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Synthesis and biological activities of turkesterone 11α-acyl derivatives

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Abstract

Turkesterone is a phytoecdysteroid possessing an 11α-hydroxyl group. It is an analogue of the insect steroid hormone 20-hydroxyecdysone. Previous ecdysteroid QSAR and molecular modelling studies predicted that the cavity of the ligand binding domain of the ecdysteroid receptor would possess space in the vicinity of C-11/C-12 of the ecdysteroid. We report the regioselective synthesis of a series of turkesterone 11α-acyl derivatives in order to explore this possibility. The structures of the analogues have been unambiguously determined by spectroscopic means (NMR and low-resolution mass spectrometry). Purity was verified by HPLC. Biological activities have been determined in *Drosophila melanogaster* B_{II} cell-based bioassay for ecdysteroid agonists and in an *in vitro* radioligand-displacement assay using bacterially-expressed *D. melanogaster* EcR/USP receptor proteins. The 11α-acyl derivatives do retain a significant amount of biological activity relative to the parent ecdysteroid. Further, although activity initially drops with the extension of the acyl chain length (C_2 to C_4), it then increases (C_6 to C_{10}), before decreasing again (C_{14} and C_{20}). The implications of these findings for the interaction of ecdysteroids with the ecdysteroid receptor and potential applications in the generation of affinity-labelled and fluorescently-tagged ecdysteroids are discussed.

Keywords: steroid, ecdysteroid, 20-hydroxecdysone, QSAR, steroid hormone receptor

Abbreviation:

CoMFA	comparative molecular field analysis
DCM	dichloromethane
DMF	dimethylformamide
DMP	2,2-dimethoxypropane
4D-QSAR	4-dimensional quantitative structure-activity relationship
EcR	ecdysteroid receptor
EcRE	ecdysteroid response element
HPLC	high-performance liquid chromatography
LBD	ligand-binding domain
NMR	nuclear magnetic resonance
ponA	ponasterone A
QSAR	quantitative structure-activity relationship
RXR	retinoid X receptor

Abbreviations continued on next page.

Abbreviations continued from previous page.

SAR	structure-activity relationship
SPE	solid-phase extraction
THF	tetrahydrofuran
TLC	thin-layer chromatography
p-TsOH	*para*-toluenesulphonic acid
USP	ultraspiracle
UV-VIS	ultraviolet-visible

Introduction

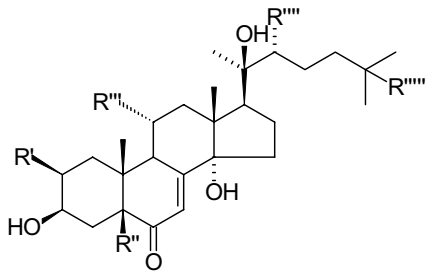
Ecdysteroids are the steroid hormones of arthropods and possibly of other invertebrate phyla too. They also occur in certain plant species, where they are known as phytoecdysteroids and are believed to contribute to the deterrence of invertebrate predators (Dinan, 2001). In insects, they regulate moulting and metamorphosis and have been implicated in the regulation of reproduction, diapause etc. (Koolman, 1989). Most actions of ecdysteroids are mediated by intracellular receptor complexes, which regulate gene expression in a tissue- and development-specific manner. In *Drosophila melanogaster*, the receptor complex consists of the ecdysteroid receptor (EcR) protein, of which there are 3 isoforms (Koelle et al., 1991; Talbot et al., 1993), and the Ultraspiracle (Usp) protein, which is a homologue of the vertebrate retinoid X receptor (RXR) protein (Oro et al., 1990). Both EcR and Usp are members of the nuclear receptor superfamily (Laudet, 1997). EcR binds ligand with high affinity only when it is complexed to Usp (Yao et al., 1993). It is not known for certain if there is a natural ligand for Usp, although it has been suggested that juvenile hormone might be a possible candidate (Jones and Jones, 2000). Owing to the critical role that the ecdysteroid system plays in regulating insect development, there is considerable interest in characterising ligand/receptor/EcRE interactions from different insect Orders and to exploit the differences to develop Order-specific pest control agents. The selectivity of the bisacylhydrazines, non-steroidal agonists, is a good example of what can be achieved (Dhadialla et al., 1998). Further, because the ecdysteroid receptor is specific to invertebrates and ecdysteroids appear to be non-toxic to vertebrates and plants (Sláma and Lafont, 1995), there is growing interest in the use of ecdysteroid systems in transgenic gene-switch systems in vertebrate or plant cells to generate medically or agronomically effective systems (Fussenegger, 2001; Lafont and Dinan, 2003).

In recent years, we have conducted an extensive SAR for ecdysteroids in a homogeneous bioassay system, based on the ecdysteroid-responsive *l(2)mbn* (B_{II}) permanent cell line of *Drosophila melanogaster* (Clément et al., 1993). The response of these cells reflects the affinity of the EcR/USP complex for the ecdysteroid analogue and can be measured in a simple microplate-based assay. In conjunction with molecular modelling methods (CoMFA and 4D-QSAR; Dinan et al., 1999; Ravi et al., 2001), SAR of an extensive range of pure ecdysteroid analogues has provided testable predictions for ecdysteroid interaction with the LBD and generated the first pharmacophore hypothesis for ecdysteroid binding. Amongst the predictions to come out of the CoMFA/4D-QSAR analyses was that a region capable of accommodating steric bulk was present around the location of C-11/C-12 of the ecdysteroid molecule. We have tested this hypothesis by generating a series of 11α-acyl derivatives of the phytoecdysteroid turkesterone (Figure 1: structure **1**) and determining their biological activities relative to the parent ecdysteroid.

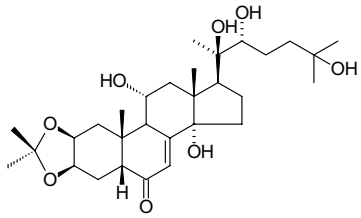
Materials and Methods

Chemicals

Turkesterone (**1**) was isolated from *Ajuga turkestanica* (Usmanov et al., 1975). 20-Hydroxyecdysone (**6**) was supplied by Dr. V. Volodin (Institute of Biology, Russian Academy of Sciences). Muristerone A (**9**) was purchased from Simes spa, Italy. Other ecdysteroids were generous gifts from other researchers (see acknowledgements in Dinan et al., 1999). Acyl anhydrides were purchased from Sigma/Aldrich. Solvents were analytical or HPLC-



	R'	R''	R'''	R''''	R'''''
1: turkesterone	-OH	-H	-OH	-OH	-OH
2: turkesterone 2-acetate	-OAc	-H	-OH	-OH	-OH
3: turkesterone 22-acetate	-OH	-H	-OH	-OAc	-OH
4: turkesterone 2, 11-diacetate	-OAc	-H	-OAc	-OH	-OH
5: turkesterone 11,22-diacetate	-OH	-H	-OAc	-OAc	-OH
6: 20-hydroxyecdysone	-OH	-H	-H	-OH	-OH
7: ponasterone A	-OH	-H	-H	-OH	-H
8: ajugasterone C	-OH	-H	-OH	-OH	-H
9: muristerone A	-OH	-OH	-OH	-OH	-H



10: 2,3-isopropylidene turkesterone

Figure 1. Structures of selected ecdysteroids. See Figure 2 for structures of further ecdysteroids mentioned in the text.

grade and used without further purification, with the exception of pyridine, which was dried over sodium hydroxide pellets for at least 24 h before use. All other chemicals were obtained from Sigma-Aldrich Co. (Poole, Dorset, U.K.) or Lancaster Synthesis (Morecambe, Lancs., U.K.).

