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## Ecdysteroid 7,9(11)-dien-6-ones as potential photoaffinity labels for ecdysteroid binding proteins

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### Abstract

Three ecdysteroid 7,9(11)-dien-7-ones (dacryhainansterone, 25-hydroxydacryhainansterone and kaladasterone) were prepared by dehydration of the corresponding 11a-hydroxy ecdysteroids (ajugasterone C, turkesterone and muristerone A, respectively). The biological activities of the dienones in the *Drosophila melanogaster* B<sub>II</sub> cell bioassay, which reflect the affinity for the ecdysteroid receptor complex, showed that the dienones retain high biological activity. Irradiation at 350 nm of the ecdysteroid dienones (100 nM) with bacterially-expressed dipteran and lepidopteran ecdysteroid receptor proteins (DmEcR/DmUSP or CfEcR/CfUSP), followed by loading with [<sup>3</sup>H]ponasterone A revealed that irradiation of dacryhainansterone or kaladasterone resulted in blocking of >70% of the specific binding sites. Thus, ecdysteroid dienones show considerable potential as photoaffinity analogues for ecdysteroid binding proteins.

**Keywords:** ecdysteroid receptor, affinity labelling, Ultraspiracle, Quantitative Structure-Activity Relationship, Comparative Molecular Field Analysis, 20-hydroxyecdysone, dacryhainansterone

### Abbreviations:

20E	20-hydroxyecdysone	HPLC	high-performance liquid chromatography
ax	axial	HR-MS	high-resolution mass-spectrometry
Cf	<i>Choristoneura fumiferana</i>	LBD	ligand-binding domain
CI-MS	chemical ionization mass spectrometry	LSIMS	liquid secondary-ion mass-spectrometry
CoMFA	comparative molecular field analysis	MS	mass-spectrometry
DCC	dextran-coated charcoal	NMR	nuclear magnetic resonance
Dm	<i>Drosophila melanogaster</i>	ponA	ponasterone A
EcR	ecdysteroid receptor protein	QSAR	quantitative structure-activity relationship
eq	equatorial	RI	relative intensity
FAB-MS	fast-atom bombardment mass-spectrometry	USP	Ultraspiracle protein
		UV	ultra-violet.

### Introduction

Affinity labelling is an important method in elucidating signal transduction mechanisms (Dorman, 2000) and has proved to be a powerful method for the characterisation of vertebrate steroid

binding proteins, including intracellular receptors, transport proteins and enzymes (Katzenellenbogen and Katzenellenbogen, 1984; Gronemeyer and Govindan, 1986; Gronemeyer, 1988). These studies have provided information about the points of attachment, the orientation of the ligand in the binding site and guidance to direct

site-directed mutagenesis studies. In contrast, the steroid hormone receptor of arthropods has received far less attention in respect to the identification of potential affinity labels. This is remarkable considering that arthropods comprise 95% of all animal species and that insights into ecdysteroid binding would provide useful information for understanding ecdysteroid-regulated gene control and identifying new possibilities for insect pest control.

Ecdysteroid receptor genes have been characterised from insects (e.g. Saleh et al., 1998, and references cited therein), crustaceans (Durica et al., 1999) and ticks (Palmer et al., 1999). The receptor complex consists of two major polypeptides, an EcR protein and a USP/RXR protein. Both are members of the nuclear receptor superfamily. The best characterised proteins are those of *Drosophila melanogaster*, where 3 isoforms of EcR are present which arise from different promoters (isoforms A and B) and alternative splicing (isoforms B1 and B2) (Koelle et al., 1991). There may also be isoforms of USP (Vögtli et al., 1999). The natural ligand of ecdysteroid receptors is generally assumed to be 20-hydroxyecdysone (**1**), but other ligands may be important in certain species or at particular stages of development (Wang et al., 2000). The ecdysteroid binds to the ligand-binding domain of the EcR protein, but only with high affinity when this is complexed with USP (Yao et al., 1993). It is currently not known with any certainty whether USP recognises a ligand, although it has been suggested that juvenile hormone may bind to this protein (Jones and Jones, 2000). In spite of extensive structure-activity relationship studies for ecdysteroids (Dinan et al., 1999; Ravi et al., 2001), the orientation and interactions of the ligand within the LBD of EcR remain unknown. The necessity for interaction between EcR and USP for high-affinity ligand binding complicates crystallographic studies on the ligand binding domain of EcR, although it has recently been possible to determine the crystal structure of the LBD of USP from *Heliothis virescens* (Billas et al., 2001). Recent modelling and virtual docking experiments (Wurtz et al., 2000) with 20E and RH5849 (a non-steroidal ecdysteroid agonist; Dhadialla et al., 1998) with models of the *Chironomus tentans* EcR ligand binding domain based on the crystal structures of the human vitamin D receptor and the human retinoic acid receptor LBDs provide conflicting information about the orientation of the ecdysteroid in the binding pocket. Consequently, the generation of an efficient ecdysteroid affinity label would provide important information on the orientation of the ligand and perhaps also facilitate crystallographic studies.

Previous approaches to affinity labelling of ecdysteroid receptors/binding proteins have comprised photoaffinity labelling based on activation of the natural 14 $\alpha$ -hydroxy-7-en-6-one ( $\lambda_{\text{max}}$  = 242nm) of ecdysteroids (Gronemeyer and Pongs, 1980; Schaltmann and Pongs, 1982) or electrophilic labelling based on brominated side-chain analogues (Strangmann-Diekmann et al., 1990). The former method has the advantage of using natural arthropod ecdysteroids (such as **1**), but appears to suffer the disadvantage of poor cross-linking yield (ca. 5%), probably as a consequence of irradiation at a wavelength (254 nm) at which the receptor proteins also absorb. No further reports of the application of the electrophilic affinity ecdysteroid analogues have appeared. The ideal affinity labelling reagent should possess i) a high affinity for the binding protein, ii)

a high cross-linking yield, iii) low non-specific binding and iv) the capacity to be detected (e.g. by radiolabelling) without detriment to the other three criteria. Extension of the natural chromophore of ecdysteroids to a dien-6-one (7,9(11) or 7,14) would shift the  $\lambda_{\text{max}}$  to longer wavelength (ca. 300 nm). The natural occurrence of 11 $\alpha$ -hydroxy ecdysteroids in certain plant species (especially *Ajuga* spp.; Imai et al., 1969; Canonica et al., 1973; Usmanov et al., 1975), provides precursors for the generation of 7,9(11)-dien-6-ones by dehydration (Canonica et al., 1977; Qui and Nie, 1983). Here we report the simple synthesis in good yield of ecdysteroid 7,9(11)-dien-6-ones from the corresponding 11 $\alpha$ -hydroxy ecdysteroid, demonstrate that the dienones retain high biological activity and provide indirect evidence that they are capable of covalently linking to ecdysteroid receptors in high yield on irradiation with long wavelength UV light (300 or 350 nm).

## Experimental

### General

Ajugasterone C (**2**) was isolated from *Leuzea carthamoides* (Píš et al., 1994). Muristerone A (**3**) was purchased from Simes spa, Milan, Italy. Turkesterone (**4**) was a generous gift from Ziyadilla Saatov. [24,25,26,27-<sup>3</sup>H]Ponasterone A (25-deoxy-20-hydroxyecdysone; 150 Ci/mmol) was obtained from American Radiochemicals Inc. ([www.arc-inc.com](http://www.arc-inc.com)).

Ultra-violet spectra were obtained in methanol using a Shimadzu ([www.shimadzu.com](http://www.shimadzu.com)) UV-2401 PC, UV-VIS dual beam spectrophotometer. Nuclear magnetic resonance (NMR) spectroscopy was performed i) on a Bruker (<http://www.Bruker.com>) AVANCE DRX400 instrument (in Exeter, UK) using standard Bruker microprograms with samples dissolved in CD<sub>3</sub>OD or ii) on a Bruker AMX500 instrument (in Paris, France) at 300 K, where samples were lyophilised and dissolved in D<sub>2</sub>O. TSPd<sub>4</sub>, 3-(trimethylsilyl)[2,2,3,3-*d*<sub>4</sub>] propionic acid, sodium salt, was used as internal reference for the proton and carbon shifts. Chemical shifts are expressed in ppm. Sep-Pak (0.5 g) C<sub>18</sub> cartridges (Millipore, [www.waters.com](http://www.waters.com)) were used for pre-HPLC purification of the samples. HPLC: (1) semipreparative, Gilson ([www.gilson.com](http://www.gilson.com)) model 806 HPLC coupled with Gilson UV-Visible detector, (2) analytical, Gilson model 811 HPLC coupled with Gilson 160 diode array detector and using Gilson Unipoint computer program; C<sub>18</sub> Spherisorb ODS-2 semipreparative and Spherisorb 5 ODS-2 analytical C<sub>18</sub> columns were used (both 5  $\mu$ m particle size; Jones Chromatography, ([www.jones-chrom.co.uk](http://www.jones-chrom.co.uk))). Chromatographic separations were monitored at 242 and 300 nm. HPLC-grade solvents were from BDH ([www.bdh.com](http://www.bdh.com)).

