	77.4
2	70.8	70.9	73.8
3	74.1	74.4	72.9
4	120.5	121.2	120.4
5	143.2	142.7	141.7
6	31.3	31.4	31.8
7	118.0	120.6	120.9
8	135.5	134.1	142.4
9	44.3	47.0	46.5
10	38.9	39.1	42.9
11	30.3	38.0	40.2
12	72.6	208.4	210.9
13	52.3	60.6	60.4
14	52.5	54.8	54.6
15	30.9	30.5	30.6
16	148.6	142.1	142.4
17	154.2	150.0	150.0
18	11.5	16.9	17.0
19	23.0	22.6	19.6
20	199.3	196.0	196.4
21	26.5	27.2	27.3

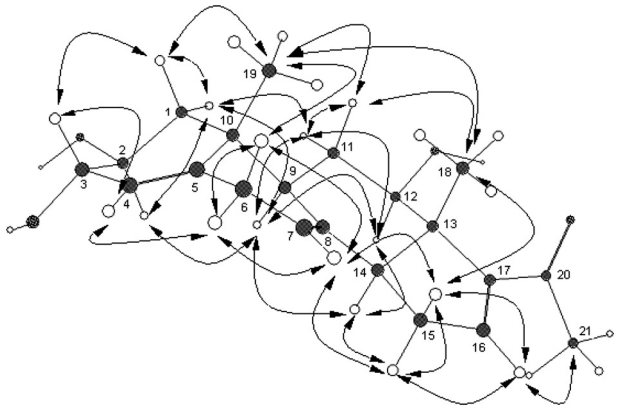


Fig. 2. Calculated Preferred Conformation of Stizophyllin (**1**) and the Observed NOESY Interactions

istry and the conformation of **1**, a detailed study including molecular modeling experiments has been performed. A key interaction between H-2 and H-9 suggested that only the half-chair conformation of the A-ring with α H-2 in a pseudo-axial position would result in a relatively short distance (*ca.* 2.8 Å) between the mentioned atoms. A similar NOE enhancement of H-2 caused by the irradiation of H-9 was previously reported for an ecdysteroid[9] isolated from a *Parazoanthus* sp. with the same A ring substitution pattern. The configuration of the hydroxyl group affixed to C-3 was assigned 3α due to the proximity of H-3 to hydrogen with δ 2.07, assigned to H-1α that is in turn proximal to the angular C-19 methyl group, and due to the diaxial relationship expected for H-2 and H-3 from the relatively large value for their coupling constant (J=8 Hz), thus placing both of the OH groups on the A-ring in pseudo-equatorial positions. Further support of the stereochemistry comes from the observed homoallylic couplings between H-6β and H-3, and H-9 and H-14 indicating a rigid structure with σ (C–H)-orbitals periplanar to the π-orbitals of Δ^4- and Δ^7-double bonds. The conformation of stizophyllin with altered stereochemistry on the A ring, 2β,3α,12β-trihydroxypregna-4,7,16-trien-20-one, was optimized to correspond to the global energetic minimum by the use of MM+, and AM1 force fields (at semi-empirical level), with the Polak-Ribiere (conjugate gradient) minimization method with an energy convergence criterion of 0.04 kJ/mol, incorporated in the HyperChem 7 software package. The ball-and-stick representation of the optimized conformation with included observed NOESY interactions is presented in Fig. 2. Arguments used to establish the relative configuration of stizophyllin (**1**) by Duh *et al.*[8] fully support the corrected stereochemistry.

Compound **2**, the details of which greatly resemble stizophyllin, was an amorphous isolate from the same fraction with a slightly smaller *Rf* value on RP TLC. The molecular formula of compound **2**, $C_{21}H_{26}O_4$, deduced from the HR-EI-MS peak at *m*/*z* 342.1831, suggested that it might be a metabolite closely related to stizophyllin (**1**), differing from which in only having two hydrogen atoms less. The IR spectrum displayed absorption bands of hydroxy (3448 cm^{-1}) and

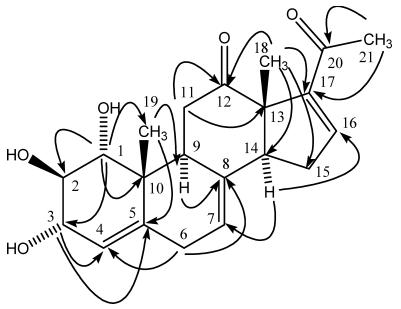


Fig. 3. Significant HMBC Correlations of Terresterone B (**3**)

two carbonyl groups (1705, 1671 cm^{-1}). The ^{1}H- and ^{13}C-NMR (Tables 1, 2) spectra of **2** showed the presence of three trisubstituted olefins (δ 121.2 d, 142.7 s, 120.6 d, 134.1 s, 142.1 d, 150.0 s), two carbonyl carbons (δ 208.4 s, 196.0 s), and two methines (δ 70.9, 74.4)-bearing an oxygen atom, as well as three tertiary methyls, four methylenes, two methines, and two quaternary carbons. The above spectral evidence and the similarity of ^{1}H-NMR spectra with stizophyllin (**1**) led us to assume that compound **2** is a pregnane derivative formed as an oxidation product of stizophyllin at C-12. The heteronuclear multiple bond coherence (HMBC) interactions of the carbonyl at δ 208.4 with H-11, H-18 and H-9 confirmed this placement. Stereochemical arguments obtained from the NOESY spectrum further permitted the assignment of the structure 2β,3α-dihydroxypregna-4,7,16-trien-12,20-dione to compound **2**, named terresterone A (**2**) (12-dehydrostizophyllin).

