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Naturally Occurring 20,26-Dihydroxyecdysone Exists as Two C-25 Epimers which Exhibit Different Degrees of Moulting Hormone Activity

Apichart Suksamrarn,* Boon-ek Yingyongnarongkul and Nart Promrangsan

Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok 10240, Thailand

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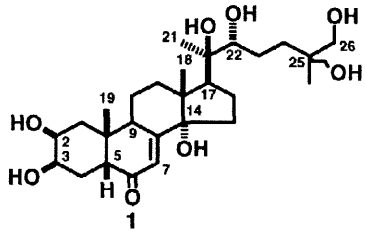
Abstract: 20,26-Dihydroxyecdysone (20,26-ECD) isolated from *Vitex canescens, V. glabrata* and *V. pinnata* has been shown to exist as two C-25 epimers. Synthesis and separation of the two epimers were accomplished and they exhibited different degree of moulting hormone activity in the *Musca* assay.

20,26-Dihydroxyecdysone (20,26-ECD, **1**) is a minor ecdysteroid, the insect moulting hormone, isolated for the first time from the pupae of the tobacco hornworm, *Manduca sexta*, by Thompson *et al.* in 1967.[1] This compound was later found in the plant *Podocarpus elatus* by Galbraith *et al.* in 1973.[2] It was then isolated from other invertebrates and plant species.[3] This ecdysteroid was also shown to be a metabolite of 20-hydroxyecdysone (20-ECD, **2**) in the blowfly *Calliphora erythrocephala*.[4] Recently we reported the isolation of a number of ecdysteroids from a complex mixture of the methanolic extract of *Vitex canescens* root bark and this included a small quantity (< 0.5 mg) of an unidentified ecdysteroid.[5] The polarity of this compound suggested that it contained one more hydroxyl group than 20-ECD (**2**). Due to the small quantity of the compound, the ^{1}H NMR spectrum did not give full information of its structure. However, from the available spectrum it could be concluded that the absence of a singlet signal of a methyl group and the presence of a hydroxymethyl (CH_2OH) group suggested that this ecdysteroid might be 20,26-ECD (**1**). This ecdysteroid has not previously been reported as a constituent of *Vitex* species.[6,7] In this paper we report the identification of this ecdysteroid in some *Vitex* species and it was found to exist as two C-25 epimers of 20,26-ECD. The synthesis and moulting hormone activity of these two C-25 epimeric ecdysteroids have also been reported.



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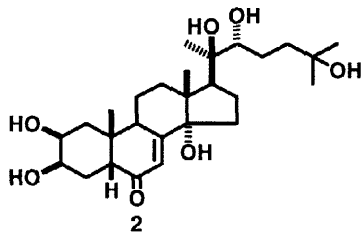

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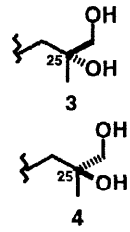

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RESULTS AND DISCUSSION

20,26-ECD from *Vitex* species

TLC of the minor ecdysteroid from *V. canescens* root bark with an authentic 20,26-ECD isolated from *Silene nutans*[8] suggested these two ecdysteroids to be the same. However, reversed-phase HPLC analysis revealed that they were different compounds. Careful HPLC analysis of these ecdysteroids indicated that each of