NMR

^{1}H and ^{13}C nuclear magnetic spectroscopy was performed on a Varian Unity 300 spectrometer (at CSIC, Barcelona) or on a Bruker DMX500 MHz spectrometer (NMR Service, University of Barcelona). Samples were dissolved in the indicated solvent. Chemical shifts are given in ppm and the coupling constants and widths at half height ($\omega_{1/2}$) are given in Hz. Spectral assignments were achieved by comparison to published data where available (Lafont et al., 2002).

Mass spectrometry

CIMS and FABMS spectra were recorded at the Ecole Normale Supérieure (Paris, France) on a Jeol MS 700 spectrometer equipped with a direct inlet probe. CIMS spectra were recorded in chemical ionisation/desorption mode using ammonia as the reagent gas.

UV spectrophotometry

Ultra-violet spectra of ecdysteroids were determined in methanol using a Shimadzu UV-2401 PC UV-VIS dual-beam spectrophotometer and quartz cells (Hellma, Essex, U.K.). Extinction coefficients were obtained from Lafont et al. (2002).

Thin-layer chromatography

TLC was performed on Merck 60 F_{254} pre-coated 10 x 10 cm plates (BDH, Poole, U.K.), with $CHCl_3$/EtOH (9:1 v/v) for development and visualisation by fluorescence quenching under short-wavelength UV light.

Solid-phase extraction

Reversed-phase (C_{18}; 0.5g) Sep-Pak cartridges (Waters/ Millipore, Watford, U.K.) were activated with methanol (5 mL), followed by water (10 mL). The sample was applied in 10% aq. MeOH and then the column was sequentially eluted with 5 mL portions of 10%, 25% and 100% aq. MeOH.

HPLC

Two HPLC systems were employed. The analytical system consisted of a Gilson model 811 HPLC coupled with a Gilson 160 diode-array detector and controlled by Gilson Unipoint program. The semi-preparative system consisted of two Gilson model 303 pumps, a model 802c manometric module, a mixing unit, a Holochrome variable wavelength detector and Rheodyne 7125 injector block. Solvent composition and flow was controlled by an Apple IIe computer with Gilson Gradient Manager program. Elution profiles were monitored at 242 nm. Three types of column were used: i) Spherisorb reversed-phase octadecyl (ODS2) column (analytical: 150 mm x 4.6 mm i.d., 5 μm particle size; semi-preparative: 250 mm x 10 mm i.d. 5 μm particle size), ii) normal-phase Zorbax SIL column (analytical: 250 mm x 4.6 mm i.d. 5 μm particle size), iii) normal-phase Apex II DIOL column (analytical: 250 mm x 4.6 mm i.d., 5 μm particle size). Columns were purchased from Jones Chromatography, Hengoed, Wales.

Table 1. ^{1}H NMR data for turkesterone and turkesterone acetates. Spectra were obtained in $CDCl_3$ on a Varian Unity 300 spectrometer (300 MHz).

	1*	**2**	**4**	**5#**
1ax-H	1.38			1.40
1eq-H	2.59			1.66
2ax-H	4.01	5.13	5.28	4.04
3eq-H	3.95	4.11	4.18	4.07
4ax-H	1.78			1.66
4eq-H	1.68			1.86
5-H	2.33		2.52	2.40
7-H	5.80	5.87	5.93 d	5.89 d
9-H	3.15	3.09	3.42	3.30
11-H	4.10	4.11	5.28	5.29
12ax-H	2.22			2.35
12eq-H	2.16			2.05
15a-H	1.95			2.06
15b-H	1.58			1.58
16a-H	2.00			2.00
16b-H	1.75			1.87
17-H	2.43			2.35
18-Me	0.88	0.878	0.925	0.884
19-Me	1.06	1.115	1.094	1.043
21-Me	1.19	1.257	1.216	1.246
22-H	3.32	3.44	3.42	4.83
23a-H	1.66			1.48
23b-H	1.29			1.68
24a-H	1.80			1.46
24b-H	1.43			1.38
26-Me	1.21	1.274	1.273	1.212
27-Me	1.22	1.274	1.268	1.189
Acetyl -CH_3	-	2.098	2.100	2.102
			2.011	2.098

*Data taken from Vokác et al. (1998; in CD_3OD)
#Spectra obtained on a Bruker DMX500 spectrometer (500 MHz) (solvent signal centered at δ 7.24 [at 7.27 for the Varian spectrometer]).

Synthesis of 11α-acyl derivatives of turkesterone

Conversion of turkesterone (**1**) to 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**)

The procedure was modified from Guédin-Vuong et al. (1985). A solution of turkesterone (10 mg; 20.8 μmol) and phenylboronic acid (2.7 mg, 22.1 μmol; 1.1 eq) in dry DMF (500 μL) was stirred at room temperature for two hours. Dry acetone (3.5 mL), dry DMP (200 μL) and fused *p*-TsOH (1 mg; 10.4 μmol; 0.5 eq) were then added and the reaction was stirred for a further 3 hours. Acetone and DMP were evaporated under nitrogen and 2 mL ethyl acetate was added to the remaining DMF. This mixture was partitioned three times with saturated aqueous sodium chloride solution leaving the 2,3-isopropylidene turkesterone 20,22-phenylboronate in the ethyl acetate phase. The product was assessed for purity by TLC ($CHCl_3$/EtOH 9:1, v/v, Rf **12**: 0.52; cf Rf **1**: 0.04) and used without further purification or assessment of yield. This reaction was repeated many times with acyl derivatization at C-11α occurring as a third step without complete purification of the

Table 2. ^{13}C NMR data for turkesterone and turkesterone acetates. Spectra were obtained in $CDCl_3$ on a Varian Unity 300 spectrometer (75 MHz).

	1*	2#	4	5
C-1	39.1	38.4	34.8	38.4
C-2	68.9	68.6	71.2	67.7
C-3	68.6	64.9	65.4	67.5
C-4	33.3	31.8	31.2	31.5
C-5	55.8	50.7	50.5	50.7
C-6	206.7	203.3	202.0	202.3
C-7	122.7	121.8	123.1	123.1
C-8	165.7	162.7	160.4	160.5
C-9	42.9	42.0	38.2	38.2
C-10	39.9	38.8	39.3	39.3
C-11	69.5	72.0	71.2	71.1
C-12	43.8	42.6	37.4	37.5
C-13	ca 49	47.3	46.9	47.2
C-14	84.9	83.9	84.2	84.1
C-15	31.9	31.0	31.7	31.7
C-16	21.5	20.5	20.4	20.5
C-17	50.4	48.8	48.8	49.3
C-18	18.9	18.0	18.0	18.0
C-19	24.6	23.9	23.9	23.8
C-20	77.8	76.2	76.8	76.6
C-21	21.0	20.6	20.7	21.2
C-22	78.4	76.4	76.4	79.3
C-23	27.4	26.0	26.0	24.8
C-24	42.4	41.1	40.6	40.2
C-25	71.3	70.0	71.0	70.6
C-26	29.7	29.5	30.0	30.2
C-27	29.0	29.3	29.5	28.7
acetyl -CH3	-	21.1	21.4	21.1
			21.2	21.7
acetyl >C=O	-	170.6	170.3	170.3
			170.0	172.4

*Data taken from Vokác et al. (1998)

spectra obtained in $CDCl_3$/Pyr

intermediates.

