Ecdysteroids of *Vitex scabra* Stem Bark

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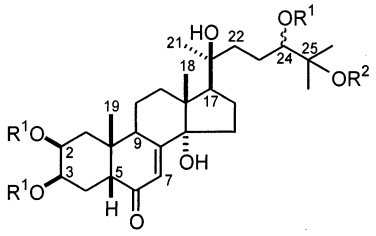
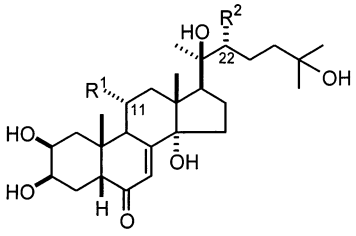
Two new ecdysteroids, 24-*epi*-pinnatasterone (**1**) and scabrasterone (**2**), together with 11 known ecdysteroids, calonysterone, pterosterone, 24-*epi*-makisterone A, 20-hydroxyecdysone (**3**), polypodine B, ajugasterone C, pinnatasterone (**4**), 11α-hydroxyecdysone, 24-*epi*-abutasterone, 20,26-dihydroxyecdysone, and turkesterone (**5**), were isolated from the stem bark of *Vitex scabra*. This plant species contained a very high concentration (1.8%) of **3** and thus provided a good source of this parent ecdysteroid and related rare ecdysteroids. Compounds **1** and **2** exhibited very low moulting activity in the *Musca* bioassay. The low biological activity of these two ecdysteroids was in agreement with those of other 22-deoxyecdysteroids.

A number of *Vitex* species have been investigated for ecdysteroids.[1−7] *Vitex scabra* Wall. ex Schauer (Verbenaceae)[8] is a moderate-sized tree endemic to the northeastern part of Thailand. No phytochemical work on this plant species has been reported to date. In this note, the isolation of two new ecdysteroids, 24-*epi*-pinnatasterone (**1**) and scabrasterone (**2**), together with 11 known ecdysteroids, is reported.

The stem bark of *V. scabra* was subjected to extraction and fractionations to yield **1** and **2** and 11 known ecdysteroids. The known compounds included calonysterone,[2] pterosterone,[6] 24-*epi*-makisterone A,[9] 20-hydroxyecdysone (**3**),[3] polypodine B,[10] ajugasterone C,[11] pinnatasterone (**4**),[1] 11α-hydroxyecdysone,[12] 24-*epi*-abutasterone,[4] 20,26-dihydroxyecdysone,[5] and turkesterone (**5**),[7,13] identified by comparison of their spectroscopic data with those of reported compounds.



1 $R^1 = R^2 = H$
4 C-24 epimer of **1**
6 R^1 = Ac; R^2 = H
7 $R^1 = R^2$ = Ac



2 R^1 = OH; R^2 = H
3 R^1 = H; R^2 = OH
5 $R^1 = R^2$ = OH

Compound **1** was obtained as a minor ecdysteroid. This compound co-chromatographed with **4** on normal-phase TLC. However, these two ecdysteroids were separated by reversed-phase HPLC. The molecular formula of **1** was established as $C_{27}H_{44}O_7$ by HRFABMS (negative ion mode, *m*/*z* 479.3008 $[M - H]^-$). IR absorption bands at 3422 and 1648 cm^{-1} indicated the presence of hydroxyl and α,β-unsaturated keto groups. The color reaction to anisaldehyde reagent as well as the ^1H NMR characteristics indicated that the isolate was an ecdysteroid. The ^1H NMR spectral features and the splitting patterns of the key resonances H-2, H-3, H-5, H-7, H-9, H-17, 18-Me, and 19-Me implied that this compound possessed the same nucleus as that of **4**. Assignments of the ^1H and ^{13}C NMR resonances were achieved by 1D and 2D techniques and comparison with the data of related compounds. Visual inspection of the ^1H NMR spectra of **1** and **4** revealed that they were nearly indistinguishable. To make an accurate comparison of the ^1H NMR spectra and to achieve precise assignments of chemical shift values of these two ecdysteroids, the ^1H NMR spectrum of a 3:1 mixture of **1** and **4** was recorded. Significant differences in chemical shift values were noted for H-17 and H-24 (0.05 and 0.02 ppm, respectively, see Experimental Section). The chemical shift difference in H-24 between the two compounds was consistent with a difference in the stereochemistry at C-24. It should be noted that a chemical shift difference of H-17 between the two epimers results from a difference in the chemical environment of the side chain, examples of which were those of abutasterone and 24-*epi*-abutasterone,[14] and pterosterone and 24-*epi*-pterosterone.[6] A different chemical environment around C-24 also caused slight shifts (0.01 ppm) for the resonances of 18-Me, 26-Me, and 27-Me. A change in the C-24 stereochemistry also affected the ^{13}C chemical shifts of the side chain. Thus, notable differences in the C-20 to C-24 resonances between **1** and **4** were observed (see Table 1).