The LSIMS spectrum of **6** was recorded by Mark Prescott and Huw Rees at the University of Liverpool on a VG Quattro triple quadrupole mass spectrometer (VG Biotech, [www.micromass.co.uk](http://www.micromass.co.uk)) using a Cs<sup>+</sup> primary beam and glycerol matrix. CI-MS spectra were recorded on a Jeol MS 700 spectrometer equipped with a direct inlet probe. Spectra were recorded in the chemical ionization/desorption mode using ammonia as the reagent gas.

## Prediction of biological activities

The biological activities of the ecdysteroid dienones were predicted based on the CoMFA QSAR models developed from the agonist activities of a training set of 71 ecdysteroid analogues in the B<sub>II</sub> bioassay (Dinan et al., 1999).

## Conversion of 11 $\alpha$ -hydroxyecdysteroids to dienones

The ecdysteroid was dissolved in 5% w/v NaOH methanolic solution (1 ml/mg ecdysteroid reacted, typically 5 mg) and left to react at room temperature. The reaction was quenched after 40 minutes with the addition of 0.8 M HCl (same volume as NaOH methanolic solution). The sample was diluted to 10% MeOH by the addition of H<sub>2</sub>O and this was applied to a C<sub>18</sub> Sep-Pak cartridge (0.5 g). The sample was eluted with 10% and 100% MeOH (5 ml of each). The 100% MeOH fraction was analysed on a C<sub>18</sub> analytical column (ODS2, 25 cm x 4.6 mm i.d., 5  $\mu$ m particle size, 1:1 v/v MeOH:H<sub>2</sub>O, 1ml/min), initially with the photodiode-array detector monitoring at 242 nm and 300 nm. This indicated further purification was necessary and this was carried out using a C<sub>18</sub> semi-preparative column (ODS2, 25 cm x 9.6 mm i.d., 5  $\mu$ m particle size, MeOH:H<sub>2</sub>O, 1:1 v/v, 2 ml/min) with detection at 300 nm. The purified product was dried and subjected to <sup>1</sup>H and <sup>13</sup>C NMR. In this way, ajugasterone C (**2**) was converted to dacryhainansterone (**5**), muristerone A (**3**) to kaladasterone (**6**) and turkesterone (**4**) to 25-hydroxydacryhainansterone (**7**) (Fig. 1).

**Dacryhainansterone (5):** UV: MeOH ( $\lambda_{\max}$  = 299 nm:  $\epsilon$  = 14200 L/mol/cm), H<sub>2</sub>O ( $\lambda_{\max}$  = 307 nm:  $\epsilon$  = 12900 L/mol/cm); <sup>1</sup>H NMR:  $\delta_{\text{H}}(\text{CD}_3\text{OD})$ : 6.28 (1H, apparent d, H-11); 5.75 (1H, s, H-7); 3.84 (1H, m, H-3); 3.73 (1H, m, H-2); 3.35 (1H, d,d, 11, 2, H-22); 2.75 (1H, broad d, 18, H-12<sub>ax</sub>); 2.5 (1H, H-12<sub>eq</sub>); 2.48 (1H, H-17); 2.45 (1H, H-5); 2.1 (1H, H-1<sub>eq</sub>); 2.05 (1H, H-16<sub>ax</sub>); 2.0 (1H, H-15<sub>ax</sub>); 1.8 (1H, H-4<sub>eq</sub>); 1.75 (1H, H-16<sub>ax</sub>); 1.7 (1H, H-1<sub>ax</sub>); 1.6 (1H, H-4<sub>ax</sub>); 1.55 (1H, H-15<sub>ax</sub>); 1.55 (1H, H-23<sub>b</sub>); 1.55 (1H, H-25); 1.46 (1H, H-24<sub>a</sub>); 1.30 (1H, H-23<sub>a</sub>); 1.24 (1H, H-24<sub>b</sub>); 1.18 (3H, s, 21-Me); 1.10 (3H, s, 19-Me); 0.94 (3H, d, 6.5, 27-Me); 0.91 (3H, d, 6.5, 26-Me); 0.89 (3H, s, 18-Me); <sup>13</sup>C NMR:  $\delta_{\text{C}}(\text{CD}_3\text{OD})$ : 205.5 (C-6); 155 (C-8); 134.7 (C-9); 132.4 (C-11); 117.8 (C-7); 83.1 (C-14); 76.6 (C-22); 76.2 (C-20); 67.4 (C-3/2); 66.9 (C-2/3); 50.2 (C-5); 49.1 (C-17); 48.0 (C-13); 46.5 (C-10); 39.4 (C-12); 37.7 (C-24); 36.2 (C-1); 36.0 (C-4); 30.2 (C-19); 29.9 (C-23); 29.1 (C-15); 27.8 (C-25); 22.0 (C-27); 21.3 (C-26); 20.3 (C-16); 19.3 (C-21); 16.7 (C-18); CI-MS m/z (% RI): 480.7 ([M+NH<sub>4</sub>]<sup>+</sup>, 11%), 463.6 ([M+H]<sup>+</sup>, 100%), 447.6 ([M-CH<sub>3</sub>]<sup>+</sup>, 51%), 445.6 ([M+H-H<sub>2</sub>O]<sup>+</sup>, 32%), 427 ([M+H-2H<sub>2</sub>O]<sup>+</sup>, 21%), 362.5 (74%), 345.5 ([M-C-20  $\rightarrow$  C-27]<sup>+</sup>, 61%).

**Kaladasterone (6):** UV: MeOH ( $\lambda_{\max}$  = 299 nm:  $\epsilon$  = 10700 L/mol/cm); <sup>1</sup>H NMR:  $\delta_{\text{H}}(\text{CD}_3\text{OD})$ : 6.3 (1H, apparent d, H-11); 5.80 (1H, s, H-7); 3.88 (1H, m, H-3); 3.83 (1H, m, H-2); 3.35 (1H, d,d, 11, 2, H-22); 2.75 (1H, broad d, 18, H-12<sub>ax</sub>); 2.46 (1H, H-12<sub>eq</sub>); 2.40 (1H, H-17); 2.1 (1H, H-1<sub>eq</sub>); 2.05 (1H, H-4<sub>eq</sub>); 2.02 (1H, H-16<sub>ax</sub>); 1.95 (1H, H-15<sub>ax</sub>); 1.8 (1H, H-4<sub>ax</sub>); 1.75 (1H, H-16<sub>ax</sub>); 1.7 (1H, H-1<sub>ax</sub>); 1.55 (1H, H-15<sub>ax</sub>); 1.55 (1H, H-23<sub>b</sub>); 1.55 (1H, H-25); 1.45 (1H, H-24<sub>a</sub>); 1.29 (1H, H-23<sub>a</sub>); 1.24 (1H, H-24<sub>b</sub>); 1.18 (3H, s, 21-

