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ECDYSTEROIDS FROM *VITEX CANESCENS*

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Key Word Index—*Vitex canescens*; Verbenaceae; ecdysteroids; canescensterone; pinnatasterone 24-*O*-(pyrrole 2-carboxylate); 20-hydroxyecdysone; turkesterone.

Abstract—In addition to 20-hydroxyecdysone and turkesterone, a new ecdysteroid, canescensterone, was isolated from the bark of *Vitex canescens*. The new compound was shown to be pinnatasterone 24-*O*-(pyrrole 2-carboxylate).

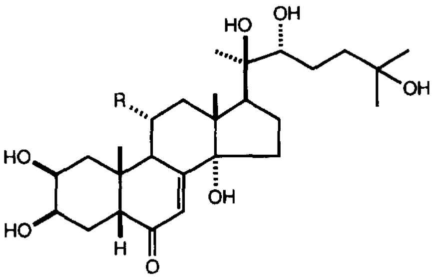
INTRODUCTION

Vitex canescens Kurz is a medium-sized tree scattered throughout the countryside of Thailand. A number of *Vitex* species have been investigated for ecdysteroids and the results have been summarized elsewhere [1, 2]. Among the *Vitex* species reported to be endemic to Thailand, only two of them, *V. pinnata* and *V. glabrata*, were reported to contain 20-hydroxyecdysone (**1**) and turkesterone (**2**) [1, 3]. The former plant species also contained pinnatasterone (**3**), an ecdysteroid lacking a C-22 hydroxyl group [1]. In this communication we wish to report the isolation of **1** and **2**, and a new ecdysteroid canescensterone (**4**), from the bark of *V. canescens*.

RESULTS AND DISCUSSION

Ecdysteroids **1**, **2** and **4** were isolated from the ethanol extract of bark of *V. canescens*. Compounds **1** and **2** were identified as 20-hydroxyecdysone and turkesterone, respectively, by TLC comparison with authentic samples and agreement of their IR and ^1H NMR data with those reported in the literature for these two ecdysteroids [3–6].

The new ecdysteroid, canescensterone (**4**), was obtained crystalline, mp 152–153°, $[\alpha]_D^{26}$ + 18.8°. The elemental analysis and ES-mass spectrum established a molecular formula of $C_{32}H_{47}O_8N$. The ^1H and ^{13}C NMR spectral features of **4** exhibited an ecdysteroid-type of spectra. The ^1H NMR spectrum of **4** was similar to those of **1** and **3** (Table 1), except for the absence of a signal at δ3.84 and 3.77 (corresponding to H-22 and H-24 in **1** and **3**, respectively). Instead, a signal, the splitting pattern of which was similar to the H-22 of an ecdysteroid with C-20 and C-22 hydroxyl groups, or the H-24 of **3**, appeared at δ5.58. Such a large downfield shift (1.74 or 1.81 ppm) suggested that the extra C_5H_4ON moiety should be



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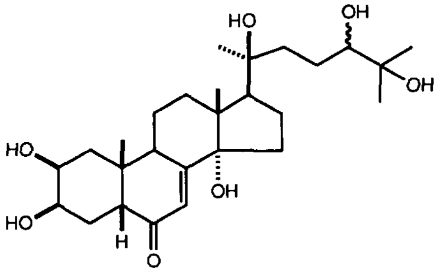
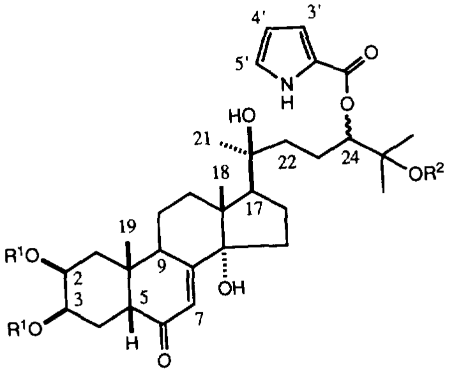
2 R = OH



3



4 $R^1 = R^2 = H$

5 R^1 = Ac, R^2 = H

6 $R^1 = R^2$ = Ac

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Table 1. ^{1}H NMR data of **1**, **3**–**6**