Acetylation of **1** furnished the triacetate **6** and the tetraacetate **7**, the H-24 resonances of which were at δ 4.80 and 5.14, respectively. A large downfield shift (0.34 ppm) of the H-24 resonance on going from the triacetate **6** to the tetraacetate **7** indicated the close proximity of H-24 of **7** to the 25-acetoxyl group. The secondary side-chain hydroxyl group should therefore be placed at the 24-position. However, the existing spectroscopic data did not permit assignment of the absolute configuration at C-24 of the ecdysteroid **1** or that of the ecdysteroid **4**.

Compound **2** gave a molecular formula of $C_{27}H_{44}O_7$ (HRFABMS), and the IR spectrum showed the presence of hydroxyl and α,β-unsaturated keto groups at 3422 and

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Table 1. ^{13}C NMR Data of Compounds **1**−**5** (in C_5D_5N)

C	1	2	3	4	5
1	37.7	39.6	38.0	37.6	39.7
2	68.0	68.3	68.3	68.0	68.3
3	67.9	68.0	68.2	67.9	68.0
4	32.3	32.8	32.5	32.3	32.8
5	51.3	52.7	51.4	51.2	52.4
6	203.6	204.1	203.5	203.5	204.0
7	121.6	122.0	121.7	121.5	122.1
8	166.3	164.5	166.1	166.3	164.2
9	34.2	42.6	34.6	34.2	42.5
10	38.5	39.4	38.8	38.8	39.4
11	20.9	68.8	21.2	20.9	68.7
12	31.7	43.8	32.1	31.7	44.0
13	47.4	47.5	48.2	47.4	48.1
14	84.3	84.4	84.4	84.3	84.1
15	31.4	31.5	31.8	31.4	31.8
16	21.8	19.5	21.6	21.9	21.4
17	53.5	53.2	50.2	53.6	49.9
18	17.8	18.7	17.9	17.8	18.8
19	24.2	24.7	24.5	24.3	24.8
20	74.4	74.2	77.0	74.2	76.7
21	26.8	26.7	21.7	27.0	21.5
22	42.6	45.4	77.7	42.3	77.4
23	26.8	21.8	27.5	26.6	27.4
24	79.9	45.6	42.6	79.8	42.6
25	72.7	69.6	69.8	72.7	69.5
26	25.9	29.6	30.1	25.9	29.9
27	25.7	29.8	30.1	25.8	30.0

1654 cm^{-1}, respectively. The ^1H NMR features of **2** were indicative of an ecdysteroid; however, a number of ^1H NMR signals revealed some significant differences in both the splitting patterns and chemical shift values as compared with those of 20-hydroxyecdysone (**3**).[3] The H-9 resonance was shifted from δ 3.55 in **3** to δ 3.84 in **2**, and the multiplicity was changed from a multiplet to a double doublet (resulting from coupling of H-9 with H-11 and H-7), indicating the presence of an 11α-hydroxyl group.[7] This was also evident from a large (8 ppm) downfield shift of the C-9 resonance in the ^{13}C NMR data. Further evidence was the presence of the C-11 carbinol proton and the carbon at δ 4.56 and 68.8, respectively. Large downfield shifts of H-12ax (δ 2.99), H-12eq (δ 2.65), H-2 (δ 4.56), and H-1 (δ 3.41) further supported the finding. Downfield shifts were also observed for the C-1 and C-12 resonances in the ^{13}C NMR spectrum of **2** (see Table 1). The existence of an 11α-hydroxyl function was further confirmed by a relatively large (0.29 ppm) downfield shift of ^1H NMR data of the 19-Me resonance[7] as compared with that of compound **3**.[3] The ^1H NMR data of **2** were in fact very similar to those of **5**.[7] The only significant difference was the absence of the C-22 carbinol resonance in both the ^1H and ^{13}C NMR spectra of **2**. Finally, HMBC correlations of H-9 (δ 3.84) to C-11 (δ 68.8) and of H-12eq (δ 2.65) to C-11 also confirmed the presence of the 11-hydroxyl group. Scabrasterone (**2**) was thus concluded to be the 11α-hydroxy analogue of taxisterone.[15] It should be noted that *V. scabra* contained a very high concentration of **3** (25.1 g or 1.8% from 1.4 kg of the dry stem bark).

