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New anti-inflammatory ergostane-type ecdysteroids from the sclerotium of *Polyporus umbellatus*

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Abstract—Bioassay-guided fractionation of the ethyl acetate extract from the sclerotium of *Polyporus umbellatus* resulted in the isolation of three new ergostane-type ecdysteroids, named polyporoid A (**1**), B (**2**), and C (**3**), together with five known ecdysteroids. The structures of the new compounds were determined on the basis of extensive spectroscopic data (IR, MS, ^{1}H and ^{13}C NMR, and 2D NMR) analyses. All compounds (**1**–**8**) exhibited potent anti-inflammatory activity in the test of TPA-induced inflammation (1 μg/ear) in mice, with ID_{50} values in the range of 0.117–0.682 μM/ear.

Polyporus umbellatus Fries (Polyporaceae) is a saprophytic fungus growing on withered beech and maple trees' roots. Its sclerotium, known as a traditional Chinese drug, is used for the promotion of diuresis. Few phytochemical studies on this fungus species have been reported to date. Previous chemical investigation revealed the presence of ergosterols, polysaccharides, and ecdysteroids.[1–6] Ecdysteroids are known as insect molting hormones and can play a role in plant defence against phytophagous insect. Recently, ecdysteroids have been reported to exhibit various biological activities including in vitro cytotoxic, in vivo antitumor-promoter, antioxidant activities, etc.[7–10] A few of ecdysteroids have been found to possess C-28 ergostane skeleton, which was reported from mushrooms and marine organisms.[11–16]

In the course of studies on the phytochemical and pharmacological constituents of *P. umbellatus*, we found that the methanol extract marked anti-inflammatory activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema in mice. As an effort to discover bioactive substances from this fungus, our studies on the EtOAc-soluble extract of sclerotium resulted in the isolation of three new ergostane-type ecdysteroids, polyporoid A (**1**), B (**2**), and C (**3**), together with five known ecdysteroids identified as polyporusterone A (**4**),[4] polyporusterone C (**5**),[4] polyporusterone B (**6**),[4] polyporusterone G (**7**),[4] and ergosta-7,22-diene-3β,5α,6β-triol (**8**).[2] Ecdysteroids **1** and **2** containing a furan ring on the side chain are rare in nature. Spectroscopic methods were used to establish the structures of compounds **1**–**8** (Fig. 1).

The dried sclerotium of *P. umbellatus* (1 kg) was ground and extracted with MeOH (3 L × 24 h × 3). After the combined extract was evaporated under reduced pressure at 40 °C, the residue (22.5 g) was suspended in H_2O and then partitioned with EtOAc. The EtOAc-soluble extract (16.9 g) was partitioned with *n*-hexane–MeOH–H_2O (19:19:2), giving *n*-hexane (6.4 g) and MeOH–H_2O (10.5 g) soluble fractions. The MeOH–H_2O extract was subjected to a silica gel column, eluting with a gradient of *n*-hexane/EtOAc, and then monitored on TLC to obtain five major fractions. Fraction 4 (1.8 g) was again chromatographed over a silica gel column (eluting with $CHCl_3$/MeOH), resulting in four fractions (fr. 4.1–4.4). Fraction 4.2 was separated by reversed-phase HPLC (52% MeOH/H_2O), yielding **3** (3 mg), **4** (10 mg), and **8** (3 mg). Fraction 4.3, eluted with $CHCl_3$/MeOH (1:1), was further purified by Sephadex LH-20 and reversed-phase HPLC (45% MeOH/H_2O) to afford **2** (7 mg), **5** (18 mg), **6** (14 mg), and **7** (3 mg). Fraction 4.4 was separated by reversed-phase HPLC (65% MeOH/H_2O) to give **1** (2 mg).

