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Isolation and Structural Characterization of Lupane Triterpenes from Polypodium Vulgare

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Abstract

Purification of the dichloromethane (CH_2Cl_2) fraction of the aqueous alcoholic extract of the rhizomes of polypodium vulgare resulted in the isolation of three lupane triterpenes namely lupenone, lupeol, and betulinic acid. The structures of isolated compounds were characterized on the basis of extensive spectral data (1D and 2D NMR; and MS) and in comparison with their physical and spectral data reported earlier.

Keywords: Polypodium vulgare, triterpenes, isolation and purification, NMR, MS, structure elucidation.

Introduction

Polypodium vulgare, the common polypody (Ooezo-denda in Japanese), is a fern widely distributed in Europe, Asia and North America. Eralier phytochemical studies of this fern resulted in the isolation of triterpenoid hydrocarbons, triterpenoid alcohols of the cycloartane group, ecdysones, and a sweet glycoside, osladin [1-4]. In Japan, Ooezo-denda is only found in Oki Island (Shimane prefecture) and at Hachinohe City (Aomori prefecture) as very small colonies. As a part of our research to discover natural sweeteners, we have recently reported several natural products from *S. rebaudiana* and *R. suavissimus* and *Siraitia grosvenorii* [5-12].

This paper describes the isolation and structure elucidation of the three lupane triterpenes 1-3 (Figure-1) on the basis of extensive NMR and mass spectroscopic data and in comparison of their physical and spectral properties reported from the literature.

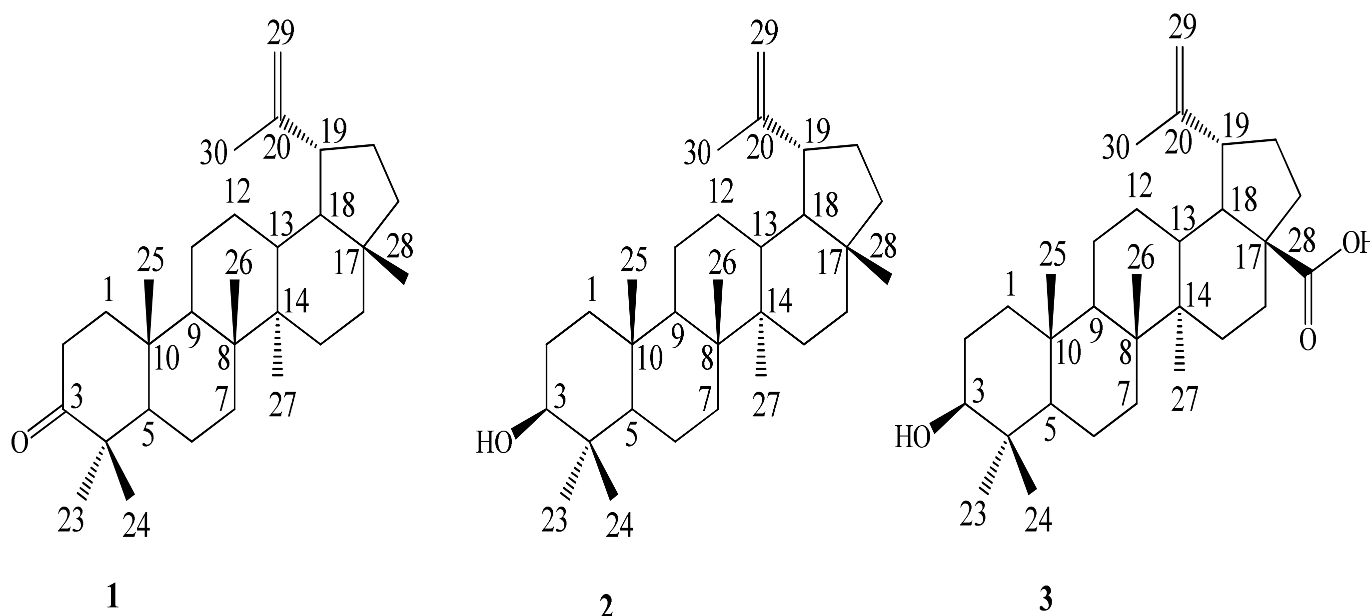


Figure-1
Structures of Lupenone (1), Lupeol (2), and Betulinic acid (3)

