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3β-*p*-HYDROXYBENZOYLDEHYDROTUMULOSIC ACID FROM *PORIA COCOS*, AND ITS ANTI-INFLAMMATORY EFFECT

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Key Word Index—*Poria cocos*; Polyporaceae; triterpene; 3β-*p*-hydroxybenzoyldehydrotumulosic acid; anti-inflammatory effect; TPA-induced inflammation; arachidonic acid-induced inflammation.

Abstract—The structure of a new triterpene derivative isolated from *Poria cocos* was determined to be 3β-*p*-hydroxybenzoyldehydrotumulosic acid by spectral and chemical methods. 3β-*p*-hydroxybenzoyldehydrotumulosic acid showed marked inhibitory activity against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- and arachidonic acid (AA)-induced ear inflammation in mice. The 50% inhibitory doses of 3β-*p*-hydroxybenzoyldehydrotumulosic acid were 0.27 and 1.25 mg per ear on TPA- and AA-induced inflammation, respectively.

INTRODUCTION

The sclerotium of *Poria cocos* Wolf Polyporaceae, is used in Chinese herbal prescriptions as a diuretic and as a sedative [1]. We have isolated previously some new lanostane-type triterpene acids from *P. cocos* [2–6], identified the triterpenes of *P. cocos* by high-performance liquid chromatography [7, 8], and have shown a considerable activity against copper sulfate induced emesis [9]. In previous papers we reported the inhibitory effects of *P. cocos* extract and its components (lanostane-type and secolanostane-type triterpene acids) on inflammatory ear oedema and tumour promotion induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse skin [10, 11]. This paper describes our continued study on the *P. cocos* sclerotium extract and the isolation and characterization of 3β-*p*-hydroxybenzoyldehydrotumulosic acid (**4a**), a new lanostane-type triterpene derivative. We have examined in this study the anti-inflammatory effect of triterpenes **1a**–**5b** against 12-*O*-tetradecanoylphorbol-13-acetate (TPA)- and arachidonic acid (AA)-induced inflammation, and have found these compounds to possess marked inhibitory effect.

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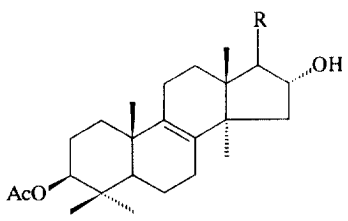
RESULTS AND DISCUSSION

Six triterpene derivatives (**1a**, **2a**, **3b**, **4a**, **5a** and **5b**) were isolated from the dichloromethane extract of the sclerotium of *P. cocos* by the procedure described in the Experimental. Triterpenes **1a**, **2a**, **3b**, **5a** and **5b** were identified as pachymic acid, dehydropachymic acid, dehydrotrametenolic acid (3β-hydroxylanosta-7,9(11),24-trien-21-oic acid) [12] and poricoic acids A and B by spectral comparison with authentic compounds [2–4]. The structure of **4a** was determined as described below.

The molecular formula $C_{38}H_{52}O_6$ of compound **4a** was established on the basis of ^{13}C NMR and DEPT spectra, and confirmed by the negative FAB mass spectrum (*m/z* 603 $[M-H]^+$). The ^1H NMR spectrum of **4a** showed signals due to five *tert*-methyl groups [δ 0.98 (3H, *s*), 1.01 (3H, *s*), 1.07 (3H, *s*), 1.08 (3H, *s*), 1.47 (3H, *s*)], an exomethylene group [δ 4.85 (1H, *br s*), 4.99 (1H, *br s*)], two olefinic methines [δ 5.41 (1H, *d*, $J = 5.9$ Hz), 5.65 (1H, *br s*)], two oxygen-bearing protons [δ 4.54 (1H, *m*), 5.12 (1H, *br s*)], benzoyl proton group [δ 7.15 (2H, *d*, $J = 8.8$ Hz), 8.24 (2H, *d*, $J = 8.8$ Hz)]. The ^{13}C NMR spectrum showed a carboxyl carbon at δ 178.6 (C-21), and ester carbonyl carbon at δ 166.1, and two methine carbons at δ 132.3 and δ 116.2, and two olefinic methines at δ 120.8 (C-7) and δ 117.0 (C-11). Two olefinic quaternary carbon resonances at δ 142.9 (C-8) and δ 146.0 (C-9) revealed



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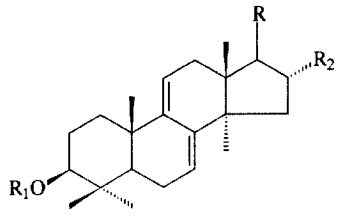
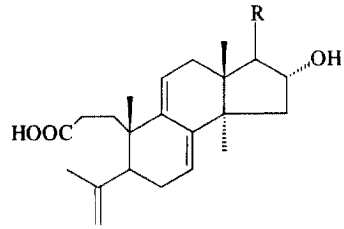


2 : R_1 : Ac R_2 : OH
3 : R_1 : H R_2: H
4 : R_1 : *p*-hydroxybenzoyl R_2 : OH



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the presence of $\Delta^{7,9(11)}$ conjugated diene system. The analysis of the DEPT spectrum revealed an exomethylene group at δ 107.0 (C-31). In addition, there were two oxymethine resonances at δ 76.4 (C-16) and δ 78.2 (C-3). These results suggested that **4a** was 3β-*p*-hydroxybenzoyldehydrotumulosic acid.