Conversion of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**) to 2,3-isopropylidene turkesterone (**10**)

To a solution of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**: synthesised from 10 mg turkesterone **1**) in a (9:1) mixture of THF/H_2O (2 mL) was added H_2O_2 (30 %; 1 mL), which had been previously adjusted to pH 7 with 0.1M NaOH. This mixture was stirred at room temperature for 2 hours. The reaction was quenched by the addition of H_2O (2 mL) and the THF was removed under reduced pressure. The residue was purified through a 0.5 g C_{18} reversed-phase Sep-Pak cartridge and assessed on TLC ($CHCl_3$/EtOH, 9:1, v/v; Rf **10**: 0.24, cf Rf **12**: 0.52). The product could be used directly in acetylation reactions without further purification.

Conversion of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**) to 11α-acyl turkesterone (**14**)

i) turkesterone 11α-acetate (**14a**)

To a solution of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**: synthesised from 5 mg turkesterone [**1**]) in dry pyridine (800 µL) at room temperature was added acetic anhydride (400 µL). The reaction was stirred at room temperature for 1 hour and quenched with the addition of H_2O (20 mL). The reaction mixture was pre-purified on a SPE cartridge and the 100% MeOH fraction was dried under nitrogen. The residue was dissolved in dioxan (700 µL) and aqueous HCl (0.1M, 700 µL) was added dropwise. The reaction mixture was stirred at room temperature for 4 hours and diluted with H_2O (20 mL). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30 %-100 % MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**12**) = 24.4 min, Rt (**14a**) = 8.3 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [45:55 v/v], 2 mL/min) and yielded turkesterone 11α-acetate (**14a**: 1.61 mg; 3.0 µmol; 30% yield).

CIMS: 556 ([M+NH_4]$^+$, 18%), 538 ([M]$^+$, 3%), 521 ([M+H-H_2O]$^+$, 10%), 503 ([M+H-2H_2O]$^+$, 8%), 479 ([M+H-AcOH]$^+$, 100%), 461 ([M+H-AcOH-H_2O]$^+$, 34%), 443 ([M+H-AcOH-2H_2O]$^+$, 13%), 425 (6%), 345 (9%)

^1H and ^{13}C NMR: see Tables 3 and 4.

ii) turkesterone 11α-propionate (**14b**)

To a solution of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**: synthesised from 5 mg turkesterone [**1**]) in dry pyridine (800 µL) at room temperature was added propionic

Table 3. ^1H NMR data for turkesterone 11α-acyl analogues. Spectra were obtained in $CDCl_3$ on a Varian Unity 300 spectrometer (300 MHz), unless otherwise indicated

	14a#$	14b@	14c	14d*	14e	14f	14g	14h
1ax-H	1.42			1.39				
1eq-H	1.72			1.70				
2ax-H	4.00	3.90	4.06	4.04	4.07	4.06	4.06	4.06
3eq-H	3.97	3.85	4.08	4.06	4.09	4.08	4.08	4.08
4ax-H	1.64			1.66				
4eq-H	1.83			1.86				
5-H	2.42		2.40	2.40				2.40
7-H	5.87	5.81	5.91	5.89	5.92	5.92	5.92	5.92
9-H	3.47	3.14	3.32	3.30	3.33	3.32	3.32	3.33
11-H	5.32	5.12	5.33	5.31	5.33	5.32	5.32	5.33
12ax-H	2.35			2.35				
12eq-H	2.16			2.05				
15a-H	2.03			2.05				
15b-H	1.49			1.53				
16a-H	2.11			2.06			2.35	2.35
16b-H	1.89			1.75				
17-H	2.45		2.34	2.35				2.36
18-Me	0.92	0.680*	0.926	0.9	0.93	0.93	0.93	0.932
19-Me	1.02	0.835*	1.069	1.05	1.07	1.07	1.07	1.073
21-Me	1.17	0.997*	1.214	1.19	1.22	1.21	1.22	1.221
22-H	usp	3.25	3.42	3.41	3.44	3.43	3.43	3.44
23a-H	1.32			1.36				
23b-H	1.67			1.60				
24a-H	1.75			1.72				
24b-H	1.45			1.60				
26-Me	1.19	0.986*	1.262	1.242	1.27	1.26	1.26	1.26
27-Me	1.20	0.986*	1.258	1.237	1.27	1.26	1.26	1.26
Acyl -CH_3	2.11	0.911	0.978	0.884	0.88	0.88	0.88	0.886
Acyl -CH_2-			2.34	2.33		2.35	2.35	2.36
				1.67				
				1.32				
				1.31				

Spectra obtained on a Bruker Advance DRX400 spectrometer (400 MHz)
*Spectra obtained on a Bruker DMX500 spectrometer (500 MHz) (solvent signal centered at δ7.24)
@ Spectra obtained in D_2O
usp: under solvent peak

Table 4. ^{13}C NMR data for turkesterone 11α-acyl analogues

	14a[#]	**14d***	**14f**[&]
C-1	37.54	38.37	38.39
C-2	67.31	67.65	67.63
C-3	67.21	67.41	67.46
C-4	31.39	31.50	31.50
C-5	51.06	50.67	50.68
C-6	203.90	202.4	202.34
C-7	122.14	123.07	123.08
C-8	162.79	160.60	160.54
C-9	38.29	38.24	38.26
C-10	38.88	39.28	39.30
C-11	71.37	70.79	70.90
C-12	37.48	37.40	37.41
C-13	usp	47.00	47.02
C-14	83.05	84.25	84.27
C-15	30.30	31.79	31.90
C-16	20.11	20.39	20.40
C-17	48.97	48.82	48.85
C-18	17.11	17.95	17.96
C-19	22.98	23.81	23.79
C-20	76.22	usp	usp
C-21	20.26	20.69	20.69
C-22	76.96	76.44	76.40
C-23	25.92	26.00	26.02
C-24	40.94	40.66	40.67
C-25	69.88	70.92	70.85
C-26	28.30	29.97	29.98
C-27	27.52	29.45	29.44
acyl-CH_3	19.53	13.94	14.11
acyl >C=O	170.88	173.09	173.07
acyl-CH_2-	-	34.71	34.77
		31.33	31.50
		24.28	24.63
		22.34	22.68
			29.60(x2)
			29.49
			20.32(x2)
			29.27

[#] spectrum obtained on a Bruker Advance DRX400 spectrometer (100 MHz) in CD_3OD

[*] spectra obtained on a Varian Unity 300 spectrometer (75 MHz) in $CDCl_3$

[&] spectra obtained on a Bruker Advance DMX500 spectrometer (125 MHz) in $CDCl_3$

usp: under solvent peak

anhydride (400 µL). The reaction was stirred at room temperature for 2 hours and quenched with H_2O (20 mL). After SPE purification, the 100% MeOH fraction was dried and the residue dissolved in dioxan (2 mL) and aqueous HCl (0.1M, 1 mL). The reaction mixture was stirred at room temperature for 4 hours and diluted with H_2O (20 mL). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30 %-100 % MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**12**) = 24.4 min, Rt (**14b**) = 11.2 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [45:55 v/v], 2 mL/min) and yielded turkesterone 11α-propionate (**14b**: 924 µg; 1.7 µmol; 17 % yield). The product was identified by ^1H NMR and mass spectrometry.