Materials and Methods

General Instrumentation: Melting points were measured using a SRS Optimelt MPA 100 instrument and are uncorrected. Optical rotations were recorded using a Rudolph Autopol V at 25°C and NMR spectra were acquired on a Varian Unity Plus 600 MHz instrument using standard pulse sequences at ambient temperature. Chemical shifts are given in δ (ppm), and coupling constants are reported in Hz. HRMS data was generated with a Thermo LTQ Orbitrap Discovery mass spectrometer in the positive positive ion mode electrospray. Instrument was mass calibrated with a mixture of Ultramark 1621, MRFA [a peptide], and caffeine immediately prior to accurate mass measurements of the samples. Samples were diluted with water:acetonitrile:methanol (1:2:2) and prepared a stock solution of 50 ul concentration for each sample. Each sample (25 ul) was introduced via infusion using the onboard syringe pump at a flow injection rate of 120 ul/min. Low pressure chromatography was performed on a Biotage Flash system using a C-18 cartridge (40+ M, 35-70 µm). TLC was performed on Baker Si-C_{18}F plates and identification of the spots on the TLC plate was carried out by spraying 10% H_2SO_4 in EtOH and heating the plate at about 80 ^oC.

Plant Material: The commercial extract of the aqueous/alcoholic (4:1) extract of the rhizomes of *P. vulgare* was purchased from Naturomic LLC, Anaheim, CA, USA. A voucher specimen was deposited at The Coca-Cola Company, No: VSPC-3166-155.

Isolation of Terpenoids (1-3): The aqueous extract of the rhizomes of *P. vulgare* (20 g) was suspended in 150 ml water and extracted successively with *n*-hexane (3 x 150 ml), CH_2Cl_2 (3 x 200 ml) and *n*-BuOH (2 x 200 ml). The CH_2Cl_2 layer was concentrated under vacuum furnished a residue (4.3 g) which was purified on a Biotage flash chromatography system using C-18 (100 g) column (solvent system: gradient from 80-20 MeOH-water to 100% MeOH at 40 ml/min, detection at UV 210 nm) for 40 min by collecting 80 fractions. Fractions 54-58, 60-64 and 68-72 were combined to get residues 0.15, 0.13 g and 0.12 g respectively, which on repeated purification using the gradient 80-90% MeOH-water on a C-18 (10 g) column at 20 ml/min for 30 min resulted lupenone (**1**, 44 mg), lupeol (**2**, 63 mg), and betulinic acid (**3**, 52 mg), respectively.

Identification of Lupenone (1), Lupeol (2) and Betulinic acid (3): Lupenone (1): Colorless needles, mp 168-170ºC; EIMS for $C_{30}H_{48}O$ *m/z*: 424 [M^+]; ^1H NMR ($CDCl_3$): δ 0.74, 0.78, 0.83, 0.91, 0.94, 1.06, 1.72 (each 3H, s, Me × 7), 4.56 (1H, s, H-29a), 4.74 (1H, s, H-29b); ^{13}C NMR ($CDCl_3$): δ 212.8 (C- 3), 150.4 (C-20), 108.8 (C-29), 59.3 (C-5), 58.0 (C-9), 53.1 (C-18), 42.6 (C-19), 42.2 (C-17), 41.6 (C-4), 41.4 (C-14, 8), 40.4 (C-22), 39.7 (C-1), 36.0 (C-10, 16), 35.6 (C-13), 35.1 (C-2), 33.1(C-7), 32.3 (C-23), 32.0 (C-24), 30.2 (C-15), 29.7 (C-21), 29.4 (C-12), 22.5 (C-11), 21.0 (C-30), 20.2 (C-28), 18.9(C-25), 18.5 (C-6), 18.0 (C-26), 15.1 (C-27) [13].

Lupeol (2): White powder, mp 212–214°C; EIMS for $C_{30}H_{50}O$ *m/z*: 426 [M+]; ^1H NMR ($CDCl_3$): δ 0.74, 0.78, 0.83, 0.91, 0.94, 1.06, 1.72 (each 3H, s, Me × 7), 3.20 (1H, *dd*, *J* = 5.4, 10.6 Hz, H-3), 4.56 (1H, s, H-29a), 4.70 (1H, s, H-29b); ^{13}C NMR ($CDCl_3$): δ 151.6 (C-20), 108.6 (C-29), 78.4 (C-3), 55.1 (C-5), 49.7 (C-9), 48.2 (C-18), 47.8 (C-19), 43.2 (C-17), 42.6 (C-14), 41.2 (C-8), 40.2 (C-22), 39.2 (C-13), 38.6 (C-4), 38.0 (C-1), 37.3 (C-10), 35.6 (C-16), 34.1 (C-7), 30.0 (C-21), 28.2 (C-23), 27.6 (C-15), 27.5 (C-12), 25.3 (C-2), 21.1 (C-11), 19.5 (C-30), 18.1 (C-6), 18.0 (C-28), 16.8 (C-25), 16.4 (C-26), 16.0 (C-24), 15.1 (C-27) [14].

