

Anti-inflammatory Lanostane-Type Triterpene Acids from *Piptoporus betulinus*

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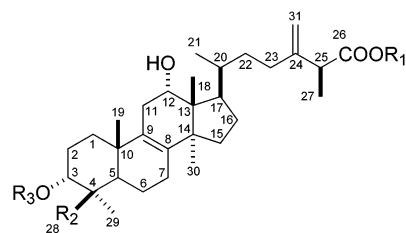
Six lanostane-type triterpene acids were isolated from the fruiting bodies of *Piptoporus betulinus*. They were identified as polyporenic acids A (**1**) and C (**2**), three derivatives of polyporenic acid A (**3**–**5**), and a novel compound, (+)-12 α ,28-dihydroxy-3 α -(3'-hydroxy-3'-methylglutaryl)-24-methyl-26-oic acid (**6**). All these compounds suppressed the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced edema on mouse ears by 49–86% with a 400 nmol/ear application.

Anti-inflammatory compounds, which suppress 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced edema, are expected to suppress tumor promotion and cell proliferation actions as well as inflammation.^{1,2} We surveyed the extracts from various species of the fruiting bodies of mushrooms to find in some species remarkable inhibitory activities against TPA-induced edema on mouse ears. *Sarcodon scabrosus* Karst. (Boraginaceae) was one of the potent species, and novel anti-inflammatory cyathane-type diterpenes were isolated from the fruiting bodies.³ In the present paper we describe isolation and identification of anti-inflammatory compounds from another potent species, *Piptoporus betulinus* Karst. (Polyporaceae).

Bioassay-guided purification of the active compounds from the methanolic extract of *P. betulinus* led to the isolation of six lanostane-type triterpene acids (**1**–**6**). Compounds **1** and **2** were identified as polyporenic acids A and C, respectively, by comparing their spectral data with those reported.^{4–8}

The ¹H NMR spectrum of **3** was similar to that of **1**, except for the presence of an additional singlet (2H) at δ 3.40 ppm. In the ¹³C NMR spectrum of **3**, signals were observed at δ 41.3 ppm (CH₂), 166.7 ppm (C=O), and 170.4 ppm (C=O) in addition to those of **1**. The HMQC and HMBC spectra indicated that these additional signals were derived from a malonyl group, meaning that **3** was an *O*-malonyl derivative of **1**. This was supported by a peak at *m/z* 555.3685 (C₃₄H₅₁O₆, [MH – H₂O]⁺) in the HR-FABMS of **3**. The malonyl group was assigned to C-3 on the basis of the HMBC spectrum, in which the correlation from –CO₂CH₂CO₂H to H-3 was observed. Alkaline hydrolysis of **3** gave **1**, confirming that **3** possesses a (25*S*)-configuration, the same as **1**.⁸ Thus, **3** was identified as (25*S*)-(+)-12 α -hydroxy-3 α -malonyloxy-24-methyl-26-oic acid.

In the ¹H and ¹³C NMR spectra of **4** all the signals assignable to polyporenic acid A were almost identical to those of **3**, but C-3 of **4** was substituted by a functional group other than the *O*-malonyl group. A signal due to an oxygen-bearing quaternary carbon was observed at δ 69.9 ppm in the ¹³C NMR spectrum. Three correlations, from the quaternary carbon to –CH₃ (3H, δ 1.39 ppm), to –CH₂– (each 1H, *d*, *J* = 15.2 Hz, δ 2.63 and 2.70 ppm), and to another –CH₂– (each 1H, *d*, *J* = 15.7 Hz, δ 2.65 and 2.73 ppm), were observed in the HMBC spectrum. These methylene groups were adjacent to carbonyl carbons (δ 172.2

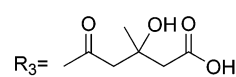
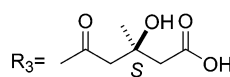


1: R₁=H, R₂=CH₃, R₃=H

3: R₁=H, R₂=CH₃, R₃=COCH₂COOH

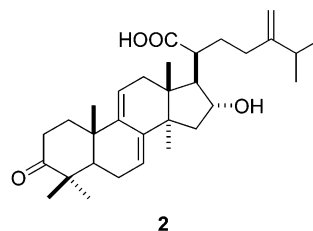
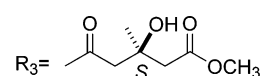
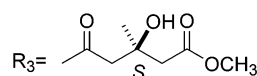
4: R₁=H, R₂=CH₃