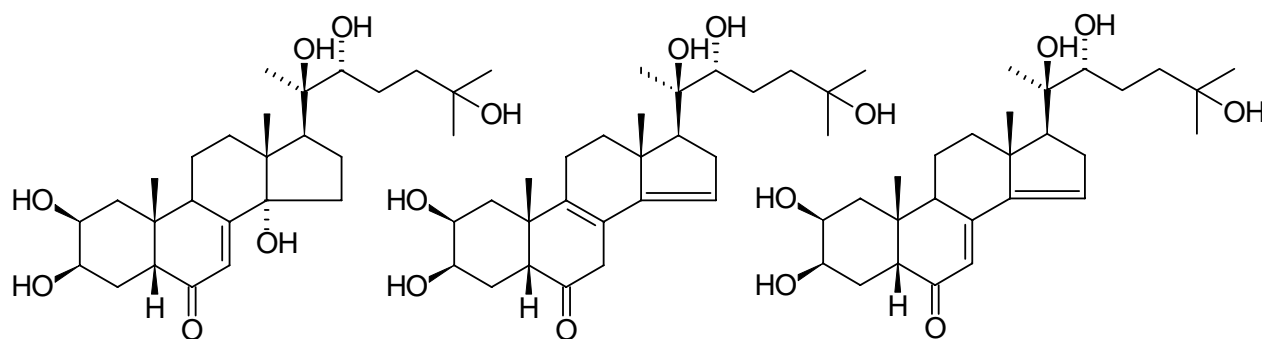
Me); 1.05 (3H, s, 19-Me); 0.94 (3H, d, 6.5, 27-Me); 0.91 (3H, d, 6.5, 26-Me); 0.89 (3H, s, 18-Me); <sup>13</sup>C NMR:  $\delta_{\text{C}}(\text{CD}_3\text{OD})$ : 201.3 (C-6); 155 (C-8); 136.3 (C-9); 132.7 (C-11); 116.3 (C-7); 82.9 (C-14); 78.97 (C-5); 76.6 (C-22); 76.2 (C-20); 68.7 (C-3/2); 67.0 (C-2/3); 49.0 (C-17); 46.5 (C-10); 44.8 (C-12); 37.7 (C-24); 36.2 (C-1); 33.0 (C-4); 29.94 (C-23); 29.1 (C-15); 27.8 (C-25); 24.97 (C-19); 22.0 (C-27); 21.3 (C-26); 20.3 (C-16); 19.3 (C-21); 16.7 (C-18); LSIMS (-ve ion-mode) m/z (%RI): 477 ([M-H]<sup>-</sup>, 80%), 460 ([M-H<sub>2</sub>O]<sup>-</sup>, 36%), 445 ([M-H<sub>2</sub>O-CH<sub>3</sub>]<sup>-</sup>, 25%), 183 (100%).

**25-Hydroxydacryhainansterone (7):** UV: MeOH ( $\lambda_{\max}$  = 299 nm:  $\epsilon$  = 10700 L/mol/cm); <sup>1</sup>H NMR:  $\delta_{\text{H}}(\text{CD}_3\text{OD})$ : 6.3 (1H, apparent t, H-11); 5.75 (1H, s, H-7); 3.84 (1H, m, H-3); 3.71 (1H, m, H-2); 3.35 (1H, d,d, 11, 2, H-22); 2.71 (1H, broad d, 18, H-12<sub>ax</sub>); 2.5 (1H, H-12<sub>eq</sub>); 2.46 (1H, H-17); 2.06 (1H, H-1<sub>eq</sub>); 2.05 (1H, H-16<sub>ax</sub>); 1.98 (1H, H-15<sub>ax</sub>); 1.83 (1H, H-24<sub>a</sub>); 1.78 (1H, H-4<sub>eq</sub>); 1.75 (1H, H-16<sub>ax</sub>); 1.73 (1H, H-1<sub>ax</sub>); 1.67 (1H, H-23<sub>a</sub>); 1.65 (1H, H-4<sub>ax</sub>); 1.60 (1H, H-15<sub>ax</sub>); 1.45 (1H, H-24<sub>b</sub>); 1.29 (1H, H-23<sub>b</sub>); 1.21 (3H, s, 26-Me); 1.20 (3H, s, 27-Me); 1.19 (3H, s, 21-Me); 1.10 (3H, s, 19-Me); 0.90 (3H, 18-Me); <sup>13</sup>C NMR:  $\delta_{\text{C}}(\text{CD}_3\text{OD})$ : 205.5 (C-6); 155 (C-8); 134.7 (C-9); 132.3 (C-11); 117.8 (C-7); 83.1 (C-14); 77.1 (C-22); 76.3 (C-20); 69.9 (C-25); 67.4 (C-3/2); 66.9 (C-2/3); 50.17 (C-5); 49.1 (C-17); 46.6 (C-10); 40.96 (C-24); 39.4 (C-12); 35.97 (C-1); 34.3 (C-4); 30.2 (C-19); 29.99 (C-15); 28.3 (C-27); 27.6 (C-26); 26.0 (C-23); 20.3 (C-16); 19.4 (C-21); 16.7 (C-18); CI-MS m/z (% RI): 496.7 ([M+NH<sub>4</sub>]<sup>+</sup>, 1%), 479 ([M+H]<sup>+</sup>, 9%), 463.6 ([M-CH<sub>3</sub>]<sup>+</sup>, 15%), 461.6 ([M+H-H<sub>2</sub>O]<sup>+</sup>, 12%), 445 ([M-CH<sub>3</sub>-H<sub>2</sub>O]<sup>+</sup>, 7%), 443 ([M+H-2H<sub>2</sub>O]<sup>+</sup>, 6%), 427 ([M+H-3H<sub>2</sub>O]<sup>+</sup>, 8%), 362.5 (64%), 345.5 ([M-C-20  $\rightarrow$  C-27]<sup>+</sup>, 45%), 116.2 ([C-22  $\rightarrow$  C-27]<sup>+</sup>, 100%).

## Preparation of podecdysone B

Podecdysone B (**8**) was prepared by thermal decomposition of 20E (100 mg) adsorbed on basic alumina (Woelm B, activity grade I; 5 g), heated at 140 °C for 3 hours. The reaction product was eluted from the alumina with methanol and then the solvent evaporated. The residue was separated by column chromatography on silica gel with chloroform/methanol (95:5 v/v). The major fraction was further purified by NP-HPLC on a Separon (Tessek) column (7  $\mu$ m particle size; 250 mm x 8 mm i.d.) eluted with n-hexane/ethanol/water (938:60:2 v/v/v) at a flow-rate of 2 ml/min. Compound **8** (40 mg) was obtained as an amorphous solid.

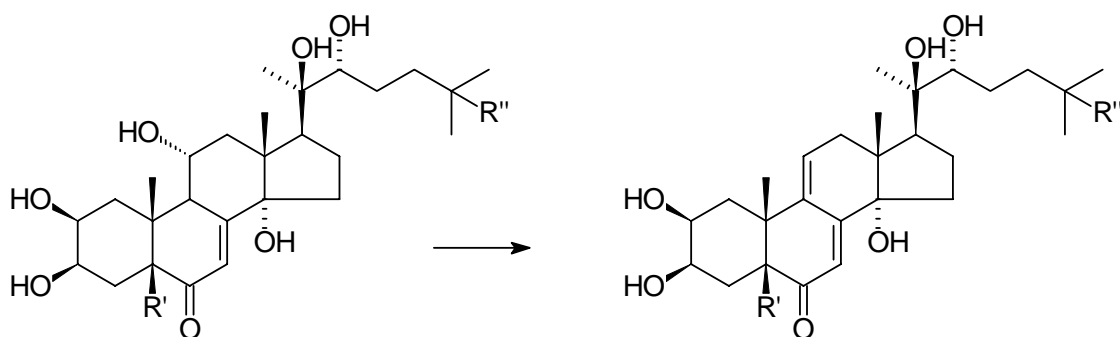
**Podecdysone B (8):** IR (KBr),  $\nu_{\max}$  (cm<sup>-1</sup>): 3415 (OH), 1711 (C=O), 1652 (C=C), 1056 (C-OH); UV (MeOH),  $\lambda_{\max}$ : 244 nm, 302 nm; <sup>1</sup>H NMR:  $\delta_{\text{H}}(\text{D}_2\text{O})$ : 1.02 (3H, s, 18-CH<sub>3</sub>); 1.03 (3H, s, 19-CH<sub>3</sub>); 1.21 (3H, s, 26-CH<sub>3</sub>); 1.23 (3H, s, 27-CH<sub>3</sub>); 1.29 (3H, s, 21-CH<sub>3</sub>); 1.33 (1H, 23Ha); 1.49 (1H, 24-Hb); 1.53 (1H, 1-Hax); 1.58 (1H, 12-Hax); 1.61 (1H, 23-Hb); 1.74 (1H, d,t,13.6; 3.7, 4-Heq); 1.78 (1H, 24-Ha); 1.86 (1H, t,d,13.6; 2.5, 4-Hax); 2.01 (1H, dd, 13.7, 3.4, 1-Heq); 2.09 (1H, d,d, 11; 7.6, 17-H); 2.23 (1H, d,d, 16; 11, 16-H); 2.26 (1H, 11-H); 2.31 (1H, d,d, 13; 4.9, 12-Heq); 2.40 (1H, broad m, 11-H); 2.42 (1H, d,d, 13.2; 3.8, 2.85-Hb); 2.54 (1H, d,d, 16; 11, 16-H); 2.82 (1H, d,d, 22; 2.8, 7-Ha); 3.46 (1H, d, 9.4, 22-H); 3.42 (1H, d, 22., 7-Hb); 3.61 (1H, dt,12.5; 3.5, 2-Hax); 4.04 (1H, m w<sub>1/2</sub> = 8, 3-Heq); 5.55 (1H, broad m, w<sub>1/2</sub> = 6, 15-H); <sup>13</sup>C NMR:  $\delta_{\text{C}}$



