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Synthesis, binding and bioactivity of γ-methylene γ-lactam ecdysone receptor ligands: Advantages of QSAR models for flexible receptors

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This paper is dedicated to the memory of Dr. Dionne Jones, deceased 5/10/2000

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ABSTRACT

Nuclear hormone receptors, such as the ecdysone receptor, often display a large amount of induced fit to ligands. The size and shape of the binding pocket in the EcR subunit changes markedly on ligand binding, making modelling methods such as docking extremely challenging. It is, however, possible to generate excellent 3D QSAR models for a given type of ligand, suggesting that the receptor adopts a relatively restricted number of binding site configurations or 'attractors'. We describe the synthesis, in vitro binding and selected in vivo toxicity data for γ-methylene γ-lactams, a new class of high-affinity ligands for ecdysone receptors from *Bovicola ovis* (Phthiraptera) and *Lucilia cuprina* (Diptera). The results of a 3D QSAR study of the binding of methylene lactams to recombinant ecdysone receptor protein suggest that this class of ligands is indeed recognised by a single conformation of the EcR binding pocket.

1. Introduction

Nuclear hormone receptors are a large family of ligand-activated proteins which bind to DNA sequences and regulate the expression of genes within the cell nucleus. These regulatory proteins control homeostasis, development and differentiation of tissues within an organism.

The ecdysone receptor protein (EcR)[1] is a nuclear hormone receptor that occurs almost exclusively in the phylum Arthropoda (e.g., insects, arachnids and crustaceans). The functional receptor for ecdysteroid hormones is a non-covalent heterodimer of EcR with a second nuclear receptor, the ultraspiracle protein (USP), the latter being a homologue of the retinoid X receptor, RXR. The functional ecdysone receptor is activated by binding to ecdysteroids. As the ecdysone receptor is absent from other organisms, it is an attractive target for the generation of safe insecticides. One class of ecdysone receptor ligands, the diacylhydrazines, has been commercialised for use as insecticides.

Due to its absence from most taxa, EcR and its functional domains are also used as components of ecdysone switches for the control of reporter and therapeutic genes in mammalian cells, and for control of transgenes more generally in agriculturally important plant and animal species. In this role, gene constructs whose expression can be controlled by the level of ecdysteroid can be prepared relatively easily. As ecdysone receptors are naturally absent from mammalian cells, they provide an orthogonal gene switch that offers great promise for the safe control of transgene expression in medical and veterinary applications.[2,3]

The ligand binding domains (LBDs) of nuclear hormone receptors frequently display structural plasticity and can bind to structurally diverse ligands by adapting the shape of their ligand binding pockets.[4,5] In solution, the unliganded receptor LBD will have a number of different conformations in dynamic equilibrium.[6] A ligand will sample these conformations and bind to the one that is complementary to its shape and other properties. This shifts the

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equilibrium towards the conformation that binds this ligand.[7] It has been demonstrated that the ecdysone receptor displays such behaviour.[8,9]

Potentially, the binding site of the ecdysone receptor might change smoothly and continuously in size and shape when different types of ligands bind. However, 3D quantitative structure–activity relationships (QSAR) studies have suggested that, for a given chemotype, the shape of the binding pocket does not change much in response to variation in substitution on the ligand.[10] This suggests that the binding pocket may be capable of adopting only a finite number of relatively stable shapes. The existence of robust chemotype-specific binding pocket conformations would provide greater confidence in binding predictions for ligand analogue optimisation programs. In addition, if all of these can be identified by structural biology, the task of predicting the activity of novel ligands by docking would be greatly facilitated.

In EcR, as in other nuclear receptors, the ligand binding pocket is likely to be open and accessible in the apo-LBD. Upon ligand binding, the energetically preferred conformation then changes to one where helix 12 is positioned over the mouth of the pocket, acting as a lid. X-ray crystallography (Fig. 1) has shown that the EcR LBD adopts a 'sock' shaped pocket when binding the natural steroid hormone, 20-hydroxyecdysone (Fig. 2). Such studies have also shown that the binding pocket for the diacylhydrazines, although utilising some of the same amino acid residues, is very different in shape and occupies a somewhat different position in the LBD, as Figure 1 illustrates.[8,11–13] Previous attempts to use docking to the ecdysteroid-bound LBD conformation to understand the mode of binding of the diacyhydrazines to the ecdysone receptor and to predict binding affinities resulted in models[14,15] which were ultimately proved incorrect by crystallography.[11]

Variability in ecdysone receptor structure between insect orders can be looked upon as providing a resource for tailoring the breadth of control spectra, potentially giving an advantage over broad-spectrum insecticides. The diacylhydrazines display remarkable selectivity for insect ecdysone receptors from different taxonomic orders. For example, Tebufenozide (Fig. 2) is two to three orders of magnitude more effective against Lepidoptera (moths/butterflies) than it is against Diptera (flies).[16] The selectivity for certain insect orders limits the environmental impact of this type of insecticide. The effectiveness of diacylhydrazines as insecticides

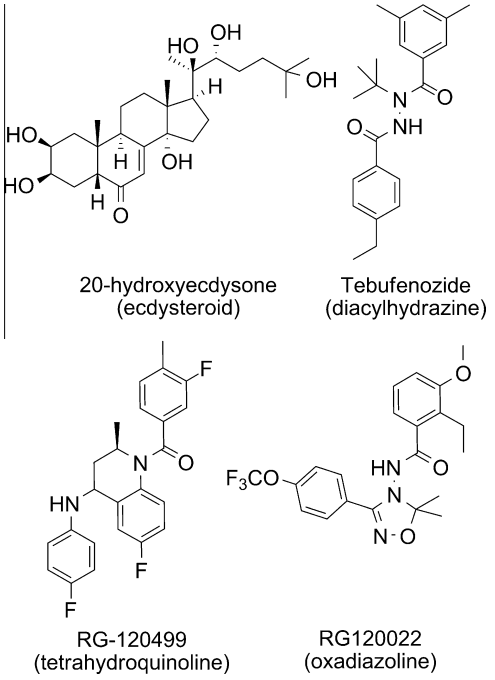


Figure 2. Examples from known ecdysone receptor ligand classes.

generally correlates with their affinities for the LBDs of the corresponding ecdysone receptors.[17,18] While all insect ecdysone receptors bind the physiological ligand (20-hydroxyecdysone) using highly conserved amino acid residues, non-ecdysteroid ligands employ interactions with other residues in the binding pocket.[19] Since these residues are less conserved in EcRs from different taxonomic orders, the binding affinity of each non-ecdysteroid chemotype can also vary greatly across orders.

Despite considerable research in this area, no class of ecdysone receptor ligand with selectivity outside the Lepidoptera and Coleoptera has yet been developed for commercial use. One hurdle in this respect is the plasticity of the EcR LBD, which makes development of a good model for binding of ligand to the receptor more

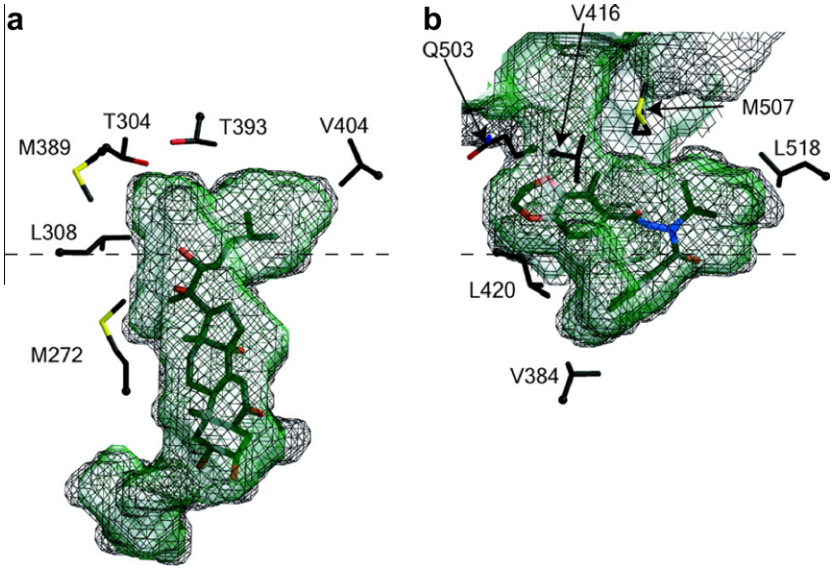


Figure 1. EcR-LBD binding pocket (a) from *Bemisia tabaci* bound to an ecdysteroid or (b) *Heliothis virescens* bound to a diacylhydrazine. Reproduced with permission from Ref. 11.

difficult. A few other classes of ecdysone receptor ligands have been discovered that have not survived the pathway to commercial insecticides, but have potential applications in ecdysone based gene switches, for example, tetrahydroquinolines[9,20,21] and oxadiazolines[22] (Fig. 2).

High-throughput screening can provide a large volume of data from which to develop QSAR to guide synthesis and optimisation of activity against a selected target. This is particularly important for a structurally adaptive receptor as it may provide a model that cannot reliably be obtained by docking procedures. Others have recently used high-throughput screens based on transgenic reporter cell lines to identify new classes of ecdysone receptor ligand.[23] We have developed an in vitro fluorescence polarisation assay for the ecdysone receptor.[24] This can be automated as a high-throughput screen to efficiently and reliably assess the binding activity of the ecdysone receptor against thousands of compounds from a chemical library.[18]

Using high-throughput screening we have discovered a new class of compounds, the γ-methylene γ-lactams, which bind to recombinant insect ecdysone receptor LBDs with high affinity.[25,26] We report the synthesis and an extensive 3D QSAR study of γ-methylene γ-lactams as ligands for the ecdysone receptor from an insect of the order Phthiraptera (lice) and another from the order Diptera. We also demonstrate that these methylene lactams can disrupt the nymphal development of *Bovicola ovis* (sheep body louse).

2. Results and discussion

2.1. Chemistry

Acetamides **1** were generally prepared from the corresponding amines by literature procedures. Procedures requiring heating were improved by use of microwave irradiation. Condensation of acetamides **1** with vicinal diones **2** provided hydroxylactams **3** which were dehydrated without further purification (Scheme 1). This procedure sometimes required protracted reaction times, but attempts to decrease these by raising the temperature resulted in generation of significantly more by-products. Dehydration of **3** provided the desired lactams **4–16**. Again the reaction time can be decreased by employing microwave irradiation.

2.2. Structure–activity relationships from ecdysone receptor binding assays

The binding of three hundred and fifty compounds of types **4–16** to recombinant ecdysone receptor LBDs from *B. ovis* and/or *L. cuprina* (sheep blowfly) was assayed by high-throughput screening in vitro. The IC_{50} of a selection of these compounds was then determined. Many of the methylene lactams were good ligands for the recombinant ecdysone receptor LBDs, binding with comparable affinity to the natural ligand 20-hydroxyecdysone. Some (e.g., **11e**) appeared to be almost as effective as the high-affinity phytoecdysteroid ponasterone A.

None of the hydroxylactams **3** demonstrated any binding to the ecdysone receptor, indicating that the methylene at position 5 is important. For *B. ovis*, replacement of a hydrogen on this methylene results in a decrease in binding as the size of the group increases, leading to complete loss of activity when R^8 is *n*-propyl.

For *B. ovis*, a variety of substituents are tolerated around the aromatic ring in **4–14**. Polysubstitution is preferred over monosubstitution and 2,5-disubstitution and 2,4,5-trisubstitution were most consistently favoured. There is clearly some room for steric bulk in the 2 position. *t*-Butyl substituents in the 2-position are favoured, but only when there is no substituent on the 6-position. Larger groups such as benzyl and phenoxy in the 2-position are also tolerated. Chlorination generally leads to the strongest binders.

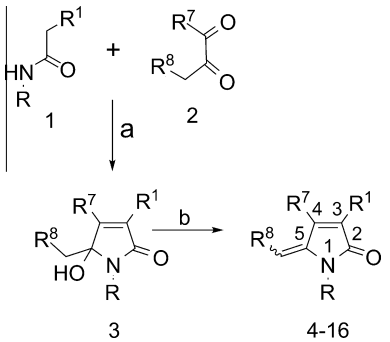
There is no clear indication of an effect of the size of the substituents R^7 on the activity. Methyl compounds **4**, phenyl compounds **12** and *n*-hexyl compound **13a** all showed similar propensity to bind. No heteroatoms were investigated at this position.

Replacement of the aromatic ring on the lactam nitrogen with an alicyclic ring generally leads to decreased binding. One significant exception to this is **15d**, where R^7 is phenyl. It is possible that this binds in the receptor in the opposite orientation. Substitution of the aromatic ring for a heterocyclic ring, benzyl moiety or an amide also generally resulted in decreased binding.