6: R₁=H, R₂=CH₂OH

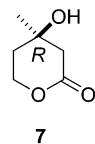


5: R₁=H, R₂=CH₃

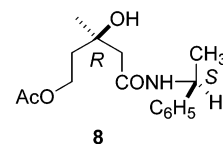
9: R₁=R₂=CH₃



2



7



8

and 174.4 ppm, respectively). The chemical shift values suggested that the carbonyl groups are carboxylic acids or esters. These observations suggested that **4** is an *O*-(3-hydroxy-3-methylglutaryl) derivative of **1**, which was supported by a peak at *m/z* 613.4108 (C₃₇H₅₇O₇, [MH – H₂O]⁺ ion) in the HRFABMS of **4**. To determine the absolute configuration, **4** was reduced using lithium borohydride to **1** and (3*R*)-mevalonic lactone (**7**), whose chirality was identified by conversion to (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]mevalonamide (**8**).^{9,10} These results confirmed that **4** is (25*S*,3'*S*)-(+)-12 α -hydroxy-3 α -(3'-hydroxy-3'-methylglutaryl)-24-methyl-26-oic acid.

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Table 1. Anti-inflammatory Activities of **1**–**6**

tested compound	dose (nmol)	IE (%)
1	400	64 ^a
2	400	49 ^a
3	400	65 ^a
4	400	76 ^a
5	400	86 ^a
6	400	75 ^a
indomethacin	560	16
glycyrrhetic acid	430	24

^a Significantly different, $P < 0.05$ in Student's t -test ($N = 5$).

The ^1H NMR spectrum of **5** was almost identical to that of **4**, except for the presence of a singlet (3H, δ 3.70 ppm), suggesting that **5** was a methyl ester of **4**. The methyl-esterified position, either the 3-hydroxy-3-methylglutaryl moiety or C-26, was elucidated to be the former on the basis of the HMBC spectrum. This presumption was supported by a peak at m/z 627.4243 ($\text{C}_{38}\text{H}_{59}\text{O}_7$, $[\text{MH} - \text{H}_2\text{O}]^+$ ion) in the HRFABMS of **5**. Methylation of **5** gave the dimethyl ester **9**, $[\alpha]^{20}_{\text{D}} +26.7^\circ$ (c 0.26, CH_2Cl), which was identical to that prepared from **4**, $[\alpha]^{20}_{\text{D}} +25.0^\circ$ (c 0.12, CH_2Cl). Thus, **5** was elucidated to be (25S,3'S)-(+)-12 α -hydroxy-3 α -(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methyl-llanosta-8,24(31)-dien-26-oic acid. This is the first isolation of **3**–**5**, although they have been isolated as methyl esters from the methylated crude extract of *P. betulinus*.¹¹

Compound **6** showed a $[\text{MH} - \text{H}_2\text{O}]^+$ ion peak at m/z 629.4081 in the HRFABMS, suggesting a molecular formula of $\text{C}_{37}\text{H}_{58}\text{O}_9$. The ^1H NMR spectrum exhibits signals similar to that of **4**, but a singlet assignable to one of the eight methyl groups was not observed. Two doublets (each 1H, d, $J = 11.3$ Hz, δ 3.52 and 3.82 ppm), which were absent in the ^1H NMR spectra of **1** and **3**–**5**, suggested the presence of a $-\text{CH}_2\text{OH}$ group. These spectral data suggested that one of the methyl groups of **4** was oxygenated, and this was supported by the HRFABMS data of **6**. The HMQC and HMBC spectra indicated that either C-28 or C-29 was a $-\text{CH}_2\text{OH}$ group. The stereochemistry of the A ring of **6** was elucidated on the basis of the HMBC and NOESY spectra. The signal assignable to H-3 was a broad singlet due to the small coupling constants between H-2 α and H-3, and H-2 β and H-3, indicating that the 3-H had the β -configuration. The oxygenated position, either C-28 or C-29, was determined to be the former, since NOE correlations were observed between H-28 and H₃-19 and between H-29 and H-5. These observations revealed that **6** is a novel lanostane-type triterpene acid, (+)-12 α ,28-dihydroxy-3 α -(3'-hydroxy-3'-methylglutaryloxy)-24-methyl-llanosta-8,24(31)-dien-26-oic acid. The absolute configuration of **6** could not be elucidated because of its small amount.