Polyporoid A (**1**)[17] was isolated as a white amorphous solid and possessed a molecular formula $C_{28}H_{44}O_7$ as determined by HRFABMS at *m/z* 493.31601 [M+H$^+$], indicating 7° of unsaturation. The IR spectrum of **1**

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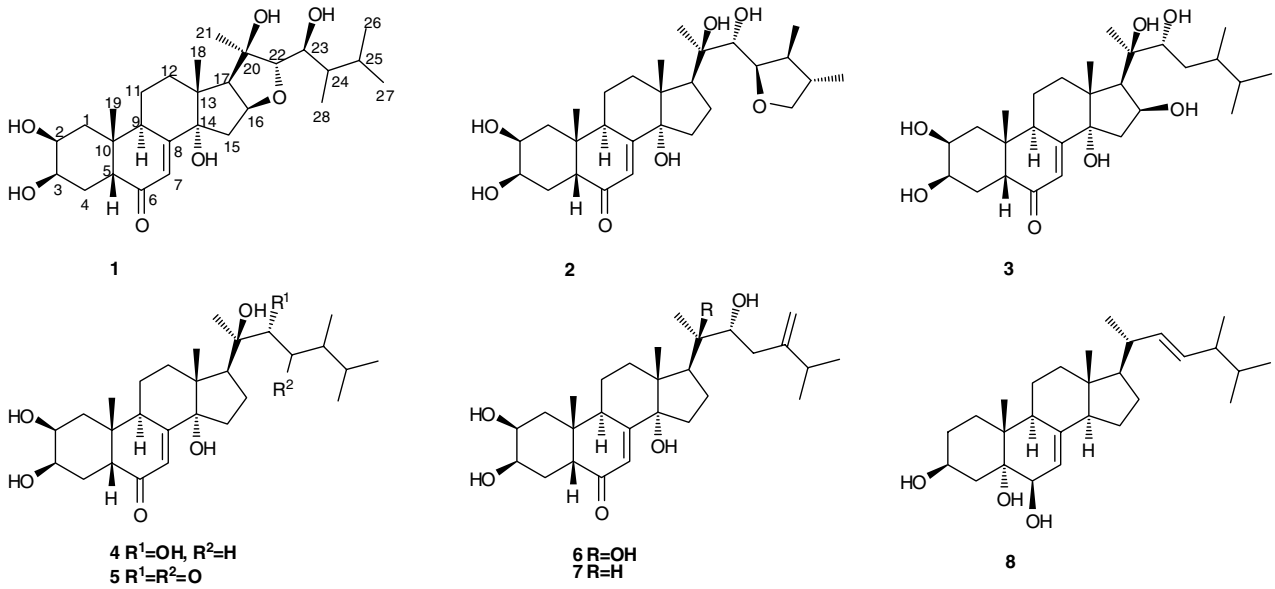

Figure 1. Structures of compounds **1**–**8**.

showed absorptions at ν_{max} 3396, 1644, and 1623 cm^{-1} due to hydroxyl and α,β-unsaturated ketone groups. The presence of the conjugated enone group was also indicated by the resonances at δ 5.76 (1H, d, J = 2.4 Hz, H-7) in the ^{1}H NMR and at δ 205.6 (C-6), 167.1 (C-8), and 122.0 (C-7) in the ^{13}C NMR. The ^{13}C NMR and DEPT spectra revealed 28 carbon signals including six methyls, five methylenes, 11 methines (five oxygenated and one olefinic), and six quaternary carbons (two oxygenated, one olefinic, and one carbonyl), thus requiring five rings in **1** to account for the seven units of unsaturation. The ^{1}H NMR showed resonances for three methyl singlets at δ 1.43 (3H, s, H-21), 1.18 (3H, s, H-18), and 0.99 (3H, s, H-19), three methyl doublets at δ 0.95 (3H, d, J = 6.7 Hz, H-26), 0.92 (3H, d, J = 6.7 Hz, H-27), and 0.87 (3H, d, J = 7.0 Hz, H-28), and five oxymethine signals at δ 4.65 (1H, m, H-16), 3.94 (1H, d, J = 9.4 Hz, H-22), 3.93 (1H, br s, H-3), 3.81 (1H, dt, J = 11.9, 4.1 Hz, H-2), and 3.78 (1H, dd, J = 9.4, 1.9 Hz, H-23). The HMQC correlations further assigned all protons and their associated carbons in the molecule. These observed data suggested that **1** was an ergostane-type ecdysteroid, and closely resembled those of **4** and **5**, which was also verified by 2D NMR.

