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Three novel degraded steroids from cultures of the Basidiomycete *Antrodiella albocinnamomea*

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ABSTRACT

Three novel degraded steroids, named albocisterols A–C (**1**–**3**), have been isolated from cultures of *Antrodiella albocinnamomea*. Their structures were defined by comprehensive spectroscopic analysis and single crystal X-ray crystallography. The mixture of compounds **2** and **3** exhibited significant inhibitory activities against protein tyrosine phosphatase 1B (PTP1B).

1. Introduction

Antrodiella albocinnamomea is a basidiomycetous fungus of the family Polyporaceae, which is wood-decaying and widely distributed in temperate to subtropical areas of China [1]. Previous studies of the genus *Antrodiella* were mainly on taxonomy, physiology and biochemistry, and biotransformation [2–4]. However, no study on the secondary metabolites of *A. albocinnamomea* was previously presented in the literature. In continuation of our ongoing investigation on structurally interesting and biologically active natural products from higher fungi [5–9], we subjected an EtOAc extract of cultures of the *A. albocinnamomea* to investigation, which resulted in the isolation of three novel degraded steroids, named albocisterols A–C (**1**–**3**). A series of highly degraded sterols have been isolated from marine creatures and from higher fungi [10–19]. In general, highly degraded sterols might be biogenetically derived from a parent sterol by a degradative loss of ring-A carbon atoms through 5, 6 and 9, 10 oxidative cleavages [10,11]. Interestingly, compounds **1**–**3** are unusual highly degraded sterols with a rare $\Delta^{20,22}$-unsaturation and a new γ-lactone or lactol ring formed by connecting C-23 and C-27 via an oxygen atom, respectively. All compounds were evaluated for their inhibitory activities against protein tyrosine phosphatase 1B (PTP1B), the mixture of compounds **2** and **3** exhibited significant inhibitory activities. Herein we report the isolation, structure elucidation, and bioactivity of these three novel sterols (Fig. 1).

2. Experimental

2.1. General experimental procedures

X-ray data were collected using a Bruker APEX DUO instrument. Melting points were surveyed with an X-4 microscopic melting point meter. Optical rotations were measured on a Jasco-P-1020 polarimeter. IR spectra were obtained by a Bruker Tensor 27 FT-IR spectrometer with KBr pellets. 1D and 2D NMR spectra were recorded on a Bruker Avance III 600 MHz spectrometer (Karlsruhe, Germany). Silica gel (200–300 mesh and 80–100 mesh, Qingdao Marine Chemical Inc., China) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography (CC). Fractions were monitored by TLC (HSGF 254, 0.2 mm, Qingdao Marine Chemical Inc., China) and spots were visualized by heating silica gel plates immersed in vanillin-H_2SO_4 in EtOH, in combination with Agilent 1200 series HPLC system (Eclipse XDB-C18 column, 5 μm, 4.6 × 150 mm). MPLC was performed on a Buchi apparatus equipped with Buchi fraction collector C-660, Buchi pump module C-605, manager C-615 and an ODS column (20 × 90 mm, 40–63 μm, YMC).

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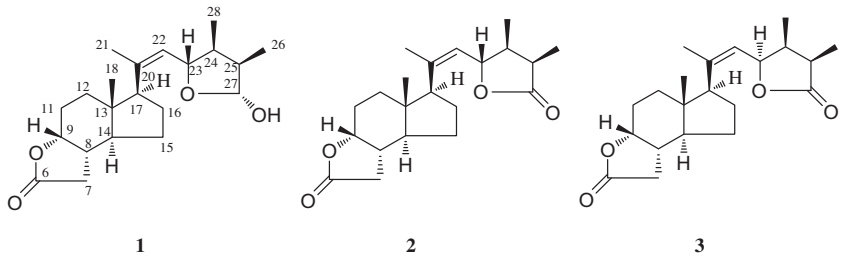

Fig. 1. Structures of compounds **1–3**.