**20-hydroxyecdysone(1)**

**podecdysone B (8)**

**stachysterone B (9)**



**ajugasterone C (2) R'=H, R''=H**

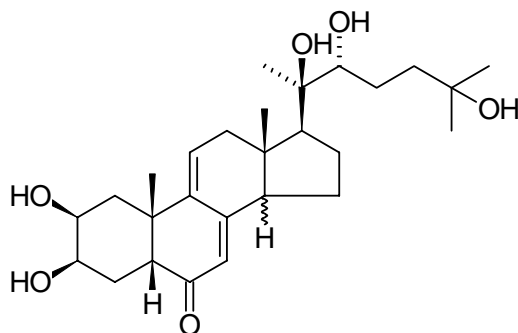
**dacyrhainansterone (5) R'=H, R''=H**

**muristerone A (3) R'=OH, R''=H**

**kaladasterone (6) R'=OH, R''=H**

**turkesterone (4) R'=H, R''=OH**

**25-hydroxycacryrhainansterone (7) R'=H, R''=OH**



**(14α-H)2β,3β,20 R,22R,25-pentahydroxy-5β-cholest-7-9(11)-dien-6-one (10)**

**(14α-H)2β,3β,20 R,22R,25-pentahydroxy-5β-cholest-7-9(11)-dien-6-one (11)**

**Figure 1:** Ecdysteroid structures and generation of ecdysteroid 7,9(11)-dien-6-ones.



(D<sub>2</sub>O): 19.7 (C-18); 21.6 (C-21); 25.2 (C-11); 28.5 (C-23); 29.8 (C-26); 31.1 (C-27); 30.8 (C-19); 33.2 (C-16); 33.5 (C-4); 38.9 (C-1); 39.5 (C-12); 41.2 (C-7); 43.1 (C-24); 46.0 (C-10); 48.8 (C-13); 55.1 (C-5); 58.6 (C-17); 69.9 (C-3); 71.3 (C-2); 74.4 (C-25); 80.1 (C-22); 80.1 (C-20); 122.1 (C-15); 139.3 (C-9); 151.5 (C-14); FAB-MS, m/z (% RI): 485 ([M+Na]<sup>+</sup>, 35%), 463 ([M+H]<sup>+</sup>, 41%), 445 ([M-H<sub>2</sub>O+H]<sup>+</sup>, 21%), 427 ([M-2H<sub>2</sub>O+H]<sup>+</sup>, 30%), 367 (12%), 343 (14%), 329 (16%), 299 (29%), 279 (38%), 181 (100%); HR-MS for C<sub>27</sub>H<sub>43</sub>O<sub>6</sub> [M+H]<sup>+</sup> found 463.30363, calculated 463.30597. These data are in accord with those previously published (Nishimoto et al., 1988).

### Preparation of stachysterone B

Stachysterone B (**9**) was prepared by acid treatment of 20E (**1**) (Harmatha et al., 2002). 20E (20 mg) were dissolved in 2 ml EtOH, and 100 µl 10N HCl was added. The mixture was left overnight at room temperature under an atmosphere of argon, then the sample was dried under a nitrogen flow and purified by NP-HPLC on a semi-preparative column (Zorbax-SIL, 250 mm long, 9.4 mm i.d., flow-rate 4 ml/min) eluted with cyclohexane/isopropanol/water, 100:40:3 v/v/v. Three peaks were collected (peak 1: Rt = 12.4 min; peak 2: Rt = 14.8 min; peak 3 = 16.0 min). These three peaks were further purified using dichloromethane/isopropanol/water, 125:30:1.5 v/v/v. Peak 1 (Rt = 11.6 min; 4.5 mg) was identified as podecdysone B (**8**). Peak 2 separated into 2 compounds: Peak 2-1 (Rt = 14.4 min; **10**; (14α-H)2β,3β,20R,22R,25-pentahydroxy-5β-cholest-7,9(11)-dien-6-one; 0.75 mg) and Peak 2-2 (Rt = 17.6 min; **11**; (14β-H)2β,3β,20R,22R,25-pentahydroxy-5β-cholest-7,9(11)-dien-6-one; ca. 0.75 mg). Peak 3 yielded stachysterone B (**9**; Rt = 14.5 min; 0.75 mg).

*Stachysterone B* (**9**): UV: MeOH (λ<sub>max</sub> = 298 nm); <sup>1</sup>H NMR : δ<sub>H</sub> (D<sub>2</sub>O): 1.00 (3H, s, 19-CH<sub>3</sub>); 1.10 (3H, s, 18-CH<sub>3</sub>); 1.21 (3H, s, 26-CH<sub>3</sub>); 1.23 (3H, s, 27-CH<sub>3</sub>); 1.28 (3H, s, 21-CH<sub>3</sub>); 1.34 (1H, 23-Ha); 1.41 (1H, t, 13.4, 1-H<sub>ax</sub>); 1.50 (1H, td, 13, 3.5, 24-Ha); 1.62 (1H, 12-H); 1.62 (1H, 23-Hb); 1.72 (1H, 4-H<sub>ax</sub>); 1.77 (1H, 4-H<sub>eq</sub>); 1.78 (1H, 24-Hb); 1.80 (1H, 11-H<sub>ax</sub>); 1.87 (1H, 11-H<sub>eq</sub>); 1.88 (1H, 1-H<sub>eq</sub>); 2.24 (1H, dd, 11, 8, 17-H); 2.30 (1H, dd, 16, 8, 16-Hβ); 2.31 (1H, 12-H<sub>eq</sub>); 2.32 (1H, 5-H); 2.53 (1H, dd, 16, 11, 16-Hα); 2.77 (1H, broad m, w<sub>1/2</sub> = 23, 9-H); 3.44 (1H, dd, 10.6, 1.4, 22-H); 3.95 (1H, dt, 11.7, 3.5, 2-H<sub>ax</sub>); 4.05 (1H, broad m, w<sub>1/2</sub> = 8, 3-H<sub>eq</sub>); 6.23 (1H, broad s, w<sub>1/2</sub> = 6.3, 7-H); 6.23 (1H, broad s, w<sub>1/2</sub> = 6.3, 15-H); <sup>13</sup>C NMR: δ<sub>C</sub> (D<sub>2</sub>O): 21.6 (C-21); 21.8 (C-18); 25.4 (C-19); 28.4 (C-23); 30.0 (C-26); 30.9 (C-27); 31.8 (C-16); 33.7 (C-4); 37.7 (C-1); 41.0 (C-9); 41.5 (C-10); 41.8 (C-12); 43.0 (C-24); 50.2 (C-13); 52.7 (C-5); 59.5 (C-17); 69.8 (C-2); 69.9 (C-3); 74.4 (C-25); 80.0 (C-20); 80.0 (C-22); 122.0 (C-7); 133.1 (C-15); 152.0 (C-14); LSIMS (-ve-ion mode, glycerol as matrix) m/z (% RI): 553 (3%, [M+glycerol-H]<sup>-</sup>), 461 (8%, [M-H]<sup>-</sup>), 183 (100%); CI-MS m/z (% RI): 480 ([M+NH<sub>4</sub>]<sup>+</sup>, 1%), 463.6 ([M+H]<sup>+</sup>, 6%), 447.6 ([M-CH<sub>3</sub>]<sup>+</sup>, 2%), 445 ([M+H-H<sub>2</sub>O]<sup>+</sup>, 1%), 429.6 ([M+H-2H<sub>2</sub>O]<sup>+</sup>, 2%), 116.2 ([C-22 → C-27]<sup>+</sup>, 100%).