In order to probe the volume around R^1, compounds **16** with acyl and aminoacyl groups at this position were synthesised. Their binding affinity as compared to the nitriles was inconsistent, being sometimes greater, sometimes less. These were not stable for extended periods and in some cases this may have affected the results.

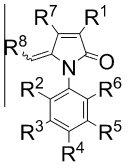
Only twenty-two IC_{50} values were determined for binding to the *L. cuprina* ecdysone receptor, insufficient to carry out a detailed SAR analysis. However, it can be seen that methylene lactams clearly exhibit binding affinity for the *L. cuprina* receptor. In many cases the IC_{50} values are similar to those for binding to the *B. ovis* protein, although some differ significantly, no doubt reflecting local differences in the ligand binding pockets. Our data add weight to the notion that new ligand chemistries can be discovered for ecdysone receptors outside the orders Lepidoptera and Coleopterea.

Given that the conjugated cyano-substituted diene motif present in the methylene lactams **4–16** represents a potential Michael acceptor, we were concerned that these compounds might inhibit the receptor by forming covalent adducts rather than by reversible binding. To investigate the likelihood of this possibility, a number of examples were treated with a variety of nucleophiles under acidic, basic and neutral conditions. Under basic conditions reaction did occur to provide complex intractable products, however under neutral and acidic conditions no reaction occurred. There was just one exception: on treatment with a considerable excess of sodium 4-toluene sulfinate in acetic acid, an adduct was isolated which corresponded to reaction at the exocyclic methylene. However, attempts to purify this material by recrystallisation resulted in loss of the sulfinyl moiety and recovery of the pure methylene lactam. As physiological pH is close to neutral, on the basis of this evidence, it is unlikely that the methylene lactams undergo a Michael reaction with the receptor. This is supported by the observation that there is no particular correlation between Michael acceptor ability and binding affinity.

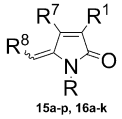


Scheme 1. Reagents and conditions: (a) piperidine, morpholine, DABCO or DBU, DMF, 1-43 days (method D); (b) formic acid, TFA or $MeSO_3H$ 18–80 °C, 4–18 h (method E); TFA or TfOH, DCM, rt, 10 min–24 h (method F); formic acid, μW, 120 °C, 10 min (method G).

Table 1
Structures and IC_{50} for binding to the *Bovicola ovis* ecdysone receptor



4a-t, 5a-r, 6a-h, 7a-i, 8a-c, 9a-f, 10a-l, 11a-g, 12a-c, 13a, 14a-c



15a-p, 16a-k

Compound	*C*log *P*	R	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	IC_{50} (*B. ovis*) (μM)	IC_{50} (*L. cuprina*) (μM)
4a	2.67		CN	Cl					Me	H	55	
4b	2.76		CN	Br					Me	H	37.5	
4c	2.45		CN	Me					Me	H	80	
4d	3.38		CN	*i*Pr					Me	H	22.5	
4e	3.78		CN	*t*Bu					Me	H	17.5	
4f	4.24		CN	Ph					Me	H	25	
4g	3.24		CN	CF_3					Me	H	27.5	48
4h	2.80		CN	MeO					Me	H	11	
4i	3.38		CN	CF_3O					Me	H	45	
4j	2.80		CN	EtO					Me	H	350	
4k	4.45		CN	PhO					Me	H	25	
4l	4.02		CN	$PhCH_2$					Me	H	22.5	
4m	3.07		CN		Cl				Me	H	19	
4n	4.45		CN		PhO				Me	H	18	
4o	2.91		CN		MeS				Me	H	25	
4p	4.24		CN		CF_3				Me	H	22	
4q	2.52		CN			Ph			Me	H	35	
4r	1.37		CN			NMe_2			Me	H	150	
4s	1.32		CN			NHCOMe			Me	H	575	
4t	3.26		CN			N(Me)COMe			Me	H	400	
5a	2.47		CN	Cl	Cl				Me	H	22.5	
5b	2.36		CN	F		F			Me	H	90	
5c	5.35		CN	MeO		MeO			Me	H	150	
5d	5.35		CN	Cl			Cl		Me	H	12.5	
5e	4.12		CN	*t*Bu			*t*Bu		Me	H	5	6.5
5f	2.70		CN	CF_3			CF_3		Me	H	4.5	
5g	3.13		CN	OH			Cl		Me	H	92.5	
5h	2.32		CN	MeO			Cl		Me	H	650	
5i	3.09		CN	MeO			NO_2		Me	H	55	
5j	4.41		CN	EtCOO			Cl		Me	H	55	
5k	3.88		CN	*i*Pr				*i*Pr	Me	H	20.8	23.5
5l	4.41		CN	*t*Bu				Me	Me	H	160	
5m	2.20		CN	*t*Bu				Et	Me	H	150	
5n	3.66		CN	NO_2				Me	Me	H	160	
5o	1.94		CN		Cl	Cl			Me	H	15	
5p	1.47		CN		NO_2	F			Me	H	20	
5q	3.75		CN		–OCH_2O–				Me	H	67.5	
5r	3.78		CN		Cl		Cl		Me	H	9.8	27.5
6a	3.85		CN	Cl	Cl	Cl			Me	H	15	
6b	3.97		CN	Cl		Cl	Cl		Me	H	7	
6c	5.21		CN	Cl		*t*Bu	Cl		Me	H	11.3	8.5
6d	3.48		CN	Cl		Cl		Me	Me	H	55	
6e	3.27		CN	Me		Cl		Me	Me	H	48	
6f	4.19		CN	*i*Pr		Cl		Me	Me	H	20	
6g	3.05		CN	Me		Me		Me	Me	H	68	
6h	4.23		CN	$CF(CF_3)_2$		Me		Me	Me	H	160	
7a	4.31		CN	*t*Bu					Et	H	40	
7b	3.91		CN	CF_3O					Et	H	26	
7c	3.79		CN	Cl	Cl				Et	H	17.5	
7d	3.70		CN	Me	Cl				Et	H	15	
7e	5.02		CN	*t*Bu			Cl		Et	H	15	11
7f	3.61		CN	Et				Me	Et	H	80	
7g	4.01		CN	*i*Pr				Me	Et	H	45	
7h	4.50		CN	Cl		Cl	Cl		Et	H	5.5	7.25
7i	4.22		CN	Cl		Cl		Cl	Et	H	160	
8a	4.44		CN	Cl			Cl		*n*Pr	H	8.5	
8b	4.14		CN	Et				Me	*n*Pr	H	48	
8c	4.54		CN	*i*Pr				Me	*n*Pr	H	10.5	
9a	4.71		CN	*t*Bu					*i*Pr	H	9.5	15
9b	5.42		CN	*t*Bu		Cl			*i*Pr	H	6.5	42
9c	4.41		CN	*i*Pr				Me	*i*Pr	H	27.5	47
9d	4.41		CN	Cl		Cl		Me	*i*Pr	H	62.5	480
9e	4.19		CN	Me		Cl		Me	*i*Pr	H	11	

Table 1 (*continued*)

Compound	Clog *P*	R	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	IC_{50} (*B. ovis*) (μM)	IC_{50} (*L. cuprina*) (μM)
9f	3.98		CN	Me		Me		Me	*i*Pr	H	55	
10a	4.97		CN	CF_3O					*n*Bu	H	15	
10b	4.75		CN	Me	Cl				*n*Bu	H	4.3	8.3
10c	4.75		CN	Me			Cl		*n*Bu	H	6	12.5
10d	6.08		CN	*t*Bu			Cl		*n*Bu	H	4	5.5
10e	4.75		CN	Cl				Me	*n*Bu	H	6	56
10f	4.14		CN	Me				Me	*n*Bu	H	35	
10g	4.67		CN	Et				Me	*n*Bu	H	46	33
10h	5.07		CN	*i*Pr				Me	*n*Bu	H	20.3	
10i	3.78		CN	Me				NO_2	*n*Bu	H	71	
10j	5.78		CN	*i*Pr		Cl		Me	*n*Bu	H	17.5	
10k	4.64		CN	Me		Me		Me	*n*Bu	H	11	
10l	3.24		CN		MeO	MeO	MeO		*n*Bu	H	21	
11a	3.91		CN	Me					*i*Bu	H	11	
11b	4.84		CN	Cl			Cl		*i*Bu	H	6.8	
11c	5.24		CN		Cl		Cl		*i*Bu	H	5.5	
11d	5.58		CN		CF_3		CF_3		*i*Bu	H	6.3	
11e	5.43		CN	Cl		Cl	Cl		*i*Bu	H	2.7	5.5
11f	4.51		CN	Me		Me		Me	*i*Bu	H	30	175
11g	5.74		CN	Cl	Cl		Cl	Cl	*i*Bu	H	45	
12a	4.05		CN	Cl			Cl		Ph	H	8.5	
12b	3.50		CN	F			Me		Ph	H	12.5	
12c	4.15		CN	*i*Pr				Me	Ph	H	43	55
13a	6.76		CN	Me		Me		Me	*n*Oct	H	22	
14a	4.22		CN	Cl		Cl		Cl	Me	78:22 *E*:*Z*-Me	85	
14b	6.18		CN	*t*Bu			Cl	Me	Me	*E*-*n*Pr	2300	
14c	2.36		CN		MeO		MeO		Me	*Z*-*i*Pr	65	
15a	1.67	Cyclopentyl	CN						Me	H	85	
15b	2.23	Cyclohexyl	CN						Me	H	50	
15c	3.82	Cyclohexyl	CN						*n*Pr	H	27.5	
15d	3.62	Cyclohexyl	CN						Ph	H	7.5	12.5
15e	3.27	2,3-Me_2cyclohexyl	CN						Me	H	250	
15f	2.51	Bicyclo[2.2.1]hept-2-yl	CN						Me	H	48	
15g	3.93	Tetrahydro-naphthalen-1-yl	CN						Me	H	26	
15h	4.24	4-Chloronaphth-1-yl	CN						Me	H	15	
15i	2.57	2-Chlorobenzyl	CN						Me	H	37.5	
15j	3.00	4-ClPhCONH	CN						Et	H	72.5	
15k	2.71	Cyclohexylcon(Me)	CN							$(CH_2)_3$	88	
15l	2.15	Piperidin-1-yl	CN						Me	H	125	
15m	1.26	5-Clpyrid-2-yl	CN						Me	H	95	
15n	2.00	3,5-Cl_2pyrid-2-yl	CN						Me	H	45	
15o	2.24	Quinolin-3-yl	CN						Me	H	26	
15p	0.70	Thiazol-2-yl	CN						Me	H	90	
16a	3.87		MeCO	Cl		Cl	Cl		Me	H	45	
16b	4.70		*i*-PrCO	Cl		Cl	Cl		Me	H	16	
16c	5.01		EtOCO	Cl		Cl	Cl		Me	H	11	
16d	5.17		PhCO		CF_3				Me	H	12.5	17.5
16e	6.54		PhCO	Cl			CF_3		Me	H	18	
16f	5.42		PhCO	*i*Pr				Me	Me	H	58	
16g	6.34		PhCO	*i*Pr				*i*Pr	Me	H	2250	
16h	5.91		PhCO	Cl		Cl	Cl		Me	H	9	
16i	4.99		PhCO	Me		Me		Me	Me	H	44	
16j	5.24		3-ClPhNHCO		Cl				Me	H	12.5	250
Ponasterone A											1.1	0.9
20-Hydroxy-ecdysone											24	5.5

2.2.1. 3D QSAR models

Both CoMFA and COMSIA models were derived from the data. The CoMFA models using steric and electrostatic fields alone, or augmented by calculated log *P*, were of higher statistical significance than the COMSIA models using a combination of steric, electrostatic, lipophilic, donor and acceptor fields. Consequently only the CoMFA results are reported here. Table 2 shows that the statistical quality of the CoMFA models was similar. The compounds in the data set were methylene lactams, with one of the most active members providing the template for 3D QSAR alignment. This compound (**6b**) was also conformationally simple, with only restricted rotation around the N-Ph bond needing to be considered (Fig. 3).

The model using steric, and electrostatic CoMFA fields had the best statistical quality. Figure 4 shows the predicted values for *B. ovis* pI_{50} values compared to the measured values. Compounds in the training set were predicted within a factor of 1.5 on average. The cross-validation predictions were within a factor of 2.7 on average. Lipophilicity appears to be important for activity, as a plot of pI_{50} against calculated log *P* (Fig. 5) clearly shows. Only compounds with *n*-butyl substituents on the methylene moiety of the methylene lactam and very large substituents on the *ortho*

Table 2
Results of the 3D QSAR modelling studies for *B. ovis*

Parameter	CoMFA	CoMFA + log *P*	CoMFA refined
SEP	0.435	0.440	0.424
# Components	7	8	8
q^2 (LOO)	0.48	0.47	0.51
SEE	0.196	0.186	0.183
r^2	0.89	0.91	0.91
F	127	125	129
Contribution %			
Steric	63	63	62
Electrostatic	37	33	38
Lipophilic/log *P*		4	
Number of compounds	113	113	113

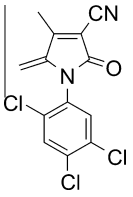


Figure 3. Methylene lactam **6b**, used for CoMFA alignments.