CIMS: 570 ($[M+NH_4]^+$, 39%), 552 ($[M]^+$, 5%), 496 ($[M+H-C_3H_5O]^+$, 90%), 479 ($[M+H-C_3H_6O_2]^+$, 100%), 461 ($[M+H-C_3H_6O_2-H_2O]^+$, 4%), 443 ($[M+H-C_3H_6O_2-2H_2O]^+$, 4%), 425 (2%), 380 (2%), 345 (1%)

^1H NMR: see Table 3.

iii) turkesterone 11α-butanoate (**14c**)

To a solution of 2,3-isopropylidene turkesterone 20,22-phenylboronate (**12**: synthesised from 10 mg turkesterone [**1**]) in dry pyridine (800 µL) at room temperature was added butanoic anhydride (400 µL). The reaction was stirred at room temperature for 6 hours and then heated to 50°C for 3 hours, after which time the reaction appeared to be complete by TLC analysis. The mixture was diluted with H_2O (20 mL) and pre-purified on a SPE cartridge, leaving the sample in methanol. This was dried under nitrogen and the residue was dissolved in dioxan (2 mL) and aqueous HCl (0.1M, 1 mL). The reaction mixture was stirred at room temperature for 4 hours and diluted with H_2O (20 mL). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30% - 100% MeOH linear gradient over 30 minutes, 1 mL/min, Rt (**12**) = 24.4 min, Rt (**14c**) = 12.5 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [50:50 v/v], 2 mL/min) and yielded turkesterone 11α-butanoate (**14c**: 1.8 mg; 3.1 µmol; 16 % yield). The product was identified by ^1H NMR and mass spectrometry.

CIMS: 584 ($[M+NH_4]^+$, 100%), 566 ($[M]^+$, 8%), 548 ($[M-H_2O]^+$, 2%), 496 ($[M+H-C_4H_7O]^+$, 58%), 479 ($[M+H-C_4H_8O_2]^+$, 72%), 461 ($[M+H-C_4H_8O_2-H_2O]^+$, 6%), 443 ($[M+H-C_4H_8O_2-2H_2O]^+$, 3%), 344 (2 %), 246 (7%)

^1H NMR: see Table 3.

iv) turkesterone 11α-hexanoate (**14d**)

2,3-Isopropylidene turkesterone 20,22-phenylboronate (**12**: synthesised from 10 mg turkesterone **1**) was dissolved in pyridine/benzene 6:5, v/v (4 mL) and hexanoic anhydride (335 µL) was added. After incubation at 50°C for 20 hours, the reaction mixture was reduced to dryness by rotary evaporation. The residue was dissolved in DCM and fractionated by silica gel chromatography. Aliquots of each fraction were analysed by TLC ($CHCl_3$/EtOH 9:1 v/v; Rf = 0.76). Fractions containing 2,3-isopropylidene turkesterone 20,22-phenylboronate 11-hexanoate were dried and redissolved in dioxan (2 mL) and aqueous HCl (0.1M, 1 mL). The reaction mixture was stirred at room temperature for 4 hours and diluted with H_2O (20 mL). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30%-100% MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**12**) = 24.4 min, Rt (**14d**) = 17.17 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [60:40 v/v], 2 mL/min) and yielded turkesterone 11α-hexanoate

Table 5. Biological activities of natural and synthetic ecdysteroids

	DmEcR/DmUSP		B_{II} Bioassay
Ecdysteroid	**IC_{50}**	**Ki**	**EC_{50}**
20-hydroxyecdysone (**6**)	1.2×10^{-7}M	5.2×10^{-8}M	7.5×10^{-9}M
turkesterone (**1**)	2.1×10^{-7}M	9.0×10^{-8}M	8.0×10^{-7}M
turkesterone 11-acetate (**14a**)	7.8×10^{-6}M	3.3×10^{-6}M	4.0×10^{-6}M
turkesterone 11α-propionate (**14b**)	2.4×10^{-5}M	1.0×10^{-5}M	8.3×10^{-6}M
turkesterone 11α-butanoate (**14c**)	9.1×10^{-5}M	3.9×10^{-5}M	4.0×10^{-5}M
turkesterone 11α-hexanoate (**14d**)	7.0×10^{-5}M	3.0×10^{-5}M	2.2×10^{-5}M
turkesterone 11α-decanoate (**14e**)	2.7×10^{-5}M	1.2×10^{-5}M	1.1×10^{-6}M
turkesterone 11α-laurate (**14f**)	nd		3.4×10^{-6}M
turkesterone 11α-myristate (**14g**)	nd		1.3×10^{-6}M
turkesterone 11α-arachidate (**14h**)	$>10^{-4}$M	n.c.	2.2×10^{-5}M
turkesterone 2-acetate (**2**)	5.2×10^{-6}M	2.9×10^{-6}M	5.2×10^{-6}M
turkesterone 2,11α-diacetate (**4**)	n.d.		2.0×10^{-4}M
turkesterone 2,22-diacetate (**5**)	n.d.		1.0×10^{-3}M

n.d.: not determined
n.c.: not calculated

(**14d**: 5.72 mg; 9.6 μmol; 48% yield). The product was identified by ^{1}H and ^{13}C NMR and mass spectrometry.

CIMS: 612 ([M+NH_4]$^+$, 2%), 597 ([M+H]$^+$, 2%), 579 ([M+H-H_2O]$^+$, 5%), 561 ([M+H-2H_2O]$^+$, 6%) 543 ([M+H-3H_2O]$^+$, 21%), 496 ([M-$C_6H_{11}O$]$^+$, 4%), 479 ([M+H-$C_6H_{12}O_2$]$^+$, 62%), 461 ([M+H-$C_6H_{12}O_2$-H_2O]$^+$, 71%), 443 ([M+H-$C_6H_{12}O_2$-2H_2O]$^+$, 33%), 427 (27%), 362 (35%), 345 (54%), 299 (5%), 178 (11%), 160 (13%), 143 (13%)

^{1}H and ^{13}C NMR: see Tables 3 and 4.

v) turkesterone 11α-decanoate (**14e**)

The production of turkesterone 11α-decanoate was completed as for **14d**, but using decanoic anhydride (474 mg). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30% - 100% MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**12**) = 24.4 min, Rt (**14e**) = 24.4 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [60:40 v/v], 2 mL/min) and yielded turkesterone 11α-decanoate (**14e**: 4.48 mg; 6.9 μmol; 34% yield). The product was identified by mass spectrometry.

