Ecdysteroids as Insect Control Agents: A New Ecdysteroid from Stem Bark of *Vitex canescens*

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Abstract

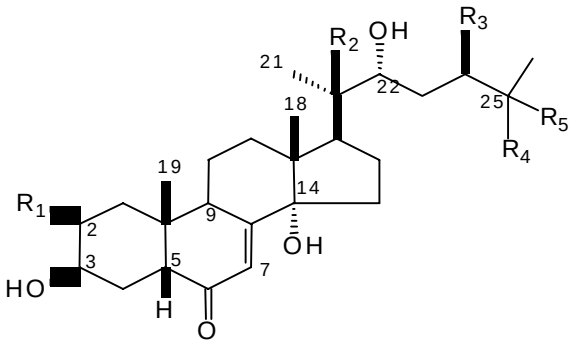
A new ecdysteroid, P5, has been isolated from the stem bark of aged trees (with flower) of *vitex canescens*. 20-Hydroxyecdysone, makisterone A, 24-epi-makisterone A, and canescensterone have also been isolated from both young trees (without flower) and aged trees (with flower) of *vitex canescens.* The methods of extraction of the ecdysteroids from *vitex canescens* are given and the melting points, infrared absorption spectra, nuclear magnetic resonance spectra and mass spectra of the ecdysteroids are reported.

Keywords : ecdysteroids, *vitex canescens.*

1. Introduction

Ecdysteroids represent a widespread family of steroids found in both animal (zooecdysteroids) and plant (phytoecdysteroids) kingdoms [1-2]. Zooecdysteroids were first detected in insects [3], then in *Crustacea* and other arthropods, and, more recently, in *Mollusca* [4], various worms [5] and even in *Coelenterata* [6]. The chemical structures of nine types of ecdysteroids are shown in Figure 1 labelled (**1**) **–** (**9**). Ecdysone, which was first isolated from silkworms, *Bombyx mori*, was shown by X-ray diffraction [7] and by synthesis to be a compound of structure (**1**) [8], and it was demonstrated later to be a secondary product of moulting glands [9-10]. Since then, an additional six zooecdysteroids have been isolated: 20-hydroxyecdysone (structure **2**), 20, 26-dihydroxyecdysone (**3**), 26-hydroxyecdysone (4), 2-deoxy-20-hydroxyecdysone (**5**), inokosterone (**6**) and makisterone C (**7**).

Ecdysteroids were initially defined as moulting hormones. However, this definition appears nowadays



(**1**) R_1 = OH, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = OH
(**2**) R_1 = OH, R_2 = OH, R_3 = H, R_4 = CH_3, R_5 = OH
(**3**) R_1 = OH, R_2 = OH, R_3 = H, R_4 = CH_2OH, R_5 = OH
(**4**) R_1 = OH, R_2 = H, R_3 = H, R_4 = CH_2OH, R_5 = OH
(**5**) R_1 = H, R_2 = OH, R_3 = H, R_4 = CH_3, R_5 = OH
(**6**) R_1 = OH, R_2 = OH, R_3 = H, R_4 = CH_2OH, R_5 = H
(**7**) R_1 = OH, R_2 = OH, R_3 = CH_2CH_3, R_4 = CH_3, R_5 = OH
(**8**) R_1 = H, R_2 = H, R_3 = H, R_4 = CH_3, R_5 = OH
(**9**) R_1 = OH, R_2 = OH, R_3 = H, R_4 = CH_3, R_5 = H

Figure 1 The Chemical Structure of Nine Types of Ecdysteroids

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to be too restricted, since these steroids are present at all stages of insect development: in newly laid eggs [11], during embryonic and postembryonic development, and in adult insects [12]. It has also been reported that isolated abdomens of several species [13] are able to synthesize ecdysteroids from cholesterol. In the ovaries of *Locusta migratoria* and other insects practically all of the ecdysteroids produced are in conjugated form, with approximately two-thirds being 2-deoxyecdysone (**8**) and the remaining one-third being further hydroxylated into ecdysone (**1**). In contrast, the prothoracic glands secrete only ecdysone (**1**), which is released into the blood [12]. Organs such as the fat body or mulphigian tubules then convert the majority of the ecdysteroid molecules by a further hydroxylation into 20-hydroxyecdysone (**2**).

The fortuitous discovery in 1966 of large amounts of ponasterone A (**9**) in the bark of *Podocarpus nakaii* [16] was the starting point for highly fruitful research which has led to the discovery of more than 100 ecdysteroids [15,17] in plants. Some of these ecdysteroids are identical with insect hormones, while others have only been detected in the plant kingdom.

