Isolation of Inhibitors of TPA-Induced Mouse Ear Edema from Hoelen, *Poria cocos*

Haruo NUKAYA,* Hirokazu YAMASHIRO, Hirotatsu FUKAZAWA, Hitoshi ISHIDA, and Kuniro TSUJI

School of Pharmaceutical Sciences, University of Shizuoka, Yada 52–1, Shizuoka 422, Japan.
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Triterpene carboxylic acids were isolated from the methanol extract of Hoelen, *Poria cocos*, and found to inhibit 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-induced mouse ear edema. Their chemical structures were identified as 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid, 16α-hydroxydehydropachymic acid, 16α-hydroxytrametenolic acid and dehydrotumulosic acid.

Key words ear edema; 12-*O*-tetradecanoylphorbol 13-acetate; Hoelen; 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid; 6α-hydroxydehydropachymic acid; 16α-hydroxytrametenolic acid

Several anti-inflammatory substances are known to inhibit the action of tumor promoters in two-stage mouse skin carcinogenesis.[1,2] Since anti-inflammatory activity may play an important role in the mechanism of anti-tumor promotion, we have searched for new anti-inflammatory substances among natural products. In this study, inhibitory activities against mouse ear edema were measured in a TPA (12-*O*-tetradecanoyl phorbol 13-acetate)-induced inflammation model, because inhibitory potency against TPA-induced inflammation may roughly parallel inhibitory activity against tumor promotion.[3,4]

It has been reported that extracts of traditional Chinese medicines used as anti-inflammatory drugs inhibited TPA-induced inflammation.[5] The extract of Hoelen was particularly effective. As active compounds in Hoelen, sixteen triterpene carboxylic acids, *i.e.*, pachymic acid, 3β-hydroxylanosta-7,9(11),24-trien-21-oic acid, *etc.*, have already been reported by Yasukawa *et al.*[6,7] Here, we describe the isolation of four active compounds from Hoelen, in addition to compounds reported previously, and the determination of their chemical structures.

In our studies on Hoelen, bioassay- and TLC-guided fractionation of the methanol extract by silica gel column chromatography led to an active fraction that did not contain pachymic acid, 3β-hydroxylanosta-7,9(11),24-trien-21-oic acid, *etc.* Compounds **1**—**5** were isolated from this fraction by means of HPLC.

Materials and Methods

Animals Male ddY mice (5 weeks old) were obtained from Japan SLC, Shizuoka, Japan. The animals were housed in an air-conditioned (22—23 °C) room lit from 08:00 to 20:00. Food and water were available *ad libitum*.

Chemicals TPA was purchased from Sigma Chemical Co., St. Louis, U.S.A. Hoelen (*Poria cocos*) was provided by Tsumura Co., Tokyo.

TPA-Induced Ear Edema TPA (0.1 μg) dissolved in acetone (20 μl) was applied to the right ear of mice by means of a micropipette. A half of the solution (10 μl) was applied to each of the inner and outer surfaces of the ear. The sample or its vehicle, $CHCl_3$–MeOH (1:1, 20 μl) as a control, was applied topically at about 30 min before the TPA treatment. For ear thickness determinations, a digital thickness meter (Sony Co., Ltd., Tokyo, Japan) with a range of 0—30 mm, was applied to the tip of the ear. The thickness of the right and left ears was measured at 6 h after TPA treatment. The edema was calculated as the difference between right and left ear thickness (*A*, TPA alone; *B*, TPA plus sample). The inhibitory activity was then calculated as follows:

$$\text{inhibitory activity (\%)} = \frac{A-B}{A} \times 100$$

Each value was calculated as the mean of individual determinations from 4 or 5 mice.

Statistical Analysis Statistical analysis was carried out by using Student's *t* test.

Results and Discussion

Pulverized Hoelen, *Poria cocos*, was extracted first with water then with methanol. The methanol extract inhibited TPA- induced ear edema more than did the water extract. The methanol extract was subjected to silica gel column chromatography and fractionated on the basis of bioassay and TLC analysis. We found that the most active fraction was eluted with $CHCl_3$–MeOH (19:1), after the fraction containing pachymic acid. This active fraction was subjected to reversed-phase preparative HPLC with 80% MeOH–H_2O containing 0.1% AcOH to obtain a mixture of compounds **1** and **2**, and compounds **3**—**5** (Fig.