Analysis of the ^{1}H–^{1}H COSY spectrum (Fig. 2) indicated the presence of a spin system (H-2/H-1, H-3; H-4/H-3, H-5) that established the connectivity of C-1 to C-5. HMBC correlations (Fig. 2) from the methine proton at δ 2.38 (H-5) to C-6 (δ 205.6), and from an olefinic proton at δ 5.76 (H-7) to C-5 (δ 51.9), C-9 (δ 34.7), and C-14 (δ 86.2) allowed the location of the α,β-unsaturated ketone group at C-6, C-7, and C-8, and a hydroxyl group at C-14. Further inspection of HMBC and ^{1}H–^{1}H COSY spectra led to the establishment of an ergostane skeleton in the side chain. HMBC correlations from the proton at δ 2.54 (1H, d, J = 7.4 Hz, H-17) to C-16 (δ 83.0), C-20 (δ 80.9), and C-22 (δ 84.7) and from the methyl protons (Me-28) to C-23 (δ 72.8) and C-24 (δ 42.5), suggesting that the oxygenated groups were located at C-16, C-20, C-22, and C-23, respectively. A weak correlation in the HMBC from the oxymethine proton at δ 4.65 (H-16) to C-22, and a remaining degree of unsaturation out of seven in the molecule suggested the presence of a new furan ring from C-16 to C-22. Additional COSY correlations displayed a spin system (H-23/H-22, H-24; H-24/H-28; and H-25/H-24, H-26, and H-27) to complete the connectivity of the side chain from C-22 to C-28.

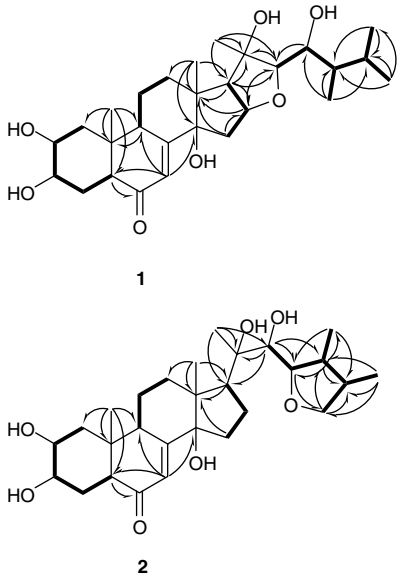

Figure 2. Key ^{1}H–^{1}H COSY (—) and HMBC (H → C) correlations for **1** and **2**.

The relative configuration of **1** was determined on the basis of NOESY experiment (Fig. 3) and ^{1}H NMR J values. The cross-peaks of H_3-19/H_2-1 and H-5, H-5/H_2-1β, and H-9/H-2 and H-4α confirmed a *cis*-fused pattern between A- and B-rings. NOESY correlations from

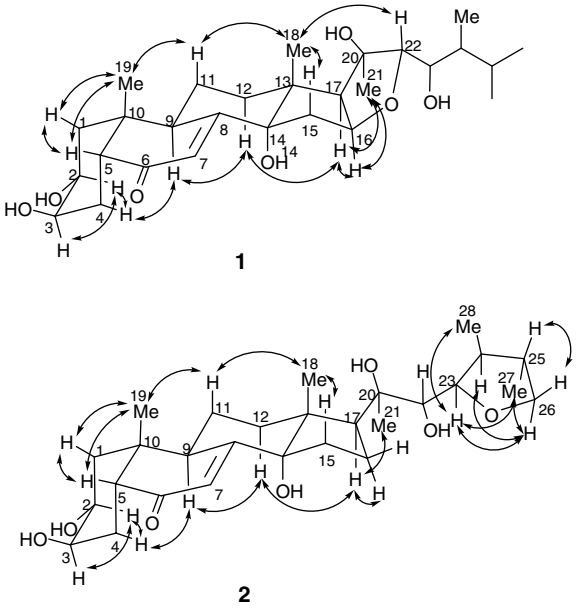

Figure 3. Key NOESY correlations (↔) of **1** and **2**.

