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Highly oxygenated ecdysteroids from *Vitex canescens* root bark

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Abstract

Highly oxygenated ecdysteroids, (24*R*)-11α,20,24-trihydroxyecdysone and 11α,20,26-trihydroxyecdysone, have been isolated from the polar fraction of *Vitex canescens* root bark. The latter exists as two C-25 epimers which could be separated by reversed-phase HPLC.

Keywords: *Vitex canescens*; Verbenaceae; Root bark; Ecdysteroids; (24*R*)-11α,20,24-Trihydroxyecdysone; 11α,20,26-Trihydroxyecdysone

1. Introduction

We previously reported the isolation of a number of ecdysteroids from a complex mixture isolated from *Vitex canescens* root bark. These included 20-hydroxyecdysone, 24-*epi*-makisterone A, shidasterone, calonysterone, turkesterone (**1**), 24-*epi*-abutasterone (**2**) (Suksamrarn, Promrangsan, Chitkul, Homvisasevongsa & Sirikate, 1997) and 20,26-dihydroxyecdysone (Suksamrarn, Yingyongnarongkul & Promrangsan, 1998). We now report the isolation and characterization of three more polar ecdysteroids from this plant species.

2. Results and discussion

Compound **3** eluted before the two epimers of **4** on reversed-phase HPLC and its high resolution FAB-mass spectrum established a molecular formula of $C_{27}H_{44}O_9$. The ^{1}H-NMR spectral data (Table 1) of this compound were similar to those of turkesterone (**1**) (Usmanov, Gorovitz & Abubakirov, 1976; Werawattanametin, Podimuang & Suksamrarn, 1986). The ^{1}H-NMR spectral features and relative positions of H-1 to H-17 resonances, as well as those of 18-Me and 19-Me (Table 1) indicated that these two ecdysteroids possess the same nucleus. The absence of a signal around δ 3.8 in the spectrum of compound **3**, as compared to that of compound **1**, suggested that H-22 has moved downfield. As compound **3** has an additional hydroxyl group compared to that of compound **1**, this last hydroxyl function should be placed on the side-chain and it should be located at the 24-position, since the splitting patterns (two broad doublet signals) at δ 4.50 and 4.35 fitted well with H-22 and H-24 signals (or vice versa). Based on the assumption that the stereochemistries at C-20 and C-22 of compounds **3** and **1** are the same, the configuration of the C-24 hydroxyl group could either be 24*R* (i.e., compound **3**) or 24*S*. By comparison of the chemical shift values and spectral features of H-22 and H-24 of compound **3** with those of 24-*epi*-abutasterone (**2**) (Table 1), it was evident that they were almost identical. The chemical shift values of these two protons were different from those of abutasterone (**5**), the C-24 epimeric ecdysteroid of **2**, which resonated at δ 4.08 and 4.24 (or vice versa) (Suksamrarn et al., 1998). It is noteworthy that the chemical shifts of 21-Me of compounds **3** and **2** were very similar, indicating that the stereochemical arrangements of the C-20 and C-22 hydroxyl groups are the same.

The structure of **3** was confirmed by ^{13}C-NMR spec-

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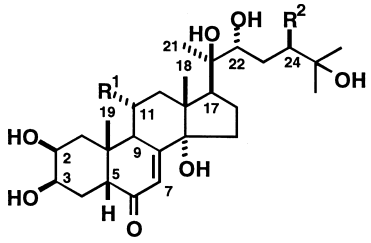

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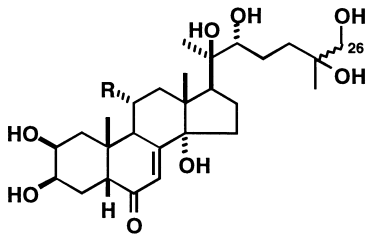
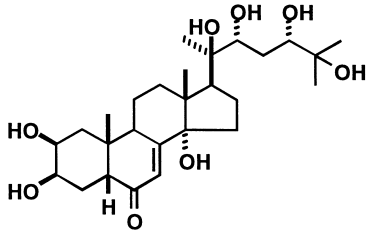
2 R^1 = H; R^2 = OH

3 R^1 = R^2 = OH



4 R = OH

6 R = H



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tral comparisons with those of compounds **1** (Werawattanametin et al., 1986) and **2** (Suksamrarn et al., 1997) (Table 2). It was evident that the ^{13}C-NMR chemical shifts of C-1 to C-21 of **3** and **1** were almost identical. However, the chemical shift values of C-22 to C-27 of these two ecdysteroids were significantly different (see Table 2). In contrast, the corresponding signals of compounds **3** and **2** were very similar. It was thus concluded that compound **3** is (24*R*)-11α,20,24-trihydroxyecdysone.