In plants, ecdysteroids are widely distributed as secondary metabolites, and they often reach concentrations several orders of magnitude greater than in insects [2, 14-15]. For example, the concentrations of phytoecdysteroids in plants may comprise between 10^{-3} % and 10^{-1}% dry weight of the plants, whereas the concentrations of zooecdysteroids in insects are usually only 10^{-5} – 10^{-9}%. However, in some plants, much higher concentrations of ecdysteroids have been found: the flowers of *Serratula inermis* contain up to 2% of 20-hydroxyecdysone (**2**) [18], the roots of the Chinese Herb *Cyanotis arachnoidae* have yielded 2.9% of 20-hydroxyecdysone (**2**) [19], and the mature stem of *Disploclisia glaucesens* has yielded 3.2% of 20-hydroxyecdysone (**2**) [20]. Ecdysteroid concentrations vary with the plant part, the season, and habitat of the plant [21-22].

In general, the occurrence of phytoecdysteroids is more likely in perennial woody and herbaceous plants than in annual herbaceous species. From an examination of the available results of plant screenings [2], it appears that among the vascular plants ecdysteroid activity has been found in 27 families of the *Pteridophyta*, 10 families of the *Fymnospermae,* and 74 families of the *Angiospermae.* In ferns the probability of finding ecdysteroids is high. The probability of finding active plant species in *Angiospermae* is typically much lower than in *Pteridophyta* or *Gymnospermae.* Among *angiosperm* families the *Verbenaceae, Labiatae (Ajuga), Asteraceae (Serratuea), Amaranthaceae (Achyranthes*, *Cyathula), Ranuculaceae (Helleborus),* and *Caryophyllaceae* are more likely sources than others.

In *Verbenaceae,* one of the best sources of ecdysteroids is *Vitex,* a genus of shrubs and small trees found in all the warmer parts of the world [23]. From 1967 onwards, a number of new ecdysteroids have been isolated from *Vitex* species, and some of these new compounds have been found to be highly active in biological systems [24-28]. The chemical structures of some of these new ecdysteroids are shown below in Figure 2. The new ecdysteroids include makisterone A (structure **10**) [27], 24-epi-makisterone A (**11**) [28], canescensterone (**12**) [27], and shidasterone (**13**) [28].

2. Experimental Details

2.1 General

Prior to use all solvents were purified according to standard procedure. Silica gel 60 F_{254} precoated plate (E.Merck) was used for thin layer chromatography (TLC). Compounds containing unsaturated bonds, especially those with conjugated systems, become visible as quenching spots under ultraviolet (UV) light at 254 nm. Anisaldehyde reagent (0.5% ethanol solution of anisaldehyde with 5% sulfuric