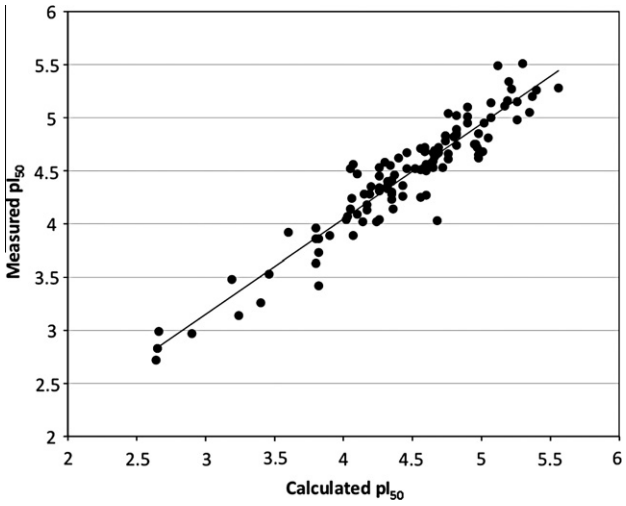


Figure 4. Predicted pI_{50} value for *B. ovis* from CoMFA model using steric and electrostatic fields.

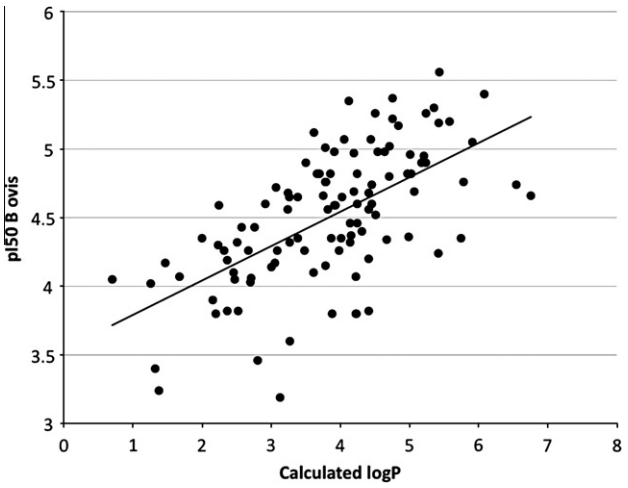


Figure 5. Relationship between *B. ovis* pI_{50} and calculated log *P*. The template molecule **6b**, and four other compounds mentioned in the text, have been omitted.

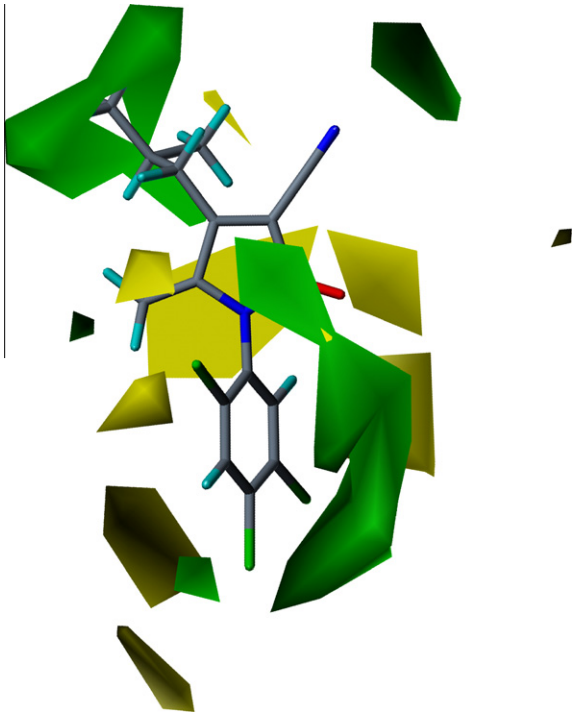


Figure 6. CoMFA steric map with an active template lactam **11e**. Green regions favour additional steric bulk, yellow regions disfavour additional steric bulk.

position of the phenyl ring, deviate markedly from this relationship. However, as log *P* (lipophilicity) and calculated molar refractivity MR (size and polarisability) also correlated ($r^2 = 0.74$) with one another, the apparent effect of lipophilicity could be due to molecular size.

Given the tendency of leave-one-out cross-validation to overestimate the predictivity of models, we also carried out a four group cross-validation study for the best QSAR model, CoMFA refined. This omits 29 compounds from the training set and predicts their activities using the QSAR model derived from the remaining compounds. We obtained an SEP of 0.46 and a cross-validated r^2 of 0.42 for six components. This is slightly inferior to the LOO model reported in Table 2, but is still statistically significant and may be a more accurate reflection of the predictivity of the model.

These structure–activity relationships are consistent with the fact that nuclear hormone receptors bind endogenous ligands that are relatively rigid and quite lipophilic. If the ecdysone receptor structure deformed continuously in response to changes in the ligand then there would be poor correlation in the model. The high degree of correlation in this model supports the conclusion from previous QSAR studies on ecdysteroids[27,28] and diacylhydrazines[29,30] that the LBD does not greatly alter its conformation on binding variously substituted ligands within each chemotype. This is consistent with the idea that the EcR LBD does indeed adopt a limited number of stable binding pocket configurations.

2.3. Molecular field maps

The molecular field maps in Figures 6 and 7 summarise the contributions that molecular size and/or lipophilicity and partial charge make to the binding of the analogues in the data set. The steric map in Figure 6 shows that there is a sterically favoured

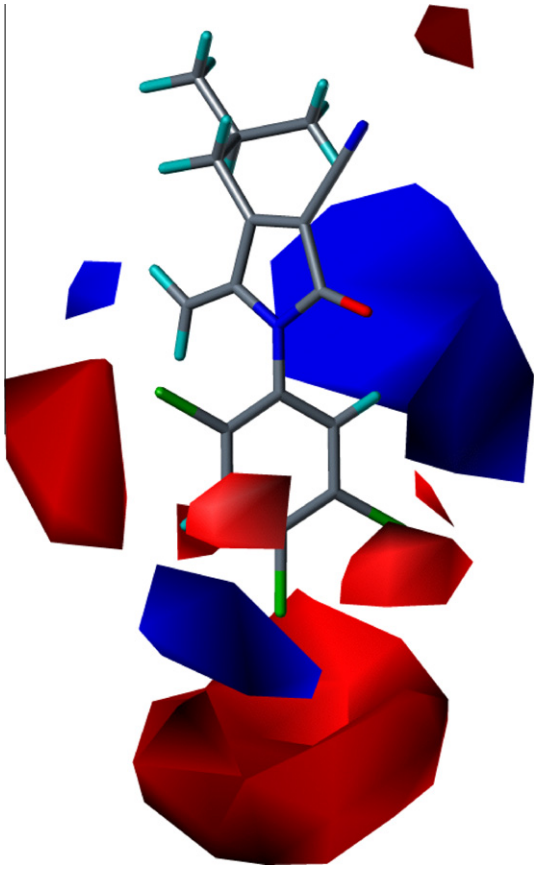


Figure 7. CoMFA electrostatic map with an active template lactam **11e**. Blue regions indicate where partial positive charge increases bioactivity, red regions where partial negative charge favours bioactivity.

(green) region above and on one face of the lactam ring. Alkyl substituents on the R^7 position interact with this region, which results in increased binding. There is another sterically favoured region below and on one side of the phenyl or other ring moiety that is attached to the lactam nitrogen atom. Sterically unfavourable (yellow) regions exist largely on the region of space on the opposite side to the sterically favoured region (which is beside and below the phenyl ring or equivalent). There is also a sterically disfavoured region in line with the methylene moiety, consistent with the poor activities of analogues that have longer alkyl substituents on the methylene group.

The electrostatic map in Figure 7 is more difficult to interpret in terms of structure–activity relationships. There are essentially two main regions, one that favours partial positive charge (blue field map) behind the lactam ring, and one that favours partial negative charge below the phenyl ring or equivalent (red field map). The latter region may reflect the favourable presence of halogens at or near the 4-position of the phenyl ring. These are electronegative atoms and so tend to adopt partial negative charges. The large blue region, favouring partial positive charge, may not be indicating that partial positive charge is beneficial, but rather, it may reflect the relatively strong binding of alkyl substituents in the 6-position and the weaker binding exhibited by analogues with partially negatively charged substituents (such as nitro, hydroxyl, or ether) in this region.

2.4. Whole insect *B. ovis* toxicity

A number of the compounds which displayed binding to the recombinant *B. ovis* ecdysone receptor LBD in vitro were tested in vivo using a *B. ovis* nymphal development assay. LC_{50} values for compounds tested in feeding assays are shown in Table 3. The values ranged from 26 to 244 μg/mL. The correlation coefficient for the association between IC_{50} (Table 1) and LC_{50} in the louse nymphal assay was $r = 0.64$ ($p < 0.1$). This correlation was influenced by a high leverage value (compound **15l**) which had markedly higher IC_{50} and LC_{50} than other values in the data set. Omitting this point still gave a correlation of $r = 0.45$, suggesting a relationship between the binding values measured in the competitive inhibition assay and toxicity measured in the louse nymphal assays.

Table 3
LC_{50} for test compounds against *B. ovis*

Compound	LC_{50} (μg/mL)	95% Confidence limits
5o	167	29–462
7e	190	46–343
8a	66	10–168
10b	175	66–888
11c	119	62–182
11d	26	6–74
15d	130	72–205
15l	244	85–458

3. Conclusion

We have discovered a new class of ligands for the ecdysone receptor in the γ-methylene γ-lactams, some of which bind with an affinity comparable to that observed for ecdysteroids. The degree of correlation in the QSAR studies on this series of molecules supports the argument that they all bind to one particular conformation of the receptor binding pocket. This in turn adds to the weight of evidence that the ecdysone receptor ligand binding pocket does not deform continuously in response to different ligand chemistries, but rather has a discrete binding conformation for each ligand chemotype.

We have demonstrated that methylene lactams bind to recombinant ecdysone receptor LBDs from more than one order of insects and, in the case of *B. ovis*, that this binding translates to developmental toxicity in vivo. Insecticidal activity towards non-lepidopteran species provides hope that new classes of ecdysone receptor modulators can be developed into commercially viable insecticides with activity spectra different from that of the diacylhydrazines.

4. Experimental section

4.1. Chemistry

4.1.1. General notes

Microwave reactions were conducted in a Biotage Initiator microwave reactor. Melting points were determined on a Bausch & Lomb hot-stage melting point apparatus and are uncorrected. ^1H and ^{13}C spectra were recorded on a Bruker AV400 at 400 and 100.6 MHz, respectively, unless otherwise stated. Chemical shifts were measured in ppm relative to $CDCl_3$ then related to tetramethylsilane ($\delta = 0.00$). Multiplicity is reported as s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Infrared spectra were recorded on a Perkin Elmer 842 spectrophotometer. Positive ion electron impact mass spectra (EIMS) and high resolution electron impact mass spectra (HR-EIMS) were recorded on a Thermoquest MAT 95XL, using an ionisation energy of 70 eV. Accurate mass measurements were obtained with a resolution of

5000–10,000 using perfluorokerosene (PFK) as the reference compound. Known compounds were prepared by the routes previously described in the literature[31] or by the methods outlined below and had physical and spectral characteristics in accordance with those reported. Cyanoacetic acid was dried at 50 °C under vacuum over $CaCl_2$ before use. Dry *N*,*N*-dimethylformamide (DMF) was obtained from a solvent dispensing system built by J. C. Meyer based on a design developed by Pangborn et al.[32] Petroleum spirit refers to the fraction boiling between 40 and 60 °C unless otherwise stated. Chromatographic purification was conducted by radial chromatography on Merck Silica Gel 60PF_{254} on a chromatotron (Harrison Research) unless otherwise stated. Dry vacuum column chromatography was conducted on Merck Silica Gel 60 15–40 μm.[33] Yields are not optimised.

4.1.2. 1-(2-Ethoxyphenyl)-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (3a) (method D)

Morpholine (five drops) was added to a solution of *N*-[2-ethoxyphenyl]cyanoacetamide (2.04 g, 10 mmol) and butane-2,3-dione (0.94 g, 6 mmol) in dry DMF (4 mL). The resulting mixture was stirred at room temperature for 6 h then diluted slowly with water (20 mL). After standing overnight the precipitate was filtered off to give crude **3a** as a solid which was used in the next step without further purification.