(14α-H)2β,3β,20R,22R,25-Pentahydroxy-5β-cholest-7,9(11)-dien-6-one (**10**): UV: MeOH (λ<sub>max</sub> = 296 nm); <sup>1</sup>H NMR: δ<sub>H</sub> (D<sub>2</sub>O): 0.79 (3H, s, 18-CH<sub>3</sub>); 1.13 (3H, s, 19-CH<sub>3</sub>); 1.22 (3H, s, 27-CH<sub>3</sub>);

1.23 (3H, s, 26-CH<sub>3</sub>); 1.25 (3H, s, 21-CH<sub>3</sub>); 1.32 (1H, 23Hb); 1.49 (1H, 24-Hb); 1.54 (1H, 4-H<sub>ax</sub>); 1.60 (1H, 15-Hβ); 1.63 (1H, 23-Ha); 1.65 (1H, t, 13.4, 1-H<sub>ax</sub>); 1.79 (1H, 16-Hα); 1.77 (1H, 24-Ha); 1.81 (1H, 4-H<sub>eq</sub>); 1.85 (1H, 16-Hβ); 1.95 (1H, 15-Hα); 1.96 (1H, broad t, 10, 17-H); 2.14 (1H, dd, 13.4, 3.4, 1-H<sub>eq</sub>); 2.37 (1H, dd, 13.3, 3.8, 5-Hβ); 2.49 (1H, broad d, 18.3, 12-H<sub>ax</sub>); 2.62 (1H, broad d, d, 10, 8, 14-Hα); 2.72 (1H, dd, 18.3, 6.7, 12-H<sub>eq</sub>); 3.42 (1H, broad d, 10.5, 22-H); 3.77 (1H, dt, 12.2, 3.4, 2-H<sub>ax</sub>); 3.94 (1H, broad m, w<sub>1/2</sub> = 8.3, 3-H<sub>eq</sub>); 5.71 (1H, broad s, w<sub>1/2</sub> = 4, 7-H); 6.45 (1H, broad d, w<sub>1/2</sub> = 6.7, 11-H); <sup>13</sup>C NMR: δ<sub>C</sub> (D<sub>2</sub>O): 13.9 (C-1); 15.0 (C-18); 21.7 (C-21); 24.0 (C-16); 24.5 (C-15); 28.3 (C-23); 29.9 (C-27); 30.8 (C-26); 32.2 (C-4); 32.9 (C-19); 41.8 (C-10); 43.0 (C-24); 45.2 (C-12); 52.2 (C-17); 52.8 (C-5); 54.5 (C-14); 69.4 (C-3); 70.0 (C-2); 74.1 (C-25); 79.6 (C-22); 80.3 (C-20); 120.2 (C-7); 137.8 (C-11); 138.5 (C-9); LSIMS (-ve-ion mode, glycerol as matrix) m/z (% RI): 553 (1%, [M+glycerol-H]<sup>-</sup>), 461 (10%, [M-H]<sup>-</sup>), 183 (100%); CI-MS m/z (% RI): 480.7 ([M+NH<sub>4</sub>]<sup>+</sup>, 0.3%), 463.6 ([M+H]<sup>+</sup>, 4%), 445.5 ([M+H-H<sub>2</sub>O]<sup>+</sup>, 1%), 346.5 (9%), 344.5 (5%), 116.2 ([C-22 → C-27]<sup>+</sup>, 100%).

(14β-H)2β,3β,20R,22R,25-Pentahydroxy-5β-cholest-7,9(11)-dien-6-one (**11**): UV: EtOH (λ<sub>max</sub> = 298 nm); <sup>1</sup>H NMR: δ<sub>H</sub> (D<sub>2</sub>O): 1.11 (3H, s, 19-CH<sub>3</sub>); 1.180 (3H, s, 18-CH<sub>3</sub>); 1.184 (3H, s, 26-CH<sub>3</sub>); 1.20 (3H, s, 27-CH<sub>3</sub>); 1.245 (3H, s, 21-CH<sub>3</sub>); 1.31 (1H, 23Ha); 1.43 (1H, 4-Hax); 1.46 (1H, 24-Hb); 1.53 (1H, 23-Hb); 1.64 (1H, d, d, 13.7, 12.5, 1-Hax); 1.76 (1H, 24-Ha); 1.78 (1H, 15-Hβ); 1.80 (2H, 16-Hα, Hβ); 1.80 (1H, 4-Heq); 1.98 (1H, m, 15-Hα); 2.01 (1H, t, 8.6, 17-H); 2.13 (1H, dd, 13.7, 4.2, 1-Heq); 2.33 (1H, dd, 19.3, 5.2, 12-Heq); 2.38 (1H, dd, 13.2, 3.7, 5-Hβ); 2.61 (1H, m, 14-Hβ); 2.64 (1H, dd, 19.3, 3.4, 12-Hax); 3.53 (1H, broad d, 10, 22-H); 3.79 (1H, dt, 12.5, 3.5, 2-Hax); 3.96 (1H, m w<sub>1/2</sub> = 8, 3-Heq); 5.89 (1H, broad s, w<sub>1/2</sub> = 4, 7-H); 6.39 (1H, t, broad, 4, 11-H); <sup>13</sup>C NMR: δ<sub>C</sub> (D<sub>2</sub>O): 21.7 (C-21); 25.5 (C-18); 26.8 (C-16); 28.4 (C-23); 29.7 (C-26); 30.6 (C-27); 31.8 (C-15); 31.9 (C-19); 36.9 (C-4); 37.8 (C-1); 40.9 (C-12); 42.3 (C-10); 42.8 (C-24); 46.1 (C-13); 52.4 (C-5); 52.8 (C-17); 55.2 (C-14); 69.4 (C-3); 70.0 (C-2); 74.0 (C-25); 79.2 (C-22); 80.9 (C-20); 122.5 (C-7); 137.5 (C-11); 136.3 (C-9); CI-MS m/z (% RI): 480.7 ([M+NH<sub>4</sub>]<sup>+</sup>, 10%), 463.6 ([M+H]<sup>+</sup>, 100%), 445.5 ([M+H-H<sub>2</sub>O]<sup>+</sup>, 10%), 427.5 (18%), 364.5 (70%), 345.4 (65%), 116.2 ([C-22 → C-27]<sup>+</sup>, 100%).

The cholest-7,9(11)-dien-6-one structures of compounds **10** and **11** were established thanks to 1D and 2D <sup>1</sup>H correlations (COSY and TOCSY) and 2D <sup>1</sup>H-<sup>13</sup>C 2D PFG-HMQC and PFG-HMBC correlation (Girault, 1998; Girault and Lafont, 1988). For compounds **10** and **11** one notes only minor modifications for the A-ring and the side-chain with respect to 20-hydroxyecdysone and the presence of two protons signals in the ethylenic region. The 18-CH<sub>3</sub> signal shows in 2D <sup>1</sup>H-<sup>13</sup>C HMBC a long-range coupling which allows the assignment of 12-C and consequently of 12-H which are *J*-coupled with the ethylenic proton signal at 6.45 ppm (1H, broad d, w<sub>1/2</sub> = 6.7, 11-H) for **10** or 6.39 ppm (1H, t, broad, 4, 11-H) for **11**. The 18-CH<sub>3</sub> signals do not show correlation with ethylenic or hydroxylated carbons, but the presence of an additional coupling with an sp<sup>3</sup> carbon (assigned to 14-C) leads to the exclusion of a double bond at positions 14-15 or 8-14. Moreover, the 19-CH<sub>3</sub> signal shows a long-range correlation in 2D <sup>1</sup>H-<sup>13</sup>C HMBC with an

ethylenic carbon assigned to 9-C. All this evidence is in agreement with the cholest-7,9(11)-dien-6-one structures proposed for compounds **10** and **11**.