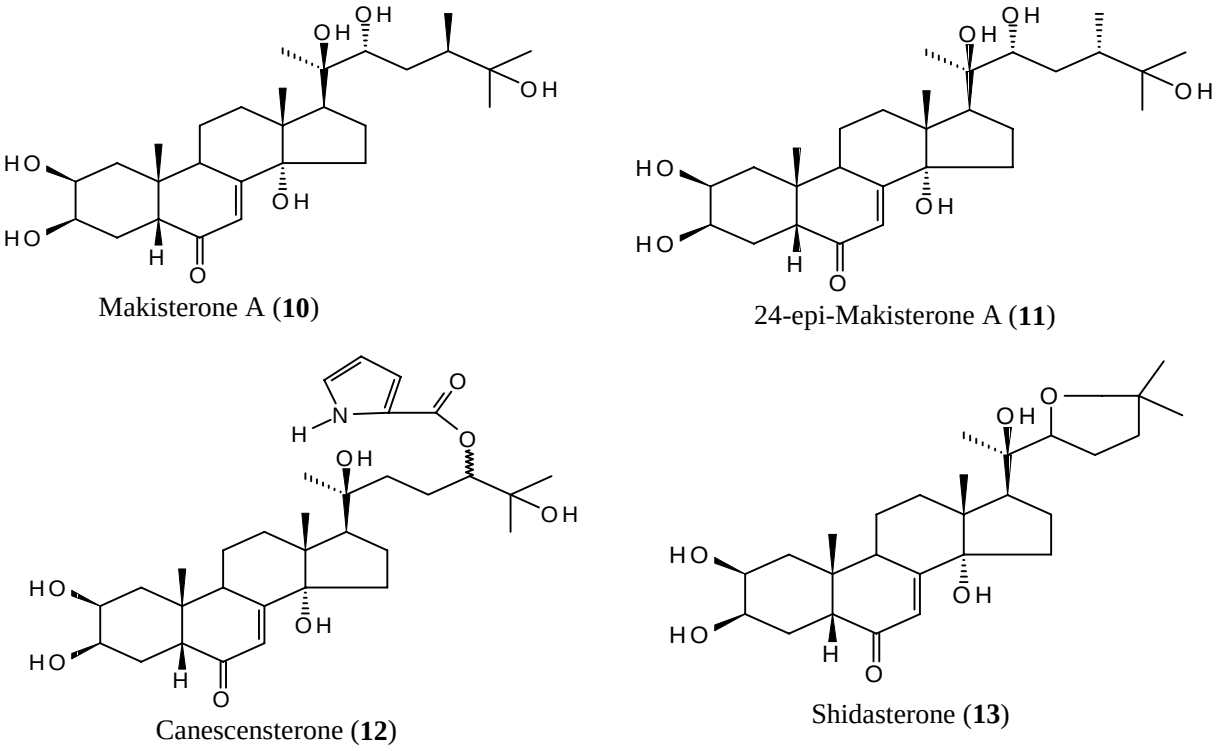

Figure 2 Chemical Structures of Some Ecdysteroids from *Vitex* Species

acid) was used as a developing reagent. Spots of organic compounds give specific colors with this reagent after heating at 100^{0}C for 2-4 min. Column chromatography was carried out using silica gel 60 (0.063-0.2 mm) and silica gel 60 of particle size less than 0.063 mm. A portion of the product to be analyzed was dissolved in a small amount of an appropriate organic solvent, mixed with silica gel of particle size 0.63-0.2mm, dried, and then added on the top of the chromatograph column.

2.2 Analytical Equipment

The melting points were determined by an electrothermal melting point apparatus.

Infrared (IR) absorption spectra were recorded with a Perkin Elmer FT-IR-2000 spectrophotometer.

Nuclear magnetic resonance (NMR) spectra were measured with a Bruker AM 400 spectrometer, using solutions in deuterated solvents with tetramethylsilane as an internal standard.

Electron impact mass spectra (EIMS) were obtained with a GC/MS Finnigan Mat. Instrument.

2.3 Plant Material

The bark was used of two samples of *Vitex canescens* obtained from Kaopra Pipoon District, Nakhon Si Thammarat Province (BKF no.96888), Thailand. Sample VC1 was from a young tree (without flowers) and sample VC2 was from an aged tree (with flowers).

2.4 Extraction and Isolation of Ecdysteroids from VC1

Three samples of air-dried pulverized bark (3.00 kg each) of the young *Vitex canescens* were ground, extracted with n-hexane (5L x 2 days), filtered, extracted with methanol (5L x 2 days) at 60^{0}C, and then filtered again. The filtrates from the three samples were combined and evaporated under reduced pressure to obtain 300g. of methanol extract.

Table 1 The selected fractions of the ethyl acetate extract of *V. canescens* (VC1)

Group No.	Fractions	Weight (g)	TLC result	
			No. of spot	Reagent coloration
VC1.1	28-36	0.1574	1	Greenish gray
VC1.2	37-43	0.2107	1	Greenish gray
VC1.3	44-46	0.3018	2	Violet, gray
VC1.4	47-54	1.6014	3	Pink, Violet, Pink
VC1.5	55-70	2.2105	2	Pink, Greenish gray

The methanol extract was diluted with a methanol: water mixture (300mL: 2000mL), and then subjected to separatory funnel extraction using chloroform (3 x 600 mL), ethyl acetate (4 x 400 mL) and butanol (3 x 500 mL) as solvents. After evaporation, the chloroform fraction yielded a residue of 14.99 g, the ethyl acetate fraction a residue of 44.95 g, and the butanol fraction a residue of 129.62 g. Tests of the ecdysteroid content of the three fractions showed that only the ethyl acetate fraction warranted further analysis.

2.5 Ethyl Acetate Extract of *V. canescens* (from VC1)

The ethyl acetate extract (44.9505 g) was first subjected to coarse separation using silica gel and column chromatography. Elution was conducted with a varying concentration mixture of chloroform and methanol. The mixture was initially pure chloroform and the proportion of methanol was then gradually increased until it was finally pure methanol. All fractions were collected and then analyzed by TLC. Five groups of combined fractions were then selected for further purification (see Table 1).

2.6 Group VC1.1 (fractions 28-36)

The first combined fraction was chromatographed (silica gel 25 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with gradual increase in the concentration of the more polar solvent MeOH. After the elution, a solid (15 mg) was obtained, which after recrystallization from methanol-chloroform gave canescensterone (6 mg) (**12**) [38].

2.7 Group VC1.2 (fractions 37-43)

The second combined fraction was chromatographed (silica gel 35 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with gradual increase in the concentration of the more polar solvent. After the elution, a solid was obtained (20 mg), which after recrystallization from methanol-chloroform again gave canescensterone (7 mg) (12)

2.8 Group VC1.3 (fractions 44-46)

The third combined fraction was chromatographed (silica gel 50 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with gradual increase in the concentration of MeOH. The first portion from the column (fractions 5-15) gave a solid (30.7 mg), which after recrystallization from methanol-chloroform gave 24-epi-makisterone A (11 mg) (**11**).