The mouse ear inflammation test was used to evaluate the anti-inflammatory activity of each isolated compound. Compounds **1**–**6** suppressed the TPA-induced edema up to 49–86% at 400 nmol application (Table 1). The activities of **1** and **3**–**6** are stronger than those of glycyrrhetic acid and indomethacin. It is known that a Chinese medicinal mushroom, *Poria cocos* Wolf (Polyporaceae), also contains various lanostane-type triterpene acids with remarkable anti-inflammatory or anti-emetic activity.^{12–14}

Experimental Section

General Experimental Procedures. Optical rotation values were measured by a JASCO DIP-1000 polarimeter; mass spectra were obtained by a JEOL JMS 700 mass spectrometer; ^1H and ^{13}C NMR spectra were recorded by a Bruker DRX500 FT-NMR spectrometer operating at 500.1

MHz for the protons and at 125.8 MHz for carbons, with TMS used as the internal standard; IR spectra were taken with a JASCO FT/IR-480 Plus spectrometer; Bond Elut SI (500 mg; Varian) was used as a cartridge column.

Fungal Material. Fruiting bodies of *P. betulinus* were collected in Nagano, Japan, in October 1996. An authenticated voucher specimen (KPM-NC0010705) has been deposited at the Kanagawa Prefectural Museum of Natural History, Kanagawa, Japan.

Extraction and Isolation of **1 and **2**.** Fruiting bodies of *P. betulinus* were extracted using MeOH at room temperature. The extract was filtered and concentrated in vacuo, and the resulting concentrate (27 g) was successively partitioned between *n*-hexane, EtOAc, and water. The major anti-inflammatory activity was found in the EtOAc layer in a bioassay using TPA-induced edema on mouse ears described below. The EtOAc layer was subjected to silica gel (150 g; Wakogel C-300) column chromatography using *n*-hexane–EtOAc as the eluent. The *n*-hexane–EtOAc (4:6) eluate was subjected to silica gel (35 g; 60 H, Merck) column chromatography using benzene–EtOAc as the eluent. The 30% EtOAc fraction was concentrated, giving **2** (14.3 mg). The *n*-hexane–EtOAc (5:5) eluate was subjected to silica gel (35 g; 60 H) column chromatography using *n*-hexane–acetone (7:3) as the eluent (12 mL/fraction). Fractions 17 and 18 were concentrated, giving **1** (16.5 mg).

Extraction and Isolation of **3–**6**.** The extract of *P. betulinus* was prepared in the same manner as mentioned above and concentrated in vacuo. The resulting concentrate (13 g) was successively partitioned between *n*-hexane and water. The insoluble materials for either *n*-hexane or water were subjected to silica gel (50 g; Wakogel C-300) column chromatography using CHCl_3 –MeOH as the eluent. The CHCl_3 –MeOH (20:0 and 19:1) eluates were then subjected to silica gel (100 g; Wakogel C-300) column chromatography using *n*-hexane–EtOAc as the eluent. The 50% EtOAc fraction was again subjected to silica gel (50 g; Wakogel C-300) column chromatography using *n*-hexane–EtOAc (6:4–5:5) as the eluent, giving **5** (55.5 mg). The 100% EtOAc fraction was again subjected to silica gel (50 g; Wakogel C-300) column chromatography using *n*-hexane–EtOAc–AcOH (70:30:0, 60:40:0, and 60:40:1) as the eluent, giving **3** (41.8 mg) and **4** (71.5 mg). The CHCl_3 –MeOH (16:4) eluate was then subjected to silica gel (25 g; Wakogel C-300) column chromatography using *n*-hexane–EtOAc as the eluent. The 100% EtOAc fraction was purified by cartridge column chromatography using *n*-hexane–EtOAc–AcOH (80:20:0, 50:50:0, 50:50:1, and 0:100:1) as the eluent, giving **6** (4.3 mg).

