Lupene-Type Triterpenes from *Periploca aphylla*

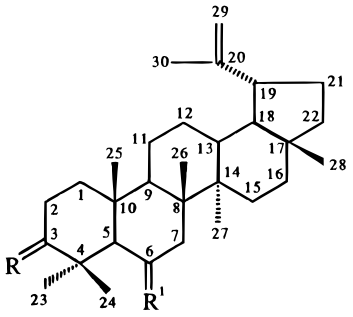
Ghulam Mustafa,[†] Erum Anis,[†] Saeed Ahmed,[†] Itrat Anis,[†] Habib Ahmed,[‡] Abdul Malik,*[,†] Syed Shahzad-ul-Hassan,[†] and M. Iqbal Choudhary[†]

International Center for Chemical Sciences, HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan, and Department of Botany, Government Degree College Matta, Swat, Pakistan

Received August 30, 1999

Two new lupane derivatives, 3β,6α-dihydroxylup-20(29)-ene (**1**) and 6α-hydroxylup-20(29)-en-3β-octadecanoate (**2**), have been isolated from the stems of *Periploca aphylla*, in addition to β-sitosterol and lupeol. The structures of **1** and **2** were determined by spectral and chemical methods. Compound **1** showed strong inhibition of α-glucosidase type VI and a moderate antibacterial activity.

The genus *Periploca* (Asclepiadaceae) consists of about 12 species, which are erect leafless shrubs, distributed in Europe, Asia, and tropical Africa. Phytochemical studies on various species of genus *Periploca* including *P. sepium*, *P. leavigatis*, and *P. gracea* have resulted in the isolation of various triterpenes, lignans, and flavonoids.[1−3] The plant *P. aphylla* Decne is widely distributed in the northern part of Pakistan and finds various medicinal uses in the indigenous system of medicine.[4] The milky juice is used as an external application to tumors and swellings and is said to be useful in cerebral fever and as a stomachic.[5] Earlier work on this plant by Mitsuhashi et al. has resulted in the isolation of several known steroids and triterpenes.[6] In the present investigation a methanolic extract of the stems of *P. aphylla* showed positive activity in the brine shrimp lethality test.[7] Further biological screening of the methanolic extract revealed strong antibacterial activity[8] against Gram-positive (*Bacillus cerus*, *Streptococcus pyogenes*) and Gram-negative (*Escherichia coli*, *Proteus mirabilis*, *Salmonella typhi*) bacteria. This prompted us to carry out bioassay-directed isolation studies on this plant. Herein, we report the isolation and structure elucidation of two new triterpenes of the lupane type, namely, 3β,6α-dihydroxylup-20(29)-ene (**1**) and 6α-hydroxylup-20(29)-en-3β-octadecanoate (**2**), along with the two known compounds β-sitosterol and lupeol.



1 R = αH, βOH; R^1 = βH, αOH
1a R = αH, βOAc; R^1 = βH, αOAc
1b R = O; R^1 = O
2 R = αH, $\beta$$CH_3$-$(CH_2)_{16}$-COO; R^1 = βH, αOH

Compound **1** was assigned the molecular formula $C_{30}H_{50}O_2$ by HRMS. It gave positive Liebermann-Burchard and $CeSO_4$ tests for a triterpene. The IR spectrum suggested that it contained hydroxyl groups (3400 cm^{-1}) and a terminal double bond (3045, 1650, and 888 cm^{-1}). The ^{1}H NMR spectrum revealed six methyl groups [δ_H 0.75, 0.80, 0.96, 0.97, 1.02, 1.21 (3H, s, each)], an isopropenyl group [δ_H 1.68 (3H, s), 4.57 and 4.67 (1H, d, J = 2.3 Hz)], and two oxymethine protons in proximity to hydroxyl groups [δ_H 3.16 (1H, dd, J = 10.8 and 5.46 Hz) and 3.81 (1H, dt, J = 10.5 and 4.8 Hz)]. The hydroxyl groups were shown to be secondary by their easy acetylation to the corresponding diacetate **1a** in which the signals at δ_H 3.16 and 3.81 were respectively shifted downfield to δ_H 4.45 and 5.51. The EIMS of **1** exhibited important peaks at *m*/*z* 442 [M^+], 427 [M − CH_3], 424 [M − H_2O], 409 [M − H_2O − CH_3], 406 [M − 2 H_2O], 236, 218, 205, 203, and 189. This fragmentation pattern supported the lup-20(29)-ene skeleton having both the hydroxyl groups in rings A and B.[9] The broad-band and DEPT ^{13}C NMR spectra showed the presence of seven methyl, 10 methylene, seven methine, and six quaternary carbon atoms, which provided further evidence for a lup-20(29)-ene skeleton for **1**. The positions of the two hydroxyl groups were determined through an HMBC experiment in which the protons of the geminal methyl groups (δ_H 1.02, 0.75) at C-4 showed ^2J correlations with C-4 (δ_C 38.8) and ^3J interactions with C-3 (δ_C 77.9) and C-5 (δ_C 55.4), confirming the position of one hydroxyl group at C-3. The position of the second hydroxyl was confirmed at C-6 since ^2J correlations of the oxymethine proton signal (δ_H 3.81) to C-5 (δ_C 55.4) and C-7 (δ_C 44.0) and ^3J interactions with C-4 (δ_C 38.8) and C-10 (δ_C 32.5) were observed in the HMBC spectrum. The magnitude of the coupling constants of the proton at C-6 allowed us to assign an α and equatorial configuration to this hydroxyl group, since when a hydroxyl group is β and axial, a $W_{1/2}$ value of J = 7.5 Hz would be expected.[10] Conclusive evidence for the structure was provided by oxidation of **1** to the diketo derivative **1b**, whose physical and spectral data were in complete agreement with those of lup-20(29)-ene-3,6-dione reported in the literature.[11] Compound **1** was therefore assigned the structure 3β,6α-dihydroxylup-20(29)-ene.