2.2. Fungal material and cultivation conditions

Fruiting bodies of *A. albocinnamomea* were collected at Changbai Mountain, Northeast of China in 1997 and were identified by Prof. Yu-Cheng Dai (Beijing Forestry University). The voucher specimen (NO. CGBWSHF00182) was deposited at herbarium of Kunming Institute of Botany. Culture medium was composed of glucose (5%), pork pepton (0.15%), yeast (0.5%), KH_2PO_4 (0.05%) and $MgSO_4$ (0.05%). Initial pH was adjusted to 6.0, the fermentation was first carried out on an erlenmeyer flask for 6 days till the mycelium biomass reached to the maximum. Then it was transferred to a fermentation tank (100 L) at 24 °C and 250 rpm for 20 days, ventilation was set to 1.0 vvm (vvm: air volume/culture volumn/min).

2.3. Extraction and isolation

The culture broth (70 L) was extracted four times with ethyl acetate (70 L × 4) under reflux. The organic layer was evaporated to give a crude extract (45 g). The crude extract was then fractionated by silica gel column chromatography (1000 g, 8 × 60 cm) eluted with a gradient of petroleum ether–acetone (100:0–0:100) to obtain eight fractions. Fraction 3 was eluted with petroleum ether–acetone (3:1), was then purified into five fractions (3A–3E) by MPLC using MeOH/H_2O as eluent. Fraction 3E was then separated by Sephadex LH-20 (MeOH) and silica gel CC ($CHCl_3$–MeOH, 40:1) to yield a mixture (4.5 mg) of **2** and **3**. Fraction 4 was eluted with petroleum ether–acetone (2:1), and then was purified by MPLC using MeOH/H_2O as eluent to afford four fractions (4A–4D). Fraction 4C was purified by Sephadex LH-20 (MeOH) and silica gel CC ($CHCl_3$–MeOH, 30:1) to afford **1** (11.0 mg).

2.3.1. Albocisterol A (*1*)

Prismatic crystals (EtOAc:MeOH = 10:1); mp 195–197 °C; $[\alpha]_D^{25}$ + 12.3 (*c* 0.12, MeOH); UV (MeOH) λ_{max} (log ε) 203.4 (4.10) nm; IR (KBr) γ_{max} 3477, 2967, 2932, 2872, 1766, 1640, 1453, 1347 cm^{-1}; ^1H and ^{13}C NMR spectra data see Table 1; HR-EI-MS (positive) *m/z* 348.2307 (calcd for $C_{21}H_{32}O_4$, 348.2301).

2.3.2. Albocisterols B and C (*2* and *3*)

Colorless oil; $[\alpha]_D^{25}$ + 14.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε): 203.6 (4.10) nm; IR (KBr) γ_{max} 3448, 2966, 2935, 1773, 1639, 1384 cm^{-1}; ^1H and ^{13}C NMR spectra data see Table 1; HR-EI-MS (positive) *m/z* 346.2139 (calcd for $C_{21}H_{30}O_4$, 346.2144).

2.4. Activity assay

Human MEG2, DPPIV and PIP1Bc with an Nterminal 6 × His-tag were recombinantly expressed in *Escherichia coli* and purified by Ni–NTA affinity chromatography [20–22]. The enzymatic assay was carried out at room temperature in 96-well plates. After the assay buffer which contained 100 mM Hepes (pH 6.0), 5 mM DTT 0.015% Brij-35 and PTPase (20 ng PTP1B, 10 ng SHP2, 10 ng SHP2 or 40 ng LAR per well) was incubated with tested compounds for 15 min, the reaction was initiated by addition of the substrate p-nitrophenol phosphate (pNPP, Sigma, P4744, St. Louis, USA) at a final concentration of 2 mM. The activity of PTPase-catalyzed hydrolysis of pNPP was determined by measuring the amount of p-nitrophenol and the absorbance at 405 nm was recorded as the amount of p-nitrophenol. The IC_{50} value was determined by the non-linear curve fitting of the percentage inhibition versus inhibitor concentration plot. Ursolic acid (Sigma, U6753, St. Louis, USA) was used for the positive control with IC_{50} vaules of 0.8, 1.2 and 1.2 μg/mL to MEG2, DPPIV and PIP1Bc, respectively [23]. All the assays were carried out in triplicate and the average results were presented.

2.5. Jones oxidation

Chromium trioxide (2.26 g, 0.023 mol) was dissolved in 7 mL water in a round bottom flask. The resulting solution was cooled to 0 °C whereupon 2 mL conc. H_2SO_4 was added dropwise. The solution was cooled to 0 °C.