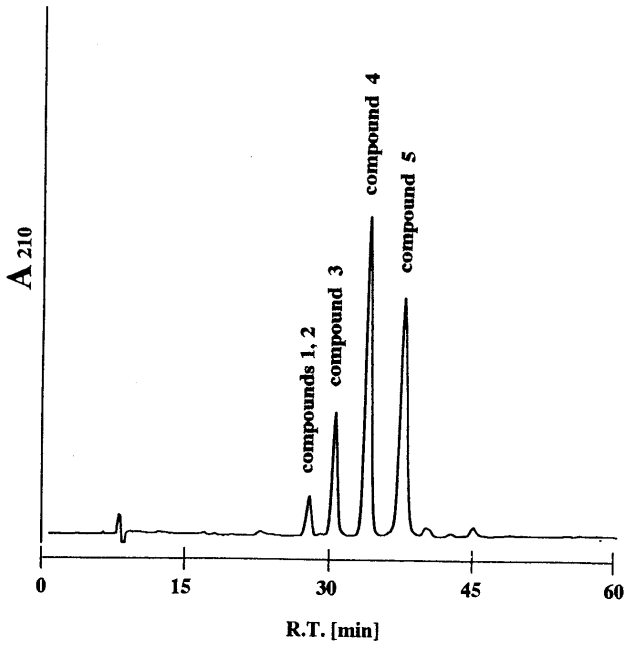


Fig. 1. HPLC Chromatogram of the Most Active Fraction
Conditions: see text.

* To whom correspondence should be addressed.

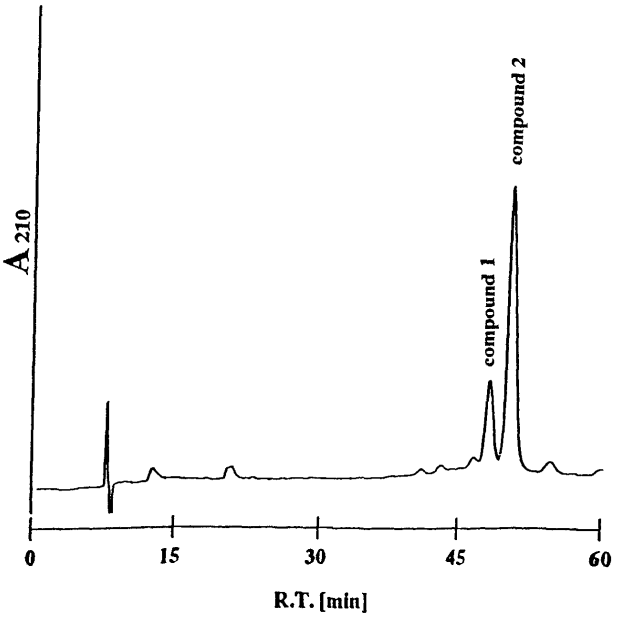


Fig. 2. HPLC Chromatogram of the Mixture of Compounds **1** and **2**
Conditions: see text.

Table 1. Inhibitory Activity of Compounds **1**—**5** and Pachymic Acid on TPA-Induced Mouse Ear Edema

Sample	Inhibitory activity (average ± S.E.)		
	Dose (μg/ear)		
	150	30	5
Compound **1**		62±17*	30±31
Compound **2**		72±14**	65±22*
Compound **3**		73±17**	44±22
Compound **4**	46±18*	31±12	9±16
Compound **5**		67±19**	13±20
Pachymic acid	44±14*	18±18	7±11

Compounds were applied before TPA (0.1 μg) treatment. Ear thickness was determinationed at 6 h after TPA treatment. * $p<0.01$, ** $p<0.05$ by Student's t test, compared with the control group.

1). Compounds **1** and **2** were isolated by HPLC with 75% MeOH–H_2O containing 0.1% AcOH from the mixture (Fig. 2).

Pachymic acid was also isolated by reversed-phase preparative HPLC from the fraction eluted before the most active fraction. The *Rf* values on a thin-layer chromatogram of compounds isolated from the active fraction were similar to, but distinctly different from, that of pachymic acid.

Inhibitory activities of compounds **1**—**5** and pachymic acid against TPA (0.1 μg/ear)-induced ear edema were tested. The results are shown in Table 1. Inhibitory activities were calculated at the time of maximum edema (6 h after TPA treatment). Compounds **1**—**3** and **5** inhibited TPA-induced ear edema more potently than pachymic acid. The activity of compound **4** was nearly equal to that of pachymic acid. Application of 5 μg/ear of compound **2** or **3** showed nearly the same inhibitory activity as that of 150 μg/ear of pachymic acid.