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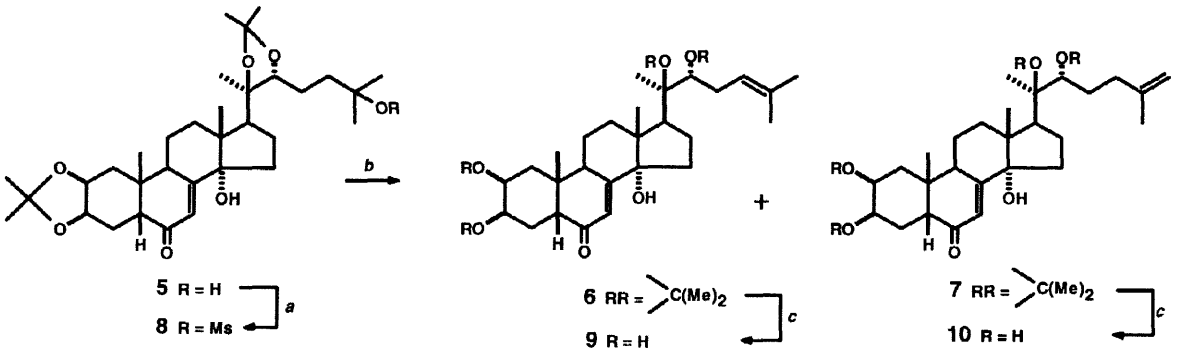
them contained a minor component, which corresponded to the major component of each other. Comparison of the ^{1}H NMR spectra of the ecdysteroids from these two sources, each of which showed only those of the major components, suggested that they should have the same oxygenation pattern, but with different stereochemical arrangement at a carbon. It has been reported that some ecdysteroids with C-25 asymmetric carbon existed as mixtures of two C-25 epimers and they included inokosterone[9] and 20-hydroxyecdysonoic acid,[10] but no separation of the two epimers was undertaken. In the case of 20,26-ECD, no report has appeared on the existence of two C-25 epimers. However, it was noticed that the ^{1}H NMR spectrum of a naturally occurring 20,26-ECD gave two slightly different chemical shift values of each of the 26-Me and 27-Me groups, presumably duing to two C-25 epimers.[3] It was thus most likely that the ecdysteroid from *V. canescens* root bark was a mixture of two C-25 epimers, the minor epimer of which was presence in about 6%. If this was the case, both of these two ecdysteroids were 20,26-ECD, one of which was 25*R* and the other was the 25*S* epimers (i.e., **3** and **4**). In order to unambiguously prove that these two ecdysteroids possessed 20,26-ECD skeleton, sufficient quantities of these ecdysteroids were needed. In order to obtain more of 20,26-ECD with the same stereochemical arrangement at the C-25 position as that found in *V. canescens* root bark, stem bark of *V. glabrata,* which has been reported to contain 20-ECD (**2**) and turkesterone[11] was chosen as a more accessible source of plant material. Fractions of the extract having similar polarity as that of 20,26-ECD have been investigated and 1 mg of 20,26-ECD has finally been isolated from 1 kg of *V. glabrata* stem bark. To our surprise, we found that the ratio of the two epimers was 1:2.5, comparing to 1:14.7 in case of *V. canescens* root bark (Table 1). We therefore investigated 20,26-ECD constituents of *V. canescens* stem barks from southern[12] and northern parts of the country and also of *V. pinnata*[13] (Table 1). Two reversed-phase HPLC systems (systems A and B) were employed in the isolation and purification of 20,26-ECD, and determination of the epimer ratio. However, in the case of *V. canescens* stem bark (sample 1) and root bark (both were from southern part of the country) and *V. pinnata*, additional normal-phase HPLC system (system C) had to be included in the separation step in order to remove 24-*epi*-abutasterone[5] from 20,26-ECD, otherwise the epimer ratio could not be accurately determined. It was interesting to note that in case of *V. canescens* stem bark from southern and northern parts of the country, the epimer ratio was approximately the same and was comparable to those from *V. glabrata* and *V. pinnata* stem barks. The significant difference in epimer ratio was, therefore, between the stem barks and the root bark.