A solution of the compound **1** (3 mg, 0.0086 mmol) in acetone (1 mL) was cooled to 0 °C and the Jones reagent (7 μL) was added slowly. Stirring was continued for 1 h at 0 °C. Sodium bisulfite was added in small portions until the brown color of the chromic acid had disappeared. The aqueous layer was extracted with Et_2O (30 mL × 2). The combined organic extracts were washed with brine and dried over Na_2SO_4, filtered and concentrated *in vacuo*. The resulting oil was purified by chromatography on silica gel with petroleum ether–acetone (2:1) as the eluent yielding the title compound as a colorless oil (2.7 mg, 90%).

2.6. X-ray diffractometry

The prismatic cystals of albocisterol A (**1**) were obtained from EtOAc/MeOH (10:1). Data were collected at 100 K on a Bruker APEX DUO diffractometer equipped with an APEX II CCD using Cu Kα radiation. Cell refinement and data reduction were performed with Bruker SAINT software. The structure was solved by direct methods using SHELXL-97. Refinements were performed with SHELXL-97 using full-matrix least-squares, with anisotropic displacement parameters for all the non-hydrogen atoms. The H atoms were placed in calculated positions and refined using a riding model. Molecular graphics were computed with PLATON. Crystallographic data have been deposited in Cambridge Crystallographic Data Centre (CCDC). Copies of the data can be obtained free of charge on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44 (0)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk).

Crystallographic data of (**1**): $C_{21}H_{32}O_4$, M = 348.47, monoclinic, a = 11.2937(3) Å, b = 6.2754(2) Å, c = 13.7441(4) Å, α = 90.00°, β = 104.8020(10)°, γ = 90.00°, V = 941.75(5) $Å^3$, T = 100(2) K, space group $P21$, Z = 2, μ(Cu Kα) = 0.664 mm^{-1}, 9251 reflections measured, 2771 independent reflections (R_{int} = 0.0387). The final R_1

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1**–**3** in methanol-d_4.

	1		**2**		**3**	
	δ_C mult	δ_H (J in Hz)	δ_C mult	δ_H (J in Hz)	δ_C mult	δ_H (J in Hz)
6	180.0, s		180.0, s		180.0, s	
7	37.0, t	2.28, m	37.0, t	2.29, d (17.0)	37.0, t	2.29, m
		2.77 dd (17.0, 6.7)		2.77, m		2.78, overlapped
8	39.4, d	2.34, m	39.3, d	2.35, m	39.3, d	2.36, m
9	81.4, d	4.62, m	81.3, d	4.62, m	81.3, d	4.62, m
11	25.2, t	2.08, m,	25.0, t	2.09, m,	25.3, t	2.12, m,
		1.90, m		1.90, m		1.90, m
12	34.7, t	1.39, m	34.5, t	1.40, m	34.5, t	1.40, m
		1.73, m		1.70, m		1.70, m
13	43.7, s		43.9, s		44.0, s	
14	49.5, d	1.27, m	49.4, d	1.29, m	49.4, d	1.29, m
15	25.5, t	1.27, m	25.5, t	1.29, m	25.5, t	1.29, m
		1.85, m		1.85, m		1.85, m
16	25.1, t	1.73, m	25.1, t	1.76, m	25.2, t	1.76, m
		1.93, m		1.90, m		1.90, m
17	59.8, d	2.14, m	59.7, d	2.20, t (9.7)	60.0, d	2.23, m
18	12.1, q	0.65, s	12.1, q	0.65, s	12.2, q	0.68, s
20	138.6, s		143.1, s		141.9, s	
21	19.2, q	1.73, d (1.0)	19.2, q	1.81, d (1.0)	18.5, q	1.77, d (1.0)
22	129.1, d	5.33, d (8.8)	124.6, d	5.35, d (8.5)	122.8, d	5.33, overlapped
23	82.3, d	4.47, dd (9.1, 8.8)	83.4, d	4.98,dd (8.5, 7.4)	80.9, d	5.31, overlapped
24	41.8, d	2.24, m	42.0, d	2.36, m	39.8, d	2.65, m
25	46.0, d	2.09, m	40.1, d	2.76, m	41.6, d	2.98, m
26	11.7, q	0.95, d (7.2)	10.6, q	1.17 d (7.2)	10.4, q	1.14 d (7.2)
27	105.0, d	4.99,br s	182.7, d		182.0, d	
28	11.4, q	0.94, d (7.2)	12.2, q	1.02 d (7.2)	9.6, q	0.85 d (7.2)

values were 0.0323 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.0863 ($I > 2\sigma(I)$). The final R_1 values were 0.0324 (all data). The final $wR(F^2)$ values were 0.0863 (all data). The goodness of fit on F^2 was 1.051. Flack parameter = −0.01(17).The Hooft parameter is −0.07(8) for 955 Bijvoet pairs. CCDC: 978675.