The configuration at 14-C was established from the following points. The 14 $\alpha$ -H configuration of **10** was established thanks to a 2D NOESY experiment where there is a strong Noe correlation of the 17-H signal ( $\alpha$ -face) with the 14-H signal and a strong Noe correlation of the 18-CH<sub>3</sub> of the  $\beta$ -face with one 12-H, which was consequently assigned as 12 $\beta$ -H (eq). Subsequently, 12 $\beta$ -H (eq) led to the assignment of the 12 $\alpha$ -H (ax) signal. ( $\alpha$ -face) which presents a strong Noe correlation with the 14-H signal. Moreover, no Noe are observed between the 14-H signal and the 18-CH<sub>3</sub> signal. The 14 $\beta$ -H configuration for **11** was established thanks to a 2D NOESY experiment where one observes a strong Noe for the 18-CH<sub>3</sub> and 15 $\beta$ -H signals (both on the  $\beta$ -face) with the 14-H signal. Moreover, no Noe is observed between the 14-H signal and 17-H signal of the  $\alpha$ -face.

Moreover, the chemical shifts observed for the 18-CH<sub>3</sub> of **10** (0.79 ppm,  $\alpha$ H) and **11** (1.18 ppm 14 $\beta$ -H) are in good agreement with the downfield shift observed for 18-CH<sub>3</sub> of 5 $\beta$ ,14 $\alpha$ -androstane (0.692 ppm) with respect to 5 $\beta$ ,14 $\beta$ -androstane (0.992 ppm) (Jackman and Sternell, 1969).

Bioassay and receptor assays

The agonist and antagonist biological activities of the products were determined with a microplate-based bioassay using the *Drosophila melanogaster* B<sub>II</sub> cell line as described previously (Clément et al., 1993). Prior to bioassay, purity of all ecdysteroid samples was assessed by HPLC and, if necessary, purified to >98%. The ecdysteroid receptor affinities of the three 7,9(11)-dien-6-ones were assessed in an *in vitro* receptor assay using crude extracts of bacterially-expressed DmEcR/DmUSP and CfEcR/CfUSP, as described previously (Dinan et al., 2001).

UV-Irradiation

Samples were irradiated in quartz vessels using a Rayonet Mini-Photochemical reactor (RMR 600; Southern New England Ultraviolet Co., Branford, CT, U.S.A). The dienones **5** – **7** (100 nM) were incubated with the EcR and USP proteins (as above) at 7 °C and allowed to reach equilibrium (3 hours for CfEcR/CfUSP and 16 hours DmEcR/DmUSP). Samples were transferred to quartz tubes and placed in a merry go-round to ensure even exposure to the UV light sources. The irradiation apparatus was operated in a refrigerated room (temperature maintained at 7 °C) and the use of an internal cooling fan ensured a large rise in temperature did not occur during the irradiation process. Samples were irradiated for 2.5 minutes at 300 nm or 20 minutes at 350 nm. Fatty-acid-free bovine serum albumin (0.4 mg) and dextran-coated charcoal (25  $\mu$ l: 500 mg acid-washed charcoal + 50 mg T70 dextran in 20 ml 10 mM Tris/HCl buffer, pH 7.2) was added to each sample and incubated for 5 minutes in ice-cold water before centrifugation at 13,000 x g for 5 minutes. The supernatant from each sample was incubated with [<sup>3</sup>H]pon A (2.6 nM) at 7 °C and allowed to reach equilibrium. A second DCC

assay was completed and aliquots of the supernatant (100  $\mu$ l) were radioassayed after the addition of 1 ml scintillation cocktail (Emulsifier-Safe; Packard Bioscience, [www.lifesciences.perkinelmer.com](http://www.lifesciences.perkinelmer.com)).

Results and Discussion

CoMFA predictions

Biological activity predictions for a range of ecdysteroid dienones were obtained using two CoMFA models based on a training set of 71 ecdysteroids (Dinan et al., 1999). Both models utilised the region-focussed electrostatic indicator field, but Model B also used the region-focussed steric indicator field. There is very good correlation between the predicted and observed EC<sub>50</sub> values for the training set in both models (A: r<sup>2</sup> = 0.903; B: r<sup>2</sup> = 0.892), while the prediction capacity of models was found to be good (A: q<sup>2</sup> = 0.631; B: q<sup>2</sup> = 0.694; Dinan et al., 1999). EC<sub>50</sub> predictions are presented in Table 1. For the 7,9(11)-dien-6-ones (**5** - **7**), the models are not in agreement, in terms of either the level or order of activity. However, both models predict that the activities of these dienones will be higher than that of 20E (**1**), which has an EC<sub>50</sub> of 7.5 x 10<sup>-9</sup>M. Both models predict a similarly high activity for the 8,14-dien-6-one, podescdysone B (**8**), but in reality this compound has a very low activity. The UV-spectrum of this compound ( $\lambda_{max}$  = 244 nm) also precludes it from consideration as a photoaffinity ligand. Both models predict that the activity of stachysterone B (**9**), a 7,14-dien-6-one, would be similar to those of the 7,9(11)-dien-6-ones.

The dehydration reaction

The three ecdysteroids used for the dehydration reaction (**2** - **4**) have all been isolated from plant sources (Usmanov et al., 1975;

**Table 1:** Predicted and determined biological activities (EC<sub>50</sub> values) of relevant ecdysteroids in the ecdysteroid agonist version of the *Drosophila melanogaster* B<sub>II</sub> cell bioassay.

EC <sub>50</sub> Ecdysteroid ( $\lambda_{max}$ nm <sup>#</sup> )	Predicted EC <sub>50</sub>		Determined
	Model A	Model B	
<b>1:</b> 20-hydroxyecdysone (242) <sup>9</sup> M*			7.5x10 <sup>-9</sup>
<b>2:</b> ajugasterone C (243) <sup>8</sup> M*			8.0x10 <sup>-9</sup>
<b>3:</b> muristerone A (236) <sup>8</sup> M*			2.2x10 <sup>-9</sup>
<b>4:</b> turkesterone (244) <sup>9</sup> M*			1.3x10 <sup>-9</sup>
<b>5:</b> dacryhainansterone (235 & 299) <sup>9</sup> M	1.8x10 <sup>-9</sup> M	5.6x10 <sup>-11</sup> M	5.2x10 <sup>-9</sup>
<b>6:</b> kaladasterone (299) <sup>7</sup> M	1.2x10 <sup>-9</sup> M	2.6x10 <sup>-10</sup> M	3.4x10 <sup>-9</sup>
<b>7:</b> 25-hydroxydacryhainansterone (299) <sup>7</sup> M	3.0x10 <sup>-9</sup> M	5.9x10 <sup>-11</sup> M	2.6x10 <sup>-9</sup>
<b>8:</b> podescdysone B (244) <sup>5</sup> M	4.6x10 <sup>-11</sup> M	1.1x10 <sup>-11</sup> M	1.2x10 <sup>-9</sup>
<b>9:</b> stachysterone B (298) <sup>8</sup> M	2.0x10 <sup>-9</sup> M	2.2x10 <sup>-10</sup> M	8.2x10 <sup>-9</sup>
<b>10:</b> (14 $\alpha$ -H)7,9(11)-dien-6-one (296) <sup>7</sup> M			2.2x10 <sup>-9</sup>
<b>11:</b> (14 $\beta$ -H)7,9(11)-dien-6-one (298) <sup>6</sup> M			1.3x10 <sup>-9</sup>

\*Data taken from Dinan et al., 1999  
#in methanol

Canonica et al., 1972; Imai et al., 1969), as have two of the intended dehydration products (**5** [Hou et al., 1982] and **6** [Canonica et al., 1973]). Dacryhainansterone (**5**) has also been synthesised previously in a dehydration reaction from ajugasterone C (Qui and Nie, 1983; Szendrei et al., 1988). Dehydration reactions were monitored by UV-spectrophotometry and a shift in the  $\lambda_{\max}$  from 242 nm to 300 nm indicated the formation of the conjugated dienone. Optimum yields occurred after 40 min and no further increase was noted up to 2 h. HPLC analysis indicated the presence of small quantities of unreacted starting material remained, so purification was necessary. Overall, the three reactions proceeded cleanly and produced yields in excess of 60%.

#### Spectroscopic identification of the 14 $\alpha$ -hydroxy conjugated dienones

In addition to the characteristic UV-spectra, the dienone products were identified unambiguously by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. NMR data for the three parent ecdysteroids have been published previously along with  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for **5** (Lafont and Wilson, 1996; Volodin et al., 1998), allowing direct comparisons with the data for the dehydration products. The introduction of the 9(11)-double bond resulted in a loss of the signal for H-9 ( $\delta$ 3.1) and a large downfield shift for the H-11 signal (from  $\delta$ 4.1 to  $\delta$ 6.3) in the  $^1\text{H}$  spectra. Similarly, large shifts in the  $^{13}\text{C}$  spectra were observed for both C-9 (from  $\delta$ 43 to  $\delta$ 135) and C-11 (from  $\delta$ 69 to  $\delta$ 132). NMR data are summarised in the Experimental section.