4.1.3. 1-(2-Ethoxyphenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (4j) (method E)

Crude 1-(2-ethoxyphenyl)-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile from the previous step was taken up in formic acid (6 mL) and heated at 60 °C for 4 h. The cooled solution was diluted with water and extracted with ether (30 mL). The ether phase was dried ($MgSO_4$), filtered and concentrated under reduced pressure. Purification by radial chromatography (dichloromethane elution) then recrystallisation from isopropanol gave **4j (1.49 g,** 59%) as colourless needles mp 110 °C; ^{1}H NMR ($CDCl_3$): δ 1.27 (t, J = 6.8 Hz, 3H, OCH_2CH_3), 2.42 (s, 3H, CH_3), 4.01 (s, J = 7.0 Hz, 2H, OCH_2CH_3), 4.78 (s, 1H, =CH), 5.17 (s, 1H, =CH), 6.99 (t, J = 6.4 Hz, 1H, Ar-H4), 7.00 (d, J = 7.7 Hz, 1H, Ar-H3), 7.15 (d, J = 7.3 Hz, 1H, Ar-H6), 7.36 (t, J = 7.6 Hz, 1H, Ar-H5); ^{13}C NMR ($CDCl_3$): δ 12.4, 14.6, 64.2, 100.2, 108.0, 112.1, 113.5, 120.9, 122.0, 130.3, 130.6, 145.3, 155.0, 157.9, 163.4; IR (KBr): ν 3420, 2985, 2224, 1715, 1633, 1506, 1391, 1290, 1170, 1048, 893, 758 cm^{-1}; HRMS (EI) m/z: calcd for $C_{15}H_{14}N_2O_2$: 254.1050 $[M]^{+\cdot}$; found: 254.1046.

4.1.4. 1-[3-Chlorophenyl]-4, 5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (3b)

1,8-Diazabicyclo[5.4.0]undec-7-ene (four drops) was added to a solution of *N*-[3-chlorophenyl)-2-cyanoacetamide (2.00, 10.0 mmol) and butane-2,3-dione (0.93 g, 10.5 mmol) in dry DMF (4 mL) (exotherm). The resulting mixture was stirred at room temperature for 30 min then water (20 mL) and diethyl ether (30 mL) were added. The aqueous layer was acidified to pH 3 with concentrated hydrochloric acid. The layers were separated and the organic layer was washed with water (20 mL). The organic layer was concentrated to give crude **3b** which was used in the next step without further purification

4.1.5. 1-[3-Chlorophenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (4m)

Prepared by method E, 90 °C, 90% formic acid, 1.5 h. After cooling, the reaction mixture was diluted with methanol (8 mL), stirred, then filtered and washed with methanol to give **4m** as a yellow solid (1.81 g, 74%). A sample was recrystallised from dichloromethane and methanol with carbon filtration to give fine, flat, pale yellow needles: mp 203 °C dec. ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, CH_3), 5.12 (d, J_{gem} = 2.7 Hz, 1H, =CH), 5.28 (d, J_{gem} = 2.7 Hz, 1H, =CH), 7.16–7.20 (m, 1H, ArH), 7.28–7.30 (m, 1H, ArH), 7.38–7.46 (m, 2H, ArH); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 12.5, 100.6, 107.9, 111.5, 125.9, 127.9, 128.8, 130.6, 134.5, 135.2, 145.2, 157.7, 163.0; IR (KBr): ν 3403, 3084, 2228, 1705, 1611, 1595, 1389, 1153, 784, 685 cm^{-1}; HRMS (EI) m/z: calcd for $C_{13}H_9Cl_2N_2O$: 244.0398 $[M]^{+\cdot}$; found: 244.0405.

4.1.6. 1-[3-(Trifluoromethyl)phenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (4p)

Prepared by method D using morpholine as the base, overnight, followed by method E, trifluoroacetic acid, 18 °C, overnight. The cooled reaction solution was diluted with aqueous isopropanol (1:1, 20 mL) to precipitate **5r (0.71 g,** 85%) as colourless needles: mp 158 °C; ^{1}H NMR ($CDCl_3$): δ 2.46 (s, 3H, CH_3), 5.11 (d, J_{gem} = 2.7 Hz, 1H, =CH), 5.33 (d, J_{gem} = 2.7 Hz, 1H, =CH), 7.48 (d, J = Hz, 1H, Ar-H), 7.54 (s, 1H, Ar-H2), 7.60–7.68 (m, 2H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 12.4, 101.0, 107.7, 111.5, 124.5 (q, J_{CF} = 4 Hz), 123.4 (q, J_{CF} = 272 Hz), 125.3 (q, J_{CF} = 4 Hz), 130.4, 131.0, 132.1 (q, J_{CF} = 33 Hz), 134.0, 144.9, 158.2, 163.1; IR (KBr): ν 3413, 2227, 1709, 1631, 1610, 1329, 1188, 1157, 1123, 808, 698 cm^{-1}; HRMS (EI) m/z: calcd for $C_{14}H_9F_3N_2O$: 278.0661 $[M]^{+\cdot}$; found: 278.0655.

4.1.7. 1-(2,4-Dimethoxyphenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (5c)

Prepared by method D using morpholine as the base, 28 days, followed by method G, chromatography eluent 1:1 dichloromethane/petroleum spirit followed by recrystallisation (methanol) to give **5c** (24%) as yellow crystals: mp 176 °C; ^{1}H NMR ($CDCl_3$): δ 2.42 (s, 3H, CH_3), 3.73 (s, 3H, OCH_3), 3.82 (s, 3H, OCH_3), 4.78 (d, J_{gem} = 2.0 Hz, 1H, =CH), 5.14 (d, J_{gem} = 2.0 Hz, 1H, =CH), 6.51–6.57 (m, 2H, Ar-H5, Ar-H6), 7.06 (d, $J_{3,5}$ = 8.4 Hz, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 12.5, 55.6, 55.8, 99.8, 99.9, 105.0, 108.1, 112.1, 114.5, 130.8, 145.7, 156.7, 157.6, 161.6, 163.6; IR (KBr): ν 3419, 2938, 2228, 1714, 1632, 1514, 1315, 1295, 1213, 1030 831 cm^{-1}; HRMS (EI) m/z: calcd for $C_{15}H_{14}N_2O_3$ 270.0999 $[M]^{+\cdot}$; found 270.1000.

4.1.8. *N*-[2,5-Bis(trifluoromethyl)phenyl]cyanoacetamide (1a) (method A)

1,3-Diisopropylcarbodiimide (DIC) (1.95 mL, 12.6 mmol) was added dropwise to a stirred, chilled (0–5 °C) suspension of 2,5-bis(trifluoromethyl)aniline (2.86 g, 12.5 mmol) and cyanoacetic acid (1.15 g, 13.5 mmol) in dry DMF (25 mL) maintained under nitrogen. The resulting mixture was stirred at room temperature for 2 days, filtered then poured into water (150 mL). The precipitated solid was filtered off, washed with water and dried under vacuum to give **1a (2.77 g,** 75%) as a white solid: mp 158 °C sub; ^{1}H NMR ($CDCl_3$): δ 3.64 (s, 2H, CH_2), 7.60 (d, $J_{3,4}$ = 8.2 Hz, 1H, Ar-H4), 7.82 (d, $J_{3,4}$ = 8.2 Hz, 1H, Ar-H3), 8.13 (br s, 1H, NH), 8.49 (s, 1H, Ar-H6); ^{13}C NMR (DMSO-d_6): δ 26.4, 116.0, 123.1 (q, J_{CF} = 274 Hz), 123.5 (q, J_{CF} = 273 Hz), 124.3 (m), 127.1 (q, J_{CF} = 3 Hz), 128.5 (q, J_{CF} = 5 Hz), 128.6 (q, J_{CF} = 29 Hz), 133.6 (q, J_{CF} = 34 Hz), 136.1 (q, J_{CF} = 2 Hz); IR (KBr) ν 3279, 1684, 1595, 1545, 1438, 1393, 1314, 1267, 1172, 1124, 1083, 1044, 897, 837, 657 cm^{-1}; HRMS (EI) m/z: calcd for $C_{11}H_6F_6N_2O$: 296.0379 $[M]^{+\cdot}$; found: 296.0375.

4.1.9. 1-[2,5-Bis(trifluoromethyl)phenyl]-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (3c)

Morpholine (seven drops) was added to a solution of *N*-[2,5-bis(trifluoromethyl)phenyl]cyanoacetamide (1.40 g, 4.7 mmol) and butane-2,3-dione (0.41 mL, 4.7 mmol) in dry DMF (1.5 mL). The resulting mixture was stood at room temperature for 4 weeks then poured into water (50 mL) and extracted with ethyl acetate

(3 × 50 mL). The combined organic phases were washed with water (3 × 50 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give crude **3c (1.65 g)** as a violet foam which was used in the subsequent step without further purification.

4.1.10. 1-[2,5-Bis(trifluoromethyl)phenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (5f) (method G)

A solution of crude 1-[2,5-bis(trifluoromethyl)phenyl]-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (0.53 g, 1.5 mmol) in formic acid (2 mL) was heated by microwave to 120 °C for 10 min then cooled, poured into water (50 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic phases were washed with water (3 × 50 mL), dried ($MgSO_4$) and concentrated under reduced pressure. The crude material was subjected to radial chromatography (10:1 dichloromethane/petroleum spirit) and recrystallised from ethyl acetate/petroleum spirit to provide **5f (67 mg,** 13% over 2 steps) as cream crystals: mp 150 °C; ^{1}H NMR ($CDCl_3$): δ 2.48 (s, 3H, CH_3), 4.69 (d, J_{gem} = 3.1 Hz, 1H, =CH), 5.28 (d, J_{gem} = 3.1 Hz, 1H, =CH), 7.59 (s, 1H, Ar-H6), 7.90 (d, $J_{3,4}$ = 8.2 Hz, 1H, Ar-H4), 8.00 (d, $J_{3,4}$ = 8.2 Hz, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 12.5, 100.9, 107.9, 111.2, 122.0 (q, J_{CF} = 274 Hz), 122.5 (q, J_{CF} = 274 Hz), 127.3 (q, J_{CF} = 4 Hz), 128.9 (q, J_{CF} = 5 Hz), 129.2 (q, J_{CF} = 4 Hz), 132.5 (q, J_{CF} = 1 Hz), 133.8 (q, J_{CF} = 32 Hz), 135.9 (q, J_{CF} = 34 Hz), 145.8, 158.5, 163.2; IR (KBr): ν 3072, 2231, 1726, 1637, 1442, 1333, 1319, 1187, 1125, 1085 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{15}H_8F_6N_2O$: 346.0535 $[M]^{+\cdot}$; found: 346.0538.

4.1.11. 1-(2-Methoxy-5-chlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (5h)

Prepared by method D using morpholine as the base, 28 days, followed by method G, chromatography eluent 3:1 dichloromethane/petroleum spirit to give **5h** (21%) as a yellow solid: mp 190 °C; ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H, CH_3), 3.76 (s, 3H, OCH_3), 4.81 (d, *J* = 2.6 Hz, 1H, =CH), 5.19 (d, *J* = 2.6 Hz, 1H, =CH), 6.96 (d, *J* = 9.0 Hz, 1H, H3), 7.18 (d, *J* = 2.6 Hz, 1H, H6), 7.37 (dd, *J* = 2.6, 9.0 Hz, 1H, H4); ^{13}C NMR ($CDCl_3$): δ 12.5, 56.1, 100.2, 108.1, 111.8, 113.5, 122.7, 125.5, 130.3, 130.6, 144.9, 154.5, 158.0, 163.1; IR (KBr): ν 3420, 3070, 2228, 1717, 1633, 1502, 1165, 816 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{14}H_{11}ClN_2O_2$: 274.0509 $[M]^{+\cdot}$; found: 274.0497.