Betulinic acid (3): Colorless amorphous powder, mp 315–317°C. EIMS for $C_{30}H_{48}O_3$ *m/z*: 456 [M+]; ^1H NMR (pyridine-*d5*): δ 0.81, 1.04, 1.06, 1.08, 1.26, 1.82 (each 3H, *s*, Me × 6), 3.42 (1H, brt, *J* = 7.2 Hz, H-3), 4.72 (1H, brs, H-29a), 4.94 (1H, brs, H-29b); ^{13}C NMR (pyridine-*d5*): δ 178.4 (C- 28), 152.0 (C-20), 109.4 (C-29), 78.2 (C-3), 56.4 (C-17), 56.2 (C-5), 51.0 (C-9), 49.8 (C-19), 47.8 (C-18), 42.9 (C-14), 41.2 (C-8), 39.6 (C-4), 39.1 (C-1), 38.7 (C-13), 37.9 (C-10), 37.4 (C-22), 34.9 (C-7), 32.6 (C-16), 31.3 (C-15), 30.6 (C-21), 28.5 (C-23), 28.3 (C-2), 26.2 (C-12), 21.3 (C-11), 19.3 (C-30), 18.8 (C-6), 16.8 (C-25), 16.4 (C-26), 16.2 (C-24), 15.0 (C-27) [14].

Results and Discussion

Compound **1** was isolated as a white powder. The mass spectral data of compound **1** gave a molecular ion peak at m/z 424 corresponding to its $(M)^+$ ion suggesting the molecular formula as $C_{30}H_{48}O$, which was supported by the ^{13}C NMR spectral data. The ^1H NMR spectra of compound **1** showed the presence of seven methyl singlets at δ 0.74, 0.78, 0.83, 0.91, 0.94, 1.06, 1.72. The ^1H NMR spectra of compound **1** also showed the presence of two protons appeared at δ 4.56 and 4.74 as singlets, representing the exocyclic double bond protons. The ^{13}C NMR spectrum of compound **1** showed a saturated carbonyl group at δ 212.8 and the alkene carbons at δ 150.4 and 108.8; suggesting the presence of a lupane triterpene having a carbonyl group in its structure. The ^1H and ^{13}C NMR values for all the protons and carbons were assigned on the basis of COSY, HMQC and HMBC correlations as reported [15-17] and were given in materials and methods. Considering lupane skeleton for compound 1 together with the absence of a hydroxyl group and the appearance of a carbonyl group in the ^{13}C NMR spectral data of 1 suggested the presence of a keto functional group and its presence was identified at C-3 position by the key HMBC correlations as shown in figure 2.

Thus, the structure of 1 was assigned as the known compound lupenone, which was confirmed by the physical and spectral data reported in the literature [13].

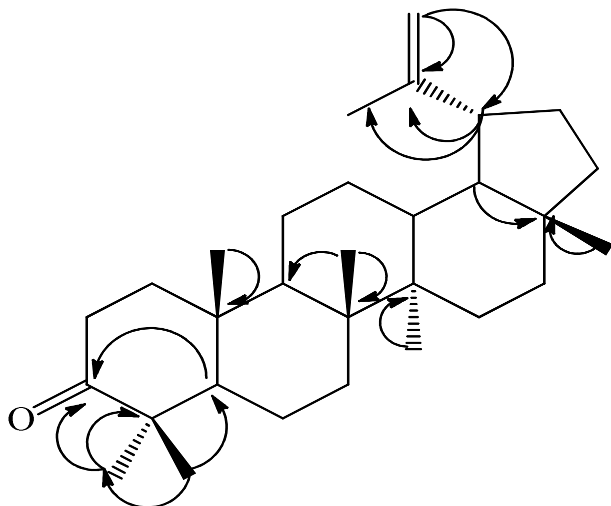


Figure-2
Key HMBC correlations of Lupenone (1)