The second portion from the column (fractions 16-29) was rechromatographed twice, eluting with CHC_{l3}, CHC_{l3}-MeOH with increasing percentage of the more polar solvent. A solid (33 mg) was obtained, which after recrystallization from methanol-chloroform gave makisterone A (**10**) (21 mg).

2.9 Group VC1.4 (fractions 47-54)

The fourth combined fraction was chromatographed (silica gel 120 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with increasing percentage of the more polar solvent. The first portion (fractions 4-7) gave a

Table 2 The selected fractions for the ethyl acetate extract of *V. canescens* (VC2)

Group No.	Fractions	Weight (g.)	TLC result	
			No. of spot	Reagent coloration
VC2.1	9-12	0.1288	1	Gray
VC2.2	13-22	1.4528	1	Gray
VC2.3	23-24	0.5174	2	Violet, gray
VC2.4	25-36	11	3	Pink, Greenish gray, Pink
VC2.5	37-50	11	3	Pink, Greenish gray, Pink

solid (40 mg) which was recrystallized from methanol-chloroform to give 24-epi-makisterone A (17mg) (**11**). The second portion (fractions 14-17) gave a solid (26 mg), which was recrystallized from methanol-chloroform to give makisterone A (14mg) (**10**).

2.10 Group VC1.5 (fractions 55-70)

The fifth combined fraction was crystallized from methanol-ethyl acetate to give20-hydroxyecdysone (300 mg) (**2**). This is a very common ecdysteroid, which occurs widely in many *Vitex canescens* [38] and was among the first ecdysteroids to be isolated. The structure of (**2**) is now well established and is usually used to relate to other ecdysteroids.

2.11 Extraction and Isolation of Ecdysteroids from VC2

Three samples of air-dried pulverized bark (4.5 kg) of the aged *V. canescens* were ground, extracted with n-hexane (5L x 2 days), filtered, extracted with methanol (6.5L x 2 days) at 60^0C, and filtered. The three filtrates were then combined and evaporated under reduced pressure to afford 900 g of methanol extract.

The methanol extract was diluted with methanol (500 mL) and water (2.5L). The mixture was then subjected to separatory funnel extraction, using chloroform (3 x 600 mL), ethyl acetate (5 x 400 mL) and butanol (4 x 400 mL) as the solvent system. After evaporation, the chloroform fraction gave a residue of 22 g, the ethyl acetate fraction a residue of 68 g, and the butanol fraction a residue of 131 g. As for the VC1 sample, only the ethyl acetate fraction gave satisfactory results for the ecdysteroid test, and therefore it was the only fraction investigated for pure ecdysteroids.

2.12 Ethyl Acetate Extract of *V. canescens* (from VC2)

The ethyl acetate extract (68.2000 g) was first subjected to coarse separation using silica gel chromatography. As in the VC1 experiments, elution was conducted with a varying concentration mixture of chloroform and methanol. The mixture was initially pure chloroform and the proportion of methanol was then gradually increased until it was finally pure methanol. All fractions were then collected, tested by TLC, and the following five groups of combined fractions were selected for further purification (see Table 2).

2.13 Groups VC2.1 (2.3 (fractions 9-24)

Tests on these groups showed that they consisted mainly of nonecdysteroid compounds. It was found to be impossible to isolate pure compounds due to the amount of available samples and some other difficulties.

2.14 Group VC2.4 (fractions 25-36)

This group was chromatographed (silica gel 100 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with gradual increase in the concentration of the more polar solvent, and two portions were obtained.

The first portion was rechromatographed twice, eluting with CHC_{l3}, CHC_{l3}-MeOH with increasing percentage of more polar solvent, to give a solid, which was found to be a new ecdysteroid **P5** (12 mg), mp 241-243 ^{0}C. The infrared spectra, NMR spectra, EIMS and ESMS properties of **P5** were measured and the following results were obtained:

IR (KBr) ν_{max} cm^{-1} : 3415(-OH), 1656 (conjugated ketone C=O)
^{1}H NMR (deuteropyridine) : see Table 5
^{13}C NMR (deuteropyridine) : see Table 6
EIMS m/z (% rel. intensity) : 440(22), 422(1), 363(8), 345(58), 327(46), 309(12), 301 (27), 157(12), 113(37)
ESMS : 499[M+H+Na]+

The second portion gave a solid (20 mg), which was recrystalized from methanol-chloroform to give canescensterone (7 mg) (**12**).