H-2 to H-4α and H-9, and a *trans*-diaxial coupling (J = 11.9 Hz) between H-2α and H-1β in the ^{1}H NMR indicated that OH-2 was β-oriented and was at equatorial position. The cross-peaks of H-3/H-2, and a broad singlet ($W_{1/2}$ = 6 Hz) observed in the ^{1}H NMR suggested an axial orientation for OH-3, which was further supported by comparing its ^{13}C NMR data with literature.[18] NOESY correlations from H-11 to both H_3-18 and H_3-19 indicated that these protons were in the same face and β-oriented. Similarly, a correlation from H-12α to H-17 placed these protons on the other face of the fused ring system, establishing a trans C/D ring fusion and an α-orientation for OH-14. Moreover, the cross-peaks of H_3-21/H-16 and H-17, and H-16/H-17 indicated that these protons were α-orientation and established the presence of a *cis*-fused D/E ring system. The stereochemistry of the side chain was successfully determined by comparing the NMR data with those of the analog Downeyoside B.[19] Further correlations of H-22 with H_3-18 confirmed that H-22 was β-oriented. A large vicinal coupling value (J = 9.4 Hz) between H-22 and H-23 supported assignment of an axial orientation for H-22 and indicated a 23S conformation according to the results of molecule dynamics and mechanics calculations in the force field CHARM.[19] From the above results, the structure of **1** was established as a new ecdysteroid as shown in Figure 1.

Polyporoid B (**2**)[20] was assigned the molecular formula $C_{28}H_{44}O_7$ (seven unsaturations) on the basis of HRFABMS data, which was the same as that of **1**. Its IR absorption bands at 3400, 1648, and 1620 cm^{-1} implied the presence of hydroxyl, conjugated carbonyl, and olefinic groups, respectively. The analysis of the NMR spectra of **2** revealed that this compound was an ergostane-type ecdysteroid similar to **1**. The significant differences were a pair of oxygenated diastereotopic hydrogen signals at δ 3.89 (1H, t, J = 7.9 Hz, H-26a) and 3.35 (1H, t, J = 7.6 Hz, H-26b) in the ^{1}H NMR spectrum. The ^{13}C NMR and DEPT spectra displayed nine carbons for the side chain, involving three methyls at δ 23.2 (C-21), 18.2 (C-28), and 15.5 (C-27), a oxymethylene at δ 75.4 (C-26), two oxymethines at δ 87.8 (C-23) and 80.2 (C-22), two methines at δ 47.9 (C-24) and 43.7 (C-25), and a quaternary oxygenated carbon at δ 78.2 (C-20). The linkage of the side chain in the molecule was established by a series of HMBC correlations (Fig. 2) from the methyl protons at δ 1.26 (H_3-21) to C-17 (δ 50.2), C-20, and C-22, from the oxymethine proton at δ 3.32 (H-22) to C-20 and C-24, and from the methine proton at δ 1.70 (H-24) to C-23, C-25, and C-28. Additional HMBC correlation from an oxymethine proton at δ 3.52 (H-23) to C-26, and the consideration of the remaining 1° of unsaturation indicated the formation of a furan ring across C-23 and C-26. The ^{1}H–^{1}H COSY spectrum revealed the presence of four spin systems (Fig. 2), and enabled us to assign the two hydroxyl groups at C-2 and C-3 as were seen in **1**.

In the NOESY spectrum (Fig. 3) of compound **2**, the detected cross-peaks of H-23/H-26α and H_3-27, H-24/H-26α indicated that these protons were α-orientations. The stereochemistry of chiral centers in the A/B and C/D rings, and the side chain (C-20 and C-22) was also assigned by NOESY spectrum, which were identical to the known compound **4**.[4]