3. Results and discussion

Compound (**1**) was obtained as colorless crystals (EtOAc:-MeOH = 10:1). Its molecular formula was determined as $C_{21}H_{32}O_4$ on the basis of HREIMS at m/z 348.2307 $[M]^+$ (calcd. for $C_{21}H_{32}O_4$, 348.2301), indicating six degrees of unsaturation. The IR spectrum suggested the presence of a hydroxyl group (3477 cm^{-1}), double bonds (1640 cm^{-1}), and a γ-lactone moiety (1767 cm^{-1}). The ^{1}H NMR spectrum showed signals for an olefinic proton at δ_H 5.33 (d, J = 8.8 Hz, H-22), three oxymethine protons at δ_H 4.47 (dd, J = 9.1, 8.8 Hz, H-23), 4.62 (m, H-9) and 4.99 (br s, H-27), four methyl groups at δ_H 0.65 (s, H-18), 0.94 (d, J = 7.2 Hz, H-28), 0.95 (d, J = 7.2 Hz, H-26) and 1.73 (d, J = 1.0 Hz, H-21). The ^{13}C NMR and DEPT spectra revealed 21 carbon signals including four methyls, five methylenes, nine methines (with two oxymethines and a hemiacetal methine), and three quaternary carbons (with one ester carbonyl).

Interpretation of the COSY spectrum gave several spin systems corresponding to substructures as shown with bold lines in Fig. 2. The above NMR data and COSY data revealed that compound **1** was a heptanpor-sterols possessing a 5/6/5 tricyclic nucleus as that of salimyxin B, a C_{21} sterol isolated from an obligate marine myxobacterium *Enhygromyxa salina* [24]. The main differences were on the side-chain. In the HMBC, correlations from H_3-21 to vinylic carbons C-20 and C-22 were indicative of a rare double bond at C-20, which also received further support by a downfield shift of H-21 at δ_H 1.73 (d, J = 1.0 Hz) and a weak correlation to H-22 in ^{1}H–^{1}H COSY spectrum. Additional HMBC correlations from H-23 to C-27 and from H-27 to C-23 in association of the downfield chemical shifts of C-23 (δ_C 82.3) and C-27 (δ_C 105.0) suggested that an oxobridge existed between C-23 and C-27, and the hemiacetal group was positioned at C-27. Thus, the planar structure of **1** was established.

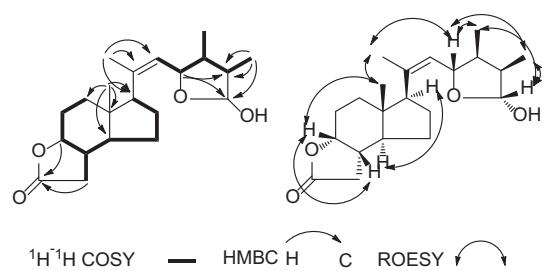

Fig. 2. Key 2D correlations of compound **1**.

The relative configuration of **1** was established by an ROESY experiment (Fig. 2), in which the correlations of H-9/18-Me, H-8 indicated that H-8 and H-9 were in a β-orientation. The ROESY correlation of H-14/H-17 and 18-Me/21-Me established that the side chain was in a β-orientation, while H-14 and H-17 were in the opposite orientation (α). Moreover, since compound **1** was a C_{21} sterol bearing an unusual side chain, a single crystal diffraction analysis of compound **1** was performed. The final refinement on Cu Kα data resulted in a Flack parameter of −0.01(17), and a Hooft parameter of −0.07(8) for 955 Bijvoet pairs allowed unambiguous assignment of the absolute configuration of **1** to be 8*S*,9*R*,13*S*,14*S*,17*R*,23*S*,24*S*,25*R*,27*R* (Fig. 3) [25,26]. Therefore, compound **1** was determined, and named albocisterol A.