Synthesis of 20,26-ECD

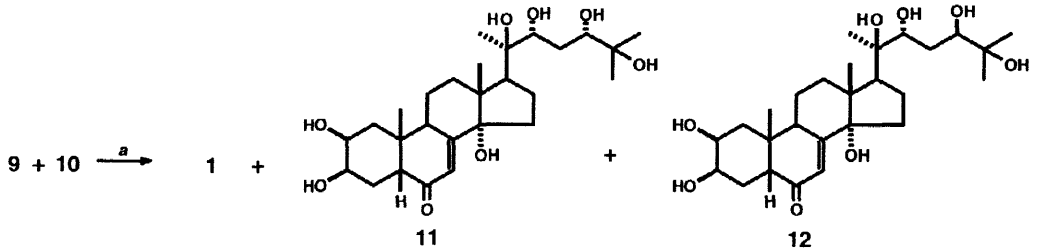
The small quantity of the available natural 20,26-ECD has led to the design of synthesis of the two epimeric 20,26-ECD. The availability of the ecdysteroids would then make it possible to study ^{1}H and ^{13}C NMR spectra and to determine the biological activity of each of these compounds. Careful selection of synthetic route could also confirm that the two ecdysteroids were the C-25 epimers of each other. Synthesis of the two epimers of 20,26-ECD has been accomplished as follows (method 1, scheme 1). Starting from the readily available ecdysteroid, 20-ECD (**2**),[11] the 2,3:20,22-diacetonide **5** was prepared according to the literature method.[14] Treatment of the pyridine solution of **5** with mesyl chloride in the presence of DMAP gave an inseparable 3:2 mixture of the olefin diacetonides **6** and **7**, presumably through the mesylate **8**.[15] The acetonide protecting groups in the products **6** and **7** were removed by treatment with 70% AcOH to the corresponding olefin mixture, which was separated by repeated column chromatography to afford stachysterone C (**9**) and 25,26-didehydroponasterone A (**10**). Dihydroxylation of the latter olefin with OsO_4 in pyridine and THF gave 20,26-ECD in 16% overall yield from the diacetonide **5**. The compound was homogeneous on TLC, but ^{1}H NMR

spectrum revealed a *ca* 1:1 mixture of two C-25 epimers. Reversed-phase HPLC indicated a 1:1.1 mixture of two epimers, which were separated into two individual epimers (system B). The first eluted component was identical to the major epimer from *S. nutans* and was assigned 20,26-ECD "epimer 1". The second component was identical to that obtained from *V. canescens* root bark and was assigned 20,26-ECD "epimer 2". From the chosen synthetic route, it was obvious that the stereochemistry of C-1 to C-24 was intact, whereas two C-25 epimers were generated. The naturally occurring ecdysteroids from two different sources, therefore, were C-25 epimeric 20,26-ECD.



Scheme 1 Reagents and conditions: *a*, MsCl, pyridine, DMAP, 0-5 °C to ambient temp.; *b*, *in situ* elimination of MsOH from **8**; *c*, 70% AcOH, EtOH; *d*, (i) OsO_4, pyridine, THF; (ii) 1% $NaHSO_3$

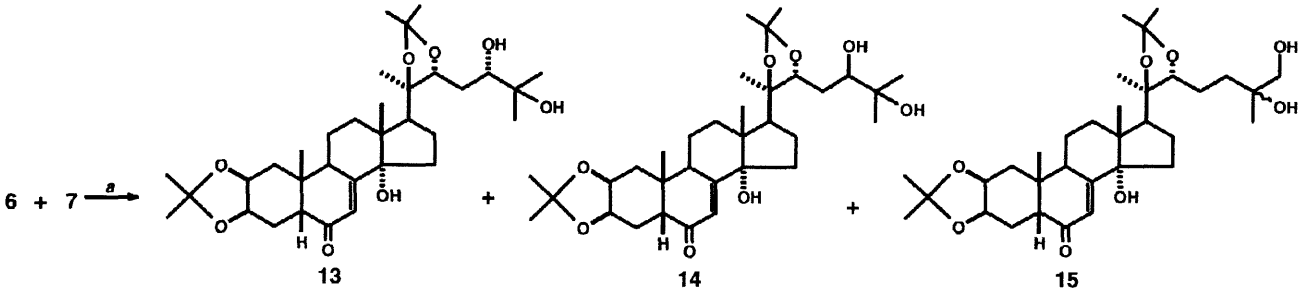
The above synthesis provided a good yield of 20,26-ECD in the dihydroxylation step of the olefin **10**. However, separation of compound **10** from its isomeric olefin **9** was quite cumbersome and this resulted in relatively low overall yield of **1**. Two alternative syntheses involving dihydroxylation of mixtures of olefins **9** and **10** or their diacetonides **6** and **7** were investigated. In the first alternative synthesis (method 2, scheme 2) the olefin mixture **9** and **10** was dihydroxylated to afford, after column chromatography, abutasterone (**11**), 24-*epi*-abutasterone (**12**) and a mixture of two epimers of 20,26-ECD, the latter of which could be separated into two epimeric 20,26-ECD by HPLC (system B). In this case the yield of 20,26-ECD from the olefin mixture **9** and **10**