The molecular formula of polyporoid C (**3**)[21] was determined to be $C_{28}H_{46}O_7$ from the molecular ion peak at m/z 495.33269 in the HRFABMS spectrum, accounting for 6° of unsaturation. This indicated the presence of two additional hydrogens and one less degree of unsaturation than those of **1**. The analysis of NMR data revealed that **3** was related to **1**, but lacked an oxymethine signal observed in the data for **1**, and instead showed a methylene signal in the ^{13}C NMR spectrum. Furthermore, all the ^{1}H NMR data for the ring system were very similar to those of **1**, suggesting that this portion of the molecule was identical to the corresponding portion of **1**. The connectivity of **3** was independently confirmed on the basis of HMBC and ^{1}H–^{1}H COSY data. Other key HMBC correlations were observed from the methine proton at δ 2.43 (H-17) to C-13, C-16, C-18, and C-20, and from the oxymethine proton at δ 4.02 (H-22) to C-20, C-21, C-23, and C-24, locating three hydroxyl groups at C-16, C-20, and C-22, respectively. ^{1}H–^{1}H COSY correlations identified a spin system (H-23/H-22 and H-24; H-24/H-25, H-28; and H-25/H-26 and H-27), suggesting the connectivity of the side chain from H-22 to H-28 in the molecule. The analysis of the ^{1}H and ^{13}C NMR data, and the NOESY correlations of **3** indicated the same relative configuration for A/B and C/D rings as were seen in **1**. The stereochemistry of the side chain was identified by comparison of **4** and those of the literature data.[4]

Eight ecdysteroids (**1**–**8**) were evaluated with respect to their anti-inflammatory activity against TPA-induced

Table 1. Inhibitory effect of ecdysteroids on TPA-induced inflammation in mice

Compound	ID_{50}[a] (μM/ear)
Polyporoid A (**1**)	0.531
Polyporoid B (**2**)	0.682
Polyporoid C (**3**)	0.184
Polyporusterone A (**4**)	0.141
Polyporusterone C (**5**)	0.289
Polyporusterone B (**6**)	0.117
Polyporusterone G (**7**)	0.207
Ergosta-7,22-diene-3β,5α,6β-triol (**8**)	0.666
Indomethacin[b]	0.838

[a] ID_{50}: The 50% inhibitory dose.
[b] Reference compound.

inflammation in mice, and the inhibitory effects were compared with indomethacin, a commercially available anti-inflammatory drug, as shown in Table 1. All the ecdysteroids exhibited potent inhibitory activity, with ID_{50} (50% inhibitory dose) values in the range of 0.117–0.682 μM/ear, of which **3**–**8** showed a potent inhibitory activity higher than indomethacin (ID_{50} 0.838 μM/ear). The results indicated that ecdysteroids **3**, **4**, and **6** with two hydroxyl substituents in the side chain possessed a more potent anti-inflammatory activity than the other ecdysteroids. Compounds **1** and **2** bearing a furan ring on the side chain showed the weakest activity. Thus, it was considered that the cyclization of the linear side chain could decrease the inhibitory activity. The data of the structure–activity relationships in these ecdysteroids could be valuable in future synthetic and pharmacological studies.

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Supplementary data

Experimental details and 1D, 2D NMR, and MS data with this article are available in the online version. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.04.008.

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17. *Compound **1**:* White amorphous solid; $[\alpha]_D^{25}$ +10.1° (*c* 0.05, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 240 (1.2); IR (KBr) ν_{max} 3396, 2961, 2873, 1644, 1623, 1562, 1413, 1384, and 1054 cm^{-1}; (+)FABMS *m*/*z* 493 $[M+H]^+$; HRFABMS: *m*/*z* 493.31601 (Calcd for $C_{28}H_{45}O_7$, 493.31653).
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20. *Compound **2**:* White amorphous solid; $[\alpha]_D^{25}$ +24.0° (*c* 0.10, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 241 (1.6); IR (KBr) ν_{max} 3400, 2960, 2870, 1648, 1620, 1565, 1418, 1385, and 1062 cm^{-1}; (+)FABMS *m*/*z* 493 $[M+H]^+$; HRFABMS: *m*/*z* 493.31668 (Calcd for $C_{28}H_{45}O_7$, 493.31653).
21. *Compound **3**:* White amorphous solid; $[\alpha]_D^{25}$ +20.5° (*c* 0.10, MeOH); UV λ_{max}^{MeOH} (nm) (log ε): 243 (0.6); IR (KBr) ν_{max} 3398, 2959, 2872, 1645, 1621, 1561, 1415, 1382, and 1057 cm^{-1}; (+)FABMS *m*/*z* 495 $[M+H]^+$; HRFABMS: *m*/*z* 495.33269 (Calcd for $C_{28}H_{47}O_7$, 495.33218).