Compounds **2** and **3** were isolated as an inseparable mixture. The HREIMS gave a pseudomolecular ion peak at m/z 346.2139 $[M]^+$, allowing a molecular formula $C_{21}H_{30}O_4$ and seven degrees of unsaturation to be assigned. The ^{1}H and ^{13}C NMR data of **2** and **3** were similar to compound **1**, suggesting compounds **2** and **3** were also degraded sterols with a C_{21} skeleton. The main difference was that the ^{13}C NMR signal at δ_C 105.0 (C-27) in **1** was down shifted to δ_C 182.7 and δ_C 182.0 in **2** and **3**, respectively, suggesting

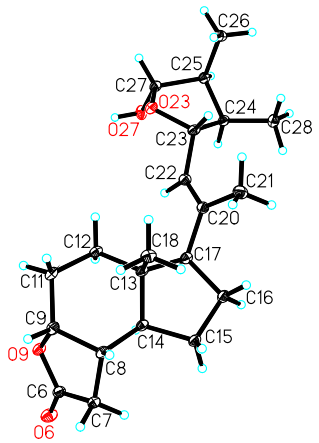

Fig. 3. X-ray crystallographic structure of compound **1**.

ester carbonyls instead of the hemiacetal group in **2** and **3**. In the HMBC spectrum, the correlations from H-23 and H-26 to C-27 further confirmed this assignment. In the ^{1}H and ^{13}C NMR spectra, the resonances appeared as pairs. However, careful analysis of the 1D and 2D NMR spectra allowed for the unambiguous assignments of the signals for each compound, which revealed that compounds **2** and **3** existed as a mixture of two stereoisomers in a ratio of 4.5:5.5 in methanol-d_4. The chemical shifts of the degraded sterol nucleus in compounds **2** and **3** were extremely similar to those of compound **1**, which suggested that compounds **2** and **3** had the same stereochemistry at the nucleus of **1**. The main differences between them are the downfield chemical shift of 2.7 ppm C-23, 2.2 ppm C-24, and 2.8 ppm for C-28 and upfield to δ 4.98 (H-23) compared to that of δ 5.31 in **3**, suggesting that compounds **2** and **3** may be isomers at C-23 or C-24.

The relative configuration of **2** and **3** was established by an ROESY experiment. In compound **2**, the ROESY correlations of H-23/21-Me and 26-Me/28-Me were exhibited, indicating that compound **2** possessed the same relative configuration as those of compound **1**. The conclusion was also supported by the Jones oxidation of compound **1** to **2**, which unambigously assigned all the configurations of the asymmetric centers. In compound **3**, the ROESY relationship was observed from H-16/H-17 and H-16/H-18, whereas the ROESY interactions between H_3-21/H-23 and H-23/H_3-28 were absent, indicating that H-23 was in an opposite orientation compared to that of **2**. Therefore, compounds **2** and **3** were isomers at C-23 instead of C-24. Since the absolute configuration of **1** was determined by X-ray analysis, the absolute configurations of **2** and **3** could be deduced on the basis of biogenetic considerations. Thus, the absolute configuration of **2** was determined as 8*S*,9*R*,13*S*,14*S*,17*R*,23*S*,24*S*,25*R*, while the absolute configuration of **3** was determined as 8*S*,9*R*,13*S*,14*S*,17*R*,23*R*,24*S*,25*R*. Therefore, the structures of compounds **2** and **3** were established and named albocisterols B and C, respectively.

The bioactivities of compounds **1**–**3** were evaluated in the enzyme inhibition assay against three protein-tyrosine phosphatases (PTPs): MEG2, DPPIV and PTP1Bc. The mixture of albocisterols B and C (**2** and **3**) showed significant inhibitory activities against protein-tyrosine phosphatase (PTP1Bc) with IC_{50} values of 1.1 μg/mL (positive control: 1.2 μg/mL). However, albocisterol A (**1**) did not show any activity against PTPs at 50 μg/mL. Additional albocisterol B (**2**), the Jones oxidation of **1**, exhibited inhibitory effect against PTP1Bc with IC_{50} values of 1.0 μg/mL. Those findings suggest that the ester carbonyl group at C-27 play a key role in mediating inhibitory activities, and provide a vivid demonstration of how subtle differences in structure impact their biological activities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.05.008.

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