H	**1***	**3***	**4***	**5**†	**6**†
2	4.15 (*m*)	4.19 (*m*)	4.15 (*br d*, 8.6)	5.07 (*ddd*, 12.2, 5, 4)	5.07 (*br d*, 12.1)
3	4.20 (*br s*)	4.26 (*br s*)	4.23 (*br s*)	5.36 (*br s*)	5.33 (*br s*)
5	2.97 (*m*)‡	3.04 (*dd*, 13, 4)	3.00 (*dd*, 13.4, 3.8)	2.39 (*dd*, 13.1, 4)	2.36 (*dd*, 13, 3.6)
7	6.22 (*d*, 2.1)	6.26 (*d*, 2.5)	6.19 (*d*, 2)	5.85 (*d*, 2)	5.87 (*d*, 2)
9	3.55 (*m*)	3.61 (*m*)	3.53 (*m*)	3.08 (*m*)	3.13 (*m*)
17	2.96 (*m*)‡	2.93 (*t*, 9)	2.90 (*t*, 9.4)	2.30 (*t*, 9.1)	2.31 (*t*, 9.1)
22	3.84 (*br d*, 8.9)				
24		3.77 (*br d*, 8.5)	5.58 (*br d*, 8.9)	4.93 (*dd*, 9.2, 3.2)	5.33 (*m*)§
18-Me	1.18 (s)	1.17 (*s*)	1.04 (s)	0.79 (s)	0.79 (*s*)
19-Me	1.02 (s)	1.09 (*s*)	1.02 (*s*)	1.01 (*s*)	1.01 (*s*)
21-Me	1.56 (s)	1.61 (*s*)	1.50 (s)	1.25 (s)	1.27 (*s*)
26-Me	1.34 (*s*)	1.47 (*s*)	1.42 (*s*)	1.27 (*s*)	1.49 (*s*)
27-Me	1.34 (*s*)	1.51 (*s*)	1.44 (*s*)	1.27 (*s*)	1.55 (*s*)
1′			13.30 (*br s*)	9.44 (*br s*)	9.42 (*br s*)
3′			7.28 (*m*)¶	6.96 (*m*)	6.92 (*m*)
4′			6.40 (*m*)¶	6.29 (*m*)	6.28 (*m*)
5′			7.28 (*m*)¶	6.98 (*m*)	7.00 (*m*)
AcO				2.02, 2.11 (each *s*)	1.95, 2.00, 2.10 (each *s*)

*Recorded in pyridine-d_5.

†Recorded in $CDCl_3$.

‡Partially superimposed signal.

§Partially obscured by H-3 signal.

¶After D_2O exchange the splitting patterns of these signals were simplified and the coupling constants were determined as follows: 7.20 (*dd*, 3.63, 1.51), 6.30 (*dd*, 3.63, 2.50), 7.23 (*dd*, 2.50, 1.51).

located at the same position as this proton. The presence of a conjugated ester function at 1683 cm^{-1} in the IR spectrum suggested that this extra group should be connected to the C-22 or C-24 hydroxyl group through an ester linkage.

The splitting patterns and the coupling constants of the aromatic protons of the D_2O-exchanged ^{1}H NMR spectrum of **4** indicated the presence of a 2-carboxyl-substituted pyrrole ring. Thus, the signal at δ7.20 (*dd*, $J = 3.63$, 1.51 Hz), 6.30 (*dd*, $J = 3.63$, 2.50 Hz) and 7.23 (*dd*, $J = 2.50$, 1.51 Hz) accounted for the H-3′, H-4′ and H-5′, respectively. The presence of the COC_4H_4N moiety was further confirmed by the EI-mass spectral peak at *m/z* 94.

The EI-mass spectral peak at *m/z* 363 corresponded to fragmentation between C-20 and C-22 to give an ion bearing an ecdysteroid ring system, and C-20 and C-21 as part of the side chain. Loss of one, two and three molecules of water from the above fragment ions gave rise to the ions at *m/z* 345, 327 and 309, respectively. These mass spectral data, together with ^{1}H NMR data of the ecdysteroid nucleus of **4** as compared to those of **1** and **3**, indicated that the nuclei of these ecdysteroids were identical. Furthermore, the presence of ions at *m/z* 211 and 210, the side-chain ions arising from fission between C-20 and C-22, also confirmed that the pyrrole 2-carboxylate moiety was not attached to the C_{21} unit of the molecule of **4**.