CIMS: 669 ([M+NH_4]$^+$, 1%), 651 ([M+H]$^+$, 1%), 634 ([M+H-H_2O]$^+$, 1.5%), 616 ([M+H-2H_2O]$^+$, 1.5%), 479 ([M+H-$C_{10}H_{20}O_2$]$^+$, 81%), 461 ([M+H-$C_{10}H_{20}O_2$-H_2O]$^+$, 80%), 443 ([M+H-$C_{10}H_{20}O_2$-2H_2O]$^+$, 57%), 425 (35%), 361 (32%), 345 (47%), 256 (14%), 190 (44%), 143 (28%)

vi) turkesterone 11α-laurate (**14f**)

The production of turkesterone 11α-laurate was completed as for **14d**, but using lauric anhydride (555 mg). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30% - 100% MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**12**) = 24.4 min, Rt (**14f**) = 26.8 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [60:40 v/v], 2 mL/min) and yielded turkesterone 11α-laurate (**14f**: 1.47 mg; 2.2 μmol; 11% yield). The product was identified by ^{1}H and ^{13}C NMR and mass spectrometry.

FABMS: 701 ([M+Na]$^+$, 11%), 661 ([M+H-H_2O]$^+$, 6%), 643 ([M+H-2H_2O]$^+$, 9%), 625 ([M+H-3H_2O]$^+$, 6%), 565(11%), 479 ([M+H-$C_{12}H_{24}O_2$]$^+$, 82%), 461 ([M+H-$C_{12}H_{24}O_2$-H_2O]$^+$, 16%), 443 ([M+H-$C_{12}H_{24}O_2$-2H_2O]$^+$, 16%), 425 ([M+H-$C_{12}H_{24}O_2$-3H_2O]$^+$, 18%), 391 (70%), 369 (36%).

^{1}H and ^{13}C NMR: see tables 3 and 4

vii) turkesterone 11α-myristate (**14g**)

The synthesis of turkesterone 11α-myristate was completed as for **14d**, but using myristic anhydride (635 mg). The deprotection reactions were diluted with H_2O as before, but this brought about precipitation of the products. The sample was centrifuged and the supernatant removed. The pellet was redissolved in methanol and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (80 %-100 % MeOH linear gradient over 20 minutes; 1 mL/min; Rt **(14g)** = 9.1 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O, 90:10 v/v, 2 mL/min) and yielded turkesterone 11α-myristate (**14g**: 1.3 mg; 1.9 μmol; 9 % yield). The product was identified by ^{1}H NMR and mass spectrometry.

FABMS: 730 ([M+Na]$^+$, 15%), 689 ([M+H-H_2O]$^+$, 14%), 672 ([M+H-2H_2O]$^+$, 24%), 654 ([M+H-3H_2O]$^+$, 17%), 493 (9%), 479 ([M+H-$C_{14}H_{28}O_2$]$^+$, 100%), 461 ([M+H-$C_{14}H_{28}O_2$-H_2O]$^+$, 66%), 443 ([M+H-$C_{14}H_{28}O_2$-2H_2O]$^+$, 52%), 425 ([M+H-$C_{14}H_{28}O_2$-3H_2O]$^+$, 55%), 391 (62%), 362 (30%), 345 (40%), 327 (32%), 309 (96%), 299 (67%), 281 (55%), 228 (92%), 211 (30%)

^{1}H NMR: see Table 3.

viii) turkesterone 11α-arachidate (**14h**)

The synthesis of turkesterone 11α-arachidate was

Table 6. Comparison between EC_{50} values determined in the B_{II} bioassay and K_d values in cell-free extracts of B_{II} cells for a range of ecdysteroids and two bisacylhydrazines. See Lafont et al. (2002) for the structures of the unnumbered ecdysteroids.

Ecdysteroid	EC50 value*	Kd value	Kd/EC50
ponasterone A (**7**)	3.1×10^{-10}M	7.2×10^{-10}M	2.3
ajugasterone C (**8**)	3.0×10^{-8}M	1.3×10^{-9}M	0.04
muristerone A (**9**)	2.2×10^{-8}M	2.2×10^{-9}M	0.1
25-fluoro-20-hydroxyecdysone	5.1×10^{-9}M	5.2×10^{-9}M	1.0
polypodine B	1.0×10^{-9}M	7.7×10^{-9}M	7.7
5-deoxykaladasterone	5.2×10^{-10}M	1.0×10^{-8}M	19.2
abutasterone	1.4×10^{-7}M	1.1×10^{-8}M	0.08
cyasterone	1.2×10^{-8}M	1.7×10^{-8}M	1.4
20-hydroxyecdysone (**6**)	7.5×10^{-9}M	2.5×10^{-8}M	3.3
inokosterone	1.1×10^{-7}M	3.5×10^{-8}M	0.17
makisterone A	1.3×10^{-8}M	6.4×10^{-8}M	4.9
turkesterone (**1**)	1.3×10^{-6}M	1.4×10^{-7}M	0.11
RH5992	5.3×10^{-7}M	2.1×10^{-7}M	0.4
ajugalactone	1.6×10^{-7}M	3.1×10^{-7}M	0.19
RH5849	1.8×10^{-6}M	1.5×10^{-7}M	0.08
ecdysone	1.1×10^{-6}M	1.7×10^{-6}M	1.55
2-deoxyecdysone	5.0×10^{-5}M	3.8×10^{-6}M	0.08
22-*epi*-ecdysone	5.3×10^{-6}M	6.7×10^{-6}M	1.26
poststerone	2.0×10^{-5}M	1.5×10^{-5}M	0.75

* values taken from Clément et al, (1993) or Dinan et al. (1999)

completed as for **14g**, but using arachidic anhydride (880 mg). The deprotection reactions were diluted with H_2O, but this brought about precipitation of the products. The sample was centrifuged and the supernatant removed. The pellet was redissolved in methanol and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (80 %-100 % MeOH linear gradient over 20 minutes; 1 mL/min; Rt **(14h)** = 19.2 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O, 95:5 v/v, 2 mL/min) and yielded turkesterone 11α-arachidate (**14h**: 2.6 mg; 3.3 μmol; 16% yield). The product was identified by ^{1}H NMR and mass spectrometry.

CIMS: 809 ([M+NH_4]$^+$, 7%), 790 ([M]$^+$, 8%), 479 ([M+H-$C_{20}H_{40}O_2$]$^+$, 55%), 461 ([M+H-$C_{20}H_{40}O_2$-H_2O]$^+$, 55%), 443 ([M+H-$C_{20}H_{40}O_2$-2H_2O]$^+$, 34%), 428 (34%), 363 (47%), 346 (65%), 331 (34%), 283 (15%), 141 (36%).

^{1}H NMR: see Table 3.