4.1.12. *N*-(3-Nitro-4-fluorophenyl)cyanoacetamide (1b)

Diphenyl phosphate (4.85 g, 24 mmol) was added to a stirred solution of 4-fluoronitroaniline (2.8 g, 20 mmol) and cyanoacetic acid (2.04 g, 24 mmol) in DMF (6 mL) followed by pyridine (1.92 g, 24 mmol). The resulting mixture was stirred overnight then slowly diluted with water (60 mL) and the precipitated solid filtered off to give **1b** as light brown crystals (2.53 g, 61%): mp 167 °C; ^{1}H NMR ($CDCl_3$): δ 3.94 (s, 2H, CH_2CN), 7.56 (dd, J_{HH} = 9.1, J_{HF} = 11.1 Hz, 1 H, Ar-H5), 7.81 (ddd, J_{HH} = 3.8, 9.1 Hz, J_{HF} = 2.9 Hz, 1H, Ar-H6), 8.42 (dd, J_{HH} = 2.7 Hz, J_{HF} = 6.8 Hz, 1H, Ar-H2), 10.75 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 27.2, 115.9, 116.1 (d, J_{CF} = 3 Hz), 119.3 (d, J_{CF} = 22 Hz), 126.9 (d, J_{CF} = 8 Hz), 135.4 (d, J_{CF} = 3 Hz), 136.7 (d, J_{CF} = 8 Hz), 151.1 (d, J_{CF} = 260 Hz), 162.1; IR (KBr): ν 3311, 2264, 1678, 1562, 1347, 1229, 839 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_9H_6FN_3O_3$: 223.0388 $[M]^{+\cdot}$; found: 223.0381.

4.1.13. 1-(3-Nitro-4-fluorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (5p)

Prepared by method D using morpholine as the base, overnight, followed by method E, 18 °C, 3 days. The reaction solution was stirred into ice/water and the precipitate filtered off to give **5o** (49%) as a solid: mp 198 °C; ^{1}H NMR ($CDCl_3$): δ 2.49 (s, 3H, CH_3), 5.15 (d, *J* = 3.1 Hz, 1H, =CH), 5.37 (d, *J* = 3.1 Hz, 1H, =CH), 7.46 (dd, $J_{5,6}$ = 9.0, J_{HF} = 10.1 Hz, 1H, Ar-H5), 7.62 (ddd, $J_{2,6}$ = 2.7, J_{HF} = 3.8, $J_{5,6}$ = 9.0 Hz, 1H, Ar-H6), 8.03 (dd, $J_{2,6}$ = 2.7, J_{HF} = 6.5 Hz, 1H, Ar-H2); ^{13}C NMR ($CDCl_3$): δ 12.5, 100.7, 107.9, 111.1, 119.9 (d, J_{CF} = 22 Hz), 125.2, 129.7 (d, J_{CF} = 4 Hz), 134.7 (d, J_{CF} = 9 Hz), 137.7 (d, J_{CF} = 4 Hz), 144.6, 154.8 (d, J_{CF} = 268 Hz), 158.2, 162.9; IR (KBr) ν 3413, 3082, 2236, 1710, 1633, 1538, 1350, 1259, 1160 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{13}H_8FN_3O_3$: 273.0544 $[M]^{+\cdot}$; found: 273.0545.

4.1.14. 1-(2,4,5-Trichlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (6b)

Prepared by method D using morpholine as the base, 2 days, followed by method E, 50 °C, 16 h. 17%, purified by recrystallisation [diethyl ether/petroleum spirit (bp 80–100 °C)] to give **6b** as a fawn solid: mp 178 °C; ^{1}H NMR ($CDCl_3$): δ 2.45 (s, 3H, CH_3), 4.82 (d, J_{gem} = 2.6 Hz, 1H, =CH), 5.31 (d, J_{gem} = 2.6 Hz, 1H, =CH), 7.41 (s, 1H, Ar-H6), 7.66 (s, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 12.6, 110.2, 107.8, 111.4, 130.4, 131.8, 132.1, 132.3, 132.7, 134.9, 143.9, 158.6, 162.7; IR (KBr): ν 3429, 2225, 1722, 1633, 1469, 901, 880 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{13}H_7Cl_3N_2O$: 311.9618 $[M]^{+\cdot}$; found: 311.9615.

4.1.15. *N*-[4-Chloro-2-isopropyl-6-methylphenyl] cyanoacetamide (1c)

Prepared by method A, 2 h, and recrystallised from chloroform to give **1c** (27%) as a white solid: mp 168 °C; ^{1}H NMR ($CDCl_3$): δ 1.20 (d, *J* = 6.8 Hz, 6H, $CH(CH_3)_2$), 2.20 (s, 3H, CH_3), 3.00 (sept, *J* = 6.8 Hz, 1H, $CH(CH_3)_2$), 3.57 (s, 2H, CH_2), 7.10 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.15 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.38 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 18.3, 23.2, 25.8, 28.9, 114.7, 124.2, 128.2, 129.6, 134.4, 137.8, 147.7, 159.9; IR (KBr): ν 3251, 2965, 2929, 2258, 1652, 1533, 1386, 1346, 1237, 1222, 892, 864 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{13}H_{15}ClN_2O$: 250.0867 $[M]^{+\cdot}$; found: 250.0861

4.1.16. 1-(4-Chloro-2-isopropyl-6-methylphenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (6f)

Prepared by method D using morpholine as the base, 7 days, followed by method E, 70 °C, 2 h, chromatography eluent 1:1 dichloromethane/petroleum spirit. Purified by recrystallisation (diethyl ether/petroleum spirit) to give **6f** (20%) as amber prisms: mp 153 °C; ^{1}H NMR ($CDCl_3$): δ 1.13 (t, *J* = 7.1 Hz, 6H, $(CH_3)_2CH$), 2.02 (s, 3H, Ar-CH_3), 2.48 (s, 3H, CH_3), 2.59 (sept, *J* = 7.0 Hz, 1H, $(CH_3)_2CH$), 4.66 (d, *J* = 2.2 Hz, 1H, =CH), 5.18 (d, *J* = 2.2 Hz, 1H, =CH), 7.16 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.23 (d, *J* = 1.8 Hz, 1H, Ar-H); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 12.5, 17.8, 23.5, 24.1, 28.9, 99.7, 108.7, 111.6, 124.9, 128.2, 128.6, 135.8, 139.2, 145.2, 150.2, 157.5, 163.0; IR (KBr): ν 3415, 3125, 2965, 1713, 1633, 1387, 1170, 901, 882, 859 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{17}H_{17}ClN_2O$: 300.1024 $[M]^{+\cdot}$; found: 300.1015.

4.1.17. *N*-{4,6-Dimethyl-2-[fluorobis(trifluoromethyl)methyl] phenyl}-cyanoacetamide (1d)

A mixture of 4,6-dimethyl-2-[fluorobis(trifluoromethyl)methyl] aniline (1.45 g, 5 mmol) and cyanoacetic acid (1.28 g, 15 mmol) in diethylcyanamide (8 mL) was heated at 60 °C for 1 h. Additional cyanoacetic acid (0.86 g, 10 mmol) was added and heating continued for 1 h. The cooled mixture was stirred into water (50 mL) and extracted with ether (30 mL). The organic phase was removed and washed with water (3 × 30 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure. The residue was subjected to radial chromatography (dichloromethane elution) to give the crude product as a pale brown oil which slowly solidified. Purification by column chromatography (eluent:gradient of ethyl acetate in 60–80 °C bp petroleum spirit) gave **1d (0.79 g,** 39%) as colourless fluffy needles: mp 141 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.26 (s, 3H, Ar-6-CH_3), 2.38 (s, 3H, Ar-4-CH_3), 3.51 (s, 2H, CH_2CN), 7.25 (br s, 1H, Ar-H), 7.30 (br s, 1H, Ar-H), 7.57 (br s, 1H, NH); ^{13}C

NMR (DMSO): δ 18.3, 20.9, 25.7, 93.3 (dsept, J_{CF} = 33, 207 Hz), 116.2, 120.7 (dq, J_{CF} = 28, 288 Hz), 122.8 (m), 125.3 (m), 132.4 (m), 135.1, 138.2 (m), 140.0, 162.1; IR (KBr): ν 3255, 2939, 2263, 1673, 1520, 1268, 1216, 981, 737 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{14}H_{11}F_7N_2O$: 356.0754 $[M]^{+\cdot}$; found: 356.0748.

4.1.18. 1-{4,6-Dimethyl-2-[fluorobis(trifluoromethyl)methyl] phenyl}-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (6h)

Prepared by method D using morpholine as the base, 6 h, followed by method E, formic acid, 80 °C, 1 h. The solid which precipitated on cooling the reaction mixture was filtered off and washed lightly with aqueous isopropanol to give **6h (0.85 g,** 73%) as colourless plates: mp 188 °C (sublimes); ^{1}H NMR ($CDCl_3$): δ 2.04 (s, 3H, CH_3), 2.42 (s, 3H, CH_3), 2.45 (s, 3H, CH_3), 4.59 (br d, 1H, =CH), 5.18 (d, *J* = 2.2 Hz, 1H, =CH), 7.32 (s, 1H, Ar-H), 7.35 (s, 1H, Ar-H); ^{13}C NMR ($CDCl_3$): δ 12.4, 17.7, 21.3, 93.0 (dsept, J_{CF} = 33, 209 Hz), 99.9, 108.2, 111.6, 120.2 (dq, J_{CF} = 28, 288 Hz), 120.6 (dq, J_{CF} = 28, 286 Hz), 124.8 (d, J_{CF} = 17 Hz), 126.1 (br m, J_{CF} = 2 Hz), 128.3, 134.9, 140.2 (d, J_{CF} = 1 Hz), 140.8, 145.1, 157.9, 162.8; IR (KBr): ν 3424, 2231, 1717, 1291, 1267, 1243, 1229, 1214, 975 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{18}H_{13}F_7N_2O$: 406.0911 $[M]^{+\cdot}$; found: 406.0907.

4.1.19. (*Z*) and (*E*)-1-(3,5-Dimethoxyphenyl)-4-methyl-5-(2-methylpropylidene)-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (14c) and (14e)

Prepared by method D using morpholine as the base, 6 days, followed by method E using formic acid, 60 °C, 24 h, chromatography eluent dichloromethane to give a 3:1 mixture of two isomers (23%). These were separated by repeated radial chromatography (dichloromethane elution) then recrystallised from methanol to give cream crystals. Compound 14c (major isomer): mp 142 °C; ^{1}H NMR ($CDCl_3$): δ 0.87 (d, *J* = 6.4 Hz, 6H, CH(CH_3)$_2$), 2.09–2.23 (m, 1H, C*H*(CH_3)$_2$), 2.39 (s, 3H, CH_3), 3.78 (s, 6H, OCH_3), 5.40 (d, *J* = 11.0 Hz, 1H, =CH), 6.39 (d, *J* = 2.4 Hz, 2H, Ar-H2, Ar-H6) , 6.52 (t, *J* = 2.4 Hz, 1H, Ar-H4); ^{13}C NMR ($CDCl_3$): δ 12.5, 22.5, 25.9, 55.5, 101.4, 105.6, 106.6, 112.3, 129.1, 136.4, 137.5, 159.4, 161.2, 164.6; IR (KBr): ν 3416, 2968, 2228, 1712, 1614, 1195, 1153, 847 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{18}H_{20}N_2O_3$: 312.1468 $[M]^{+\cdot}$; found: 312.1467. Compound 14e (minor isomer): mp 167 °C; ^{1}H NMR ($CDCl_3$): δ 1.08 (d, *J* = 6.6 Hz, 6H, CH(CH_3)$_2$), 2.62 (s, 3H, CH_3), 3.04–3.17 (m, 1H, C*H*(CH_3)$_2$), 3.79 (s, 6H, OCH_3), 5.47 (d, *J* = 11.0 Hz, 1H, =CH), 6.32 (d, *J* = 2.2 Hz, 2H, Ar-H2, Ar-H6) , 6.51 (t, *J* = 2.2 Hz, 1H, Ar-H4); ^{13}C NMR ($CDCl_3$): δ 16.5, 23.2, 27.2, 55.5, 100.9, 107.0, 109.3, 112.2, 132.2, 135.1, 137.2, 156.1, 161.3, 162.4; IR (KBr): ν 3419, 2969, 2225, 1713, 1613, 1194, 1155, 1059, 847 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{18}H_{20}N_2O_3$: 312.1468 $[M]^{+\cdot}$; found: 312.1464.

4.1.20. *E*- and *Z*-*N*-Methylcyclohexanecarbohydrazide

A solution of cyclohexanecarboxylic chloride (14.4 g, 98 mmol) in dry dichloromethane (28 mL) was added dropwise to a stirred, chilled (-35 °C) solution of methylhydrazine (15.5 mL, 294 mmol) in dry dichloromethane (28 mL). The addition rate and cooling bath (acetone/CO_2) temperature were controlled so as to maintain the internal reaction temperature between −35 °C and −40 °C. The reaction mixture was then allowed to warm to 10 °C and ice (50 g) added. The reaction mixture was stirred for 30 min then the organic layer was removed and the aqueous layer extracted with 2 M hydrochloric acid (3 × 100 mL). The combined acidic extracts were washed with dichloromethane (100 mL), basified with 10% aqueous sodium hydroxide solution and extracted with dichloromethane (3 × 100 mL). These combined dichloromethane extracts were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a pale yellow solid, which was recrystallised from diethyl ether to give an inseparable mixture of E- and Z-*N*-*Methylcyclohexanecarbohydrazide* (4.51 g, 29%) as colourless crystals: mp 112 °C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ 1.08–1.80 (m, 10H, cyclohexyl-H), 2.35 (tt, *J* = 3.1, 11.1 Hz, 0.5H, cyclohexyl-H1), 3.06, 3.14 (2*s*, 3H, NCH_3), 4.06 (br s, 2H, NH_2); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 25.6, 25.7, 25.9, 28.9, 38.0, 38.4, 39.2, 40.3, 174.1, 178.5; IR (KBr): ν 3222, 3219, 2936, 2855, 1661, 1621, 1446, 1392, 1217, 1102, 995 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_8H_{16}N_2O$: 156.1257 $[M]^{+\cdot}$; found: 156.1255.