Scheme 2 Reagents and conditions : *a*, (i) OsO_4, pyridine, THF; (ii) 1% $NaHSO_3$

was 25%. The second alternative synthesis (method 3, scheme 3) involved dihydroxylation of the olefin diacetonides **6** and **7** mixture to give a mixture of abutasterone 2,3:20,22-diacetonide (**13**), 24-*epi*-abutasterone 2,3:20,22-diacetonide (**14**) and two C-25 epimers of 20,26-ECD 2,3:20,22-diacetonide (**15**). Column chromatography of the mixture afforded pure **13**, part of pure **14**, part of pure 20,26-ECD 2,3:20,22-diacetonide "epimer 1", a mixture of **14** and 20,26-ECD 2,3:20,22-diacetonide "epimer 2", and a mixture of 20,26-ECD 2,3:20,22-diacetonide "epimer 1" and "epimer 2". The latter mixture was rechromatographed and this resulted in partial separation of the two epimers. Deacetonation of **13**, **14**, 20,26-ECD 2,3:20,22-diacetonide "epimer 1" and "epimer 2" respectively yielded compounds **11**, **12**, 20,26-ECD "epimer 1" and 20,26-ECD

"epimer 2". Removal of the acetonide protecting group in the mixture of **14** and 20,26-ECD 2,3:20,22-diacetonide "epimer 2", followed by column chromatography, gave **12** and 20,26-ECD "epimer 2". It should be mentioned that in this case the two epimers of 20,26-ECD could be separated through their diacetonides without using HPLC, but the separation procedures were relatively complicated. The first alternative method (method 2) seemed to be most convenient, since it required less separation steps and compounds **11** and **12** were easily removed from the two epimeric 20,26-ECD.



Scheme 3 Reagents and conditions: *a*, (i) OsO_4, pyridine, THF; (ii) 1% $NaHSO_3$

Comparison of the ^1H NMR spectra of 20,26-ECD "epimer 1" to that of the 20,26-ECD "epimer 2", it appeared that they were almost identical. The only different feature of the two spectra was the presence of a multiplet signal at δ2.27 in the spectrum of the former isomer. The relative vicinity of the H-2 and H-3 around δ4.19-4.25, and H-7 and OH around δ6.25-6.31 were more far apart in the latter isomer. For accurate assignment of the ^1H chemical shift values of both epimers, it should be made on the same spectrum of the mixture of the two epimers. The ^1H NMR spectrum of a *ca* 1:1 mixture of the two epimers revealed some further differences: there were 0.004, 0.01 and 0.01 ppm differences in the chemical shift values of the 18-Me, 27-Me and 21-Me signals, respectively, of these two isomers. However, assignments of the proton signals to individual isomer could not be made with confidence, since their chemical shift values were very close and the two isomers were present in the mixture in equal or almost equal quantity. A portion of each of the pure epimers were therefore remixed in a ratio of 8:5 and the ^1H NMR spectrum of the two epimers determined. The assignments of individual proton signals could then be made (Table 2). The ^{13}C NMR spectral data of each epimer (Table 3) were almost identical; the only significant differences were those of 18-Me and 26-Me. From these findings it was evident that care must be taken in interpreting NMR spectral data of ecdysteroids that could exist as two epimers. It is noteworthy that the existing data does not permit the assignment of absolute configuration of both epimers of 20,26-ECD (i.e., **3** and **4**).