Synthesis of turkesterone acetates

Conversion of turkesterone (**1**) to turkesterone 2-monoacetate (**2**)

To a solution of turkesterone (**1**: 5 mg; 10.4 μmol) in dry pyridine (400 μL) at room temperature was added acetic anhydride (200 μL; 2.08 mmol; 200 eq.). The reaction was stirred at room temperature for 1 hour and quenched with H_2O (20 mL). The residue was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30%-100% MeOH linear gradient over 30 minutes, 1 mL/min; Rt (**1**) = 6.3 min, Rt (**2**) = re8.4 min]. The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [45:55 v/v], 2 mL/min) and yielded turkesterone 2-acetate (**2**: 613 μg; 1.1 μmol; 12%). The product was identified by mass spectrometry.

CIMS: 557 ([M+NH_4]$^+$, 62%), 539 ([M+H]$^+$, 32 %), 522 ([M+H-H_2O]$^+$, 61%), 486 ([M+H-3H_2O]$^+$, 65%), 462 (40%), 444 (33%), 439 (74%), 423 (84%), 405 (47%), 362 (32%), 344 (28%), 328 (16%), 178 (18%), 160 (18%).

Conversion of turkesterone (**1**) to turkesterone 2,11-diacetate (**4**)

To a solution of turkesterone (**1**: 10 mg; 20.8 μmol) in dry pyridine (800 μL) at room temperature was added acetic anhydride (400 μL; 4.16 mmol; 200 eq.). The reaction was stirred at room temperature for 1 hour and quenched with the addition of H_2O (20 mL). The residue was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30%-100% MeOH linear gradient over 30 minutes; 1 mL/min; Rt (**1**) = 6.3 min, Rt (**4**) = 11.3 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [45:55 v/v], 2 mL/min) and yielded turkesterone 2,11-diacetate (**4**: 5.6 mg; 9.7 μmol; 48% yield). The product was identified by ^{1}H and ^{13}C NMR and mass spectrometry.

CIMS: 598 ([M+NH_4]$^+$, 8%), 580 ([M]$^+$, 6%), 563 ([M+H-H_2O]$^+$, 10%), 545 ([M+H-2H_2O]$^+$, 31%), 538 ([M-Ac]$^+$, 12%), 485 ([M+H-Ac-3H_2O]$^+$, 48%), 480 ([M-2Ac-H_2O]$^+$, 28%), 464 (70%), 420 (38%), 404 (82%), 387 (100%), 344 (24%), 325 (16%), 128 (65%)

^{1}H and ^{13}C NMR: see Tables 1 and 2.

Conversion of 2,3-isopropylidene turkesterone (**10**) to turkesterone 11,22-diacetate (**5**)

To a solution of 2,3-isopropylidene turkesterone (**10**: synthesised from 10 mg turkesterone [**1**]) in dry pyridine (800 μL) at room temperature was added acetic anhydride (400 μL). The reaction was stirred at room temperature for 3 hours and quenched with H_2O (20 mL). The reaction mixture was pre-purified on a SPE cartridge and the 100% MeOH fraction dried under nitrogen. The residue was dissolved in dioxan (2 mL) and aqueous HCl (0.1M, 1 mL). The reaction mixture was stirred at room temperature for 4 hours and was diluted by the addition of H_2O (20 mL). The product was pre-purified on a SPE cartridge and analysed by HPLC using an ODS2 analytical column with detection at 242 and 300 nm (30% - 100% MeOH linear gradient over 30 minutes, 1 mL/min, Rt (**10**) = 12.5 min, Rt (**5**) = 11.2 min). The product was purified on an ODS2 semi-preparative column (MeOH/H_2O [45:55 v/v], 2 mL/min) and yielded turkesterone 11,22-diacetate (**5**: 5.4 mg, 9.3 μmol, 46% yield). The product was identified by ^{1}H and ^{13}C NMR and mass spectrometry.

CIMS: 598 ([M+NH_4]$^+$, 27%), 563 ([M+H-H_2O]$^+$, 8%), 545 ([M+H-2H_2O]$^+$, 5%), 538 ([M-Ac]$^+$, 5%), 521 ([M+H-Ac-H_2O]$^+$, 100%), 503 ([M+H-Ac-2H_2O]$^+$, 40%), 485 ([M+H-Ac-3H_2O]$^+$, 22%), 461 (18%), 443 (35%), 425 (41%), 344 (19%), 176 (20%), 136 (52%), 128 (25%).

^{1}H and ^{13}C NMR: see Tables 1 and 2.

Purity of test compounds

All compounds for bioassay were purified by RP-HPLC using water/methanol mixtures. Purity of samples for bioassay was >98%.

B_{II} bioassay

The microplate-based B_{II} bioassay, based on the ecdysteroid-responsive *D. melanogaster l(2)mbn* permanent cell line, was performed as described previously (Clément et al., 1993).

Competitive radioligand binding assay with in vitro-expressed receptor complexes

Bacterially-expressed DmEcR and DmUSP proteins were used in a competitive binding assay with [24,25,26,27-^{3}H]ponasterone A (0.2 nM; 150 Ci/mmol; ARC Inc.) as previously described (Dinan et al., 2001). K_i values were calculated from IC_{50} values by means of the Cheng-Prusoff equation ($K_i = IC_{50}/\{1 + [\text{radiolabelled ligand}]/K_d\}$; Cheng and Prusoff, 1973), with the K_d for [^{3}H]ponA being 0.15 nM.

Extraction of receptor complexes from B_{II} cells and cell-free receptor assays

Ecdysteroid receptor complexes were extracted from B_{II} cells by sonication as described previously (Dinan, 1985). After centrifugation (20,000 x g for 30 min), aliquots of the supernatant were incubated with [^{3}H]ponA (0.2 nM; 183 Ci/mmol) and known

concentrations of the test compounds. Specifically-bound radiolabel was determined by dextran-coated charcoal assay (Dinan, 1985). K_d values were determined by the LIGAND program.

Results and Discussion

Regioselective synthesis of 11α-acyl derivatives of turkesterone

Owing to the presence of several reactive secondary hydroxyl groups in turkesterone, it was necessary to protect the diols at C-2/C-3 and C-20/C-22 in order to selectively acylate the 11α-hydroxyl. The acylation sequence of the hydroxyls in 20-hydroxyecdysone has been investigated previously (Galbraith and Horn, 1969) and found to be: 2>22>3>>25. Acetylation experiments comparing turkesterone and 20-hydroxyecdysone indicated that the sequence for turkesterone was 2>11>22>3>>25 (data not shown). Figure 2 demonstrates the protection strategy employed. The 20,22-phenylboronate was first produced, followed by the 2,3-acetonide derivative. The 11α-hydroxyl group could then be derivatised with the appropriate acyl anhydride. Conditions for this reaction had to be modified to take account of the solid state and lower solubility of the higher acyl anhydrides relative to their lower homologues. The diol-protecting groups were readily removed with dilute acid. Overall yields for the 11α-acyl derivatives were variable. The synthesis of turkesterone 11α-benzoate was attempted, but the product proved unstable, degrading rapidly back to turkesterone.

