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Semi-Synthetic Ecdysteroids as Gene-Switch Actuators: Synthesis, Structure–Activity Relationships, and Prospective ADME Properties

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The ligand-inducible, ecdysteroid receptor (EcR) gene-expression system can add critical control features to protein expression in cell and gene therapy. However, potent natural ecdysteroids possess absorption, distribution, metabolism and excretion (ADME) properties that have not been optimised for use as gene-switch actuators in vivo. Herein we report the first systematic synthetic exploration of ecdysteroids toward modulation of gene-switch potency. Twenty-three semi-synthetic O*-alkyl ecdysteroids were assayed in both a natural insect system (*Drosophila B_{II} *cells) and engineered gene-switch systems in mammalian cells using* Drosophila melanogaster, Choristoneura fumiferana*, and* Aedes aegypti *EcRs. Gene-switch potency is maintained, or even enhanced, for ecdysteroids methylated at the 22-position in favourable cases. Furthermore, trends toward lower solubility, higher permeability, and higher blood–brain barrier penetration are supported by predicted ADME properties, calculated using the membrane-interaction (MI)-QSAR methodology. The structure–activity relationship (SAR) of alkylated ecdysteroids indicates that 22-OH is an H-bond acceptor, 25-OH is most likely an H-bond donor, and 2-OH and 3-OH are donors and/or acceptors in network with each other, and with the EcR. The strategy of alkylation points the way to improved ecdysteroidal actuators for switch-activated gene therapy.*

Introduction

In essence, gene therapy is the in situ administration of a therapeutic biological polymer. Just as the safety and efficacy of small-molecule drugs rely on an accurate understanding of dose–response relationships and adjustment of dose in view of a therapeutic window, so does the safety and effectiveness of gene therapy increase with the ability to modulate the level, time course, and spatial/tissue distribution of expression of the therapeutic gene.[1–6] This type of control can be achieved by use of a ligand-inducible gene-expression system (gene switch). In such systems, a small-molecule drug binds to, and modulates the action of an engineered transcription factor on a regulating promoter that is linked to the transcription factor through a complementary DNA binding site, and also to the gene of interest through sequence and spatial proximity. The insect-derived ecdysteroid receptor (EcR)-based system is a candidate for this task. Owing to the ability to control protein expression levels, the incorporation of gene switches into gene therapeutic regimens may render those therapies currently under development more effective, and also enable wider extension among indications in cancer,[7] cardiovascular diseases,[8,9] diabetes, neurodegenerative disorders,[10,11] motor neuron diseases, muscular dystrophy,[12] cystic fibrosis,[13,14] neuropathic pain,[15] rheumatoid arthritis[16,17] and regenerative medicine in general.[18,19] Additionally, gene switches have biopharmaceutical applications in areas such as cell-based assays and animal models for developmental drug testing, as well as biotherapeutics and biomaterials production.[20] Among the various available gene-switch platforms, the insect ecdysteroid-regulated switches are refractory to human endogenous steroids, and typically show very low basal transgene expression, high inducibility, and broad dose–response gradation—usually outperforming other systems in each of these aspects.[21–23] The EcR gene switch functions through assembly of 1) the nuclear receptor EcR with a heteropartner protein (the vertebrate retinoid X receptor, RXR, its insect orthologue, the ultraspiracle protein, USP, or a variant); 2) a DNA response element complementary to the DNA binding domains of EcR and the hetero-

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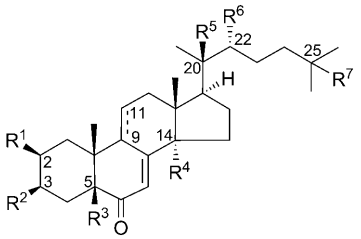
partner; and 3) a ligand for the EcR.[24] Complete assembly triggers downstream organisation of transcription factors and initiation of transcription of the programmed gene. From a medicinal point of view, the entire cascade may be actuated by the EcR ligand, that is to say, an otherwise non-medicinal substance that acts as the control regulator for the previously implanted—by viral vector or transfected autologous cell administration—gene switch/therapeutic gene programme. The ligand dose determines the magnitude of therapeutic transcription. Moreover, the pharmacodynamics of the therapeutic gene transcript are largely relayed through the pharmacodynamics of the actuating ligand; therefore, to a significant extent, one may modulate the gene behaviour by design of the drug. Consequently, in analogy to conventional drugs, ADME characteristics and pharmacokinetic profile are critical properties of a gene-switch actuator.