The 6 triterpenes isolated from *P. cocos* in this study were examined for their inhibitory effect on AA- and TPA-induced inflammation in mice. The inhibitory effects were compared with those of the commercially available anti-inflammatory drugs, indomethacin and hydrocortisone (Table 1).Compound **4a** inhibited the TPA-induced inflammation with 0.274 mg per ear of the 50% inhibitory dose, corresponding to that observed with indomethacin. The inhibitory effect evaluated for five triterpenes; **1a**, **2a**, **3b**, **5a** and **5b**, in this study were consistent with our previous results [10]. Compounds **2a**, **3b**, **4a** and **5a** markedly inhibited AA-induced inflammation with 0.8–1.3 mg per ear of the 50% inhibitory dose, corresponding to that seen with indomethacin.

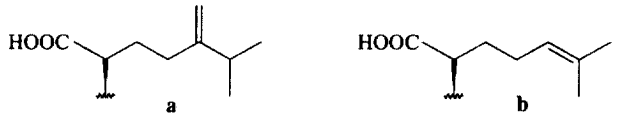
EXPERIMENTAL

Mp: uncorr. HPLC: C_{18} silica column (Pegasil ODS column, 25 cm × 20 mm i.d.; Senshu Scientific Co.), MeOH as mobile phase (flow rate 3 ml min^{-1}). UV Spectrophotometric detector (Shimadzu Co., Ltd., Tokyo, Japan). NMR spectra were measured with a JEOL JNM-GSX 400 (^{1}H, 400 MHz; ^{13}C, 100 MHz) spectrometer, Japan, and chemical shifts are presented as *d* values relative to tetramethylsilane as an internal standard. Mass spectra were measured with a Hitachi M-2000 double-focusing mass spectrometer, Japan, at an ionization voltage of 70 eV. Hydrocortisone and arachidonic acid were purchased from Sigma Chemical Co., Ltd., St Louis, Mo, USA. 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was purchased from Cancer Research Inc., Minnesota, USA. Acetone, chloroform and methanol were purchased

Table 1. ^{13}C NMR spectral data for dehydropachymic acid and 3β-*p*-hydroxybenzoyldehydrotumulosic acid

C	**2a**	**4a**
1	35.6	31.3
2	24.5	23.6
3	80.6	78.2
4	37.8	37.8
5	49.7	45.3
6	34.1	23.2
7	120.6	120.8
8	142.8	142.9
9	145.8	146.0
10	37.6	37.2
11	117.0	117.0
12	36.3	36.1
13	49.4	49.5
14	45.1	45.1
15	44.4	44.4
16	76.4	76.4
17	57.6	57.6
18	17.6	17.7
19	22.8	22.6
20	48.4	48.6
21	178.5	178.6
22	31.4	31.4
23	33.2	33.2
24	156.0	156.1
25	34.1	34.1
26	22.0	22.0
27	21.9	21.9
28	28.2	28.2
29	17.1	22.5
30	26.5	26.6
31	107.0	107.1
1′	—	122.2
2′,6′	—	132.3
3′,5′	—	116.2
4′	—	163.5
7′	—	166.1
MeCO	21.1	—
MeCO	170.5	—

Side chains (R)



from Tokyo Kasei Kogyo Co., Ltd., Japan. The sclerotium of *P. cocos* was obtained from the market in Tokyo in April 1994. This was identified as the sclerotium of *P. cocos* by description, chemical analysis and purity test [1, 13].

Isolation of triterpenes from the dichloromethane extract of P. cocos

P. cocos (12 kg) was extracted by refluxing with CH_2Cl_2 for 3 h. The organic solvent was evaporated *in vacuo* to dryness. The yield of extract was 36 g and this was subjected to CC on Wakogel C-300 (Wako-Jyunyaku Co., Ltd., Japan), using $CHCl_3$–MeOH to obtain eight fractions. The fractions (2, 5 and 6) were further subjected to CC (Wakogel C-300, Wako-Jyunyaku Co., Ltd., Japan) using the solvent gradient system $CHCl_3$–MeOH (1:0–1:1) to obtain several fractions. Finally, compounds **1a**, **2a** and **3b** were obtained from fraction 2 by high pressure liquid chromatography (HPLC). Compound **4a** was isolated from fraction 5 by the same method. Compounds **5a** and **5b** were isolated from fraction 6 by the same method. Yield **1a** (628 mg), **2a** (226 mg), **3b** (106 mg), **4a** (44 mg), **5a** (136 mg) and **5b** (105 mg).