From reversed-phase HPLC analysis it was concluded that, for all *Vitex* species investigated, the second component, 20,26-ECD "epimer 2", was always the major ecdysteroid. The epimer ratios in stem barks were significantly different from that in the root bark.

Moulting Hormone Activity

In insects, it was proposed that 20,26-ECD was a deactivation product of 20-ECD (**2**).[1,16] The role of 20,26-ECD in plants, however, has not yet been speculated upon. If this ecdysteroid served as a defensive chemical against non-adapted herbivorous insects as well as that expected for 20-ECD,[7] different epimer ratios could possibly lead to selective activity of the ecdysteroid. It was interesting, therefore, to compare moulting hormone activity of the two epimers of 20,26-ECD. The two C-25 epimeric ecdysteroids exhibited lower activity than that of the parent ecdysteroid **2** in the *Musca* assay. However, the two epimers showed different degrees of activity in this bioassay system. 20,26-ECD "epimer 2" exhibited approximately two-fold higher activity than

20,26-ECD "epimer 1". The result suggested that binding of the "epimer 2" to the insect receptor was more specific than that of the "epimer 1". Our study provided more information concerning a better understanding of binding of ecdysteroids to receptors. Further chemical work should focus on the determination of absolute configuration of each epimer of 20,26-ECD.

Table 1 Ratio of two C-25 epimers of 20,26-ECD in *Vitex* species[a]

Entry	Plant material	"Epimer 1" : "Epimer 2"
1	*V. glabrata*[b]	1 : 2.5
2	*V. canescens* (sample 1)[b]	1 : 4.0
3	*V. canescens* (sample 2)[b]	1 : 3.7
4	*V. pinnata*[b]	1 : 2.1
5	*V. canescens*[c]	1 : 14.7

[a]Reversed-phase HPLC analysis (column: Hypersil BDS C18, 5 μm, 4.6x250 mm; mobile phase: *i*-PrOH-H_2O (5:95); flow rate: 1.4 ml/min; detector: 242 nm)
[b]Stem bark; [c]Root bark

Table 2 ^{1}H NMR data of two C-25 epimers of 20,26-ECD

H	"Epimer 1"	"Epimer 2"
2	4.19 (*m*)	4.18 (*m*)
3	4.23 (br *s*)	4.23 (br *s*)
5	3.01[a]	3.01[d]
7	6.25 (*d*, 2.4)	6.25 (*d*, 2.4)
9	3.58 (*m*)	3.58 (*m*)
17	3.03[a]	3.08[d]
22	3.92 (br *d*, 10.3)	3.90 (*dd*, 10.6, 1.8)
26	3.87[b] and 3.88[c]	3.86[e]
OH	6.29 (*s*)	6.31 (*s*)
18-Me	1.204 (*s*)	1.208 (*s*)
19-Me	1.06 (*s*)	1.06 (*s*)
21-Me	1.58 (*s*)	1.57 (*s*)
27-Me	1.47 (*s*)	1.46 (*s*)