The second ecdysteroid, compound **4**, has the same molecular formula as compound **3** from the HR-FAB-MS. The ^{1}H-NMR spectral data of H-1 to H-17, as well as 18-Me and 19-Me of this ecdysteroid and **1** were almost identical, thus suggesting presence of the ecdysteroid nuclei for both compounds. However, the ^{1}H-NMR spectral data of H-22 to H-27 of **4** differed from those of compounds **1** and **3**, but were similar to those of 20,26-dihydroxyecdysone (**6**) (Suksamrarn et al., 1998) (Table 1), especially those related to H-22, H-26 and H-27. The structure of this ecdysteroid was thus proposed to be **4**, which was confirmed by analysis of the ^{13}C-NMR spectral data (Table 2). The ^{13}C-NMR chemical shifts of C-1 to C-19 of **4** were almost identical to those of compound **1**. The C-20, C-21 and C-22 chemical shifts of **4** were also almost the same as those of **1**, indicating that the spatial arrangements of the hydroxyl groups at C-20 and C-22 are the same. The C-23 to C-27 chemical shifts agreed very well with the side-chain structure of compound **4**. The structure

Table 1
^{1}H-NMR spectral data of compounds **1**–**4** and **6**[a]

H	1[b]	2[c]	3	4	4	6[c]	6[c]
				'Epimer 1'	'Epimer 2'	'Epimer 1'	'Epimer 2'
1	3.40		3.43 (*dd*, 12.5, 3.8)	3.43 (*dd*, 12.4, 3.5)	3.44 (*dd*, 12.5, 3.5)		
2	4.56 (*m*)[d]	4.18 (*m*)	4.57 (*m*)[f]	4.58 (*m*)[f]	4.58 (*m*)[d]	4.19 (*m*)	4.18 (*m*)
3	4.19 (*br s*)	4.23 (*br s*)	4.20 (*br s*)	4.20 (*br s*)	4.21 (*br s*)	4.23 (*br s*)	4.23 (*br s*)
5	3.00[f]	2.99 (*dd*, (12.9, 3.5)	3.00 (*dd*, 12.7, 3.2)	3.00[f]	3.00[f]	3.01[f]	3.01[f]
7	6.26 (*d, ca* 2)	6.22 (*d*, 2.1)	6.25 (*d, ca* 2)	6.28 (*d, ca* 2)	6.28 (*br s*)	6.25 (*d*, 2.4)	6.25 (*d*, 2.4)
9	3.83(*m*)[f]	3.57 (*m*)	3.84 (*br d*, 7)	3.85–3.89[f]	3.85–3.89[f]	3.58 (*m*)	3.58 (*m*)
11	4.56 (*m*)[d]		4.60 (*m*)[f]	4.58 (*m*)[f]	4.58 (*m*)[d]		
12ax	3.00[f]		3.04[e,f]	3.02[f]	3.02[f]		
17	3.06	3.08 (*t*, 9.1)	3.16 (*t*, 9.1)[e]	3.09 (*t*, 9.1)	3.09 (*t*, 8.9)	3.03[f]	3.08[f]
22	3.83 (*m*)[f]	4.51 (*dd*, 9.6, 2.3)[g]	4.50 (*br d*, 8.6)[e]	3.85–3.89[f]	3.85–3.89[f]	3.92 (*br d*, 10.3)	3.90 (*dd*, 10.6, 1.8)
24		4.37(*dd*, 9, 2.7)[g]	4.35 (*br d*, 8.8)[e]				
26	–	–	–	3.85–3.89[f]	3.85–3.89[f]	3.87[f] and 3.88[f]	3.86[f]
18-Me	1.23 (*s*)[e]	1.22 (*s*)	1.26 (*s*)	1.24 (*s*)	1.24 (*s*)	1.204 (*s*)	1.208 (*s*)
19-Me	1.28 (*s*)[e]	1.06 (*s*)	1.29 (*s*)	1.30 (*s*)	1.30 (*s*)	1.06 (*s*)	1.06 (*s*)
21-Me	1.55 (*s*)	1.63 (*s*)	1.61 (*s*)	1.56 (*s*)	1.55 (*s*)	1.58 (*s*)	1.57 (*s*)
26-Me	1.33 (*s*)	1.46 (*s*)	1.45 (*s*)	–	–	–	–
27-Me	1.33 (*s*)	1.47 (*s*)	1.45 (*s*)	1.45 (*s*)	1.44 (*s*)	1.47 (*s*)	1.46 (*s*)

[a] All spectra were recorded in pyridine-d_5.
[b] Taken from supplementary data of Werawattanametin et al. (1986).
[c] Data taken from Suksamrarn et al. (1997, 1998), respectively.
[d] Two superimposed signals.
[e] Assignments may be reversed for signals with the same superscript.
[f] Partially overlapping signals.
[g] Assignments may be reversed for signals with the same superscript.

of this ecdysteroid was therefore 11α,20,26-trihydroxyecdysone.