Compound **5** was tumulosic acid, which has been reported by Cort *et al.*[8] Its inhibitory activity has already been reported by Yasukawa *et al.*[7]

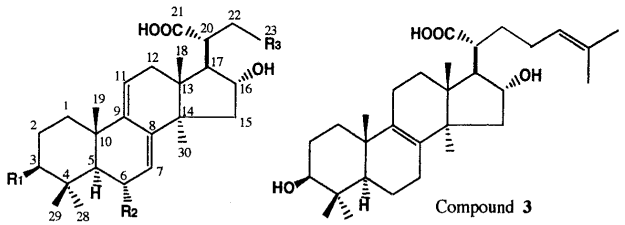
Table 2. ^{13}C-NMR Spectral Data for Compounds **1**—**4** and Pachymic Acid in ($CDCl_3$: CD_3OD = 2 : 1)

C	Pachymic acid	**1**	**2**	**3**	**4**
1	34.8	35.0	34.9	35.1	35.0
2	23.7	26.9	23.3	26.7	27.0
3	81.0	78.2	81.0	78.0	78.2
4	37.4	38.3	37.6	38.3	38.3
5	50.2	48.8	54.8	50.0	48.9
6	16.8	22.6	67.7	17.7	22.7
7	26.0	120.5	125.8	26.0	120.5
8	134.1	141.5	141.0	133.9	141.5
9	133.9	145.3	143.8	133.8	145.4
10	36.6	37.0	37.5	36.5	37.1
11	20.1	115.5	116.8	19.9	115.6
12	28.5	35.4	35.0	28.4	35.5
13	46.9	48.2	48.0	47.5	48.3
14	45.4	44.1	44.1	45.3	44.2
15	42.0	42.7	42.5	41.8	42.8
16	76.2	76.0	76.0	76.0	76.0
17	55.9	56.2	55.9	55.7	56.2
18	16.7	16.6	16.6	16.6	16.7
19	18.6	22.1	23.2	18.3	22.3
20	46.9	46.6	46.7	47.0	47.1
21	178.8	178.8	179.1	179.1	179.1
22	30.1	31.6	29.9	31.5	30.1
23	31.9	25.8	31.8	25.7	32.0
24	155.0	123.5	154.9	123.4	155.1
25	33.4	131.5	33.4	131.1	33.5
26	21.3	25.0	21.2	24.8	21.4
27	21.2	17.0	21.1	16.7	21.3
28	27.4	27.6	29.8	27.2	27.7
29	16.0	15.3	16.3	14.7	15.3
30	24.6	25.4	25.3	24.3	25.6
31	106.1	—	106.1	—	106.2
MeCO	20.6	—	20.6	—	—
MeCO	171.5	—	171.5	—	—

A comparison of the spectral data of pachymic acid[9] and the other compounds indicated that compounds **1**—**4** have pachymic acid-like structures. The mass number of compound **4** is 2 amu smaller than that of tumulosic acid in the FAB-MS. The ^{13}C-NMR spectrum shows two olefinic methines, and two olefinic quaternary carbon resonances, revealing the presence of a $\Delta^{7,9(11)}$ conjugated diene system. Thus, compound **4** is the dehydro derivative of tumulosic acid, dehydrotumulosic acid, which has been isolated as its methyl ester from *Poria cocos* by Valisolalao *et al.*,[10] though its activity has not been reported.

The ^{13}C-NMR spectrum of **2** is similar to that of dehydropachymic acid, except for the 6 position, suggesting the presence of a hydroxyl group there. An H–H correlation spectroscopy (COSY) correlation between H-5 and H-6 (J = 10.5 Hz) and an nuclear Overhauser effect (NOE) correlation between H-19 and H-6 were apparent, confirming that **2** is 6α-hydroxydehydropachymic acid.

The spectral data of **3** are similar to those of tumulosic acid, except for the side chain. Compound **3** has an isopropylidene group at the 24 position in place of the exomethylene group of tumulosic acid. The spectral data of **3** are similar to those of trametenolic acid,[11] except for the hydroxyl group at the 16 position. Accordingly compound **3** is 16α-hydroxytrametenolic acid. This conclusion was supported by the ^{1}H-NMR, ^{13}C-NMR



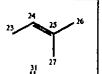
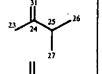
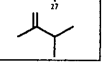
	R_1	R_2	R_3
7,9 diene type			
Compound 1	OH	H	
Compound 2	CH_3COO	OH	
Compound 4	OH	H	

Fig. 3. Chemical Structures of Compounds **1**—**4**

and FAB-MS data.