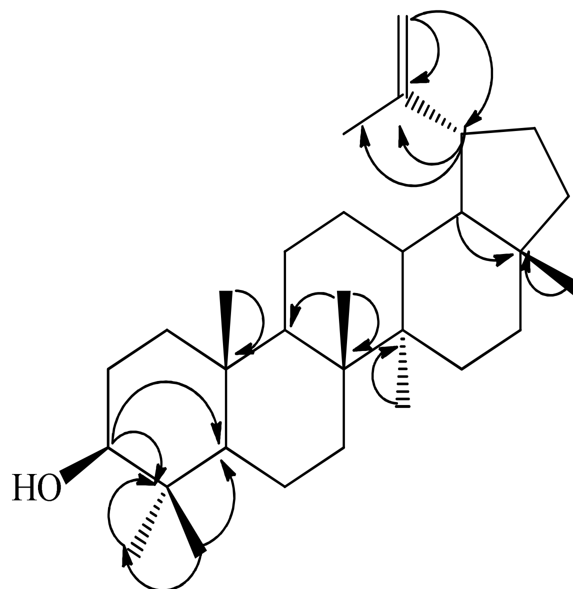


Figure-3
Key HMBC correlations of Lupeol (2)

Compound 2 was also isolated as a white powder and its mass spectral data suggested the molecular formula as $C_{30}H_{50}O$, two atomic mass units (amu) more to 1. The ^{1}H NMR spectrum showed seven tertiary methyl singlets at δ 0.74, 0.78, 0.83, 0.91, 0.94, 1.06, 1.72; and one secondary hydroxyl group as doublet of doublets at δ 3.20. It also showed two olefinic protons at δ 4.56 and 4.70 representing the exocyclic double bond as in 1. The ^{13}C NMR of the compound showed 30 signals for the terpenoid of lupane skeleton which includes a carbon bonded to the hydroxyl group at C-3 position appeared at δ 78.4, while the olefinic carbons of the exocyclic double bond appeared at δ 151.6 and 108.6. The above spectral data (NMR and mass) suggested that compound 2 is also a lupane triterpene having a secondary hydroxyl group in place of the keto group in 1, which was supported by the key HMBC correlations as shown in figure-3. Thus, the structure of 2 was assigned as lupeol that was consistent to the reported literature values [14]. The ^{1}H NMR spectrum of lupeol (1) was shown in Figures 5a and 5b.

Compound **3** was isolated as a colorless amorphous powder and its mass spectral data suggested the molecular formula as $C_{30}H_{48}O_3$. The ^{1}H NMR spectrum showed six tertiary methyl singlets at δ 0.81, 1.04, 1.06, 1.08, 1.26, 1.82; and one secondary hydroxyl group as a broad triplet at δ 3.42, and two olefinic protons at δ 4.72 and 4.94 representing the exocyclic double bond. In the absence of an additional methyl singlet as in **1** and **2** together with the appearance of a carbonyl group at δ 178.4 in the ^{13}C NMR spectrum of compound **3** suggested the presence of an acid group in its structure which was identified at C-28 position by the key HMBC correlations as shown in Figure 4. Based on the above spectral data, the structure of **3** was assigned as betulinic acid further supported by the physical and spectral data reported from the literature [14].