Compounds **1** and **2** were subjected to in vivo *Musca* assay for moulting hormone activity,[16] and it was found that these two compounds exhibited very low activity (EC_{50} were 5.2×10^{-4} and 1.0×10^{-3} M, respectively) based on the activity of the ecdysteroid **3** (1.6×10^{-5} M). The low biological activity of these two ecdysteroids was in agreement with those lacking a 22*R*-hydroxyl group, including the ecdysteroid **4** (EC_{50} was 6.9×10^{-4} M).[1,16,17]

Experimental Section

General Experimental Procedures. Melting points were determined on an Electrothermal apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer FT-IR Spectrum BX spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 and a JEOL JNM-A500 spectrometer operating at 400 and 100, and 500 and 125 MHz, respectively. The chemical shifts (δ) are reported in ppm, and coupling constants (J) are given in Hz. For the spectra taken in C_5D_5N and $CDCl_3$, the residual nondeuterated solvent signals at δ 8.71 and 7.24 and the solvent signals at δ 149.90 and 77.00 were used as references for ^1H and ^{13}C NMR spectra, respectively. FABMS and ESMS were measured with a Finnigan MAT 90 and a Bruker Esquire-LC mass spectrometer, respectively. Unless indicated otherwise, Merck Si gel 60 (finer than 0.063 mm) was used for column chromatography. TLC was conducted on plates precoated with Merck Si gel 60 F_{254}. The eluting solvent system for column chromatography used throughout the experiments was $CHCl_3$−MeOH, with increasing percentage of the more polar solvent. Reversed-phase column chromatography was conducted using Merck Si gel 60 RP-18 (40−63 μm). Spots on TLC were visualized under UV light and by spraying with anisaldehyde−H_2SO_4 reagent followed by heating. Reversed-phase HPLC was performed on a Spherisorb S10ODS2 column (5 μm, 250 × 10 mm) with MeOH−H_2O as a mobile phase at a flow rate of 2 mL min^{-1}, using a UV detector at 254 nm.

Plant Material. *V. scabra* stem bark was collected from Muong Samsib, Ubon Ratchathani Province, Thailand. A voucher specimen of this plant, No. 0030 (RU), is deposited at the Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand.

Extraction and Isolation. The dried stem bark (1.4 kg) was pulverized and extracted with MeOH in a Soxhlet extraction apparatus. The filtered solution was concentrated under reduced pressure to a volume of ca. 250 mL, and H_2O (1.8 L) was added. The solution was partitioned successively with $CHCl_3$ (500 mL × 3) and *n*-BuOH (500 mL × 5). The solvents were evaporated to give $CHCl_3$ (1.7 g) and BuOH (98.7 g) extracts.

The BuOH extract was chromatographed (Merck Si gel, 0.063−0.200 mm); the eluates were examined by TLC, and 17 fractions were obtained. Fraction 3 was subjected to column chromatography to yield calonysterone (5 mg), the ^1H NMR data of which were identical to those reported previously.[2] Fraction 4 was similarly chromatographed to give pterosterone[6] (5 mg) and 24-*epi*-makisterone A[9] (13 mg). Spectroscopic (^1H NMR and mass spectral) data were consistent with the literature values.