IR (KBr) ν_{max} cm^{-1} : 3380 (-OH), 1640 (conjugated ketone C=O)
^{1}H NMR (deuteropyridine) : see Table 3
^{13}C NMR (deuteropyridine) : see Table 4
EIMS m/z (% rel. intensity) : 426(0.5), 411(0.5), 393(2), 363(30), 345(100), 327(95), 309(45), 291(16), 211(30), 210(12), 209(18), 193(9), 192(6), 191(10), 183(14), 182(7), 143(13), 125(10), 111(14), 109(29), 107(8), 94(4), 93(5), 67(20), 66(1), 65(2).

2.15 Group VC2.5 (fractions 37-50)

The fifth combined fractions were chromatographed (silica gel 150 g), eluting with CHC_{l3}, CHC_{l3}-MeOH with gradual increase in the concentration of more polar solvent, and three portions were obtained.

The first portion (fractions 2-9) gave a solid (55 mg) which was recrystallized from methanol-chloroform to give 24-epi-makisterone A (31 mg) (**11**) [40].

IR (KBr) ν_{max} cm^{-1} : 3400(-OH), 1850 (conjugated ketone C=O)
^{1}H NMR (deuteropyridine) : see Table 3
^{13}C NMR (deuteropyridine) : see Table 4
EIMS m/z (% rel. intensity) : 441[M+H-3H2O]$^+$(2), 423(1), 363(76), 345(100), 327(65), 301(8), 283(5), 175(4), 157(4), 131(3), 113(7), 95(12), 70(3)

The second portion (fractions 12-16) gave a solid (60 mg), which was recrystalized from methanol-chloroform to give makisterone A (39 mg) (**10**) [40].

IR (KBr) ν_{max} cm^{-1} : 3415 (-OH), 1656 (conjugated ketone C=O)
^{1}H NMR (deuteropyridine) : see Table 3
^{13}C NMR (deuteropyridine) : see Table 4
EIMS m/z (% rel. intensity) : 477[M+H-H2O]$^+$(1), 459(0.5), 363(65), 345(100), 327(84), 175(7), 157(6), 113(7)

The third portion (fractions 24-32) gave a solid (800 mg) which was recrystalized from methanol-chloroform to give hydroxyecdysone (400 mg) (2).

3. Results and Discussion

Using ethyl acetate as solvent, five ecdysteroids (labelled **P1–P5**) were extracted from the bark of a young tree (sample VC1) and an aged tree (sample VC2) of *Vitex canescens*. The extracted ecdysteroids and their properties were found to be as follows:

3.1 P1 (from VC1 and VC2): Canescensterone (12)

P1 was obtained as a crystalline solid, mp 152-

Table 3 Comparison of ^{1}H NMR data for **P2** and **P3** with those reported for 24-epi-makisterone A makisterone A, respectively.

H	P2	24-epi-Makisterone A	P3	Makisterone A
2	4.19(m)	4.20(br. d,11Hz)	4.17(m)	4.2(br. d, 11Hz)
3	4.24(br. s)	4.24(br. s)	4.22(br. s)	4.24(br. s)
5	3.03(m)	3.10(m)	3.02(m)	3.04(dd, 13, 4Hz)
7	6.26(d,1.6Hz)	6.26(d, 2Hz)	6.31(d, 2.3Hz)	6.30(d, 2Hz)
9	3.60(t, 8.3Hz)	3.59(m)	3.59(s)	3.62(m)
17	3.03(m)	3.10(m)	3.00(m)	2.99(t, 8Hz)
22	4.07(br. d, 8.7Hz)	4.08(br. d, 8.2Hz)	3.98(br. d, 10.6Hz)	4.02(br. d,11Hz)
18	1.21	1.22	1.27	1.27
19	1.07	1.08	1.23	1.10
21	1.59	1.59	1.59	1.62
26	1.34	1.35	1.32	1.34
27	1.39	1.40	1.29	1.32
28	1.26(d, 6.9Hz)	1.27(d, 7Hz)	1.07(d, 6.8Hz)	1.09(d, 6.6Hz)

153^{0}C. The ^{1}H NMR and ^{13}C NMR spectral features of **P1** exhibited an ecdysteroid type of spectra. The ^{1}H NMR spectrum of P1 was similar to that of (**1**) and (**3**), except for the absence of the signal at δ 3.87, which corresponds to H-22 in (**2**), and the signal at δ 3.77, which corresponds to H-24 in (**10**). Instead, a signal was obtained at δ 5.58 which had a splitting pattern similar both to the H-22 of an ecdysteroid with C-20 and C-22 hydroxyl groups and to the H-24 of pinnatasterone. Such a large downfield shift (1.7 or 1.81 ppm) suggested that an extra C_5H_4ON group should be located at the same position as H-24. The presence of a conjugated ester function at 1640 cm^{-1} in the IR spectrum suggested that this extra portion could be connected to the C-22 or C-24 hydroxyl group through an ester linkage.