(25S)-(+)-12 α -Hydroxy-3 α -malonyloxy-24-methyl-llanosta-8,24(31)-dien-26-oic acid (3**):** colorless powder; $[\alpha]^{22}_{\text{D}} +24.1^\circ$ (c 1.97, CHCl_3); IR (film) ν_{max} 3480, 2946, 1711, 1645, 1457, 1345, 1207 cm^{-1} ; FABMS m/z 555 $[\text{MH} - \text{H}_2\text{O}]^+$ (78), 451 $[\text{MH} - \text{OCOCH}_2\text{COOH} - \text{H}_2\text{O}]^+$ (100); HRFABMS m/z 555.3685 (calcd for $\text{C}_{34}\text{H}_{51}\text{O}_6$ $[(\text{MH} - \text{H}_2\text{O})^+]$, 555.3686).

Alkaline Hydrolysis of **3.** NaOH (6 N, 0.1 mL) was added to a solution of **3** (5 mg) in MeOH (0.5 mL) at room temperature, and the reaction mixture was stirred for 12 h. The solution was purified using a cartridge column eluted with EtOAc to afford **1** quantitatively.

(25S,3'S)-(+)-12 α -Hydroxy-3 α -(3'-hydroxy-3'-methylglutaryloxy)-24-methyl-llanosta-8,24(31)-dien-26-oic acid (4**):** colorless powder; $[\alpha]^{22}_{\text{D}} +14.7^\circ$ (c 1.20, CHCl_3); IR (film) ν_{max} 3480, 2948, 1711, 1645, 1458, 1375, 1208 cm^{-1} ; FABMS m/z 613 $[\text{MH} - \text{H}_2\text{O}]^+$ (6), 451 $[\text{MH} - \text{OCOCH}_2\text{C}(\text{OH})(\text{CH}_3)\text{CH}_2\text{COOH} - \text{H}_2\text{O}]^+$ (36); HRFABMS m/z 613.4108 (calcd for $\text{C}_{37}\text{H}_{57}\text{O}_7$ $[(\text{MH} - \text{H}_2\text{O})^+]$, 613.4104).

Reduction of **4.** Compound **4** (25 mg) in 2 mL of LiBH_4 solution (2.0 M in THF; Aldrich) was stirred at room temperature for 12 h. The reaction was quenched with H_2O and then aqueous HCl (1 N). The solution (pH 7) was extracted with EtOAc to give **1** (15 mg). The solution was acidified to pH 1 with aqueous HCl (1 N), stirred for 3 days, and then extracted with EtOAc. The extract was purified by HPLC with an ODS column (YMC RS-323, 250 $\times$ 10 mm) eluting with MeOH– H_2O (1:1) at a flow rate of 3.0 mL/min with detection at 210

nm to give **7** (0.7 mg).¹⁰ Compound **7** was treated with (S)-1-phenylethylamine (2 μ L) in THF (50 μ L), followed by acetylation with Ac₂O in pyridine, to give **8**.⁹ Identification of **8** was carried out using HPLC with a silica gel column (YMC A-004, 300 $\times$ 4.6 mm) eluting with *n*-hexane–CH₂Cl₂–*i*-PrOH (20:20:1) at a flow rate of 2.0 mL/min with detection at 254 nm. The *t*_R's of authentic (3R)-5-*O*-acetyl-1-[(S)-phenylethyl]mevalonamide and (3S)-5-*O*-acetyl-1-[(S)-phenylethyl]mevalonamide were 14.7 and 15.8 min, respectively, and **8** was identical to the former on the chromatogram.

(25S,3'S)-(+)-12 α -Hydroxy-3 α -(3'-hydroxy-4'-methoxycarbonyl-3'-methylbutyryloxy)-24-methylallanosta-8,24-(31)-dien-26-oic acid (5): colorless powder; $[\alpha]_D^{25} +17.0^\circ$ (c 2.62, CHCl₃); IR (film) $\nu_{\max}$ 3483, 2948, 1710, 1645, 1457, 1376, 1204 cm⁻¹; FABMS *m/z* 627 [MH – H₂O]⁺ (8), 451 [MH – OCOCH₂C(OH)(CH₃)CH₂COOCH₃ – H₂O]⁺ (78); HRFABMS *m/z* 627.4243 (calcd for C₃₈H₅₉O₇ [(MH – H₂O)⁺], 627.4261).