The third ecdysteroid possessed the same elemental composition as that of compound **3** as determined from the the HR-FAB-mass spectrum. The ^{1}H-NMR spectrum of this compound (Table 1) was almost indistinguishable from that of compound **4**. The ^{13}C-NMR spectra of both compounds (Table 2) were almost identical; the only significant differences involved C-26 and C-27, suggesting that **3** and **4** were C-25 epimeric ecdysteroids. The occurrence of two C-25 epimers in compound **6** has recently been detected in a number of *Vitex* species including *V. canescens* and two C-25 epimers of **6** have been synthesized (Suksamrarn et al., 1998). The structure of this ecdysteroid was thus concluded to be **4**, but with a different C-25 configuration from that of the second new ecdysteroid. The existing spectroscopic data does not permit the assignment of absolute configuration at C-25 of both epimers of compound **4**. This compound was designated as 11α,20,26-trihydroxyecdysone 'epimer 2', and the above-mentioned second new compound as 11α,20,26-trihydroxyecdysone 'epimer 1'.

It should be noted that introduction of an 11α-hydroxyl group to an ecdysteroid molecule caused downfield shifts to many signals in the ^{1}H-NMR spectrum. Significant and diagnostic shifts were observed by the presence of a carbinol proton signal around δ 4.56–4.60 and downfield shifts of H-9 and H-12ax of ca 0.26 and 0.46 ppm, respectively (Girault & Lafont, 1988). Further downfield shifts of the remote protons were also observed: ca 1.3 and 0.39–0.40 ppm shifts for H-1eq and H-2ax, and a significant downfield shift (0.23–0.24 ppm) was observed for the 19-Me signal. It should also be noted that the presence of an 11α-hydroxyl group caused a 1.6–1.7 ppm downfield shift of the C-1 resonance, in addition to the expected downfield shift of the C-11 resonance, in the ^{13}C-NMR spectrum. Significant downfield shifts were also observed for C-9 and C-12 resonances (ca 8 and 12 ppm, respectively) in the ^{13}C-NMR spectrum as compared to those of compounds **2** and **6**.

Table 2
^{13}C-NMR spectral data of compounds **1**–**4** and **6**[a]

C	**1**[b]	**2**[b]	**3**	**4**	**4**	**6**[b]	**6**[b]
				'Epimer 1'	'Epimer 2'	'Epimer 1'	'Epimer 2'
1	39.63	37.97	39.64	39.65	39.64	37.95[c]	37.95[c]
2	68.25[c]	68.12[c]	68.19	68.20	68.20	68.05[d]	68.05[d]
3	68.04[c]	68.05[c]	67.94	67.94	67.94	68.12[d]	68.14[d]
4	32.69	32.39	32.61	32.64	32.66	32.41[e]	32.44[e]
5	52.27	51.37	52.25	52.26	52.26	51.37	51.39
6	204.01	203.43	203.73	203.74	203.76	203.48	203.50
7	122.19	121.62	122.00	122.02	122.01	121.62	121.62
8	164.27	166.12	164.11	164.07	164.09	166.10	166.12
9	42.40	34.40	42.54	42.53	42.53	34.40	34.45
10	39.35	38.66	39.31	39.30	39.30	38.62	38.64
11	68.73	21.10	68.63	68.62	68.62	21.40	21.45
12	43.96	31.71	43.96	43.93	43.93	31.68[e]	31.70[e]
13	48.06	48.12	48.00	47.96	47.96	48.08	48.08
14	84.16	84.23	84.06	84.01	84.01	84.18	84.18
15	31.72	31.99	31.64	31.65	31.65	31.96[e]	31.98[e]
16	21.41	21.32	21.18	21.40	21.41	21.66	21.70
17	49.88	50.04	49.73	49.78	49.78	50.07	50.09
18	18.74	17.91	18.72	18.68	18.68	17.35	17.86
19	24.66	24.44	24.65	24.65	24.65	24.52[f]	24.76[f]
20	76.76	76.87	76.62	76.62	76.61	76.87	76.87
21	21.41	21.76	21.47	21.29	21.32	21.09	21.09
22	77.44	73.76[d]	73.53[c]	77.39	77.43	77.65	77.68
23	27.28	35.20	35.00	26.57	26.51	26.76	26.70
24	42.59	75.94[d]	75.73[c]	37.35	37.31	37.55[e]	37.52[c]
25	69.58	72.63	72.40	72.41	72.37	72.66	72.60
26	29.84	26.14	25.85	70.63	70.44	70.79	70.62
27	29.84	26.09	25.92	24.32	24.52	24.41[f]	24.43[f]

[a] All spectra were recorded in pyridine-d_5.
[b] Data taken from Werawattanametin et al. (1986), Suksamrarn et al. (1997, 1998), respectively.
[c–f] Assignments may be reversed for signals with the same superscript.