The EI-mass spectral peak at m/z 363 was identified as being due to a fragmentation between C-20 and C-22. This fragmentation would produce an ion containing an ecdysteroid ring system and with C-20 and C-21 as part of the side chain. Loss of one, two or three molecules of water from the above fragment ions would then give rise to the ions at m/z 345, 327 and 309, respectively. Furthermore, the presence of ions at m/z 211 and 210, which correspond to the side-chain ions arising from fission between C-20 and C-22, confirmed that the pyrrole 2-carboxylate moiety was not attached to the C-21 unit of the molecule of (**12**).

The presence of the fragment ions at m/z 183 and 182 was taken as evidence that C-24 was the point of attachment of the pyrrole 2-carboxylate moiety, since fragments with these m/z ratios would be produced by fission between C-23 and C-24. Comparison of ^{13}C NMR data of (**12**) with those of (**2**) suggested that the oxygen function was located at C-24 rather than at C-22.

TLC comparison of **P1** with that of authentic canescensterone (**12**) (previously isolated from *Vitex canescens*) also confirmed the above interpretations. Furthermore, the ^{1}H NMR and ^{13}C NMR spectral data of P1 were consistent with those of reported canescensterone (**12**) [28].

P1 was therefore identified as the ecdysteroid canescensterone (**12**).

Table 4 Comparison of ^{13}C NMR data for **P2** and **P3** with those reported for 24-epi-makisterone A and makisterone A, respectively.

C	**P2**	24-epi-Makissterone A	**P3**	Makisterone A
1	38.0	38.1	38.0	38.0
2	68.1	68.1	68.1	68.1
3	68.2	68.1	68.0	68.1
4	32.5	32.5	32.5	32.4
5	51.4	51.4	51.4	51.4
6	203.5	203.3	203.5	203.3
7	121.6	121.6	121.7	121.7
8	166.3	166.1	166.1	165.9
9	34.5	34.6	34.5	34.5
10	38.7	38.7	38.7	38.7
11	21.1	21.2	21.2	21.2
12	31.7	31.8	31.9	31.8
13	48.1	48.2	48.2	48.2
14	84.2	84.2	84.2	84.2
15	32.0	32.1	32.1	32.1
16	21.5	21.6	21.4	21.4
17	50.0	50.1	50.0	50.0
18	17.9	17.9	17.9	17.9
19	24.5	24.5	24.5	24.5
20	77.1	77.1	77.0	77.0
21	21.3	21.4	21.6	21.6
22	76.2	76.2	74.7	74.8
23	35.1	35.3	34.6	34.7
24	43.6	43.5	41.8	41.9
25	72.3	72.3	72.1	72.1
26	26.9	27.0	26.5	26.5
27	29.0	29.0	28.3	28.3
28	16.8	16.8	15.4	15.5

3.2 P2 (from VC1 and VC2): 24-Epi-makisterone A (11)

P2 was obtained as a white solid, mp 234-236 ^{0}C. The IR spectrum indicated a strong absorption band of the hydroxyl group (3400 cm^{-1}) and of the carboxyl group of α , β-unsaturated ketone (1650 cm^{-1}). The ^{1}H NMR spectrum of the tetracyclic ring system of **P2** was very similar to that of 20-hydroxyecdysone (**2**). The EI-mass spectral peak of **P2** at m/z 363 was associated with a fragmentation between C-20 and C-22. Loss of one, two and three molecules of water from this ion would give ions at the observed m/z 345, 327, and 309, respectively. The peaks at m/z 301 and 283 were attributed to ions produced by fission between C-17 and C-20 and with loss of one and two molecules of water, respectively. The mass spectral data confirmed the existence of the same tetracyclic ring system in **P2** as that in 20-hydroxyecdysone (**2**) and indicated that the observed differences between P2 and (**2**) were due to the side chains.

A comparison of the ^{1}H NMR spectrum of the side chain of **P2** with that of (**2**) showed that the only significant difference was the presence of a three-proton doublet (J = 6.9 Hz) at δ 1.26 in **P2.** The following mass spectral data also confirmed the presence of a methyl group at the side chain portion. A peak at m/z 175 was associated with a side chain ion arising from cleavage between C-20 and C-22. Respective loss of one and two molecules of water would then give ions with the observed ratios at m/z 113 and 95. A comparison of ^{1}H NMR

Table 5 Comparison of ^{1}H NMR data for **P5** with those reported for shidasterone, canescensterone and makisterone A.