The evidence that C-24 was the point of attachment of the pyrrole 2-carboxylate moiety was the presence of the fragment ions at *m/z* 183 and 182, arising from fission between C-23 and C-24. Comparison of the ^{13}C NMR data (Table 2) of **4** with those of **1** and **3** suggested that the oxygen function was located at C-24 rather than C-22. Acetylation of **4** afforded the diacetate **5** and triacetate **6**. The ^{1}H NMR spectra of these two acetate derivatives were very similar. The differences were, apart from the number of acetyl groups, downfield shifts of the C-26 and C-27 methyl resonances and, more importantly, a relatively large downfield shift (0.4 ppm) of the H-24 signal of **6**, as compared to those of **5**. The acetylation shift in the latter case indicated the proximity of the C-24 carbinol proton to the C-25 acetoxyl group.

Conclusive evidence for the structure was obtained from a hydrolysis study of **4**. In order to hydrolyse the ester group in **4**, a methanolic soln of **4** was treated with aqueous sodium hydroxide. The result, however, was disappointing; a rather complex mixture of products was obtained. This finding was not surprising, since it was observed that **1** in basic solution suffered from a number of reactions, including base-catalysed autoxidation [7]. We also found that potassium carbonate and some other bases did not give promising results. However, we eventually found that guanidine [8] could hydrolyse **4** and the product was identical (TLC, IR and ^{1}H NMR) to **3** [1]. This evidence not only confirmed the position of the hydroxyl group to be at C-24, but also suggested that the configuration at C-24 of **3** and **4** should be the same. Canescensterone (**4**) was thus concluded to be pinnatasterone 24-*O*-(pyrrole 2-carboxylate). It should be noted that this is the first report of an ecdysteroid with a pyrrole

Table 2. ^{13}C NMR data of **1**, **3** and **4**

C	1	3	4
1	38.0	37.6	37.9
2	68.3	68.0^a	68.1^e
3	68.2	67.9^a	68.0^e
4	32.5	32.3	32.4
5	51.4	51.2	51.3
6	203.5	203.5	203.5
7	121.7	121.5	121.5
8	166.1	166.3	166.1
9	34.6	34.2	34.3
10	38.8	38.8	38.5
11	21.2	20.9^b	20.9^f
12	32.1	31.7^c	31.7^g
13	48.2	47.4	47.3
14	84.4	84.3	84.3
15	31.8	31.4^c	31.4^g
16	21.6	21.9^b	21.9^f
17	50.2	53.6	53.0
18	17.9	17.8	17.8
19	24.5	24.3	24.3
20	77.0	74.2^d	74.0^h
21	21.7	27.0	25.6
22	77.7	42.3	41.7
23	27.5	26.6	25.0
24	42.6	79.8	80.6
25	69.8	72.7^d	71.7^h
26	30.1	25.9	27.3
27	30.2	25.8	26.9
2′			123.9
3′			115.9^i
4′			110.2^i
5′			124.1
COO			161.9

All compounds were recorded in pyridine-d_5.

[a–i] Assignments may be reversed for signals with the same superscript.

ring in the molecule [9, 10]. This ecdysteroid exhibited high moulting hormone activity in the *Musca domestica* bioassay, based on the activity of **1** [11–13]. The result was in contrast to the low activity of pinnatasterone (**3**) [1]. The high moulting hormone activity of **4** might be due to the presence of the pyrrole 2-carboxylate moiety at the 24-position.

EXPERIMENTAL

General. Mps: uncorr. ^{1}H and ^{13}C NMR spectra were recorded at 300 and 75.5 MHz, respectively. Unless indicated otherwise, Merck silica gel 60 (> 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.

Plant material. The bark of *V. canescens* was collected in Pipoon District, Nakhon Si Thammarat Province. A voucher specimen of this plant (BKF No. 96888) is deposited at the Forest Herbarium, Royal Forest Department, Ministry of Agriculture and Cooperatives, Bangkok.