Methylation of 4 and 5. Methylation of **5** (5 mg) in MeOH (1 mL) with ethereal diazomethane for 2 h gave **9** quantitatively. Compound **9** was also prepared from **4** in the same manner.

(+)-12 α ,28-Dihydroxy-3 α -(3'-hydroxy-3'-methylglutaryl-oxy)-24-methylallanosta-8,24(31)-dien-26-oic acid (6): colorless powder; $[\alpha]_D^{25} +25.6^\circ$ (c 0.43, MeOH); ¹H NMR (CDCl₃, 500 MHz) δ 5.19 (1H, brs, H-3), 5.00, 4.93 (each 1H, brs, H-31), 3.98 (1H, d, *J* = 8.1 Hz, H-12), 3.82, 3.52 (each 1H, d, *J* = 11.3 Hz, H-28), 3.20 (1H, q, *J* = 7.1 Hz, H-25), 2.77, 2.71 (each 1H, d, *J* = 15.3 Hz, H-2'), 2.77, 2.65 (each 1H, d, *J* = 15.6 Hz, H-4'), 2.58, 2.08 (each 1H, m, H-11), 2.26, 2.08 (each 1H, m, H-23), 2.05 (1H, m, H-17), 2.03 (2H, m, H-7), 2.04, 1.28 (each 1H, m, H-16), 1.89, 1.70 (each 1H, m, H-2), 1.68, 1.28 (each 1H, m, H-22), 1.67, 1.21 (each 1H, m, H-15), 1.64 (1H, m, H-5), 1.62, 1.45 (each 1H, m, H-6), 1.52, 1.44 (each 1H, m, H-1), 1.44 (1H, m, H-20), 1.42 (3H, s, 3-CH₃), 1.34 (3H, d, *J* = 7.1 Hz, H-26), 1.09 (3H, s, H-30), 1.02 (1H, d, *J* = 6.4 Hz, H-21), 1.02 (3H, s, H-29), 0.94 (3H, s, H-19), 0.58 (3H, s, H-18); ¹³C NMR (CDCl₃, 125 MHz) δ 177.4 (C, C-27), 174.6 (C, C-5'), 171.8 (C, C-1'), 149.5 (C, C-24), 134.8 (C, C-8), 132.9 (C, C-9), 110.5 (CH₂, C-31), 73.9 (CH, C-3), 73.2 (CH, C-12), 69.7 (C, C-3'), 65.1 (CH₂, C-28), 49.7 (C, C-14), 49.6 (C, C-13), 46.2 (CH, C-5), 46.0 (CH, C-25), 45.7 (CH₂, C-2'), 45.1 (CH₂, C-4'), 43.1 (CH, C-17), 42.3 (C, C-4), 36.6 (C, C-10), 36.0 (CH, C-20), 34.3 (CH₂, C-22), 32.6 (CH₂, C-11), 32.0 (CH₂, C-15), 31.7 (CH₂, C-23), 30.6 (CH₂, C-1), 27.7 (CH₂, C-16), 27.3 (CH₃, 3'-CH₃), 26.4 (CH₂, C-7), 24.5 (CH₃, C-30), 23.2 (CH₂, C-2), 21.7 (CH₃, C-29), 19.4 (CH₃, C-19), 18.2

(CH₂, C-6), 17.9 (CH, C-21), 16.5 (CH₃, C-26), 16.3 (CH₃, C-18); FABMS *m/z* 629[MH – H₂O]⁺ (4), 467[MH – OCOCH₂C(OH)(CH₃)CH₂COOH – H₂O]⁺ (25); HRFABMS *m/z* 629.4081 (calcd for C₃₇H₅₇O₈ [(MH – H₂O)⁺], 629.4053).

Anti-inflammatory Test. The mouse ear inflammatory test was conducted according to Gschwendt's method.¹⁵ The experiment complied with regulations concerning animal experimentation and the care of experimental animals of the Faculty of Agriculture at Shinshu University.

Acknowledgment. We thank Ms. K. Hashimoto of Shinshu University for providing the NMR spectra.

Supporting Information Available: Table of ¹³C and ¹H NMR spectra for **1**, **3**–**5**, and **9** and a figure showing NOEs and coupling constants of the A ring of **6**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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