4.1.21. *E*- and *Z*-*N*′-(2-Cyanoacetyl)-*N*-methylcyclohexanecarbohydrazide (1e)

A mixture of *E*- and *Z*-*N*-methylcyclohexanecarbohydrazide (1.00 g, 6.4 mmol), cyanoacetic acid (0.55 g, 6.4 mmol) and phosphorus oxychloride (1.96 g, 12.8 mmol) in dichloroethane (10 mL) was heated at 90 °C (oil bath) for 2.5 h. It was then cooled to room temperature, diluted with ice (10 g) and stirred for 30 min. The layers were separated then the aqueous layer extracted with dichloromethane (3 × 20 mL). The combined organic extracts were extracted with 10% sodium hydroxide (2 × 20 mL). The sodium hydroxide extracts were acidified with concentrated hydrochloric acid and then extracted with dichloromethane (2 x20 mL). The dichloromethane layers were dried ($MgSO_4$), filtered and concentrated under reduced pressure to give a yellow oil (1.15 g, 86%). Purification by dry vacuum column chromatography, eluting with a gradient of methanol in chloroform gave **1e** as a pale yellow oil (0.81 g, 57%). ^{1}H NMR ($CDCl_3$): δ 1.04–1.39 (m, 5H, CH_2), 1.57–1.79 (m, 5H, CH_2), 2.46 (tm, *J* = 11.2 Hz, 1H, CH), 3.06 (s, 2H, CH_2CN), 3.27, 3.53 (2s, 3H, N-CH_3), 9.44, 9.92 (2s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 24.4, 25.4, 25.6, 28.9, 29.3, 35.5, 38.5, 39.9, 40.7, 113.9, 114.4, 161.4, 161.8, 175.9, 179.0; IR (KBr): ν 3312. 3015, 2304, 1800, 1710, 1504, 1441, 1393, 1319, 1128 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{11}H_{17}N_3O_2$: 223.1315 $[M]^{+\cdot}$; found: 223.1313.

4.1.22. *E*- and *Z*-*N*-(3-Cyano-2-oxo-2,4,5,6-tetrahydro-1*H*-indol-1-yl)-*N*-methylcyclohexanecarboxamide (15k)

Prepared by method D using morpholine as the base, 3 days, followed by method E, 90 °C, 4 h, chromatography eluent 1:1:2 ethyl acetate: dichloromethane: petroleum spirit to give **15k** (45%) as a pale yellow gum which was an inseparable 6:1 mixture of isomers: ^{1}H NMR ($CDCl_3$): δ 1.03–1.92 (m, 10H, cyclohexyl-H), 1.97 (quintet, *J* = 6.0 Hz, 2H, H5), 2.03 (m, 1H), 2.42, 2.51 (2q, *J* = 5.1 Hz, 2H, H6), 2.83, 2.91 (2t, *J* = 6.4 Hz, 2H, H4), 3.08, 3.36 (2s, 3H, NCH_3), 5.80, 6.00 (2t, *J* = 4.6 Hz, 1H, H7); ^{13}C NMR ($CDCl_3$): δ 22.3, 24.1, 24.2, 24.3, 25.3, 25.4, 25.5, 28.4, 29.0, 29.3, 29.7, 34.0, 38.1, 39.7, 40.6, 100.5, 111.3, 118.1, 119.6, 135.1, 135.2, 157.1, 157.8, 161.9, 175.2, 178.8; IR (KBr): ν 3480, 2934, 2859, 2230, 1723, 1680, 1602, 1452, 1377, 1342, 1148, 735 cm^{-1}; HRMS (EI) *m*/*z*: calcd for $C_{17}H_{21}N_3O_2$: 299.1628 $[M]^{+\cdot}$; found: 299.1621.

4.1.23. 1-(5-Chloropyrid-2-yl)-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (3d)

Prepared by method D using morpholine as the base, 16 h, to give the crude product which was triturated with isopropanol, filtered off and used in the next step without further purification.

4.1.24. 1-(5-Chloropyrid-2-yl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (15m) (method F)

Trifluoromethanesulfonic acid (1.5 mL, 17 mmol) was added to a stirred, chilled (0–5 °C) solution of 1-(5-chloropyrid-2-yl)-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (460 mg, 1.7 mmol) in dichloromethane (5 mL). The resulting solution was stirred at 0–5 °C for 2 h then diluted with ice-water (25 mL). The organic layer was removed and washed with water (2 × 25 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to a mustard solid. Recrystallisation from methanol gave 1-(5-chloropyrid-2-yl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole-3-carbonitrile (0.25 g, 60%) as beige needles:

mp 173 °C; ^{1}H NMR ($CDCl_3$): δ 2.44 (s, 3H, CH_3), 5.50 (d, J_{gem} = 2.5 Hz, 1H, =CH), 6.04 (d, J_{gem} = 2.0 Hz, 1H, =CH), 7.66 (d, $J_{3,4}$ = 8.6 Hz, 1H, Py-H3), 7.80 (dd, $J_{4,6}$ = 2.4 Hz, $J_{3,4}$ = 8.6 Hz, 1H, Py-H4), 8.44 (d, $J_{4,6}$ = 2.4 Hz, 1H, Py-H6); ^{13}C NMR ($CDCl_3$): δ 12.4, 105.2, 107.1, 111.5, 120.9, 130.1, 138.2, 142.6, 146.6, 147.2, 159.2, 163.2; IR (KBr): ν 3426, 2231, 1723, 1473, 1389, 1112, 891, 762 cm^{-1}; HRMS (EI) m/z: calcd for $C_{12}H_8ClN_3O$: 245.0350 $[M]^{+\cdot}$; found: 245.0351.

4.1.25. 3-Acetyl-1-(2,4,5-trichlorophenyl)-4,5-dimethyl-5-hydroxy-2-oxo-2,5-dihydro-1*H*-pyrrole (3e) (method D)

DABCO (1,4-diazabicyclo[2.2.2]octane) (20 mg, 0.18 mmol) was added to a mixture of 3-oxo-*N*-(2,4,5-trichlorophenyl)butanamide (1.40 g, 5 mmol) and butanedione (0.44 mL, 5.0 mmol) in dry DMF (1.5 mL). The resulting solution was stirred at room temperature for 39 days then poured into water (25 mL) and extracted with ethyl acetate (3 × 25 mL). The combined organic phases were washed with water (2 × 25 mL), dried ($MgSO_4$), filtered and concentrated under reduced pressure to give crude **3e (2.16 g)** as a brown gum which was used in the next step without further purification.

4.1.26. 3-Acetyl-1-(2,4,5-trichlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16a)

Prepared by Method G, chromatography eluent 1:1 dichloromethane: petroleum spirit to provide **16a** (6% over 2 steps) as a pale yellow gum: ^{1}H NMR ($CDCl_3$, 200 MHz): δ 2.51 (s, 3H, CH_3), 2.62 (s, 3H, $COCH_3$), 4.76 (d, J_{gem} = 2.4 Hz, 1H, =CH), 5.26 (d, J_{gem} = 2.4 Hz, 1H, =CH), 7.43 (s, 1H, Ar-H6), 7.68 (s, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$, 50 MHz): δ 10.4, 29.4, 99.6, 127.0, 131.4, 131.5, 131.7, 132.6, 133.0, 133.9, 145.0, 154.6, 166.7, 195.8; IR (neat): ν 2932, 1719, 1630, 1466, 1134, 1081 cm^{-1}; HRMS (EI) m/z: calcd for $C_{14}H_{10}Cl_3NO_2$: 328.9772 $[M]^{+\cdot}$; found: 328.9773.

4.1.27. 4-Methyl-3-oxo-*N*-(2,4,5-trichlorophenyl) pentanamide (1f)

Pyridine (0.1 mL, 1.2 mmol) was added to a solution of 2,4,5-trichloroaniline (3.92 g, 20 mmol) and ethyl isobutyryl acetate (5.2 mL, 32 mmol) in xylene (30 mL). The resulting solution was stirred and heated to reflux for 1 h then additional pyridine (0.1 mL) added and the mixture heated to reflux for 1 h. A portion of the xylene (25 mL) was distilled off then the mixture allowed to cool to room temperature. The precipitated solid was filtered off and dried under vacuum over $CaCl_2$ to give **1f (2.49 g,** 40%) as pale grey needles: mp 96 °C; ^{1}H NMR ($CDCl_3$): δ 1.19 (d, J = 6.4 Hz, 6H, CH_3), 2.73 (sept, J = 6.4 Hz, 1H, $CH(CH_3)_2$), 3.66 (s, 2H, CH_2), 7.48 (s, 1H, Ar-H6), 8.60 (s, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 17.7, 42.3, 46.5, 121.8, 122.5, 127.4, 129.9, 131.5, 134.1, 164.0, 211.1; IR (KBr): ν 3201, 2979, 1707, 1686, 1570, 1496, 1360, 1074, 886 cm^{-1}; HRMS (EI) m/z: calcd for $C_{12}H_{12}Cl_3NO_2$: 306.9928 $[M]^{+\cdot}$; found: 306.9943.

4.1.28. 3-Isobutyryl-1-(2,4,5-trichlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16b)

Prepared by method D using DABCO as the base, 39 days, followed by method G, chromatography eluent 1:3 dichloromethane/petroleum spirit to give **16b** (51%) as a colourless solid; mp 103 °C; ^{1}H NMR ($CDCl_3$): δ 1.13 (d, J = 6.8 Hz, 3H, $CH(CH_3)_2$), 1.14 (d, J = 6.8 Hz, 3H, $CH(CH_3)_2$), 2.46 (s, 3H, CH_3), 3.67 (sept, J = 6.8 Hz, 1H, $CH(CH_3)_2$), 4.74 (d, J_{gem} = 2.7 Hz,1H, =CH), 5.22 (d, J_{gem} = 2.7 Hz, 1H, CH), 7.44 (s, 1H, Ar-H6), 7.67 (s, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 11.7, 17.8, 17.9, 39.1, 98.5, 127.4, 131.4, 131.7, 131.9, 132.5, 132.9, 134.3, 145.1, 154.2, 166.0, 202.6; IR (KBr): ν 3092, 2976, 2936, 1679, 1631, 1591, 1474, 1407, 1389, 1354, 1167, 1128, 1082, 888, 871, 828 cm^{-1}; HRMS (EI) m/z: calcd for $C_{16}H_{14}Cl_3NO_2$: 357.0085 $[M]^{+\cdot}$; found: 357.0075.

4.1.29. 3-Ethoxycarbonyl-1-(2,4,5-trichlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16c)

Prepared by method D using morpholine as the base, 38 days, followed by method G, chromatography eluent 1:1 dichloromethane/petroleum spirit then recrystallised from methanol to give **16c** (34%) as a pale yellow solid: mp 121 °C; ^{1}H NMR ($CDCl_3$): δ 1.36 (t, J = 7.1 Hz, 3H, OCH_2CH_3), 2.48 (s, 3H, CH_3), 4.35 (q, J = 7.1 Hz, 2H, OCH_2CH_3), 4.67 (d, J_{gem} = 1.6 Hz, 1H, =CH), 5.19 (d, J_{gem} = 1.6 Hz, 1H, =CH), 7.39 (s, 1H, Ar-H6), 7.63 (s, 1H, Ar-H3); ^{13}C NMR ($CDCl_3$): δ 11.7, 14.2, 61.2, 98.3, 122.1, 131.4, 131.6, 131.9, 132.5, 132.8, 134.2, 144.6, 154.3, 162.3, 164.5; IR (KBr): ν 3128, 2981, 1736, 1629, 1473, 1403, 1357, 1266, 1156, 1131, 1101, 1082, 1034, 912 cm^{-1}; HRMS (EI) m/z: calcd for $C_{15}H_{12}Cl_3NO_3$: 358.9877 $[M]^{+\cdot}$; found: 358.9862.