Compound **2** was assigned the molecular formula $C_{48}H_{84}O_3$ by HREIMS. It also gave positive Liebermann-Burchard and $CeSO_4$ tests for a triterpene. Analysis of the IR spectrum suggested the presence of a hydroxyl group (3385 cm^{-1}), a terminal double bond (3050, 1630, 888 cm^{-1}),

* To whom correspondence should be addressed. Tel.: (92-21) 4968733. Fax: (92-21) 4963373,4963124. E-mail: gm4u@cubexs.net.pk.
[†] HEJ Research Institute of Chemistry.
[‡] Government Degree College Matta.

10.1021/np990426v CCC: $19.00
Published on Web 05/11/2000

and an ester group (1735 cm^{-1}). The ^1H NMR spectrum exhibited signals for six methyl groups [δ_H 0.76, 0.81, 0.84, 0.89, 1.0, 1.30 (3H, s, each)], an isopropenyl group [δ_H 1.65 (3H, s), 4.56, 4.68 (1H, d, J = 2.3 Hz)], an oxymethine proton in proximity to an ester moiety [δ_H 4.48 (1H, dd, J = 5.2, 10.1 Hz)], and another oxymethine proton in proximity to a hydroxyl group [δ_H 3.95 (1H, dt, J = 5.0, 10.7 Hz)]. The ester function was also confirmed by the ^{13}C NMR spectrum of **2**, which showed an ester carbonyl carbon signal at δ_C 170.2. The ^{13}C NMR spectrum (broad-band and DEPT) of **2** corroborated the presence of eight methyl, 26 methylene, seven methine, and six quaternary carbons. These were assigned on the basis of comparison with **1** as well as from HMQC and HMBC experiments. The mass spectrum of **2** exhibited peaks at m/z 708 [M^+], 409 [$M - C_{18}H_{36}O_2 - CH_3$], 391 [$M - C_{18}H_{36}O_2 - CH_3 - H_2O$], 218, 205, 203, and 189. This fragmentation pattern suggested that compound **2** was also of the lup-20(29)-ene type, having an ester moiety and a hydroxyl group in rings A and B.

The alkaline hydrolysis of **2** in methanolic KOH yielded **2a** and methyl octadecanoate, with the latter confirmed by comparison with an authentic sample from its GC retention time. The spectral and physical data of **2a** were in complete conformity to those of compound **1**. The attachment of the octadecanoate moiety in **2** was determined through a comparison of the ^1H NMR spectrum of **2** with that of **1** and also through an HMBC experiment in which the protons of the geminal methyl groups (δ_H 1.02, 0.76) at C-4 showed ^2J correlations to C-4 (δ_c 38.5) and ^3J interactions with C-3 (δ_C 81.4) and C-5 (δ_C 56.0), confirming the position of the octadecanoate moiety at C-3. The oxymethine proton (δ_H 4.48) at C-3 also showed ^2J correlations to C-2 (δ_C 23.6) and C-4 (δ_C 38.5) and a ^3J correlation to the carbonyl carbon (δ_C 170.2) of the octadecanoate moiety in the HMBC spectrum. The hydroxyl group at C-6 was assigned an α and equatorial configuration for the same reasons as described earlier for compound **1**. On the basis of the above accumulated evidence the structure of **2** was established as 6α-hydroxylup-20(29)-en-3β-octadecanoate.