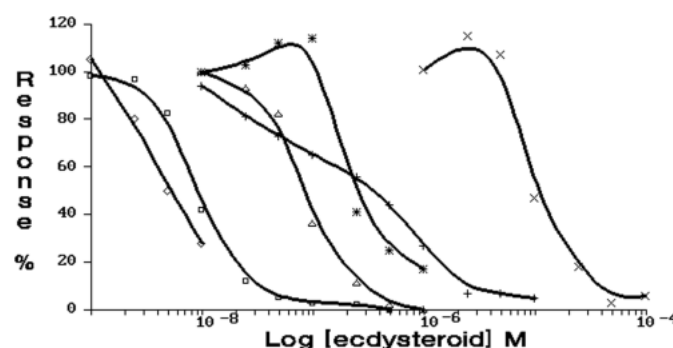
#### Acid treatment of 20-hydroxyecdysone

In addition to the expected products of podescdysone B and stachysterone B, this reaction yielded the previously unknown ecdysteroids **10** and **11**. We hypothesize these compounds arise by sequential rearrangements of the diene following dehydration at C-14: 7,14-dien-6-one (stachysterone B; **9**)  $\rightarrow$  8(9),14-dien-6-one (podescdysone B; **8**)  $\rightarrow$  8(14),9(11)-dien-6-one (hypothetical intermediate)  $\rightarrow$  (14 $\alpha$ -H)7,9(11)-dien-6-one (**10**) + (14 $\beta$ -H)7,9(11)-dien-6-one (**11**).

#### Biological activity

$B_{11}$  bioassay data (Table 1) demonstrate that the three 14 $\alpha$ -hydroxy conjugated dienones all displayed high levels of agonist activity in the sequence dacryhainansterone > 25-hydroxydacryhainansterone > kaladasterone. Both dacryhainansterone and 25-hydroxydacryhainansterone displayed higher activities than the 11 $\alpha$ -hydroxyecdysteroids from which they were derived, but the converse was true for kaladasterone. The activity curve for kaladasterone appears bi-phasic when compared to the other ecdysteroids (Fig. 2). Although the activity levels were lower than those predicted by both CoMFA models, the order of activity was correctly predicted by model B and dacryhainansterone (**5**) had a slightly higher activity than 20E (**1**). It is interesting to note that earlier structure-activity relationship studies suggested the presence of a 5 $\beta$ -hydroxyl group and the absence of a C-25 hydroxyl group should potentiate biological activity (Dinan, 1989), and the CoMFA data from Model A predicted that kaladasterone should display the highest biological activity. However, kaladasterone actually exhibited the lowest  $\text{EC}_{50}$  value, suggesting that the presence of a 5 $\beta$ -hydroxyl together with

the absence of a 25-hydroxyl somehow result in an interaction, which is detrimental to activity. Further evidence for this can be seen with 25-deoxypolypodine B (Dinan et al., 1999). Polypodine B contains both 5 $\beta$  and C-25 hydroxyl groups and has an  $\text{EC}_{50}$  in the  $B_{11}$  bioassay of  $1.0 \times 10^{-9}\text{M}$ . 25-Deoxypolypodine B lacks the C-25 hydroxyl group, but is otherwise structurally identical to polypodine B and subsequently a higher  $\text{EC}_{50}$  could be assumed. However the  $\text{EC}_{50}$  of this compound was actually 10-fold lower ( $1.0 \times 10^{-8}\text{M}$ ). The discovery of such an interaction may also explain the bi-phasic effect seen in the biological activity curve for kaladasterone. Absence of a 14 $\alpha$ -hydroxyl group (compound **10**) did not affect the biological activity significantly (compare **7** and **10**), which is in accord with recent findings (Harmatha et al., 2002) which showed that the configuration at C-14 is more important than the substituent (14-H, 14-OH or 14-OOH; compare **10** and **11**).



**Figure 2:** Concentration-dependence of agonist activity of selected ecdysteroids in the *Drosophila melanogaster*  $B_{11}$  cell bioassay: 20-hydroxyecdysone (**1**:  $\square$ ), dacryhainansterone (**5**:  $\Delta$ ), kaladasterone (**6**:  $+$ ), 25-hydroxydacryhainansterone (**7**:  $*$ ), podescdysone B (**8**:  $'$ ) and stachysterone B (**9**:  $\Delta$ ). Data points are the means of 4 replicates.

Both CoMFA models dramatically overpredict the activity of podescdysone B, and also overpredict the activity of stachysterone B. This probably arises from the lack of structural variety in the region of C-8 to C-11 amongst the members of the CoMFA training set.

#### Stability of receptor complexes to irradiation with UV light

Ecdysteroid receptor complexes (CfEcR/CfUSP) were irradiated with UV light at 300 or 350 nm and the level of specific binding capacity for [ $^3\text{H}$ ]pon A (2.6 nM) remaining was determined (Fig. 3). Irradiation at 350 nm for 20 min left 87% of the specific binding capacity intact, while irradiation at 300 nm resulted in substantial degradation even by 5 min. Subsequent experiments involved receptor irradiation for 20 min at 350 nm or 2.5 min at 300 nm.

#### Stability of dacryhainansterone to irradiation with UV light

Samples of **5** (50  $\mu\text{g}$ ) were dissolved in water (100  $\mu\text{l}$ ) and irradiated at 350 nm for up to 3 h or at 300 nm for up to 1 h. Aliquots of the solutions (10  $\mu\text{l}$ ) were assessed by gradient RP-HPLC with monitoring by photo-diode array detector. Dacryhainansterone was unaffected by irradiation under these conditions (data not shown).

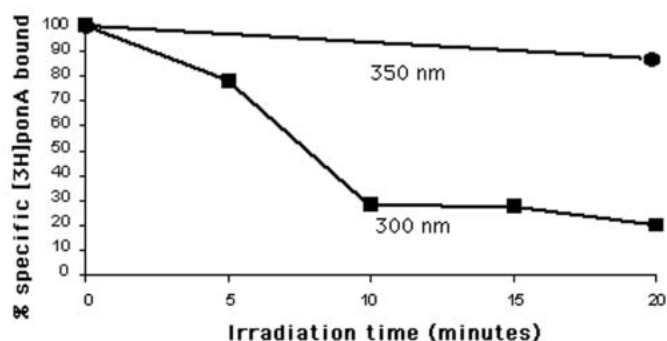
#### Irradiation of receptor proteins in the presence of ecdysteroid dienones



Ecdysteroid receptor complexes were incubated with the dienone (100 nM) and irradiated at 350 nm for 20 mins. Unbound steroid was removed with DCC and then the receptor proteins were incubated with [<sup>3</sup>H]ponA (2.55 nM ± 100-fold excess unlabelled ponA) to determine the remaining specific binding. The results are presented graphically in Fig. 4A for DmEcR/DmUSP and Fig. 4B for CfEcR/CfUSP and compared to the  $K_d$  values for the dienones with the dipteran and lepidopteran receptor proteins (Table 2) and their  $EC_{50}$  values in the  $B_{II}$  bioassay (Table 1). These results indicate that the blocking efficiency is related to the affinity of the dienone for the receptor.

Irradiation of receptor proteins in the presence of 20-hydroxyecdysone or dactryhainansterone

Ecdysteroid receptor complexes were incubated with 20-hydroxyecdysone or dactryhainansterone (100 nM) and irradiated at 350 nm for 20 mins. Unbound steroid was removed with DCC and the receptor proteins were then incubated with [<sup>3</sup>H]ponA (2.55 nM) to determine the remaining binding capacity. The results are presented graphically in Fig. 5A for DmEcR/DmUSP and Fig. 5B for CfEcR/CfUSP. 20-Hydroxyecdysone and dactryhainansterone had very similar  $EC_{50}$  values in the  $B_{II}$  bioassay:  $7.5 \times 10^{-9}$ M and  $5.2 \times 10^{-9}$ M, respectively. Following irradiation with 20-hydroxyecdysone the specific binding capacity for [<sup>3</sup>H]ponasterone A was only slightly reduced (which may reflect incomplete removal of 20E by the DCC), whereas after irradiation with dactryhainansterone, the binding capacity was significantly reduced. This is in accord with the dienone 5 blocking the binding site by the production of covalent linkages.