Compound 4 (**4a**). Colourless needles (pyridine–*n*-hexane), mp 242–244°. $[\alpha]_D$: +40 (c = 0.2, MeOH). IR ν_{max}^{KBr} cm^{-1}:3399 (OH), 1706 (COOH). UV λ_{max}^{MeOH} nm: 243, 251. N-FAB m/z: 603 [M−H]. ^{1}H NMR δ (pyridine-d_5): 0.98 (3H, *s*, 28-CH_3), 0.99 (3H, *d*, J = 3.7 Hz, 26-CH_3), 1.00 (3H, *d*, J = 3.7 Hz, 27-CH_3), 1.01 (3H, *s*, 29-CH_3), 1.07 (3H, *s*, 19-CH_3), 1.08 (3H, *s*, 18-CH_3), 1.47 (3H, *s*, 30-CH_3), 1.74 (1H, *br d*, J = 13.0 Hz, 1-H), 1.92 (1H, *dd*, J = 13.0 Hz, J = 5.9 Hz, 2-H), 1.94 (1H, *d*, J = 13.2 Hz, 15-H), 1.96 (1H, *br d*, J = 7.3 Hz, 5-H), 1.98 (1H, *dd*, J = 13.0 Hz, J = 7.0 Hz, 2-H), 2.13 (1H, *m*, 6-H), 2.29 (1H, *m*, 25-H), 2.40 (1H, *m*, 23-H), 2.41 (1H, *m*, 22-H), 2.44 (1H, *t*, J = 5.9 Hz, J = 17.6 Hz, 12-H), 2.45 (1H, *br dd*, J = 13.0 Hz, J = 2.9 Hz, 1-H), 2.46 (1H, *m*, 15-H), 2.56 (1H, *br d*, J = 11.7 Hz, 23-H), 2.65 (1H, *d*, J = 17.6 Hz, 12-H), 2.66 (1H, *m*, 22-H), 2.86 (1H, *dd*, J = 11.7 Hz, J = 5.9 Hz, 17-H), 2.96 (1H, *td*, J = 11.7 Hz, J = 8.1 Hz, 20-H), 4.54 (1H, *m*, 16-H), 4.85 (2H, *br s*, 31-CH_2), 5.12 (1H, *br s*, 3-H), 5.41 (1H, *d*, J = 5.9 Hz, 11-H), 5.65 (1H, *br s*, 7-H), 7.15 (2H, *d*, J = 8.8 Hz, 3′,5′-H), 8.24 (2H, *d*, J = 8.8 Hz, 2′,6′-H). ^{13}C NMR: Table 1.

Assay of TPA and AA-induced inflammation

Female ICR mice (Japan SLC, Inc., Shizuoka, Japan) were housed in an air-conditioned specific pathogen free room (22–23°) with light from 08:00 to 20:00. Food and water were available *ad libitum*. TPA (1 μg) and AA (1 mg) dissolved in Me_2CO (20 μl) was applied to the right ear only of ICR mice using a micropipette; 10 μl was delivered to both the inner

Table 2. Inhibitory effect of some lanostane-type triterpenes and reference compounds on TPA- and AA-induced inflammation in mice*

Compound	TPA		AA	
	ID_{50} (mg per ear)	I.R. (%)	ID_{50} (mg per ear)	I.R. (%)
Pachymic acid (**1a**)	0.044	94	N.D.	13
Dehydropachymic acid (**2a**)	0.020	96	1.2	40†
Dehydrotrametenolic acid (**3b**)	0.027	99	0.8	55†
3β-*p*-Hydroxybenzoyldehydrotumulosic acid (**4a**)	0.274	40	1.3	38†
Poricoic acid A (**5a**)	0.028	99	1.2	38†
Poricoic acid B (**5b**)	0.017	95	1.3	37†
Indomethacin	0.300	96†	1.0	55†
Hydrocortisone	0.030	99†	N.D.	8†

* The samples were applied at 30 min before TPA and AA treatment, respectively. Ear thickness determined at 6 h (1 h) after TPA (AA) treatment, respectively. I. R. Inhibitory ratio at 0.25 mg per ear and †1.0 mg per ear.

and outer surfaces of the ear. A sample of the test chemical or its vehicle, MeOH–$CHCl_3$ (1:1, 20 μl), as a control, was applied topically about 30 min before each TPA treatment. The resulting oedema was measured 6 h after TPA treatment. For ear thickness determinations, a pocket thickness gauge (Mitsutoyo Co., Ltd., Japan) with a range of 0–9 mm, graduated at 0.01 mm intervals and modified so that the contact surface area was increased, thus reducing the tension, was applied to the tip of the ear.

The ear thickness was determined before treatment (a). The oedema was measured 6 h and 1 h after TPA and AA treatment (b: TPA and AA alone; b': TPA and AA plus sample), respectively. The inhibitory ratio (I.R.) was calculated as follows: oedema A was induced by TPA and AA alone (b–a); oedema B was induced by TPA and AA plus sample (b'–a).

$$\text{I.R.}(\%) = \frac{A-B}{A} \times 100$$

Each value was the mean of individual determinations from 5 mice, and the 50% inhibitory dose (ID_{50}) was calculated by probit graphic interpolation of data for at least 4 dose levels.

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