Enzyme inhibitory activity of **1** against α-glucosidase type VI[12−14] showed that it was a potent inhibitor, giving an IC_{50} value of 42.5 μM. The in vitro antibacterial activity of **1** was tested against Gram-positive (*B. cereus*, *Staphylococcus pyogenes*, and *Streptococcus pyogenes*) and Gram-negative (*E. coli*, *P. mirabilis*, and *S. typhi*) bacteria using an agar well diffusion method.[15] Amoxcillin and ampillicin were used as standard drugs for comparison. Compound **1** showed moderate activity against *S. pyogenes* (zone of inhibition 18 mm compared to 35 mm for the standard drugs at the same concentration level). Compound **2** was inactive in the assays used in this investigation.

Experimental Section

General Experimental Procedures. Melting points were determined in glass capillary tubes using a Buchi 535 melting point apparatus, and specific rotations were measured on a Schimdt and Haensch Polartronic D polarimeter. IR spectra were recorded on a JASCO A-302 spectrophotometer. ^1H and ^{13}C NMR spectra were performed on a Bruker AM-400 NMR spectrometer in $CDCl_3$ solution with tetramethylsilane (TMS) as internal standard. EIMS and HRMS were taken on Finnigan MAT-112 and Finnigan MAT-312 instruments, respectively. Si gel (Merck 254, 70−230 mesh) was used for column chromatography.

Plant Material. The dried plant material was collected from Swat (North West Frontier Province, Pakistan) and identified as *Periploca aphylla* by Mr. Habib Ahmed. A voucher specimen is deposited in the herbarium (accession no. 1997-1294) of the Department of Botany, Government Degree College Matta, Swat, Pakistan.

Extraction and Isolation. The dried plant material (28 kg) was extracted three times with MeOH at room temperature. The methanolic extract was evaporated in vacuo, yielding a brownish residue, which was suspended in H_2O and then extracted with *n*-hexane. The aqueous layer was further extracted with chloroform. The fractionation was controlled through a brine shrimp lethality test. The chloroform fraction, which showed potent cytotoxicity, was chromatographed over Si gel using mixtures of *n*-hexane and EtOAc of increasing polarity as eluants. The fraction obtained from *n*-hexane and EtOAc (19:1) showed strong cytotoxicity, and its further chromatography over Si gel using mixtures of *n*-hexane and EtOAc (9:1) as eluent yielded 6α-hydroxylup-20(29)-en-3β-octadecanoate (**2**, 65 mg) and lupeol (80 mg) from less polar fractions and 3β,6α-dihydroxylup-20(29)-ene (**1**, 55 mg) and β-sitosterol (65 mg) from polar fractions. The physical and spectral data of β-sitosterol and lupeol showed complete agreement with those reported in the literature.[16,17] Strong brine shrimp toxicity was observed for compound **1** compared to very weak activity of **2**.

3β,6α-Dihydroxylup-20(29)-ene (1): gummy solid; $[\alpha]^{20}_D$ +14° (c 0.4, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3400, 3045, 1650, 888 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 3.16 (1H, dd, J = 5.5, 10.8 Hz, H-3), 3.81 (1H, dt, J = 4.8, 10.5 Hz, H-6), 1.02 (3H, s, H-23), 0.75 (3H, s, H-24), 0.96 (3H, s, H-25), 1.21 (3H, s, H-26), 0.97 (3H, s, H-27), 0.80 (3H, s, H-28), 4.57 (1H, d, J = 2.2 Hz, H-29), 4.67 (1H, d, J = 2.3 Hz, H-29), 1.68 (3H, s, H-30); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 38.6 (t, C-1), 27.5 (t, C-2), 77.9 (d, C-3), 38.8 (s, C-4), 55.4 (d, C-5), 67.8 (d, C-6), 44.0 (t, C-7), 38.2 (s, C-8), 50.5 (d, C-9), 32.5 (s, C-10), 22.1 (t, C-11), 25.4 (t, C-12), 37.9 (d, C-13), 41.8 (s, C-14), 27.4 (t, C-15), 35.7 (t, C-16), 43.2 (s, C-17), 48.5 (d, C-18), 48.2 (d, C-19), 150.9 (s, C-20), 30.0 (t, C-21), 40.2 (t, C-22), 28.1 (q, C-23), 18.5 (q, C-24), 16.7 (q, C-25) 16.0 (q, C-26), 14.6 (q, C-27), 18.0 (q, C-28), 109.3 (t, C-29), 19.3 (q, C-30); EIMS (70 eV) m/z 442 $[M]^+$, 424 (21), 409 (3), 406 (18), 236 (5), 218 (18), 205 (35), 203 (10), 189 (55); HREIMS m/z 442.6404 (calcd for $C_{30}H_{50}O_2$, 442.64019).

