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, (+)-12α,28-dihydroxy-3α-(3′-hydroxy-3′-methylglutaryloxy)-24-methyllanosta-8,24(31)-dien-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 ^{1}H 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 ^{13}C NMR spectrum of **3**, signals were observed at δ 41.3 ppm (CH$_2$), 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 (C$_{34}$H$_{51}$O$_6$, [MH − H$_2$O]$^+$) 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 −CO$_2$CH$_2$CO$_2$H 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*)-(+)-12α-hydroxy-3α-malonyloxy-24-methyllanosta-8,24(31)-dien-26-oic acid.

In the ^{1}H and ^{13}C 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 ^{13}C NMR spectrum. Three correlations, from the quaternary carbon to −C*H*$_3$ (3H, δ 1.39 ppm), to −C*H*$_2$− (each 1H, d, *J* = 15.2 Hz, δ 2.63 and 2.70 ppm), and to another −C*H*$_2$− (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
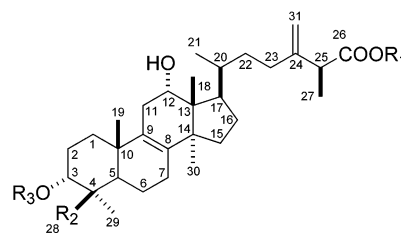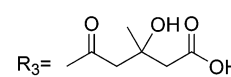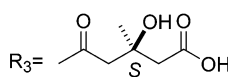

1: R$_1$=H, R$_2$=CH$_3$, R$_3$=H

3: R$_1$=H, R$_2$=CH$_3$, R$_3$=COCH$_2$COOH

4: R$_1$=H, R$_2$=CH$_3$ **6**: R$_1$=H, R$_2$=CH$_2$OH

5: R$_1$=H, R$_2$=CH$_3$ **9**: R$_1$=R$_2$=CH$_3$
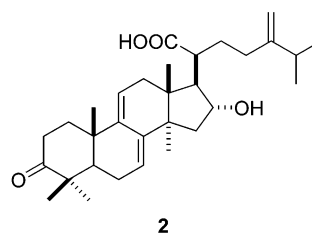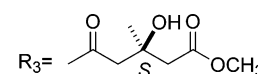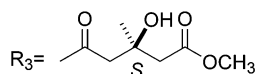
and 174.4 ppm, respectively). The chemical shift values suggested that the carbonyl groups are carboxylic acids or esters. These observations suggested that **4** is an *O*-(3-hydroxy-3-methylglutaryl) derivative of **1**, which was supported 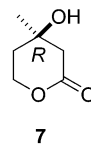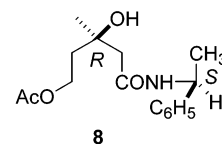by a peak at *m/z* 613.4108 (C$_{37}$H$_{57}$O$_7$, [MH − H$_2$O]$^+$ ion) in the HRFABMS of **4**. To determine the absolute configuration, **4** was reduced using lithium borohydride to **1** and (3*R*)-mevalonic lactone (**7**), whose chirality was identified by conversion to (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]mevalonamide (**8**).[9,10] These results confirmed that **4** is (25*S*,3′*S*)-(+)-12α-hydroxy-3α-(3′-hydroxy-3′-methylglutaryloxy)-24-methyllanosta-8,24(31)-dien-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 ^{1}H 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 ($C_{38}H_{59}O_7$, $[MH - H_2O]^+$ ion) in the HRFABMS of **5**. Methylation of **5** gave the dimethyl ester **9**, $[\alpha]^{20}_D$ +26.7° (c 0.26, CH_3Cl), which was identical to that prepared from **4**, $[\alpha]^{20}_D$ +25.0° (c 0.12, CH_3Cl). Thus, **5** was elucidated to be (25S,3′S)-(+)-12α-hydroxy-3α-(3′-hydroxy-4′-methoxycarbonyl-3′-methylbutyryloxy)-24-methyllanosta-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 $[MH - H_2O]^+$ ion peak at m/z 629.4081 in the HRFABMS, suggesting a molecular formula of $C_{37}H_{58}O_9$. The ^{1}H 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 ^{1}H NMR spectra of **1** and **3**−**5**, suggested the presence of a −CH_2OH 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 −CH_2OH 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-2α and H-3, and H-2β 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 H$_3$-19 and between H-29 and H-5. These observations revealed that **6** is a novel lanostane-type triterpene acid, (+)-12α,28-dihydroxy-3α-(3′-hydroxy-3′-methylglutaryloxy)-24-methyllanosta-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; ^{1}H 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)-(+)-12α-Hydroxy-3α-malonyloxy-24-methyllanosta-8,24(31)-dien-26-oic acid (3): colorless powder; $[\alpha]^{22}_D$ +24.1° (c 1.97, $CHCl_3$); IR (film) ν_{max} 3480, 2946, 1711, 1645, 1457, 1345, 1207 cm^{-1}; FABMS m/z 555 $[MH - H_2O]^+$ (78), 451 $[MH - OCOCH_2COOH - H_2O]^+$ (100); HRFABMS m/z 555.3685 (calcd for $C_{34}H_{51}O_6$ $[(MH - H_2O)^+]$, 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)-(+)-12α-Hydroxy-3α-(3′-hydroxy-3′-methylglutaryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (4): colorless powder; $[\alpha]^{22}_D$ +14.7° (c 1.20, $CHCl_3$); IR (film) ν_{max} 3480, 2948, 1711, 1645, 1458, 1375, 1208 cm^{-1}; FABMS m/z 613 $[MH - H_2O]^+$ (6), 451 $[MH - OCOCH_2C(OH)(CH_3)CH_2COOH - H_2O]^+$ (36); HRFABMS m/z 613.4108 (calcd for $C_{37}H_{57}O_7$ $[(MH - H_2O)^+]$, 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 × 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-phenyletheylamine (2 μL) in THF (50 μL), followed by acetylation with Ac$_2$O in pyridine, to give **8**.[9] Identification of **8** was carried out using HPLC with a silica gel column (YMC A-004, 300 × 4.6 mm) eluting with *n*-hexane−CH$_2$Cl$_2$−*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 (3*R*)-5-*O*-acetyl-1-[(*S*)-phenylethyl]mevalonamide and (3*S*)-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.