The third steroidal isolated, terresterone B (**3**), as implied by its molecular formula, $C_{21}H_{26}O_5$, the molecular ion at *m/z* 358.1779 (Δ-0.0001 mmu of calcd) in the EI-HR-MS, had an extra oxygen atom compared to terresterone A (**2**). The pregnane framework of **3** was substantiated by correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and HMBC correlations, and by comparing the ^{1}H- and ^{13}C-NMR data with those of **1** and **2**. The hydroxy and three oxymethine groups in **3** were indicated by an IR band at 3311 cm^{-1} and by the three relatively significant peaks at *m/z* 340, 322 and 304 formed by consecutive losses of water from the molecular ion in the EI-MS spectrum, and also by the resonances of three methine signals at δ 77.4, 73.8, and 72.9 in the ^{13}C-NMR. These three hydroxyl groups were concluded to be adjacent to each other, since a correlation between the carbinol proton at δ 3.58 (dd) and the other two at δ 3.45 (d) and 4.10 (m) was observed in the COSY spectrum. Further inspection of ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) spectra of **3** (Fig. 3) suggested that a further oxymethine proton (δ 3.45 d), absent in terresterone A (**2**), should be assigned at C-1. The value of the coupling constant (J=10.4 Hz) between H-1 and H-2 was indicative of a *trans*-diaxial relationship. Thus, terresterone B (**3**) (1α-hydroxy-12-dehydrostizophyllin) was given a structure 1α,2β,3α-trihydroxypregna-4,7,16-trien-12,20-dione.

This is the first evidence of isolation of pregnane-type steroids (**1**—**3**) from the Fungi.

Compounds **1**—**3** were tested for antimicrobial activity in a disk diffusion assay, however, they showed neither antibacterial nor antifungal activity against a panel of common pathogenic microorganisms at a dose of 50 μg per disk. Previously, stizophyllin was isolated in a bioactivity guided phytochemical study[8] and was found to exhibit cytotoxic activity that was related to the unsaturated carbonyl moiety of the molecule. Terresterones A, B (**2**, **3**), thus may have potential cytotoxic activity and deserve further study in that direction.

Experimental

Column chromatography was carried out on silica gel 60 (0.2—0.5 mm, 0.04—0.063 mm, Merck) and Cosmosil 75 C_{18}. Preparative HPLC separations were conducted on a Shimadzu liquid chromatograph (LC-10AS) with RID-6A and SPD-10A detectors using a Waters 5C 18-AR-II column. UV spectra were obtained on a Shimadzu UV-1650PC instrument in MeOH. CD spectra were recorded on a Jasco J-725 spectropolarimeter in MeOH. IR spectra were measured on a JASCO FT/IR-5300 spectrophotometer. Optical rotations were measured on a JASCO DIP-1000 polarimeter with $CHCl_3$ as solvent. 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 solvent. Chemical shifts are given relative to TMS δ 0.00 (ppm) as an internal standard (^{1}H) and δ 77.03 (ppm) from $CDCl_3$ as a standard (^{13}C-NMR). Mass spectra were obtained on a JEOL JMS AX-500 instrument or a JEOL Mstation JMS 700 instrument. TLC was performed on silica gel plates (Kieselgel 60 F_{254}, Merck) and RP C_{18} silica gel plates (Merck). The spots on TLC were visualized by UV light (254 nm) and by spraying with 30% H_2SO_4 and Godin's reagent,[10] followed by heating.

Fungal Material Fruiting bodies of *T. terrestris* were collected in Aichi prefecture, Japan in October 2003 and identified by M. Nukada. A voucher specimen (KN0101) has been deposited in Faculty of Food Culture, Kurashiki Sakuyo University, Kurashiki 710–0290, Japan.

Extraction and Isolation The MeOH extract (2.98 g) of dried fruiting bodies of *T. terrestris* (10.36 g) was subjected to open column silica gel chromatography. On elution with chloroform–methanol in a gradient in order of increasing polarity (from 10 : 1 to 3 : 1 $CHCl_3$: MeOH and afterwards by washing the column with methanol) ten fractions were obtained (fr. 1—10). Fraction 4 (289.9 mg) was further chromatographed on an open RP C-18 column using an H_2O–MeOH gradient (from 70% to 100% MeOH) to give ten subfractions. Subfraction 4-2 (4.2 mg) and 4-3 (12.1 mg) were purified by reverse-phase preparative HPLC with the solvent system CH_3CN : H_2O (13 : 7) to give terresterone A (**2**) (1.7 mg) and stizophyllin (**1**) (7.0 mg). Fraction 5 (547.0 mg) was purified by the same method as mentioned above to afford sixteen subfractions. Subfractions 5-1 (23.7 mg) and 5-2 (31.3 mg) after RP C-18 preparative HPLC (CH_3CN : H_2O=1 : 1) yielded terresterone B (**3**) (17.4 mg).