Recorded in pyridine-d_5
[a-e]Obscured signals

Table 3 ^{13}C NMR data of two C-25 epimers of 20,26-ECD

C	"Epimer 1"	"Epimer 2"
1	37.95[a]	37.95[e]
2	68.05[b]	68.05[f]
3	68.12[b]	68.14[f]
4	31.68[c]	31.70[g]
5	51.37	51.39
6	203.48	203.50
7	121.62	121.62
8	166.10	166.12
9	34.40	34.45
10	38.62	38.64
11	21.40	21.45
12	32.41[c]	32.44[g]
13	48.08	48.08
14	84.18	84.18
15	31.96[c]	31.98[g]
16	21.66	21.70
17	50.07	50.09
18	17.35	17.86
19	24.52[d]	24.76[h]
20	76.87	76.87
21	21.09	21.09
22	77.65	77.68
23	26.76	26.70
24	37.55[a]	37.52[e]
25	72.66	72.60
26	70.79	70.62
27	24.41[d]	24.43[h]

Recorded in pyridine-d_5
[a-h]Assignments may be reversed for signals with the same superscript

EXPERIMENTAL

General experimental details have been described previously.[17] HPLC was used in the analyses and separations of two epimers of 20,26-ECD. The following conditions were used in this study. System A- column: Spherisorb S10ODS2, 10 μm, 10×250 mm; mobile phase: MeOH-H_2O (35:65); flow rate: 2.0 ml/min; detector: 254 nm; t_R of turkesterone and 20,26-ECD were 20.8 and 27.5 min, respectively. System B- column: Hypersil BDS C18, 5 μm, 4.6×250 mm; mobile phase: *i*-PrOH-H_2O (5:95); flow rate: 1.4 ml/min; detector: 242 nm; t_R of 20,26-ECD "epimer 1" and "epimer 2" were 38.5 and 44.5 min, respectively. System C- column: Hypersil Si, 5 μm, 4.6×250 mm; mobile phase: $CHCl_3$-MeOH (93:7); flow rate: 1.2 ml/min; detector: 254 nm; t_R of 24-*epi*-

abutasterone (**12**) and 20,26-ECD were 10.5 and 15.0 min, respectively. *Vitex glabrata*,[11] *V. pinnata*[13] and *V. canescens* (sample 1, from southern part of the country)[12] stem barks were from the same sources as described previously. *V. canescens* stem bark (sample 2, from northern part of the country) was collected from Prae district and a voucher specimen is deposited at the Plant Collection Centre, Faculty of Science, Ramkhamhaeng University. 20,26-ECD from *V. canescens* root bark was obtained from ref. 5. 20,26-ECD from *Silene nutans* was a generous gift from Professor R. Lafont, Ecole Normale Superieure, Paris.

Isolation of 20,26-ECD from *Vitex glabrata*

Pulverized, dry bark of *V. glabrata* (1 kg) was successively extracted with *n*-hexane and MeOH in a Soxhlet extraction apparatus. The methanolic extract, after being diluted with water, was successively extracted with EtOAc and *n*-BuOH. The BuOH extract was subjected to column chromatography, using $CHCl_3$-MeOH as eluting solvents. Fractions eluted by $CHCl_3$-MeOH (83:17) contained mainly turkesterone, previously isolated from this plant species,[11] and a minor ecdysteroid which was contaminated with other compounds. Repeated column chromatography, followed by reversed-phase HPLC (system A) resulted in the isolation of 20,26-ECD (**1**, 1 mg). The ratio of two epimers of 20,26-ECD were determined (system B, Table 1).

20,26-ECD from *V. canescens* (sample 2) was similarly isolated; the ratio of two epimers of 20,26-ECD from this plant species were determined (Table 1).

Isolation of 20,26-ECD from *V. canescens* (sample 1)

The pulverized, dry bark of *V. canescens* (sample 1, 1 kg) was similarly subjected to extraction as described for *V. glabrata*. The BuOH extract, after being chromatographed to remove most of turkesterone and other components, was subjected to HPLC separation (system A) to remove the remaining turkesterone from 20,26-ECD and 24-*epi*-abutasterone (**12**), followed by normal-phase HPLC separation (system C) to remove **12** from 20,26-ECD. This resulted in the separation of *ca* 0.5 mg of 20,26-ECD and the ratio of two epimeric 20,26-ECD was determined (system B, Table 1).