(25*S*,3′*S*)-(+)-12α-Hydroxy-3α-(3′-hydroxy-4′-methoxycarbonyl-3′-methylbutyryloxy)-24-methyllanosta-8,24-(31)-dien-26-oic acid (5): colorless powder; [α]^{23}$_D$ +17.0° (*c* 2.62, CHCl$_3$); IR (film) ν_{max} 3483, 2948, 1710, 1645, 1457, 1376, 1204 cm^{-1}; FABMS *m/z* 627 [MH − H$_2$O]$^+$ (8), 451 [MH − OCOCH$_2$C(OH)(CH$_3$)CH$_2$COOCH$_3$ − H$_2$O]$^+$ (78); HRFABMS *m/z* 627.4243 (calcd for C$_{38}$H$_{59}$O$_7$ [(MH − H$_2$O)$^+$], 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.

(+)-12α,28-Dihydroxy-3α-(3′-hydroxy-3′-methylglutaryloxy)-24-methyllanosta-8,24(31)-dien-26-oic acid (6): colorless powder; [α]^{21}$_D$ +25.6° (*c* 0.43, MeOH); ^{1}H NMR (CDCl$_3$, 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-C*H*$_3$), 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); ^{13}C NMR (CDCl$_3$, 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 (CH$_2$, C-31), 73.9 (CH, C-3), 73.2 (CH, C-12), 69.7 (C, C-3′), 65.1 (CH$_2$, C-28), 49.7 (C, C-14), 49.6 (C, C-13), 46.2 (CH, C-5), 46.0 (CH, C-25), 45.7 (CH$_2$, C-2′), 45.1 (CH$_2$, C-4′), 43.1 (CH, C-17), 42.3 (C, C-4), 36.6 (C, C-10), 36.0 (CH, C-20), 34.3 (CH$_2$, C-22), 32.6 (CH$_2$, C-11), 32.0 (CH$_2$, C-15), 31.7 (CH$_2$, C-23), 30.6 (CH$_2$, C-1), 27.7 (CH$_2$, C-16), 27.3 (CH$_3$, 3′-CH$_3$), 26.4 (CH$_2$, C-7), 24.5 (CH$_3$, C-30), 23.2 (CH$_2$, C-2), 21.7 (CH$_3$, C-29), 19.4 (CH$_3$, C-19), 18.2 (CH$_2$, C-6), 17.9 (CH, C-21), 16.5 (CH$_3$, C-26), 16.3 (CH$_3$, C-18); FABMS *m/z* 629 [MH − H$_2$O]$^+$ (4), 467 [MH − OCOCH$_2$C(OH)(CH$_3$)CH$_2$-COOH − H$_2$O]$^+$ (25); HRFABMS *m/z* 629.4081 (calcd for C$_{37}$H$_{57}$O$_8$ [(MH − H$_2$O)$^+$], 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.

Supporting Information Available: Table of ^{13}C and ^{1}H 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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