H	P5	Shidasterone	Canescensterone	Makisterone A
2	4.18 (br. t)	4.18 (m)	4.15(br. d, 8.6Hz)	4.2(br. t)
3	4.26 (br. s)	4.26 (m)	4.23(br. s)	4.24(br. s)
5	3.03 (dd, 13.1Hz, 3.5Hz)	3.02 (dd, 13Hz, 3.7Hz)	3.00(dd, 13.4Hz, 3.8Hz)	3.04(dd, 13Hz, 4Hz)
7	6.22 (d, 2.1Hz)	6.25 (d, 2.2Hz)	6.19(d, 2Hz)	6.30(d, 2Hz)
9	3.61 (m)	3.60 (m)	3.53(m)	3.62(m)
17	2.82 (8.89)	2.84	2.90(t, 9.4Hz)	2.99(t, 8Hz)
18 - Me	1.07 (s)*	1.07 (s)	1.04(s)	1.27
19 - Me	0.99 (s)*	1.07 (s)	1.02(s)	1.10
21 - Me	1.39 (s)	1.41 (s)	1.50(s)	1.62
22	4.09 (dd, 8.9Hz, 5.1Hz)	4.10 (t, 7.5Hz)	-	4.02
24	-	-	5.58(br. d, 8.9Hz)	-
26 - Me	1.09 (s)*	1.21 (s)	1.42(s)	1.34
27 - Me	1.20 (s)*	1.23 (s)	1.44(s)	1.32
28-Me	0.80 (d, 6.8Hz)	-	-	1.09(d, 6.6Hz)
1'	-	-	13.30(br. s)	-
3'	-	-	7.28(m)	-
4'	-	-	6.40(m)	-
5'	-	-	7.28(m)	-

All compounds were recorded in pyridine - d_5
* Assignment may be reversed for signals with the same superscript

spectra of 24-epi-makisterone A (**11**) with that of 20-hydroxyecdysone (**2**) indicated that an extra methyl group was located at C-24 in **P2.** A mass spectral peak at m/z 70, which could be associated with the ion $(C_5H_{10})^+$, also supported the above observation.

The foregoing evidence prompted us to compare spectroscopic data of 24-epi-makisterone A (**11**) with that of previously isolated makisterone A (**10**). Although the ^{1}H NMR spectra of these two ecdysteroids (Table 3) were very similar, there were some differences in signals from some protons, and in particular with the chemical shift values of the 24-Me groups of (**11**) (δ 1.26) and (**10**) (δ 1.09). The ^{13}C NMR spectra of these two compounds (Table 4) also showed some differences. A TLC comparison of **P2** with two samples of authentic makisterone A (**10**) (the previously isolated sample and a sample from Sigma Chemicals USA) also confirmed the above conclusion. The ^{1}H NMR and ^{13}C NMR spectral data of **P2** were all consistent with the reported spectral data reported for 24-epi-makisterone A (**11**) [29].

P2 was therefore identified as the ecdysteroid 24-epi-makisterone A (**11**).

3.3 P3 (from VC1 and VC2): Makisterone A (10)

P3 was obtained as a colorless solid, mp 263-265 ^{0}C. The IR spectrum showed strong absorption bands of the hydroxyl group (3415 cm^{-1}) and of the carboxyl group of α,β-unsaturated ketone (1656 cm^{-1}). The ^{1}H NMR spectra of **P3** and of 20-hydroxyecdysone (**2**) were very similar. The presence of fragment ions at m/z 363, 345 and 327 confirmed the identity of the tetracyclic ring system of **P3** with that of (**2**). The only significant different was the presence of an additional methyl resonance in **P3** which appeared as a doublet (J = 6.8 Hz) at δ 1.07. This data suggests that the methyl group should be located in the side chain portion of the molecule.

A fragment ion at m/z 175 in the EI-mass spectrum was associated with the side-chain ion arising from cleavage between C-17 and C-20. Loss of one molecule of water from this ion would then

give another fragment ion at the observed m/z 157. The prominent peak at m/z 113 was attributed to side chain fission between C-20 and C-22.

From the splitting pattern and the chemical shift value, the possible locations of the extra methyl group in **P3** could be C-23 or C-24. However, from the similarity of the spectral feature of the H-22 signal of (**10**) as compared to that of (**2**), it was concluded that C-23 was unsubstituted. The only position for locating the methyl group was therefore at C-24.

A comparison of the ^{1}H NMR and ^{13}C NMR spectral data of **P3** with those reported for makisterone A (**10**) [40] (see Table 4) led to the conclusion that these two compounds were identical. Moreover, a TLC comparison of **P3** with authentic makisterone A (**10**) (previously isolated from *V.* canescens) also confirmed the identification of **P3** as makisterone A (**10**).

3.4 P4 (from VC1 and VC2): 20- hydroxyecdysone (2)

P4 was obtained as a white solid, which was identified as 20-hydroxyecdysone.