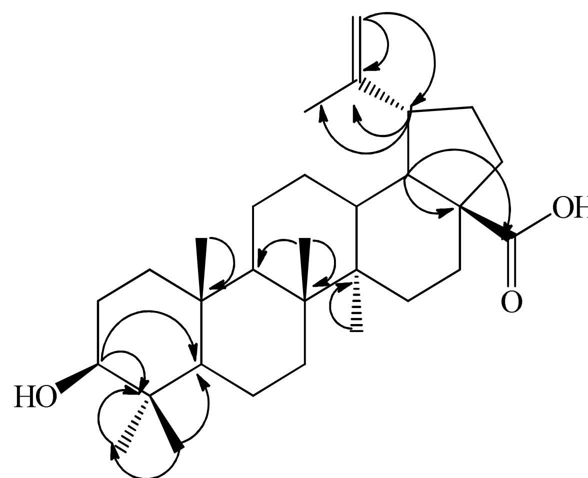
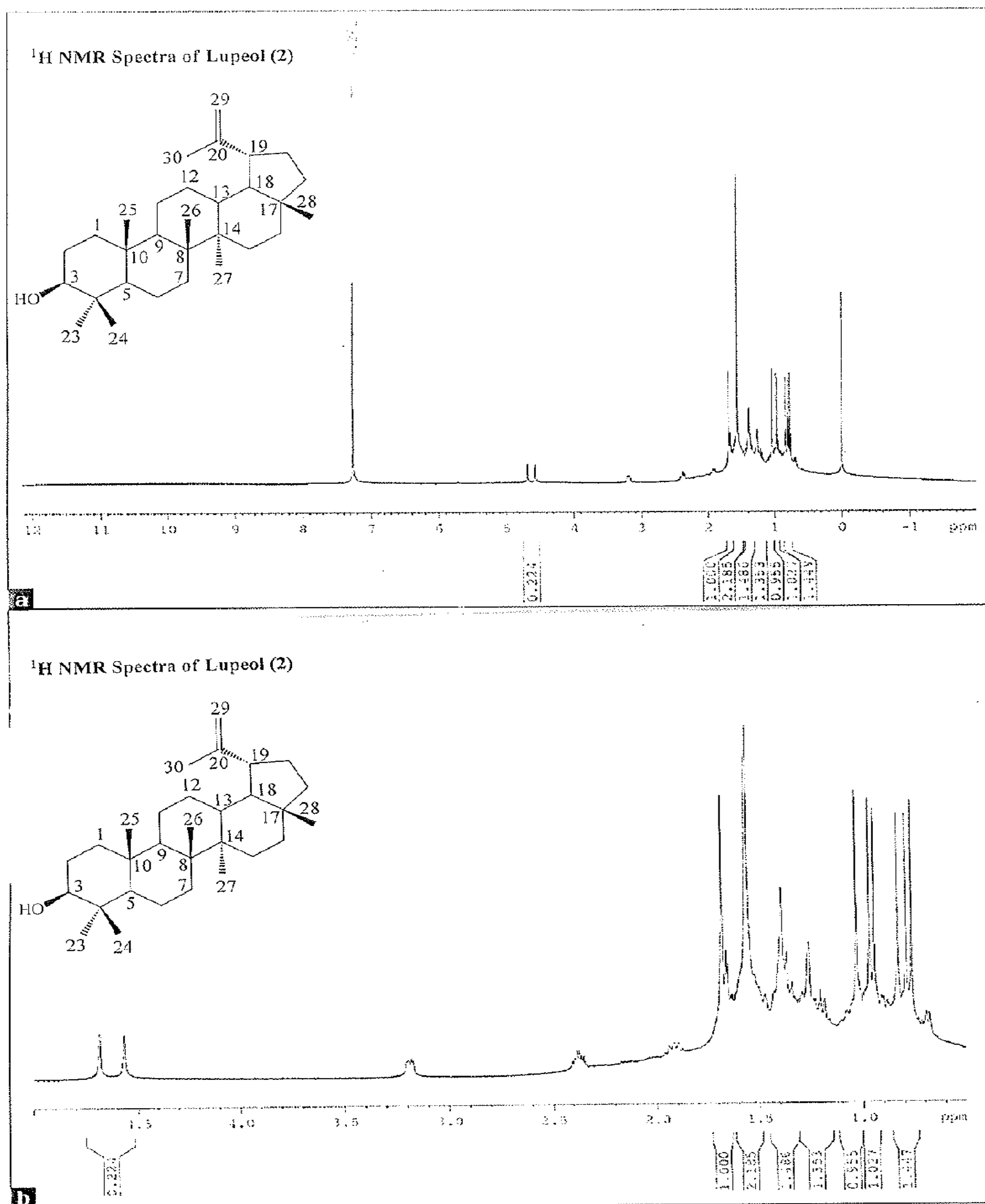


Figure-4
Key HMBC correlations of Betulinic acid (3)

Conclusion

Three lupane triterpenes were isolated from the commercial aqueous alcoholic extract of the rhizomes of *P. vulgare*. The structures of the three triterpene compounds were identified as lupenone (**1**), lupeol (**2**), and betulunic acid (**3**) on the basis of spectroscopic and by comparing their physical properties and spectral data reported in the literature.



Figures 5a and 5b
^{1}H NMR Spectra of Lupeol (2) in $CDCl_3$

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