Identification of turkesterone derivatives

Turkesterone derivatives were identified by a combination of ^{1}H and ^{13}C NMR and mass spectrometry. The major distinctive ^{1}H-NMR features amongst the compounds were the presence of one or more singlet absorptions at ca. δ 2.0 due to the acetyl groups for the acetates, and the shifts of ca. 1 ppm for the acylated oxymethine in acyl analogues. In the homogeneous series of 11α-acyl analogues, significant differences were only observed in the number of signals in the ^{13}C-NMR associated with the length of the acyl chain. Diagnostic absorptions are reported for all compounds and selected representatives were fully characterized by NMR. Minor differences were observed in the 11-*O*-acyl series and the expected shifts upon acylation of other positions.

^{1}H NMR for 11α-acyl derivatives

The most noticeable change from the spectrum for turkesterone was the shift in the H-11 signal from δ4.1 to δ5.3. The signal for the acyl methyl (CH_3) was present at δ0.8-0.9 and signals at δ1.3-2.3 related to the acyl CH_2 groups.

^{13}C NMR for 11α-acyl derivatives

A small shift in the C-11 signal from δ69.5 in turkesterone to δ71-72 was apparent for all acyl derivatives. The acyl carbonyl (C=O) was identified by a signal at δ171-173, the acyl methyl was identified by a signal at δ14 and the acyl CH_2 groups were identified by a sequence of signals around δ22-35.

^{1}H NMR for acetate derivatives

2ax-H and 3eq-H seen at δ4.01 and δ3.95, respectively, in turkesterone displayed downfield shifts to δ5.1-5.3 and δ4.1-4.2, respectively, upon derivatization at C-2. Similarly the peak for 11-H seen at δ4.1 in turkesterone was seen at δ5.3-5.6 in derivatives containing an acetate at C-11, and the signal for 22-H, seen at δ3.44 in turkesterone, shifted to δ4.8 upon derivatization at C-22. Acetyl (CH_3) signals were apparent at δ2.0-2.1.

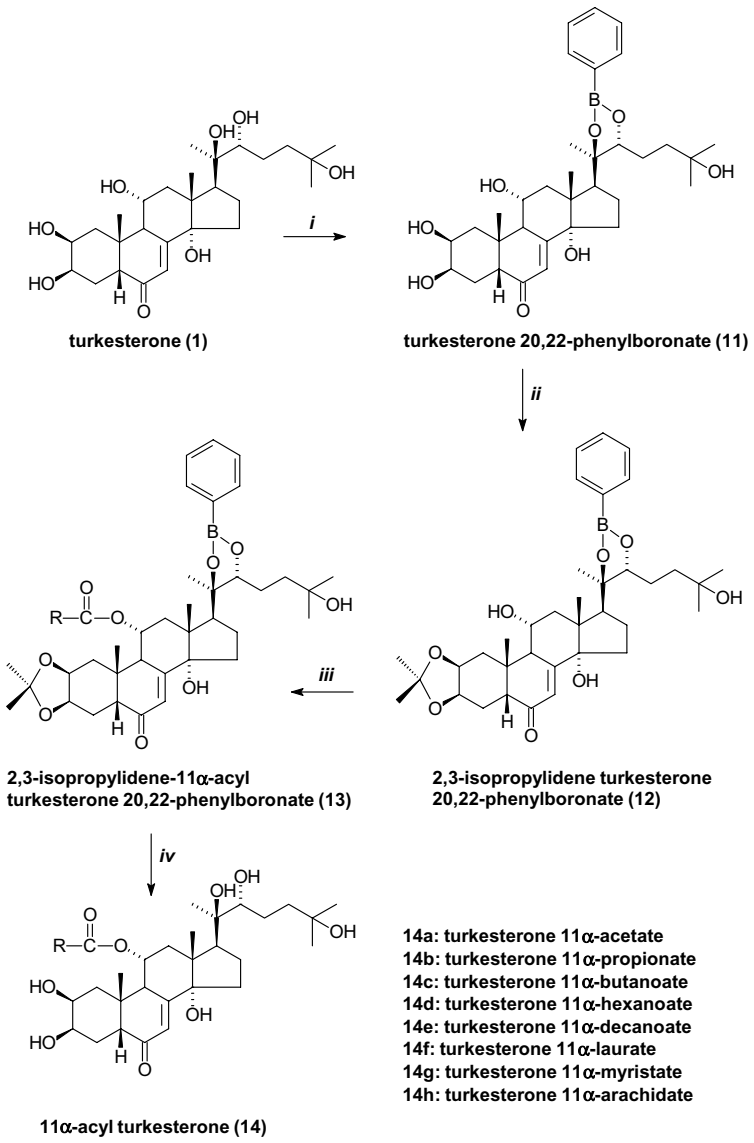


Figure 2. Scheme for the synthesis of turkesterone 11α-acyl derivatives: i) phenylboronic acid, dry DMF, r.t. 2 h, ii) addition of dry DMP, dry acetone and fused *p*-TsOH, r.t. 3h, iii) R-COOCO-R, dry pyridine (or pyridine/benzene 6:5 v/v), r.t. (or 50°C) for 2-20 h and iv) 0.1M HCl/dioxan (1:1 v/v), r.t. 4 h.

^{13}C NMR for acetate derivatives

Only minor shifts were observed for signals corresponding to C-2, C-3, C-11 and C-22 for compounds derivatised at C-2, C-11 and/or C-22 (from δ68.9, 68.6, 69.5 and 78.4 for turkesterone to δ71.2, 65, 71 and 79, respectively, for derivatised compounds). The acetyl carbonyl (C=O) was the most obvious addition to the spectra at ca. δ170 while the acetyl (CH_3) signals could be identified at ca. δ21.

Biological activity of turkesterone 11α-acyl derivatives

B_{II} bioassay: The biological activity of turkesterone in the B_{II} bioassay is ca. 100-fold lower than that of 20E (Table 5). It is notable that all the tested derivatives of turkesterone retain quantifiable biological activity in this bioassay, but their activities range from almost identical to over 1000-fold less than that of turkesterone. When one compares the activities of the various 11α-acyl homologues, biological activity initially decreases with increasing acyl chain length (C_2 to C_4) with the acetate, propionate and butanoate being 5-, 10- and 50-fold less active than turkesterone. However, with intermediate length acyl groups (C_6 to C_{14}) the activity increases again, such that the activities of the 11α-decanoate, -laurate and -myristate are similar to that of turkesterone. Activity is again lower with the C_{22} arachidate ester (27.5-fold lower than turkesterone). These data indicate that the ligand binding pocket of EcR is capable of accepting steric bulk around C-11 of the ecdysteroidal ligand and that, further, interaction between the ligand and the binding pocket is enhanced with medium-chain length acyl groups (C_{10} - C_{14}), relative to short- and long-chain acyl derivatives. Turkesterone 2-acetate has a biological activity similar to that of turkesterone 11α-acetate, in accord with previous observations that small substituents at C-2 lower activity, but do not abolish it. The diacetates, 2,11- and 2,22-, have very much lower activities than turkesterone, but the 2,11-diacetate is more active than the 2,22-diacetate, again revealing that derivatisation at 11α is less detrimental than at C-22.