3.5 P5 (from VC2)

Compound **P5** was obtained as a colorless solid, mp 241-243^{0}C. The IR spectrum showed strong absorption bands of the hydroxyl group (3400 cm^{-1}) and the carbonyl group of α, β-unsaturated ketone (1650 cm^{-1}). The ecdysteroid mass spectrum (positive ion mode) established a molecular formula of $C_{28}H_{44}O_6$. The ^{1}H NMR spectrum of this compound was very similar to that of shidasterone (13) [40] (see Table 6). The spectral features and relative positions of H-2, H-3, H-5, H-7, H-9 and H-17 as well as those of 18-Me and 19-Me indicated that these two compounds possessed the same nucleus.

The EI-mass spectrum of **P5** lacked the molecular ion peak of shidasterone (13) at m/z 476. Instead there was a peak at m/z 458, corresponding to $[M-H_2O]^{+}$, and peaks at m/z 440 and 422 corresponding to further losses of a second and a third molecule of water, respectively.

A peak at m/z 363 was attributed to a fragmentation between C-20 and C-22, since this fragmentation would give an ion consisting of an ecdysteroid ring system with C-20 as part of the side chain, and with three hydroxyl groups attached to the ring system and a fourth hydroxyl group at the C-20 position. The reason for locating the fourth hydroxyl group at the C-20 position was that the ^{1}H NMR chemical shift of the C-21 methyl group appeared as a singlet, δ = 1.39. Fragmentation between C-20 and C-22 was also supported by the existence of signals at m/z 345 (the highest mass number), 327 and 309 which could be attributed to loss of one, two and three molecules of water respectively from a fragment ion.

Two peaks at m/z 301 and 283 were attributed to ions produced by fission between C-17 and C-20 with loss of one and two molecules of water, respectively.

The structure of compound **P5** was further confirmed by a comparison of ^{13}C NMR spectral data from **P5** with data for shidasterone (**13**) and makisterone A (**10**) (see Table 6). The ^{13}C NMR of C-22 in **P5** was similar to that of (**13**), but C-24 was similar to that of makisterone A (**10**). It was therefore concluded that **P5** differed from shidasterone (**13**) only at the methyl group of C-24. The proposed structure of **P5** is shown in Figure 3.

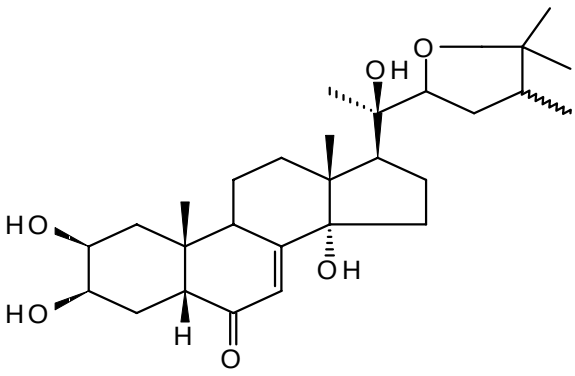

Figure 3 Proposed Structure for New Ecdysteroid P5

Table 6 Comparison of ^{13}C NMR data for **P5** with those reported for shidasterone, canescensterone and makisterone A.

C	P5	Shidasterone	Canescensterone	Makisterone A
1	38.0	37.9	37.9	38.0
2	68.16	66.1	68.09	68.1
3	68.24	66.0	68.03	68.1
4	32.56	32.4	32.4	32.4
5	51.47	51.3	51.3	51.4
6	203.67	20.3	203.5	203.3
7	121.76	121.6	121.5	121.7
8	166.28	166.1	166.1	165.9
9	34.50	34.3	34.3	34.5
10	38.76	38.6	38.5	38.7
11	21.7	21.0	20.9	21.2
12	31.82	31.6	31.7	31.8
13	47.77	47.5	47.3	48.2
14	84.24	84.6	84.3	84.2
15	31.8	31.6	31.4	32.1
16	21.7	21.6	21.9	21.4
17	50.8	51.3	53.0	50.0
18	18.0	17.6	17.8	17.9
19	24.5	24.4	24.3	24.5
20	76.29	75.5	74.0	77.0
21	21.1	21.1	25.6	21.6
22	81.60	84	41.7	74.8
23	35.5	38.9	25.0	34.7
24	42.96	27.6	80.6	41.9
25	82.50	80.3	71.7	72.1
26	21.0	28.2	27.3	26.5
27	27.4	28.7	26.9	28.3
28	15.5	-	-	15.5
2'	-	-	123.9	-
3'	-	-	115.9	-
4'	-	-	110.2	-
5'	-	-	124.1	-
COO	-	-	161.9	-

All compounds were recorded in pyridine – d_5

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