Fractions 6 and 7 were combined and crystallized from MeOH−EtOAc to afford 20-hydroxyecdysone (**3**, 15.1 g), mp 240−241 °C (lit. 240−242 °C).[13] TLC comparison with an authentic sample and ^1H NMR spectral comparison with reported values[3] revealed the identity of this compound. The filtrate was combined with fraction 8 and chromatographed to give, after crystallization from MeOH−EtOAc, additional **3** (8.1 g). Another two crops of **3** (1.4 and 0.5 g) were obtained from the filtrate. After removal of compound **3**, the filtrate was evaporated and the residue subjected to repeated column chromatograghy, and two subfractions were selected for further column chromatography followed by reversed-phase column chromatography, eluted with H_2O and H_2O−MeOH with increasing MeOH content, to afford polypodine B (2 mg) and ajugasterone C (5 mg), the ^1H NMR data of which were consistent with the literature values.[18]

Fraction 9, after two repeated column chromatographies, gave nine subfractions. Subfraction 4 was subjected to reversed-phase HPLC separation, using MeOH−H_2O (40:60). The collected effluent with t_R of 27.97 min yielded 24-*epi*-pinnatasterone (**1**, 3 mg), and that with t_R of 32.72 min yielded pinnatasterone (**4**, 2 mg). The latter ecdysteroid was identical to compound **4** isolated previously[1] by direct reversed-phase HPLC comparison and by ^1H NMR spectral comparison. The ^1H NMR spectrum of a 3:1 mixture of **1** and **4** was also recorded. Subfraction 5 was similarly purified by reversed-phase HPLC (MeOH−H_2O, 40:60, t_R = 23.37 min) to give 11α-

hydroxyecdysone (1 mg). The ^{1}H and ^{13}C NMR spectral data were consistent with those reported previously.[12]

Fraction 10 was subjected to three repeated column chromatographies to yield seven subfractions. Subfractions 2 and 3 were purified by reversed-phase HPLC in the same manner described above to give, respectively, 24-*epi*-abutasterone (2 mg, MeOH−H_2O, 40:60, t_R = 17.53 min) and scabrasterone (**2**, 2 mg, MeOH−H_2O, 30:70, t_R = 18.76 min). The former was identical (IR, ^{1}H NMR, and mass spectral data) to that obtained from *V. canescens* root bark.[4] Fraction 11 was chromatographed twice, followed by reversed-phase column chromatography, and the second subfraction, eluted by H_2O−MeOH (60:40), yielded 20,26-dihydroxyecdysone (3 mg). The first subfraction was further chromatographed to give turkesterone (**5**, 9 mg). The two compounds were identified by direct TLC comparison with authentic compounds and ^{1}H NMR and, in the case of compound **5**, ^{13}C NMR spectral comparisons with those of authentic samples.[5,7,13]

Compound 1: IR (KBr) ν_{max} 3422, 2929, 1648, 1384, 1057 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz)[a] δ 1.00 (3H, s, H_3-19), 1.08 (3H, s, H_3-18), 1.42 (3H, s, H_3-26), 1.46 (3H, s, H_3-27), 1.55 (3H, s, H_3-21), 2.90 (1H, t, J = 9.3 Hz, H-17), 2.96 (1H, dd, J = 13.1, 3.6 Hz, H-5), 3.56 (1H, m, H-9), 3.76 (1H, br d, J = 8.8 Hz, H-24), 4.15 (1H, m, H-2), 4.23 (1H, br s, H-3), 6.19 (1H, d, J = 2.1 Hz, H-7), [a]chemical shift values were assigned from the spectrum of a 3:1 mixture of **1** and **4**, whereas coupling constants were calculated from those of individual compounds; ^{13}C NMR, see Table 1; HRFABMS (negative ion mode) m/z 479.3001 [M − H]$^-$ (calcd for $C_{27}H_{43}O_7$, 479.3008).

Compound 2: IR (KBr) ν_{max} 3422, 2925, 1654, 1384 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) δ 1.18 (3H, s, H_3-18), 1.29 (3H, s, H_3-19), 1.33 (2 × 3H, s, H_3-26, H_3-27), 1.53 (3H, s, H_3-21), 2.65 (1H, dd, J = 11.9, 5.8 Hz, H-12eq), 2.93 (1H, t, J = 9 Hz, H-17), 2.99 (1H, m[a], H-12ax), 3.00 (1H, dd, J = 13.1, 3.6 Hz, H-5), 3.41 (1H, dd, J = 12.8, 4.2 Hz, H-1), 3.84 (1H, dd, J = 8.5, 2.1 Hz, H-9), 4.21 (1H, m, $W_{1/2}$ = 7 Hz, H-3), 4.56 (2H, m[a], H-2 and H-11), 6.26 (1H, d, J = 2.1 Hz, H-7), [a]obscured signal; ^{13}C NMR, see Table 1; HMBC correlations: H-1 (C-2), H-7 (C-5, C-9, C-14), H-9 (C-8, C-11), H-12eq (C-11, C-13, C-14), 18-Me (C-12, C-13, C-14, C-17), 19-Me (C-1, C-10, C-5), 21-Me (C-17, C-20, C-22), 26-Me (C-24, C-25, C-27), 27-Me (C-24, C-25, C-26); ESMS (positive ion mode) m/z 503 [M + Na]$^+$(100); HRFABMS (negative ion mode) m/z 479.3011 [M − H]$^-$ (calcd for $C_{27}H_{43}O_7$, 479.3008).