Following the above procedure, 20,26-ECD from *V. pinnata* stem bark and *V. canescens* root bark were isolated and the two C-25 epimers determined (Table 1).

Synthesis of two C-25 epimers of 20,26-ECD

Method 1. 1) **25,26-Didehydroponasterone A.** 20-ECD 2,3:20,22-diacetonide (**5**) (112 mg, 0.2 mmol), prepared according to ref. 14, was dissolved in dry pyridine (0.5 ml) and the solution stirred at 0-5 °C for 10 min. Mesyl chloride (0.2 ml, 2.57 mmol) and DMAP (5 mg) were added and the reaction mixture was left to stir at 5 °C for 20 min, then slowly allowed to warm up to ambient temperature during the period of 4 h. Water was added and the mixture extracted with $CHCl_3$ (3×25 ml). The combined $CHCl_3$ layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness to yield a mixture of stachysterone C 2,3:20,22-diacetonide (**6**) and 25,26-didehydroponasterone A 2,3:20,22-diacetonide (**7**). The crude mixture, the ^1H NMR of which indicated a 3:2 mixture of **6** and **7**, was dissolved in EtOH (0.5 ml); 70% AcOH (2 ml) was added and the mixture was stirred for 4 days. Water was added and the mixture extracted with *n*-BuOH (4×20 ml). The combined organic layer was washed with water, evaporated by co-distillation with water under reduced pressure. The crude mixture which contained stachysterone C (**9**) and 25,26-didehydroponasterone A (**10**) in a ratio of 3:2 (from ^1H NMR analysis) was chromatographed, using $CHCl_3$-MeOH as eluent, to afford pure compound **9**, a mixture of compounds **9** and **10**, and pure compound **10**. The compounds **9** and **10** mixture was chromatographed to yield more of pure compound **9** and **10**, together with a mixture of compounds **9** and **10**, the latter of which was subjected to another column chromatography. This resulted in the separation of 23 mg (25% yield from the diacetonide **5**) of stachysterone C (**9**), 16 mg (18% yield from the diacetonide **5**) of 25,26-

didehydroponasterone A (**10**). Compounds **9** and **10** were identical to stachysterone C and 25,26-didehydroponasterone A prepared by a different method.[15]

2) **Dihydroxylation of 25,26-didehydroponasterone A (10). Synthesis and separation of two C-25 epimeric 20,26-ECD.** A solution of OsO_4 (300 µl, 0.058 mmol, prepared by dissolving 250 mg of OsO_4 in 5 ml of THF) was added to a solution of compound **10** (16 mg, 0.035mmol) in pyridine (0.7 ml) and the reaction mixture stirred for 10 min. 1% $NaHSO_3$ solution (5 ml) was then added and the mixture stirred for 20 min. Saturated brine (50 ml) was added and the mixture repeatedly extracted with *n*-BuOH until no products were detected in the aqueous phase. The combined organic phase was evaporated and the residue chromatographed, with $CHCl_3$-MeOH as eluent, to afford a mixture of two C-25 epimeric 20,26-ECD (15 mg, 87%) which were homogeneous on TLC. The ^1H NMR spectrum indicated the presence of two C-25 epimers in a ratio of *ca* 1:1. The two isomers were separated from each other by HPLC (system B) to give 20,26-ECD "epimer 1" (6 mg) and 20,26-ECD "epimer 2" (6 mg) which were respectively identical to the major epimer obtained from *S. nutans* and *V. canescens*. ^1H NMR data of each of the epimeric ecdysteroids (Table 2) were assigned by comparison with the ^1H NMR spectrum of a 8:5 mixture of the two epimers. The ^{13}C NMR data of the two epimers were also obtained (Table 3).