The natural ligands for the EcR are the insect moulting hormone, 20-hydroxyecdysone **25** (20E), and its 25-deoxy derivative, ponasterone A **26** (PoA). Several hundred analogues, mostly plant-derived, are known. Several natural ecdysteroids have been demonstrated to be effective EcR gene-switch actuators in mouse models using non-therapeutic reporter genes.[21,25] Furthermore, ecdysteroids appear toxicologically benign, and otherwise seem to have salutary pharmacological effects.[26,27] Low levels of ecdysteroids are normally present in certain vegetables of a healthy human diet. For use as drugs, however, despite promising molecular pharmacology, the physicochemical properties of these compounds are dubious. In general, ecdysteroids are too hydrophilic and water soluble, with numerous hydroxy groups that lend themselves to metabolism or conjugation. The half-life in humans is estimated to be as low as 4 h (ecdysone) and 9 h (20E).[28] Additionally, the specific H-bond acceptor/H-bond donor role of each of these hydroxy groups in EcR binding and gene-switch potency is not entirely transparent, in spite of the availability of crystal structures of several ligand–EcR complexes,[29–32] as well as multi-dimensional QSAR modelling.[33,34]

Herein we report the first systematic synthetic exploration of ecdysteroids toward modulation of gene-switch potency. Hydroxy groups, individually and severally, were methylated or otherwise alkylated (Table 1). Thereby, new semi-synthetic ecdysteroids, twenty-three in all, were synthesised, purified and assigned structurally. We hypothesised that alkylation might be tolerated or even beneficial at those positions where the hydroxy group does not function as a hydrogen donor in H-bonding interactions with the EcR. To examine this hypothesis, the steroids were assayed in gene-switch systems in murine cells. Furthermore, the ADME properties of the resultant structure, in particular, membrane permeability and resistance to metabolism, might improve overall.[35] Classic precedents in which pharmacokinetics or metabolism is modulated by the methylated analogue include morphine/codeine,[36,37] amphetamine/methamphetamine,[37,38] erythromycin/clarithro-

Table 1. Structures, names, and numbering of ecdysteroid ether analogues (**1**–**23**) and reference compounds (**24**–**30**).



No.	Structure	R^1	R^2	R^3	R^4	R^5	R^6	R^7	C9–C11[a]
1	20E 2-methyl ether	OCH_3	OH	H	OH	OH	OH	OH	1
2	20E 3-methyl ether	OH	OCH_3	H	OH	OH	OH	OH	1
3	20E 14-methyl ether	OH	OH	H	OCH_3	OH	OH	OH	1
4	20E 22-methyl ether	OH	OH	H	OH	OH	OCH_3	OH	1
5	20E 22-ethyl ether	OH	OH	H	OH	OH	OCH_2CH_3	OH	1
6	20E 22-*n*-propyl ether	OH	OH	H	OH	OH	OnPr	OH	1
7	20E 22-allyl ether	OH	OH	H	OH	OH	$OCH_2CH{=}CH_2$	OH	1
8	20E 22-*n*-butyl ether	OH	OH	H	OH	OH	OnBu	OH	1
9	20E 22-benzyl ether	OH	OH	H	OH	OH	OCH_2Ph	OH	1
10	20E 25-methyl ether	OH	OH	H	OH	OH	OH	OCH_3	1
11	20E 2,22-dimethyl ether	OCH_3	OH	H	OH	OH	OCH_3	OH	1
12	20E 3,22-dimethyl ether	OH	OCH_3	H	OH	OH	OCH_3	OH	1
13	20E 14,22-dimethyl ether	OH	OH	H	OCH_3	OH	OCH_3	OH	1
14	20E 22,25-dimethyl ether	OH	OH	H	OH	OH	OCH_3	OCH_3	1
15	20E 2,3,14,22-tetra methyl ether	OCH_3	OCH_3	H	OCH_3	OH	OCH_3	OH	1
16	PoA 2-methyl ether	OCH_3	OH	H	OH	OH	OH	H	1
17	PoA 14-methyl ether	OH	OH	H	OCH_3	OH	OH	H	1
18	PoA 22-methyl ether	OH	OH	H	OH	OH	OCH_3	H	1
19	PoA 2,22-dimethyl ether	OCH_3	OH	H	OH	OH	OCH_3	H	1
20	PoA 3,22-dimethyl ether	OH	OCH_3	H	OH	OH	OCH_3	H	1
21	PoA 14,22-dimethyl ether	OH	OH	H	OCH_3	OH	OCH_3	H	1
22	DaH 22-methyl ether	OH	OH	H	OH	OH	OCH_3	H	2
23	20E 22-[(2′R/S)-2′-ethyl oxiran-2′-yl)]ether	OH	OH	H	OH	OH	$OC(cyclo\text{-}OCH_2)CH_2CH_3$	OH	1
24	ecdysone	OH	OH	H	OH	H	OH	OH	1
25	20-hydroxyecdysone	OH	OH	H	OH	OH	OH	OH	1
26	ponasterone A	OH	OH	H	OH	OH	OH	H	1
27	dacryhainansterone	OH	OH	H	OH	OH	OH	H	2
28	muristerone A	OH	OH	OH	OH	OH	OH	H	1; 11α-OH
29	polypodine B	OH	OH	OH	OH	OH	OH	OH	1
30	3,5-dimethylbenzoic acid *N*-*tert*-butyl-*N*′-(2-ethyl-3-methoxybenzoyl)hydrazide								