Acetylation of 1. Compound **1** (20 mg) was dissolved in pyridine (1 mL) with Ac_2O (3 mL), and the mixture left overnight at room temperature. Ice was added to the reaction mixture, and it was extracted with EtOAc. The EtOAc layer was evaporated to yield the diacetate **1a**. Gummy solid: IR ($CHCl_3$) ν_{max} 1740, 1665, 1250, 880 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz) δ 4.45 (1H, m, H-3), 5.51 (1H, m, H-6), 0.90 (6H, s, H-23, 27), 1.32, 1.24, 1.02 (3H each, s, H-20, 25, 24), 1.78 (3H, s, H-28), 2.16 (6H, s, $COCH_3$-3,6); EIMS (70 eV) m/z 526 $[M]^+$ (9), 466 (8), 406 (6), 258 (15), 218 (100), 189 (15); HREIMS m/z 526.4827 (calcd for $C_{34}H_{54}O_4$ 526.4821).

Oxidation of 1. Chromium trioxide−pyridine complex (5 mL) and **1** (45 mg) were stirred at room temperature for 12 h. Dilution of the reaction mixture with cold water, extraction with diethyl ether, and successive washing with dilute hydrochloric acid (2 M) and water afforded white needles of lup-20(29)-ene-3,6-dione (**1b**) from chloroform−hexane: mp 170 °C; $[\alpha]^{20}_D$ −33° (c 1.5, $CHCl_3$). The physical and spectral data of **1b** showed complete agreement with the literature values.[11]

6α-Hydroxylup-20(29)-en-3β-octadecanoate (2): gummy solid; $[\alpha]^{20}_D$ +26° (c 0.4, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3385, 3050, 1735, 1630, 1380, 1230, 888 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 4.48 (1H, dd, J = 5.2, 10.1 Hz, H-3), 3.95 (1H, dt, J = 5.0, 10.7 Hz, H-6), 1.02 (3H, s, H-23), 0.76 (3H, s, H-24), 0.84 (3H, s, H-25), 1.30 (3H, s, H-26), 0.89 (3H, s, H-27), 0.81 (3H, s, H-28), 4.56 (1H, d, J = 2.2 Hz, H-29a), 4.68 (1H, d, J = 2.3 Hz, H-29b), 1.65 (3H, s, H-30), 2.35 (2H, t, J = 5.7 Hz, H-2′), 1.21−1.29 (30H, br s, H-3′-H-17′), 0.74 (3H, t, J = 3.5 Hz, H-18′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 38.5 (t, C-1), 23.6 (t, C-2), 81.4 (d, C-3), 38.5 (s, C-4), 56.0 (d, C-5), 68.2 (d, C-6), 43.9 (t, C-7), 38.1 (s, C-8), 50.3 (d, C-9), 31.9 (s, C-10), 21.9 (t, C-11), 25.2 (t, C-12), 36.9 (d, C-13), 41.7 (s, C-14), 27.4 (t, C-15), 35.6 (t, C-16), 43.0 (s, C-17), 48.3 (d, C-18), 48.0 (d, C-19), 150.7 (s, C-20), 29.9 (t, C-21), 40.0 (t, C-22), 28.1 (q, C-23), 18.2 (q, C-24), 16.6 (q, C-25), 16.0 (q, C-26), 14.5 (q, C-27), 18.2 (q, C-28), 109.3

(t, C-29), 19.3 (q, C-30), 170.2 (s, C-1′), 34.6 (t, C-2′), 27.1 (t, C-3′), 25.5 (t, C-4′), 29.4−29.9 (t, C-5′-C-16′), 22.7 (t, C-17′), 14.1 (q, C-18′); EIMS (70 eV) *m*/*z* 708 $[M]^+$ (20), 409 (80), 391 (15), 218 (18), 205 (30), 203 (100), 189 (55); HREIMS *m*/*z* 708.6410 (calcd for $C_{30}H_{50}O_2$, 708.6419).