The mass number of compound **1** is 2 amu smaller than that of **3**. The ^{13}C-NMR spectrum indicated the presence of a $\Delta^{7,9(11)}$ conjugated diene system. Compound **1** is thus 3β,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid. This conclusion was supported by the ^{1}H-NMR, ^{13}C-NMR and FAB-MS data.

The ^{13}C-NMR spectral data and the chemical structures of compounds **1**—**4** are shown in Table 2 and Fig. 3, respectively.

In conclusion, we have isolated active compounds **1**—**5** that inhibit TPA-induced mouse ear edema, from Hoelen and determined their chemical structures. Compounds **1**—**3** have not previously been reported. The inhibitory activities against TPA-induced ear edema of **1**—**3** were more potent than that of pachymic acid. We are planning to test the anti-promoting activities of these compounds.

Experimental

Extraction and Isolation of Compounds 1—5 and Pachymic Acid Pulverized Hoelen (513 g) was extracted with water (1.2 l) under reflux three times. The residual Hoelen was extracted with MeOH (1.2 l) under reflux twice. The MeOH solutions were combined and evaporated *in vacuo* to give an extract (2.74 g), which was subjected to silica gel column chromatography with $CHCl_3$–MeOH (19 : 1) to afford an active fraction (59.1 mg). The fraction was detected by TLC on Kieselgel 60 (Merck) with $CHCl_3$–MeOH (19 : 1) by spraying Vaughn's reagent and heating.

Compounds **3**—**5** and a mixture of compounds **1** and **2** were separated from the above fraction by HPLC (YMC-pack ODS-A, 10 × 300 mm) with 80% MeOH–H_2O containing 0.1% AcOH at a flow rate of 2.0 ml/min, with monitoring of the absorbance at 210 nm, to yield **3** (9.4 mg), **4** (20.1 mg) and **5** (15.6 mg). Compounds **1** and **2** were isolated from their mixture by HPLC with 75% MeOH–H_2O containing 0.1% AcOH to yield **1** (0.8 mg) and **2** (1.4 mg). Pachymic acid was isolated by HPLC from the fraction eluted before the above active fraction on silica gel column chromatography. The HPLC was performed under the same conditions as described above, except for the use of 85% MeOH–H_2O containing 0.1% AcOH.

^{1}H-NMR and ^{13}C-NMR spectra were measured with a JEOL GSX-500 spectrometer (500 MHz). All signals are expressed as ppm downfield from tetramethylsilane (TMS) used as an internal standard (δ value), and coupling constants (J) are given in Hz. The following abbreviations are used: singlet (s), doublet (d), triplet (t), multiplet (m), broad (br). Mass spectra were measured with a JEOL JMS-SX102 mass spectrometer.

Compound **1**: Amorphous powder. FAB-MS m/z: 471 [M+H]. ^{13}C-NMR ($CDCl_3$: CD_3OD = 2 : 1): see Table 2.

Compound **2**: Amorphous powder. FAB-MS m/z: 543 [M+H], ^{1}H-NMR ($CDCl_3$: CD_3OD = 2 : 1) δ: 0.63 (3H, s, H-18), 1.01, 1.03 (each 3H, d, J = 7 Hz, H-26,27), 1.04 (3H, s, H-19), 1.15 (each 3H, s, H-29, 30), 1.18 (3H, s, H-28), 1.21 (1H, d, J = 10.5 Hz, H-5), 1.53 (1H, d, J = 13.5 Hz, H-15α), 2.08 (3H, s, acetyloxy), 2.42 (1H, br t, J = 11 Hz, H-20), 4.08 (1H, dd, J = 6.5, 10 Hz, H-16), 4.38 (1H, d, J = 10.5 Hz, H-6), 4.46 (1H, dd, J = 6, 10 Hz, H-3), 4.73, 4.76 (each 1H, s, H-31), 5.32 (1H, s, H-7), 5.36 (1H, d, J = 6 Hz, H-11). ^{13}C-NMR ($CDCl_3$: CD_3OD = 2 : 1): see Table 2.

Compound **3**: Amorphous powder. FAB-MS m/z: 473 [M+H], ^{13}C-NMR ($CDCl_3$: CD_3OD = 2 : 1): see Table 2.

Compound **4**: Amorphous powder. FAB-MS m/z: 485 [M+H]. ^{13}C-NMR ($CDCl_3$: CD_3OD = 2 : 1): see Table 2.

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