20,26-ECD "epimer 1": Amorphous; IR ν_{max} 3400, 2924, 1649, 1443, 1383, 1315, 1120, 1055, 880 cm^{-1}; ^1H NMR and ^{13}C NMR data are given in Tables 2 and 3; HR-FABMS (-ve) m/z 495.2962 $[M-H]^-$. $C_{27}H_{43}O_8$ requires 495.2957.

20,26-ECD "epimer 2": Amorphous; IR ν_{max} 3400, 2928, 1648, 1382, 1185, 1105, 1052, 875 cm^{-1}; ^1H NMR and ^{13}C NMR data are given in Tables 2 and 3; HR-FABMS (-ve) m/z 495.2945 $[M-H]^-$. $C_{27}H_{43}O_8$ requires 495.2957.

Method 2. A 3:2 mixture of compounds **9** and **10** (53 mg) prepared according to the method 1, was subjected to dihydroxylation in the same manner as described for the dihydroxylation of compound **10** (method 1) to yield a mixture of abutasterone (**11**), 24-*epi*-abutasterone (**12**) and two C-25 epimers of 20,26-ECD. Column chromatography resulted in the isolation of compound (**11**) (8 mg), compound (**12**) (14 mg) and a mixture of two C-25 epimers of 20,26-ECD (14 mg). The yields of compounds **11**, **12** and **1** from the olefin mixture **9** and **10** were 14, 25 and 25%, respectively. The ecdysteroids **11** and **12** were identical (^1H NMR and TLC comparisons) to the previously prepared compounds.[15]

Method 3. A mixture of the olefin diacetonides **6** and **7** (180 mg) was subjected to dihydroxylation as described for compound **10** (method 1) to give a mixture of abutasterone 2,3:20,22-diacetonide (**13**), 24-*epi*-abutasterone 2,3:20,22-diacetonide (**14**) and two C-25 epimers of 20,26-ECD 2,3:20,22-diacetonide (**15**). Column chromatography gave pure **13** (20 mg), part of pure **14** (9 mg), part of pure 20,26-ECD 2,3:20,22-diacetonide "epimer 1" (2 mg), a mixture of compound **14** and 20,26-ECD 2,3:20,22-diacetonide "epimer 2" (70 mg) and a mixture of 20,26-ECD 2,3:20,22-diacetonide "epimer 1" and 20,26-ECD 2,3:20,22-diacetonide "epimer 2" (36 mg). The latter mixture was rechromatographed to give pure 20,26-ECD 2,3:20,22-diacetonide "epimer 1" (10 mg), 20,26-ECD 2,3:20,22-diacetonide "epimer 2" (5 mg) and a mixture of the two epimers (18 mg). Among the diacetonides, that of the "epimer 1" was the most polar component. The diacetonides **13**, **14** and each of the pure epimer of the diacetonide **15** were separatedly subjected to deacetonation using 70% AcOH to afford compounds **11** (11mg), **12** (5 mg), 20,26-ECD "epimer 1" (5 mg) and 20,26-ECD "epimer 2" (3 mg). A portion (23 mg) of compounds **14** and 20,26-ECD 2,3:20,22-diacetonide "epimer 2" mixture were similarly treated with 70%AcOH and subsequently chromatographed to give compounds **12** (7 mg) and 20,26-ECD

"epimer 2" (4 mg). Compounds **11 and 12** were identical to those obtained from the method 2. HPLC analysis revealed that 20,26-ECD, part of the pure 2,3:20,22-diacetonide of which was isolated earlier from the mixture of diacetonides and was assigned 20,26-ECD 2,3:20,22-diacetonide "epimer 1", was identical to 20,26-ECD "epimer 1", whereas that obtained at the latter step of the separation procedure and was assigned 20,26-ECD 2,3:20,22-diacetonide "epimer 2", was identical to 20,26-ECD "epimer 2".

Biological activity testing. The moulting hormone bioassay using *Musca domestica* larvae has been performed and evaluated by the established methods.[18,19]

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