Hydrolysis of 2. Compound **2** (25 mg) was refluxed with 5% methanolic KOH for 3 h. The reaction mixture was extracted with $CHCl_3$. The $CHCl_3$ solution, after washing with H_2O and drying over anhydrous Na_2SO_4, was evaporated and passed over a Si gel column using *n*-hexanes−EtOAc (9:1) as eluent to afford **2b** (13 mg) and methyl octadecanoate (8 mg). The latter was identified by comparison with an authentic sample of methyl octadecanoate (Aldrich Chemical Co., St Louis, MO), by its GC retention time, while the spectral data of **2b** were completely comparable with those of **1**.

Enzyme Inhibitory Activity. Enzyme inhibitory activity against α-glucosidase type VI (Sigma G6136) was observed spectrophotometrically at pH 6.8 and at 37 °C using 0.7 mM *p*-nitrophenyl α-D-glucopyranoside (PNP-G) as a substrate and 0.017 units/mL enzyme, in 50 mM sodium phosphate buffer containing 100 mM NaCl, with 0.3 mM 1-deoxynojirimycin used as positive control (IC_{50} = 0.3 mM).[13] The absorption increase at 400 nm due to the hydrolysis of PNP-G by α-glucosidase was studied continuously with a spectrophotometer (Molecular Devices, Sunnyvale, CA).[14]

Antibacterial Activity. Antibacterial activity of **1** and **2** was determined by the disk diffusion method[15] using Nutrient Agar medium. For this purpose, sterile disks 6 mm in diameter of filter paper were prepared. From a stock solution of 10 mg/mL, 10 μL, i.e., 100 μg of the sample was applied to each disk. The disks were air-dried and then placed on a lawn of the test organism. The plates were incubated at 37 °C for 24 h, and then zones of inhibition were observed around each disk, measured, and recorded.

References and Notes

(1) Zorina, L. G.; Matyukhino, L. G.; Pyabinin; A. A. *Khim. Prir. Soedin.* **1996**, *2*, 291−295.
(2) Barrera, J. B.; Funes, J. B.; Fuente, G. D.; Gonzales, G. A. *An. R. Soc. Espan. Fis. Quim., Ser. B* **1966**, *82*, 859−864.
(3) Askri, M.; Bui, A. M.; Mighri, Z. *J. Soc. Chim. Tunis.* **1982**, *8*, 23−28.
(4) Shastri, B. N. *Wealth of India*; Sree Saraowaty: New Dehli, 1966; p 313.
(5) Baquar, S. R. *Medicinal and Poisonous Plants of Pakistan*; Printas Press: Karachi, 1989.
(6) Mitsuhashi, H.; Tomitomo, K. *Shoyakugaku Zasshi* **1971**, *25*, 7−10.
(7) Meyer, B. N.; Ferrigni, J. E.; Jacobsen, L. B.; Nicholas, P. E.; McLaughlin, J. L. *Planta Med.* **1982**, *45*, 31−34.
(8) Rahman, A.-u.; Choudhary, M. I.; Thomson, W. J. *Manual of Bioassay Techniques for Natural Product Research*; Harwood Academic Press: Karachi, 1999; p 82.
(9) Budzikiewicz, H.; Welson, J. M.; Djerassi, C. *J. Am. Chem. Soc.* **1963**, *85*, 3688−3699.
(10) Kuroyanagi, M.; Shidotsu, M.; Ebihara, T.; Kawai, H.; Ueno, A.; Fukushima, S. *Chem. Pharm. Bull.* **1986**, *34*, 4012−4017.
(11) Dantanarayana, A. P.; Kumar, N. S.; Sultanbawa, M. U. S. *J. Chem. Soc., Perkin Trans. 1* **1981**, 2717−2723.
(12) Bischoff, H. *Eur. J. Clin. Invest.* **1994**, *24*, 3−10.
(13) Asano, N.; Oseki, K.; Keneko, E.; Matsui, K. *Carbohydr. Res.* **1994**, *258*, 255−266.
(14) Matsui, T.; Yoshimoro, C.; Osajima, K.; Oki, T.; Osajima, Y. *Biosci. Biotech. Biochem.* **1996**, *60*, 2019−2022.
(15) Baur, A. W.; Kirby, W. J.; Sherris, M. T. *Am. J. Clin. Pathol.* **1996**, *45*, 493−496.
(16) Holland, H. L.; Diakow, P. R. P.; Taylor, G. J. *Can. J. Chem.* **1978**, *56*, 3121−3127.
(17) Ageta, H.; Ageta, T. *Chem. Pharm. Bull.* **1984**, *32*, 369−376.

NP990426V