3. Experimental

3.1. General

^{1}H-NMR and ^{13}C-NMR spectra were recorded at 400 and 100 MHz, respectively. Unless indicated otherwise, Merck silica gel 60 (finer than 230 mesh) was used for CC. TLC was conducted on plates precoated with Merck silica gel 60 F_{254}. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

3.2. Extraction and isolation

Pulverized, dry root bark (4 kg) of *V. canescens*, obtained from the same source as described previously (Suksamrarn et al., 1997), was extracted successively with *n*-hexane and EtOH in a Soxhlet extraction apparatus. The concd. EtOH extract was diluted with H_2O and the filtered soln. extracted successively with $CHCl_3$ and EtOAc, using a continuous liquid–liquid extraction apparatus and *n*-BuOH, using separatory funnels. The BuOH extract (19.64 g) was chromatographed (Merck silica gel, 70–230 mesh) using CH_2Cl_2–MeOH as eluent, with increasing MeOH content, and 6 frs. were obtained. The 5th fr. (3.77 g), eluted by CH_2Cl_2–MeOH (60:40), was subjected to CC with the same adsorbent and eluting solvent system. Fr. eluted by CH_2Cl_2–MeOH (83:17 to 75:25) (580 mg) gave positive colouration of ecdysteroids with anisaldehyde reagent on TLC and was subjected to reversed-phase HPLC separation [column: Spherisorb S10ODS2, 5 μm, 250 × 10 mm; mobile phase: MeOH–H_2O (30:70); flow rate: 2.0 ml min^{-1}; detector: 254 nm]. This resulted in the separation of turkesterone (**1**), previously isolated from the EtOAc extract (Suksamrarn et al., 1997), from a number of more polar ecdysteroids which were only partially separated from each other. The combined polar fr. was subjected to HPLC separation [column: μBondapak C18, 10 μm, 300 × 3.9 mm; mobile phase: MeOH–H_2O (18:82); flow rate: 1.0 ml min^{-1}; detector: 254 nm] and three frs., with R_t of 26.0, 34.4 and 40.8 min, respectively, were collected. TLC examinations indicated that each of the frs. contained 3–5% of slightly less polar components.

Normal-phase HPLC purification of each of the above frs. [column: Hypersil Si, 5 μm, 250 × 4.6 mm; mobile phase: $CHCl_3$–MeOH (90:10); flow rate: 1.2 ml min^{-1}; detector: 254 nm] afforded, respectively, compounds **3** (R_t 13.0 min, 5 mg), compound **4** 'epimer 1' (R_t 16.4 min, 2 mg) and compound **4** 'epimer 2' (R_t 16.4 min, 3.5 mg).

3.3. (24*R*)-11α,20,24-Trihydroxyecdysone (*3*)

IR: ν_{max}^{KBr} cm^{-1}: 3408, 2967, 1654, 1384, 1052. ^{1}H and ^{13}C-NMR spectral data are given in Tables 1 and 2, respectively. HR-FABMS (negative ion mode): *m*/*z* 511.2912 [M-H]$^-$ (calc. for $C_{27}H_{44}O_9$-H: 511.2907).

3.4. 11α,20,26-Trihydroxyecdysone 'epimer 1' (*4*)

IR: ν_{max}^{KBr} cm^{-1}: 3448, 2952, 1660, 1385, 1053. ^{1}H and ^{13}C-NMR spectral data are given in Tables 1 and 2, respectively. HR-FABMS (negative ion mode): *m*/*z* 511.2906 [M-H]$^-$ (calc. for $C_{27}H_{44}O_9$-H: 511.2907).

3.5. 11α,20,26-Trihydroxyecdysone 'epimer 2' (*4*)

IR: ν_{max}^{KBr} cm^{-1}: 3448, 2927, 1660, 1385, 1053. ^{1}H and ^{13}C-NMR spectral data are given in Tables 1 and 2, respectively. HR-FABMS (negative ion mode): *m*/*z* 511.2908 [M-H]$^-$ (calc. for $C_{27}H_{44}O_9$-H: 511.2907).

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