4.1.30. *N*-[2-Chloro-5-(trifluoromethyl)phenyl]-3-oxo-3-phenyl propanamide (1g) (method C)

3-Amino-4-chlorobenzotrifluoromethane (1.00 g, 5.1 mmol) and ethyl benzoylacetate (1.20 g, 6.2 mmol) were subjected to microwave irradiation at 200 °C for 2 h. This reaction was carried out five times. Solid from the combined reaction mixtures was precipitated out with 75% ethanol in water to give **1g (1.36 g,** 16%). A sample recrystallised from diethyl ether and light petroleum gave colourless, fluffy needles: mp 135 °C. ^{1}H NMR ($CDCl_3$): δ 4.19 (s, 2H, CH_2), 7.30 (br d, J = 8.1 Hz, 1H, NHAr-H4), 7.47–7.56 (m, 3H, NHAr-H3, Ph-H3, -H5), 7.65 (t, J = 7.3 Hz, 1H, Ph-H4), 8.03 (d, J = 7.9 Hz, 2H, Ph-H2, -H6), 8.78 (1H, NHAr-H6), 10.17 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 45.0, 118.6 (q, J_{CF} = 4 Hz), 121.3 (q, J_{CF} = 4 Hz), 123.6 (q, J_{CF} = 272 Hz), 126.6, 128.5, 129.0, 129.6, 130.0 (q, J_{CF} = 33 Hz), 134.5, 135.3, 135.8, 164.3, 196.1; IR (KBr): ν 3191, 1693, 1662, 1587, 1532, 1428, 1332, 1219, 1167, 1123, 1083 cm^{-1}; HRMS (EI) m/z: calcd for $C_{16}H_{11}ClF_3NO_2$: 341.0425 $[M]^{+\cdot}$; found: 341.0427.

4.1.31. 3-Benzoyl-1-[2-chloro-5-(trifluoromethyl)phenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16e)

Prepared by method D using 1,8-diazabicyclo[5.4.0]undec-7-ene as the base, 7 days, followed by method E, formic acid, 45 °C, 4 h, dry column vacuum chromatography eluent 1:9 ethyl acetate/bp 60–80 °C petroleum spirit to give **17f** (44%) as a pale yellow semicrystalline mass: ^{1}H NMR ($CDCl_3$): δ 2.32 (s, 3H, CH_3), 4.71 (d, J_{gem} = 2.7 Hz, 1H, =CH), 5.19 (d, J_{gem} = 2.7 Hz, 1H, =CH), 7.49 (tm, J = 7.7 Hz, 2H, COPh-H3, -H5), 7.61 (tm, J = 7.4 Hz, 1H, COPh-H4), 7.65 (br s, 1H, N-Ar-H6), 7.67 (t, 1H, N-Ar-H4), 7.69 (t, 1H, N-Ar-H3), 7.69 (t, J = 8.2/8.8 Hz, 2H, Ar-H), 7.92 (dm, J = 7.7 Hz, 2H, COPh-H2, -H6); ^{13}C NMR ($CDCl_3$): δ 11.4, 97.5, 123.1 (q, J_{CF} = 272 Hz), 127.1 (q, J_{CF} = 4 Hz), 128.47 (q, J_{CF} = 4 Hz), 128.54, 129.7, 130.5 (q, J_{CF} = 34 Hz), 130.6, 131.4, 132.8, 133.9, 136.8, 138.0 (q, J = 1 Hz), 145.3, 150.3, 165.7, 190.8; IR (KBr): ν 3391, 3057, 1698, 1652, 1619, 1492, 1443, 1326, 1262, 1215, 1187, 1119, 1086, 1026, 951, 836, 687 cm^{-1}; HRMS (EI) m/z: calcd for $C_{20}H_{13}ClF_3NO_2$: 391.0581 $[M]^{+\cdot}$; found: 391.0567.

4.1.32. *N*-(2-Isopropyl-6-methylphenyl)-3-oxo-3-phenylpropanamide (1h) (method B)

A solution of 2-isopropyl-6-methylaniline (14.92 g, 100 mmol) and ethyl benzoylacetate (21.14 g, 110 mmol) in DMF (60 mL) was stirred and heated at 150 °C for 4 h then cooled to 80 °C. Water was added until the mixture was turbid. It was stirred for 30 min, water and ethanol added and then stirred for a further 1 h. The precipitated solid was filtered off to give **1h (20.4 g,** 72%). A sample was recrystallised from methanol to give a colourless solid: mp 289 °C sub; ^{1}H NMR ($CDCl_3$, 40 °C, 500 MHz): δ 1.14 (d, J = 6.9 Hz, 6H, $CH(CH_3)_2$), 2.18 (s, 3H, CH_3), 3.03 (sept, J = 6.9 Hz, 1H, $CH(CH_3)_2$), 4.15 (s, 2H, CH_2), 7.05 (br d, J = 7.6 Hz, 1H, NHAr-H3), 7.13 (br d, J = 7.6 Hz, 1H, NHAr-H5), 7.17 (t, J = 7.6 Hz, 1H, NHAr-H4), 7.50 (t, J = 7.6 Hz, 2H, COPh-H3, H5), 7.62 (t, J = 7.6 Hz, 1H, COPh-H4), 8.05

(d, J = 7.6 Hz, 2H, COPh-H2, H6), 8.46 (br s, 1H, NH); ^{13}C NMR ($CDCl_3$, 40 °C, 125 MHz): δ 18.6, 23.4, 28.7, 45.6, 123.5, 127.9, 128.1, 128.7, 129.0, 132.4, 134.3, 135.9, 136.3, 145.6, 164.4, 196.5; IR (KBr): ν 3258, 2963, 1625, 1550, 1469, 1257, 778 cm^{-1}; HRMS (EI) m/z: calcd for $C_{19}H_{21}NO_2$: 295.1567 [M]$^{+\cdot}$; found: 295.1567.

4.1.33. 3-Benzoyl-1-[2-isopropyl-6-methylphenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16f)

Prepared by method D using DABCO as the base, 21 days, followed by method E, 18 °C, 1 h to give **16f** (55%) as a yellow solid: mp 125 °C; ^{1}H NMR ($CDCl_3$): δ 1.17 (d, J = 6.8 Hz, 3H, CH(CH_3)$_2$), 1.18 (d, J = 6.8 Hz, 3H, CH(CH_3)$_2$), 2.13 (s, 3H, Ar-CH_3), 2.30 (s, 3H, CH_3), 2.78 (sept, J = 6.8 Hz, 1H, CH(CH_3)$_2$), 4.57 (d, J = 1.3 Hz, 1H, =CH), 5.07 (d, J = 1.3 Hz, 1H, =CH), 7.14 (d, J = 7.3 Hz, 1H, Ar-H3), 7.25 (d, J = 7.3 Hz, 1H, Ar-H5), 7.31 (t, J = 7.7 Hz, 1H, Ar-H4), 7.45 (t, J = 7.3 Hz, 2H, 7.7 Hz, COPh-H3, -H5), 7.57 (t, J = 7.3 Hz, 1H, COPh-H4), 7.90 (d, J = 7.3 Hz, 2H, COPh-H2, -H6); ^{13}C NMR ($CDCl_3$): δ 11.3, 18.1, 23.6, 24.3, 28.6, 97.0, 124.2, 128.3, 128.4, 129.4, 129.7, 130.4, 131.0, 133.8, 137.0, 137.3, 146.3, 148.3, 149.4, 166.3, 191.4; IR (KBr): ν 3423, 2963, 1692, 1654, 1628, 1472, 1449, 1164 cm^{-1}; HRMS (EI) m/z: calcd for $C_{23}H_{23}NO_2$: 345.1723 [M]$^{+\cdot}$; found: 345.1715.

4.1.34. 3-Benzoyl-1-[2,6-diisopropylphenyl]-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16g)

Prepared by method D using DABCO as the base, 21 days, followed by method E, formic acid, 18 °C, 1 h, chromatography eluent 1:19 ethyl acetate/petroleum spirit. Purified by recrystallisation (ethanol/water) to give **16g** (23%) as pale yellow needles: mp 164 °C; ^{1}H NMR ($CDCl_3$): δ 1.19 (d, J = 6.8 Hz, 6H, CH(CH_3)$_2$), 1.20 (d, J = 6.8 Hz, 6H, CH(CH_3)$_2$), 2.33 (s, 3H, CH_3), 2.73 (quint, J = 6.8 Hz, 2H, CH(CH_3)$_2$), 4.58 (d, J_{gem} = 1.5 Hz, 1H, =CH), 5.08 (d, J_{gem} = 1.5 Hz, 1H, =CH), 7.26 (d, J = 7.7 Hz, 2H, Ar-H3, -H5), 7.41 (t, J = 7.7 Hz, 1H, Ar-H4), 7.45 (t, J = 7.7 Hz, 2H, COPh-H3, -H5), 7.57 (t, J = 7.3 Hz, 1H, COPh-H4), 7.91 (d, J = 7.3 Hz, 2H, COPh-H2, -H6); ^{13}C NMR ($CDCl_3$): δ 11.2, 23.8, 24.3, 28.9, 97.1, 124.0, 128.3, 129.0, 129.8, 130.9, 133.7, 137.0, 147.5, 148.0, 149.5, 166.8, 191.5; IR (KBr): ν 3385, 2963, 2869, 1693, 1660, 1629, 1610, 1386, 1345, 1157, 860, 822, 712, 689 cm^{-1}; HRMS (EI) m/z: calcd for $C_{25}H_{27}NO_2$: 373.2036 [M]$^{+\cdot}$; found: 373.2027.

4.1.35. *N*-(2,4,5-Trichlorophenyl)-3-oxo-3-phenylpropanamide (1i)

Prepared by method B, to give **1i** (28%) as near colourless solid which was a mixture of the ketone and enol forms in solution: mp 168 °C; ^{1}H NMR (DMSO-d_6): δ 4.31 (s, 2H, $COCH_2$), 6.32 (s, 1H, COCH=C), 7.46–7.59 (m, 5H, COPh-H3, -H4, -H5 enol form), 7.55 (t, J = 7.5 Hz, 2H, COPh-H3, H5 ketone form), 7.67 (t, J = 7.3 Hz, 1H, COPh-H4 ketone form), 7.76 (d, J = 5.9 Hz, 2H, COPh-H2, H6 enol form), 7.89 (s, NHAr-H6 ketone form), 7.91 (NHAr-H6 enol form), 7.99 (d, J = 7.3 Hz, 2H, COPh-H2, H6 ketone form), 8.21 (s, 1H, NHAr-H3 enol form), 8.22 (s, 1H, NHAr-H3 ketone form); ^{13}C NMR (DMSO-d_6): δ 48.0, 89.9, 124.5'125.3, 126.0, 127.3, 128.8, 129.2, 129.3, 130.3, 131.0, 131.8, 133.8, 134.1, 134.9, 135.4, 136.5, 167.0, 170.1, 171.4, 194.9; IR (KBr): ν 3446, 3186, 2355, 1691, 1577, 1517, 1360, 1075, 756 cm^{-1}; HRMS (EI) m/z: calcd for $C_{15}H_{10}Cl_3NO_2$: 340.9772 [M]$^{+\cdot}$; found: 340.9786.

4.1.36. 3-Benzoyl-1-(2,4,5-trichlorophenyl)-4-methyl-5-methylene-2-oxo-2,5-dihydro-1*H*-pyrrole (16h)

Prepared by method D using DABCO as the base, 43 days, followed by method G, chromatography eluent 1:1 dichloromethane/petroleum spirit to give **16h** (61%) as a yellow solid: mp 166 °C; ^{1}H NMR ($CDCl_3$): δ 2.27 (s, 3H, CH_3), 4.72 (d, J = 2.6 Hz, 1H, =CH), 5.17 (d, J = 2.6 Hz, 1H, =CH), 7.46 (t, J = 7.7 Hz, 2H, COPh-H3, -H5), 7.48 (s, 1H, NAr-H6), 7.58 (t, J = 7.1 Hz, 1H, COPh-H4), 7.66 (s, 1H, N-Ar-H3), 7.89 (d, J = 7.7 Hz, 2H, COPh-H2, -H6); ^{13}C NMR ($CDCl_3$): δ 11.3, 97.6, 128.5, 129.6, 130.5, 131.4, 131.7, 131.9, 132.5, 132.8, 133.9, 134.2, 136.7, 145.1, 150.2, 165.6, 190.7; IR (KBr): ν 3416, 3082, 3061, 1713, 1659, 1469, 1394, 1360, 889, 845, 708, 693 cm^{-1}; HRMS (EI) m/z: calcd for $C_{19}H_{11}Cl_3NO_2$ [M-H]$^+$: 389.9850; found: 389.9848.