Stizophyllin (**1**): Amorphous yellowish solid, $[\alpha]_D^{20}$ +79.1° (c=0.58, $CHCl_3$). UV λ_{max} (MeOH) nm (log ε): 241 (3.94), 202 (4.13). CD (MeOH): λ_{ext} nm ($\Delta\varepsilon$) 329 (−0.63), 242 (+57.22). IR (KBr) cm^{-1}: 3354, 1648, 1583, 1375, 1241, 1057, 1014, 753, 660. HR-MS-FAB *m/z*: 367.1892 ($[M+Na]^+$, Calcd for $C_{21}H_{28}O_4Na$: 367.1885). EI-MS *m/z* (int.): 344 $[M]^+$ (28), 326 (38), 308 (23), 293 (27), 265 (20), 252 (16), 223 (15), 197 (13), 195 (16), 159 (21), 143 (24), 123 (20), 105 (31), 91 (36), 77 (23) 43 (100). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2.

Terresterone A (**2**): Amorphous yellowish solid, $[\alpha]_D^{20}$ +502.5° (c=0.04, $CHCl_3$). UV λ_{max} (MeOH) nm (log ε): 222 (3.95), 202 (4.09). CD (MeOH): λ_{ext} nm ($\Delta\varepsilon$) 287 (+24.50), 248 (−22.47), 215 (+101.96). IR (KBr) cm^{-1}: 3448, 1705, 1671, 1595, 1508, 1376, 1229, 1060, 752. HR-MS-EI *m/z*: 342.1826 ($[M]^+$, Calcd for $C_{21}H_{26}O_4$: 342.1831). EI-MS *m/z* (int.): 342 $[M]^+$ (99), 324 (42), 298 (29), 281 (24), 238 (32), 237 (18), 195 (18), 159 (27), 131 (29), 129 (25), 121 (21), 115 (25), 105 (29), 91 (40), 77 (28), 43 (100). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2.

Terresterone B (**3**): Amorphous colorless solid, $[\alpha]_D^{20}$ +108.6 (c=1.0, $CHCl_3$). UV λ_{max} (MeOH) nm (log ε): 225 (3.78), 201 (4.04). CD (MeOH): λ_{ext} nm ($\Delta\varepsilon$) 290 (+0.14), 249 (−0.13), 216 (+0.56). IR (KBr) cm^{-1}: 3311, 1705, 1670, 1593, 1428, 1371, 1297, 1256, 1231, 1070, 824, 750. HR-MS-EI *m/z*: 358.1779 ($[M]^+$, Calcd for $C_{21}H_{26}O_5$: 358.1780). EI-MS *m/z* (int.): 358 $[M]^+$ (9), 340 (82), 322 (30), 304 (16), 279 (29), 251 (20), 261 (28), 233 (21), 219 (18), 199 (13), 193 (14), 178 (24), 165 (22), 149 (20), 141 (19), 129 (24), 105 (27), 91 (92), 77 (34), 65 (16), 43 (100). ^{1}H- and ^{13}C-NMR ($CDCl_3$): see Tables 1 and 2.

Acknowledgments We thank Miss Y. Okamoto (TBU) for the mass spectra measurements.

References

1) Brown G., *Nat. Prod. Rep.*, **6**, 653—696 (1998).
2) Ourisson G., *J. Plant Physiol.*, **143**, 434—439 (1994).
3) Gonzales A., Leon F., Rivera A., Padron J., Gonzales-Plata J., Zuluaga J., Quintana J., Estevez F., Bermejo J., *J. Nat. Prod.*, **65**, 417—421 (2002).
4) Quang N., Hashimoto T., Nukada M., Yamamoto I., Hitaka Y., Tanaka M., Asakawa Y., *Phytochemistry*, **62**, 109—113 (2003).
5) Quang N., Hashimoto T., Hitaka Y., Tanaka M., Nukada M., Yamamoto I., Asakawa Y., *Phytochemistry*, **63**, 919—924 (2003).
6) Quang N., Hashimoto T., Hitaka Y., Tanaka M., Nukada M., Yamamoto I., Asakawa Y., *Phytochemistry*, **65**, 1179—1184 (2004).
7) Blunt J., Stothers J., *Org. Magn. Reson.*, **9**, 439—464 (1977).
8) Duh C., Pezzuto J., Kinghorn D., Leung S., Farnsworth N., *J. Nat. Prod.*, **50**, 63—74 (1987).
9) Searle P., Molinski T., *J. Nat. Prod.*, **58**, 264—268 (1995).
10) Godin P., *Nature* (London), **174**, 134 (1954).