Compound 4: ^{1}H NMR (C_5D_5N, 400 MHz)[a] δ 1.00 (3H, s, H_3-19), 1.09 (3H, s, H_3-18), 1.43 (3H, s, H_3-26), 1.47 (3H, s, H_3-27), 1.55 (3H, s, H_3-21), 2.85 (1H, t, J = 9 Hz, H-17), 2.96 (1H, dd, J = 13, 4 Hz, H-5), 3.56 (1H, m, H-9), 3.74 (1H, br d, J = 8.5 Hz, H-24), 4.15 (1H, m, H-2), 4.23 (1H, br s, H-3), 6.19 (1H, d, J = 2.5 Hz, H-7), [a]chemical shift values were assigned from the spectrum of a 3:1 mixture of **1** and **4**, whereas coupling constants were calculated from those of individual compounds; ^{13}C NMR, see Table 1.

Acetylation of Compound 1. Compound **1** (1 mg) was dissolved in dry pyridine (1 mL), Ac_2O (1 mL) was added, and the mixture was stirred at ambient temperature for 36 h. H_2O (30 mL) was added and the mixture extracted with $CHCl_3$ (15 mL × 3). The combined organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to dryness. The crude products were separated by column chromatography, with $CHCl_3$−MeOH (85.5:1.5) as eluting solvent, to give the acetates **6** (0.2 mg) and **7** (0.6 mg).

Compound 6: IR (KBr) ν_{max} 3444, 2924, 2853, 1740, 1662, 1463, 1372, 1247, 1045, 1027 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.821 (3H, s, H_3-18), 1.01 (3H, s, H_3-19), 1.20 (2 × 3H, s, H_3-26, H_3-27), 1.26 (3H, s, H_3-21), 1.99, 2.09, 2.10 (each 3H, each s, AcO), 2.25 (1H, t, J = 9.4 Hz, H-17), 2.38 (1H, dd, J = 13.2, 3.8 Hz, H-5), 3.09 (1H, m, H-9), 4.80 (1H, dd, J = 10.2, 2.3 Hz, H-24), 5.06 (1H, m, H-2), 5.35 (1H, br s, H-3), 5.86 (1H, d, J = 2.1 Hz, H-7); HRFABMS (negative ion mode) m/z 605.3324 [M − H]$^-$ (calcd for $C_{33}H_{49}O_{10}$, 605.3325).

Compound 7: IR (KBr) ν_{max} 3359, 2922, 2851, 1724, 1658, 1632, 1467, 1423, 1266, 1135, 1112 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.819 (3H, s, H_3-18), 1.01 (3H, s, H_3-19), 1.26 (3H, s, H_3-21), 1.43 (3H, s, H_3-26), 1.47 (3H, s, H_3-27), 1.95, 1.99, 2.087, 2.09 (each 3H, each s, AcO), 2.25 (1H, t, J = 9.3 Hz, H-17), 2.38 (1H, dd, J = 13.2, 4 Hz, H-5), 3.09 (1H, m, H-9), 5.14 (1H, dd, J = 10.2, 2.1 Hz, H-24), 5.06 (1H, m, H-2), 5.35 (1H, br s, H-3), 5.86 (1H, d, J = 2.1 Hz, H-7); HRFABMS (negative ion mode) m/z 647.3433 [M − H]$^-$ (calcd for $C_{35}H_{51}O_{11}$, 647.3431).

Biological Assay. Compounds **1** and **2** were subjected to the *Musca* bioassay as described previously.[16] Both ecdysteroids exhibited very low moulting activity, based on the activity of ecdysteroid **3**.

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