4.1.37. *N*,1-Bis(3-chlorophenyl)-4-methyl-5-methylene-2-oxo-*N*-phenyl-2,5-dihydro-1*H*-pyrrole-3-carboxamide (16j)

Prepared by method D using morpholine as the base, overnight, followed by method E, 18 °C, overnight. The reaction solution was diluted with aqueous isopropanol (1:1) to precipitate the product which was filtered off and dried to give **16j** (75%) as pale yellow needles: mp 164 °C; ^{1}H NMR ($CDCl_3$): δ 2.70 (s, 3H, CH_3), 5.15 (d, J = 2.6 Hz, 1H, =CH), 5.39 (d, J = 2.6 Hz, 1H, =CH), 7.07 (dm, J = 7.9 Hz, 1H, Ar-H), 7.20–7.25 (m, 2H, Ar-H), 7.33 (t, J = 1.8 Hz, 1H, Ar-H), 7.38–7.48 (m, 3H, Ar-H), 7.88 (t, J = 2.0 Hz, 1H, Ar-H), 10.57 (s, 1H, NH); ^{13}C NMR ($CDCl_3$): δ 11.5, 100.8, 117.9, 120.1, 120.5, 124.4, 125.9, 128.0, 128.8, 129.9, 130.5, 134.61, 134.63, 135.1, 139.0, 145.9, 154.6, 160.0, 168.3; IR (KBr): ν 3072, 1701, 1660, 1631, 1588, 1543, 875, 780 cm^{-1}; HRMS (EI) m/z: calcd for $C_{19}H_{14}Cl_2N_2O_2$: 372.0427 [M]$^{+\cdot}$; found: 372.0417.

4.2. Receptor screening

The ligand-binding regions from the EcR and USP proteins of the insect pests, *B. ovis*[34] ($BoEcR_{DEF}$/$BoUSP_{DEF}$) and *Lucilia cuprina*, ($LcEcR_{DEF}$/$LcUSP_{DEF}$) were co-expressed using a baculovirus vector, purified as a recombinant heterodimer by immobilised metal affinity chromatography,[35] and used in fluorescence polarisation assays.[24]

The standard fluorescence polarisation (FP) assay buffer used was 50 mM sodium phosphate, 100 mM NaCl, pH 7.4[24] with a final volume of 200 μL for all assays. The assays were set up in opaque black flat-bottomed 96-well plates and were incubated at 4 °C overnight, then re-equilibrated at room temperature for 3 h before reading the polarisation (mP) values. The plate reader was a PHERAstar instrument (BMG Labtech, Offenburg, Germany) with the fluorescence polarisation optic module FP 485 520A 520B (installed according to manufacture's instructions) and operated by the PHERAstar software. The standard instrument setup used the default FP basic parameters for a 96-well plate and involved a 3 s, 3 mm linear shake before each cycle.

4.2.1. High-throughput screening

All preparative pipetting steps for the high-throughput polarisation assays were carried out by the TECAN Genesis 200 robot run by Gemini software V.4.2.0.0 and using TECAN 200 μL disposable conductive tips (TECAN Cat. No. 10612510). The plate reader was normalised to the polarisation value of 100mP for the unbound fluorescein–inokosterone A conjugate (MB4628)[24] and the gain and focal height were adjusted against a blank (lacking recombinant heterodimer) at the start of each test run for each plate.

Each plate contained 6 μL of 60 different compounds in dimethyl sulfoxide (DMSO) randomly chosen from the CSIRO compound library[36] that had been dispensed into the centre wells of the 96-well plate for the high-throughput screening of a total of three thousand compounds. The plates were stored at −20 °C until ready for use and were allowed to thaw at room temperature for three hours prior to commencement of the assay. A script was written for the TECAN robot to pipette 194 μL of a buffer mixture into the wells of the plates containing the library compounds. The buffer mixture contained 2.51 mg/mL of purified $BoEcR_{DEF}$/$BoUSP_{DEF}$ heterodimer or 1.14 mg/mL $LcEcR_{DEF}$/$LcUSP_{DEF}$ heterodimer, 0.5 mg/mL of

bovine serum albumin (BSA) and 36 nM fluorescein–inokosterone A conjugate in the standard FP assay buffer.

Ten wells on each plate were manually set up to contain the native hormone (20-hydroxyecdysone) as positive controls at three different concentrations, in triplicate, and one well was used for the heterodimer-free blank. Buffer mixture was manually added to the controls and contained 2.51 mg/mL of purified $BoEcR_{DEF}$/$BoUSP_{DEF}$ or 1.14 μg/mL $LcEcR_{DEF}$/$LcUSP_{DEF}$ receptor, 0.5 mg/mL of bovine serum albumin (BSA), 36 nM fluorescein–inokosterone A conjugate and 6 μL of DMSO in the standard FP assay buffer. The blank contained 0.5 mg/mL of bovine serum albumin (BSA), 36 nM fluorescein–inokosterone A conjugate and 6 μL of DMSO in the standard FP assay buffer.

The compounds were screened at a rate of 10 plates (600 compounds) per day. The plates were set up in the afternoon, stored at 4 °C overnight and were then equilibrated at room temperature for three hours before reading the mP values. Data were plotted in Excel and used to calculate the IC_{50} for the binding activity of each compound.

4.2.2. IC_{50} determinations

A 30 mM stock solution of the test compound (i.e., the putative ligand) in DMSO was used to prepare a dilution series covering the assay range of 3 nM to 3 mM (final concentration). The diluent for the test compound was DMSO. These preparative pipetting steps were performed in 0.6 mL microfuge tubes. Each 200 μL assay included 6 μL of test compound in DMSO (3% v/v final concentration DMSO in each assay), 0.5 mg/mL bovine serum albumin (BSA), 36 nM fluorescein–inokosterone A conjugate and 2.51 mg/mL $BoEcR_{DEF}$/$BoUSP_{DEF}$ receptor or 1.14 mg/mL $LcEcR_{DEF}$/$LcUSP_{DEF}$ receptor in standard FP assay buffer.

The plate reader was normalised to 100 mP using a 200 μL sample containing 36 nM fluorescent conjugate, 0.5 mg/mL BSA and 3% v/v DMSO in standard FP assay buffer. Data were plotted in Excel (Microsoft Corporation). All samples were assayed in triplicate on a single plate and IC_{50} values were determined from the mean. Random samples of 10% of the IC_{50} determinations on the $BoEcR_{DEF}$/$BoUSP_{DEF}$ receptor and the $LcEcR_{DEF}$/$LcUSP_{DEF}$ receptor displayed a coefficient of variation of 1–6% and 1–4%, respectively, with the exception of one sample which displayed a coefficient of variation of 18% in the IC_{50} for binding to the $BoEcR_{DEF}$/$BoUSP_{DEF}$ receptor. Another compound was assayed on two separate occasions and displayed a coefficient of variation of 9% in the IC_{50} for binding to the $BoEcR_{DEF}$/$BoUSP_{DEF}$ receptor.

4.3. *B. ovis* toxicity assay

Wool snippets 1–1.5 cm in length were weighed to 20 mg amounts, added to flat bottom vials and treated with 200 μL of acetonic solutions of test compounds at concentrations ranging from 0.01 to 1000 μg/mL. Louse diet[37] was weighed to 10 mg quantities, added to 75 mm × 12 mm borosilicate culture tubes and treated with 50 μL of similar concentrations of test compound. Diet and wool were allowed to dry overnight at 5 °C. Lice were collected from sheep penned adjacent to the laboratory, provided with untreated louse diet and held overnight in an incubator at 36.5 °C and 68% relative humidity. The next morning the treated wool snippets were added to the culture tubes containing diet treated with corresponding concentrations of test compound together with ten third instar *B. ovis* nymphs. Four replicate tubes were used for each concentration and diet and wool in the control tubes were treated with acetone. All tubes were held in an incubator at 36.5 °C and 68% relative humidity for nine days to allow time for the nymphs to moult. On day nine lice were removed from the tubes and the numbers of dead lice recorded. LC_{50} values and 95% confidence limits were calculated using PoloPlus© LeOra Software (2002).[38] LC_{50} values were corrected for the mortality observed in solvent-only control experiments.

4.4. Computational methods

Molecular modelling was carried out using Sybyl 8.1 on Silicon Graphics Fuel and Red Hat Linux PC workstations. Structures were constructed using modelling and molecular mechanics minimisation in Sybyl using the Tripos forcefield and default values. Gasteiger–Huckel charges were used in the minimisation calculations. Structures were energy minimized using the default Sybyl energy minimizer and Gasteiger–Huckel charges.

4.4.1. Alignment and choice of conformation

The highly active and relatively rigid molecule, lactam **6b**, was used as a template molecule. Molecules were set to a conformation that most closely matched that of the template, and the Sybyl default minimizer was used to relieve any strain in the molecules. The molecules were aligned on the template to maximize the overlap between the methylene lactam ring and the aromatic ring attached to the lactam nitrogen atom. Where two conformations of similar energy existed for rotation around the lactam-aromatic ring bond, molecules were placed in a consistent conformation with placement of any *ortho* substituents done consistently so that moieties with similar properties were given maximum opportunity to overlap. Where it was not clear from energetic or conformational considerations, which of two possible conformers is the correct one, both conformations were used to develop an initial CoMFA model, which was then used to eliminate the conformation whose activity was predicted least accurately. The stereochemistry of the γ-methylene substituent in compounds with non-hydrogen substituents was fixed to *E*. A final CoMFA model was then developed using the conformation that was most consistent with the activities of other members of the data set. The conformations of the alkyl substituents on the methylene lactam ring were set to near orthogonality to the lactam ring, to minimize the energies of the ecdysone agonists. The conformations of other substituents on the lactam ring were set to be extended, then energy minimized. The alignment of all molecules on the template is illustrated in Figure 8.

4.4.2. 3D QSAR modelling

3D QSAR models were built using both the comparative molecular field analysis method (CoMFA)[39] and the comparative molecular similarity analysis method (COMSIA).[40] COMSIA dissects molecular interactions into five properties, and uses a Gaussian function to probe the similarity of each molecule with respect to the probe within the lattice. In contrast, CoMFA used only steric and electrostatic fields, which may explain why CoMFA models that included log *P* were generally also significant. Sybyl 8.1 was used to generate the CoMFA fields using Gasteiger–Huckel charges, with an sp^3 hybridized carbon probe with a charge of +1.0 for the CoMFA fields, and a grid spacing of 2 Å. The default values for the field cut offs were used. The log *P* (octanol–water) values calculated using the Pomona College *C*log *P* method.[41] The COMSIA fields were also generated using Sybyl 8.0 and Gasteiger–Huckel charges. The standard COMSIA probe with a unit radius, +1.0 charge, and unit hydrophobicity were used. Steric, electrostatic, lipophilic, donor, and acceptor fields were generated.

The QSAR models were derived using the partial least squares method. The sampls method was used to generate a cross-validated the leave-one-out (LOO) predictivity estimate of the models, and to determine the correct number of principal components for the final non-cross-validated models. Region focusing was also used to refine the CoMFA models, which resulted in a small improvement in statistical quality.

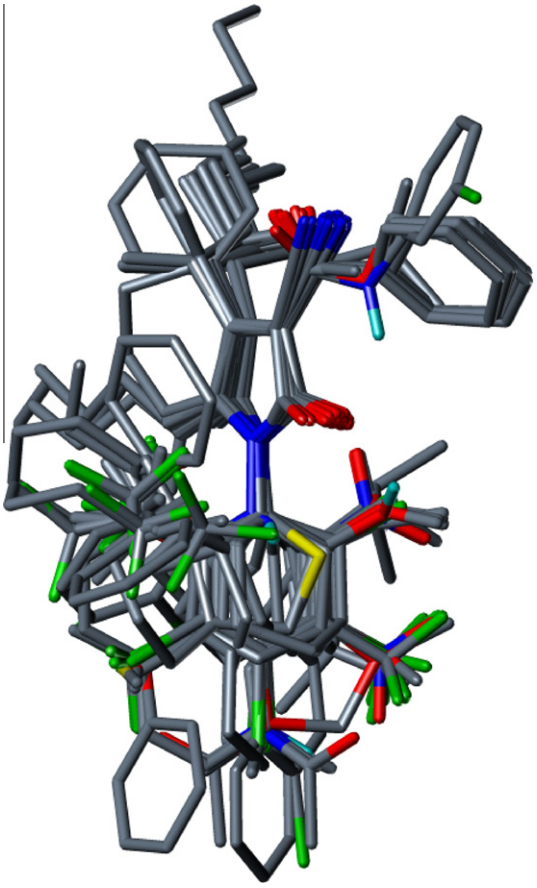


Figure 8. Alignment of methylene lactam ecdysone receptor ligands on template.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.020.

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