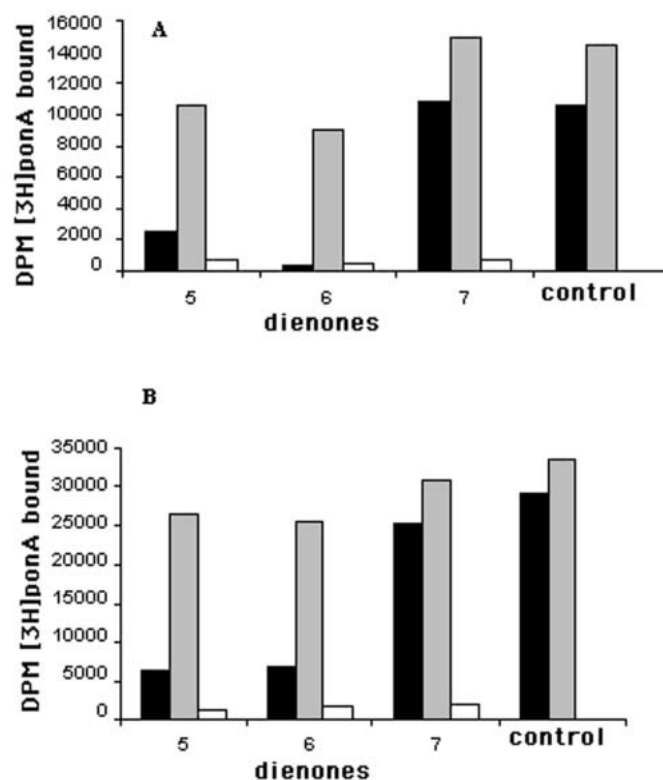
**Figure 3:** Stability of the ligand binding capacity of CfEcR/CfUSP complexes to irradiation at 300 (squares) or 350 nm (circles). Receptor complexes were irradiated in the absence of ligand and remaining specific binding was determined by subsequent addition of [<sup>3</sup>H]ponasterone A (± 250-fold excess of unlabelled ponasterone A). Results shown are the mean values of duplicate samples; variation between replicates is 5% or less.

## Implications and prospects

Molecular modelling has considerable potential for identifying high affinity ligands for targeted synthesis, and thus may save a large amount of synthetic effort. The CoMFA models accurately predicted that 7,9(11)-dien-6-ones would retain high biological activity even if both models did overpredict their activities. In contrast, both models predicted that podedcysone B (8) should be a highly active molecule, when it in fact possesses a rather low activity. Inclusion

of the data for the various dienones into the CoMFA analysis should result in models with improved prediction capabilities.

Ecdysteroid 7,9(11)-dien-6-ones were pursued as photoaffinity labels, based on their UV-spectra and high agonist activity in the  $B_{II}$  bioassay. Amongst these, dactryhainansterone appears the most promising, because it possessed higher activity than 25-hydroxydactryhainansterone and because it did not show the biphasic activity curve presented by kaladasterone. However, the covalent blocking efficiencies of these 3 dienones indicate that kaladasterone may be more effective than dactryhainansterone for DmEcR/DmUSP and as effective for CfEcR/CfUSP. The covalent blocking of the ligand binding site of ecdysteroid site is specific, as no blocking



**Figure 4:** Effect of irradiation (350 nm for 20 min) in the presence/absence of ecdysteroid dienones (5: dactryhainansterone, 6: kaladasterone and 7: 25-hydroxydactryhainansterone; each at 100 nM) on the capacity of DmEcR/DmUSP (A) and CfEcR/CfUSP (B) ecdysteroid receptor complexes subsequently to bind [<sup>3</sup>H]ponasterone A specifically. Solid bars - UV + [<sup>3</sup>H]ponasterone A; shaded bars - no UV + [<sup>3</sup>H]ponasterone A; open bars - no UV + [<sup>3</sup>H]ponasterone A + a 250-fold excess unlabelled ponasterone A. Results shown are the mean values of duplicate samples; variation between replicates is 7% or less.

was seen on irradiation of receptor complexes with 20E, and of very high efficiency in the cases of dactryhainansterone and kaladasterone. 25-Hydroxydactryhainansterone gives a much lower blocking efficiency, in line with its lower affinity for the LBD of the EcR/USP complex. We are currently generating side-chain labelled [<sup>3</sup>H]dactryhainansterone to assess directly the photoaffinity labelling ability of ecdysteroid dienones. This will provide a valuable tool for probing the ligand-binding domain of ecdysteroid receptors from insect and other arthropods, which may reveal differences between pest and non-pest species that might be exploited in the design of more specific pest control agents. Also, the availability of an effective

ecdysteroid affinity label could be used to search for ecdysteroid receptor proteins in invertebrate species of other Classes (e.g.

**Table 2:** Comparison of the apparent irradiation-induced covalent blocking efficiency (CBE) of ecdysteroid dienones with the  $K_d$  values for each compound with DmEcR/DmUSP and CfEcR/CfUSP.

Dienone	CfEcR/CfUSP		DmEcR/DmUSP	
	CBE	$K_d$	CBE	$K_d$
dacryhainansterone (5) <sup>9</sup> M	78%	$5 \times 10^{-9}$ M	77%	$1.3 \times 10^{-9}$ M
kaladasterone (6) <sup>10</sup> M	79%	$1.3 \times 10^{-9}$ M	97%	$3.6 \times 10^{-10}$ M
25-hydroxydacryhainansterone (7) <sup>7</sup> M	13%	$2.7 \times 10^{-7}$ M	0%	$2.3 \times 10^{-7}$ M

nematodes), where previous physiological, biochemical and molecular biological investigations on the hormonal role of ecdysteroids have been inconclusive. This could shed further light on whether arthropods and nematodes form a Ecdysozoa clade (Manuel et al., 2000).

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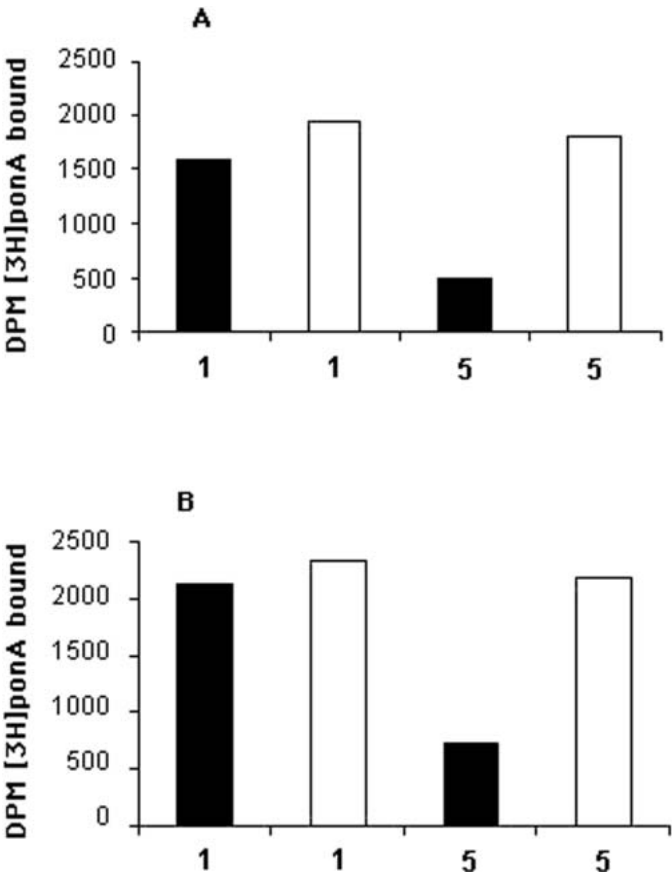
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**Figure 5:** Effect of irradiation (350 nm for 20 min) in the presence of 100 nM 20-hydroxyecdysone (1) or dacryhainansterone (5) on the capacity of DmEcR/DmUSP (A) and CfEcR/CfUSP (B) ecdysteroid receptor complexes subsequently to bind [<sup>3</sup>H]ponasterone A; Filled bars, samples irradiated at 350 nm for 20 minutes; open bars samples not irradiated. Results shown are the mean values of duplicate samples; variation between replicates is 5% or less.

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