In vitro receptor assays: In addition to receptor affinity, a number of factors (e.g. penetration into the cells, sequestration, hydrolysis of ester-containing compounds) might affect potency in the B_{II} bioassay. Consequently, the ability of certain of the test compounds to displace [^{3}H]ponA from *in vitro*-expressed DmEcR/DmUSP receptor complex was assessed (Table 5). Owing to limited quantities of the DmEcR and DmUSP proteins, it was not possible to assess all of the analogues. Comparison of the receptor assay with the B_{II} bioassay reveals a distinct difference between 20E and turkesterone. While 20E is more potent in the B_{II} bioassay (EC_{50} = 7.5 x 10^{-9}M) than in the receptor assay (K_i = 5.2 x 10^{-8}M), turkesterone is, conversely, ca. 9-fold less potent in the B_{II} bioassay (EC_{50} = 8.0 x 10^{-7}M) than in the receptor assay (K_i = 9.0 x 10^{-8}M). This implies that other factors (entry, efflux, sequestration or metabolism) affect turkesterone and 20E differentially. It is known that the B_{II} cells do not metabolise 20E (Dinan, 1985). The differential activities in the two systems may relate to previous findings that turkesterone is a potent ecdysteroid in some insect systems, but not in others (Sláma et al., 1993). Alternatively, it may indicate that the *in vitro* preparation has the capacity to dehydrate 11α-hydroxyl ecdysteroids to 7,9(11)-dien-6-ones (which are known to be biologically active) by a chemical reaction which occurs readily (Bourne et al., 2002).

However, there is good agreement between the K_i and EC_{50} values for the 11α-acyl derivatives of turkesterone and, as far as the somewhat restricted range of analogues assessed permits, the activity pattern in the receptor assay is similar to that in the B_{II} bioassay with the activity decreasing with increasing acyl length from C_2 to C_4 and then increasing again with C_6 and C_{10} acyl derivatives. The C_{20} derivative has a very low activity. There is also good agreement between the K_i and EC_{50} values for turkesterone 2-acetate. Thus, although these data reveal inconsistencies for turkesterone and 20E, they support the notion that steric bulk can be accommodated around C-11 of the ecdysteroid within the ligand binding pocket of EcR.

Since the receptor complex almost certainly consists of other protein components in addition to EcR and USP, which are unlikely to be present in the *in vitro* translated complexes, we include data (Table 6) on the comparison of the structure-activity relationships for the B_{II} bioassay and a cell-free receptor assay based on ecdysteroid receptor complexes extracted from B_{II} cells, where other endogenous transcription factors and binding proteins could be part of the extracted receptor complex. In general, there is reasonable agreement between the EC_{50} and K_d values determined for most of the compounds. However, it is noticeable that the three 11α-hydroxy compounds included here (ajugasterone C, muristerone A and turkesterone) all have very low K_d/EC_{50} ratios, as seen above for turkesterone in the comparison of the B_{II} bioassay and *in vitro*-expressed DmEcR/DmUSP.

Implications

Enhanced activity of 25-deoxy compounds: With the *D. melanogaster* B_{II} bioassay and *in vitro* DmEcR/DmUSP receptor assays, turkesterone shows considerably lower activity than 20E, although in other systems turkesterone is apparently more potent. Turkesterone was used in this study as a model compound, because it was the most readily available ecdysteroid possessing an 11α-hydroxyl group. However, to exploit the potential of 11α-derivatives it would be preferable to use higher potency analogues lacking the 25-hydroxyl group e.g ajugasterone C (EC_{50} = 3.0 x 10^{-8}M) or muristerone A (EC_{50} = 2.2 x 10^{-8}M), which should generate 11α-derivatives with ca. 100-fold greater activity than those listed in Table 5.

Future synthesis of 11β-acyl derivatives: It would be desirable to synthesise a parallel series of 11β-acyl derivatives in order to compare their activities to those of the 11α-acyl analogues. Realisation of this goal will be complicated by the absence of any known 11β-hydroxy ecdysteroids (Lafont et al., 2002) and the ready tendency of 11α-hydroxy ecdysteroids to dehydrate, generating 7,9(11)-dien-6-ones, under a variety of chemical conditions (Canonica et al., 1977; Qui and Nie, 1983; Bourne et al., 2002), making sequential oxidation and reduction of the 11-hydroxyl group difficult.

Relationship to other steroid hormones: The availability of hydrophobic space in the ligand-binding pocket in the region of C-11 of the steroid appears to be a common feature of steroid hormone receptors. 11β-Amidoalkoxyphenyl estradiols are potent antioestrogens (Nique et al., 1994). The hydrophobic pocket has been exploited to develop 11β-substituted 19-norsteroids as fluorescent steroid derivatives for the glucocorticoid and progesterone receptors (Teutsch et al., 1994). The antiprogesterone

RU486 (mifepristone), used as an abortifacient, is an 11β-aryl-19-nor steroid (Rosen et al., 1995).

Application for synthesis of tagged ecdysteroids: The studies shown here reveal that there is considerable steric space in the ligand binding pocket of DmEcR extending out from C-11 of the ecdysteroid, as was predicted from the CoMFA models. This area would thus be a good candidate for the preparation of tagged ecdysteroids, for example the attachment of a fluorescent moiety for the generation of an alternative, non-radioactive receptor assay ligand. Currently, [^{3}H]ponA is almost exclusively used as the ligand for receptor assays, but it is expensive and, owing to its very high specific activity (120 - 180 Ci/mmol), requires regular repurification. C-11 could also be a good location for an affinity labelling moiety as an alternative to the photoreactive endogenous Δ^7-en-6-one (Schaltmann and Pongs, 1982) or an extended conjugated system (7,9[11]-dien-6-one; Bourne et al., 2002). For example, bromoacetate derivatives have proved effective electrophilic affinity agents for vitamin D binding protein (Swamy et al., 2000) and the human progesterone receptor (Holmes et al., 1981). Also, a successful photoaffinity label for human multidrug resistance protein has been based on the steroid agosterol A (Ren et al., 2001), which is structurally similar to ecdysteroids and possesses an 11α-hydroxyl group, on to which the azidophenyl photoaffinity label is attached. Further, it has been found that insect EcR proteins when expressed in mammalian cells and partnered by RXR have altered ligand specificity and affinity, such that 20E, the endogenous hormone in insect systems, is virtually inactive. However, muristerone A, possessing an 11α-hydroxyl group, is active, but micromolar concentrations are required. For gene switching it would be highly desirable to identify ecdysteroids with higher affinity than muristerone A. Production of 11α-derivatives may enhance affinity by providing closer contact to the ligand binding pocket.

Conclusion

The prediction from CoMFA modelling of ecdysteroid QSAR data that the ligand binding domain of *D. melanogaster* EcR proteins possesses a hydrophobic pocket in the vicinity of C-11 of the steroid is substantiated by the biological activity of a series of 11α-acyl derivatives of turkesterone. All the 11α-acyl derivatives retain some biological activity and activity is higher with medium-chain acyl derivatives. The presence of a hydrophobic pocket in this region is a feature shared with several other steroid hormone receptors. The presence of this pocket may be exploited to generate fluorescently-tagged and affinity-labelled ecdysteroids which retain high biological activity.

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