Extraction and isolation. Pulverized, dry bark (1.80 kg) was extracted successively with *n*-hexane and EtOH in a Soxhlet extraction apparatus. The concd EtOH extract was subjected to continuous liquid–liquid extraction using EtOAc. The EtOAc extract (32 g) was chromatographed (Merck silica gel 60, 70–230 mesh) using $CHCl_3$–MeOH as eluent, with increasing MeOH content, and 3 main frs were selected by TLC examination of the eluate. The first fr., eluted by $CHCl_3$–MeOH (19:6) was repeatedly chromatographed using EtOAc–MeOH (49:1 to 24:1) as eluting solvent followed by crystallization in MeOH– EtOAc to yield canescensterone (**4**, 23 mg), mp 152–153°. Found: C, 62.81; H, 8.25; N, 2.17; $C_{32}H_{47}O_8N \cdot 2H_2O$ requires: C, 63.05; H, 8.37; N, 2.29% $[\alpha]_D^{26}$ + 18.8° (MeOH; *c* 0.09). UV λ_{max}^{MeOH} nm (log ε): 240sh (4.10), 259 (4.18). IR ν_{max}^{KBr} cm^{-1}: 3412 (OH), 1683 (conj. ester C=O), 1643 (conj. ketone C=O). ESMS *m/z* (rel. int.): 597 $[M + H + Na]^{+\cdot}$ (8), 596 $[M + Na]^+$ (25). EIMS *m/z* (rel. int.): 444 $[M + H - COC_4H_4N - 2H_2O]^{+\cdot}$ (2), 426 (2), 411 (3), 393 (2), 363 (1), 345 (3), 327 (5), 309 (1), 291 (1), 211 (6), 210 (6), 209 (5), 193 (4), 192 (1), 191 (4), 183 (4), 182 (1), 143 (81), 125 (100), 111 (88), 109 (15), 107 (26), 94 (72), 93 (82), 67 (21), 66 (19), 65 (23). ^{1}H and ^{13}C NMR data: Tables 1 and 2, respectively.

The second fr., eluted by $CHCl_3$–MeOH (18:7 to 7:3), was chromatographed and crystallized from MeOH–EtOAc to give 20-hydroxyecdysone (**1**, 1.77 g), mp 240–242° (lit. mp 240–242° [3]). TLC comparison with authentic sample [3], and IR and ^{1}H NMR data were consistent with those reported for **1** [3, 4].

The third fr., eluted by $CHCl_3$–MeOH (17:8), was rechromatographed to yield crude **2** (303 mg). A portion of the crude **2** was purified by CC to afford pure turkesterone (**2**) as an amorphous solid (lit. amorphous solid [6]). TLC comparison with authentic sample [3], and IR and ^{1}H NMR data were consistent with those reported for **2** [3, 6].

Acetylation of canescensterone (**4**). Ac_2O (0.4 ml) was added to a soln of **4** (7 mg) in pyridine (1 ml) and the mixt. stirred at 30–34° for 2 days. More Ac_2O (0.2 ml) was added and stirring continued for another 3 days. The reaction mixt. was cooled in an ice bath and H_2O (10 ml) was added. The mixt. was extracted with EtOAc (4 × 10 ml); the combined organic phase was washed with H_2O, dried over anhydrous Na_2SO_4 and evapd *in vacuo*. The crude products were subjected to CC using $CHCl_3$–MeOH (49:1) as eluent to give the acetates **5** (3 mg) and **6** (3 mg).

Canescensterone 2,3-diacetate (**5**). UV λ_{max}^{MeOH} nm (log ε): 240sh (4.23), 259 (4.30). IR ν_{max}^{KBr} cm^{-1}: 3428 (OH), 1739 (acetate C=O), 1656 (conj. ketone C=O). ^{1}H NMR data: Table 1.

Canescensterone 2,3,25-triacetate (**6**). UV λ_{max}^{MeOH} nm (log ε): 240sh (4.20), 259 (4.28). IR ν_{max}^{KBr} cm^{-1}: 3468 (OH), 1740 (acetate C=O), 1658 (conj. ketone C=O). ^{1}H NMR data: Table 1.

Hydrolysis of canescensterone (**4**). A 0.5 ml soln consisting of 1:1 mixt. of 0.15 M guanidine hydrochloride and 0.15 M $KHCO_3$ was added to **4** (3.5 mg) in MeOH (0.1 ml), and the mixt. kept stirring at 60–62° for 4 days. H_2O (5 ml) was added and the mixt. extracted with *n*-BuOH (2 × 10 ml). The organic phase was washed with H_2O and the solvent evapd to dryness. The crude product was subjected to prep. TLC with $CHCl_3$–MeOH (5:1) as the mobile phase (3 developments) to afford pinnatasterone (**3**, 1 mg) and recovered **4** (1.5 mg). TLC comparison of **3** with authentic sample, and IR and ^1H NMR data were consistent with those reported for **3** [1].

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