[a] 1 = single bond, 2 = double bond.

mycin,[39,40] among others.[41–44] To explore this concept, we report ADME calculations based on the established membrane-interaction QSAR (MI-QSAR) methodology and discuss the new steroids in comparison with their nonalkylated counterparts. Finally, the new alkylated structures might illuminate ecdysteroid SAR, in particular the H-bonding role of specific hydroxy groups. To test this idea, we performed a SAR analysis in several gene-switch assays and the natural *Drosophila* B_{II} cell system. The composite information gained from the novel semi-synthetic ecdysteroids indicates maintained potency and points toward potentially improved ADME properties of specific derivatives, constituting valuable progress toward gene-switch activator molecules suitable for drug development.

Results

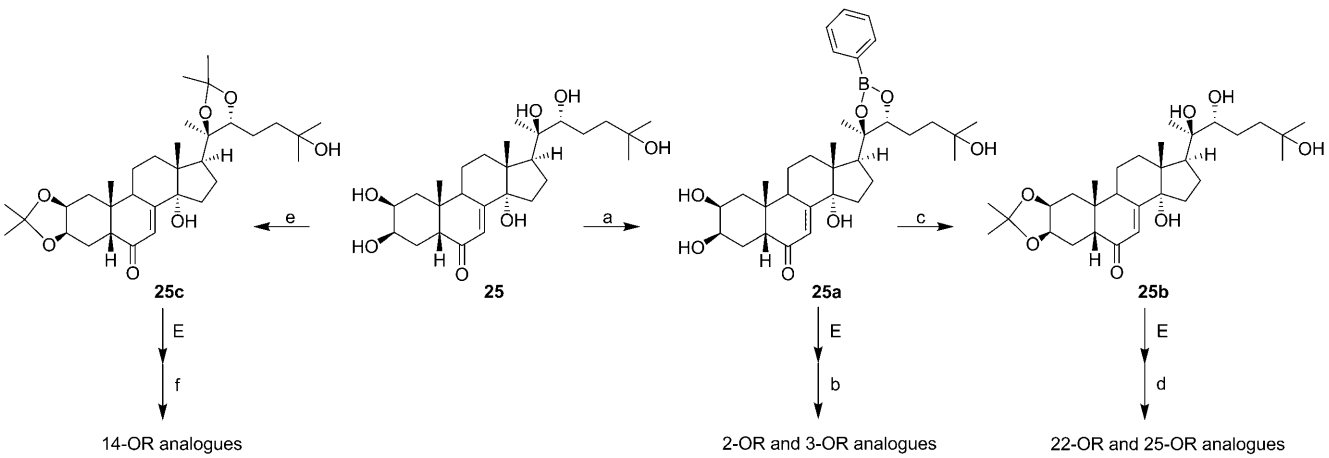
Synthesis

Twenty-three ecdysteroid *O*-alkyl ethers were synthesised (Table 1), including derivatives of 20E (**25**), the most abundant insect moulting hormone, PoA (**26**), one of the most potent natural ecdysteroids, and dacryhainansterone (DaH; **27**), a moderately strong agonist with an unusual core structure. The derivatives of 20E are five monomethyl ethers at the 2-, 3-, 14-, 22- or 25-OH (**1**–**4**, **10**), four dimethyl ethers at the 22-OH and one each of 2-, 3-, 14-, and 25-OH (**11**–**14**), and one tetramethyl ether (**15**). PoA derivatives include three mono-*O*-methyl ethers at the 2-, 14- or 22-OH (**16**–**18**), and three dimethyl ethers (**19**–**21**). Additionally, several 20E 22-*O*-ether analogues were prepared, including compounds with *O*-*n*-alkyl groups up to a four-carbon chain (**5**, **6** and **8**) and the allyl (**7**), benzyl (**8**) and 2′-ethyloxiranyl (**23**) ether groups. Selective introduction of a methyl group on individual hydroxy positions was obtained using the protection/deprotection strategy depicted in Scheme 1, which involves the transformation of the 2,3-*cis*- and/or 20,22-diol groups into acetonide or phenylboronate groups.[45,46]

Simultaneous preparation of singly and multiply methylated analogues (**16**, **17**, **19**, **20**) was achieved using a one-pot reaction approach starting with the unprotected ecdysteroid. In methylation reactions involving Ag_2O and CH_3I, the reactivity sequence of ecdysteroid hydroxy groups is 22-OH > 2-OH > 3-OH ⋙ 14-OH. While methylation of the tertiary hydroxy group at the 25-position of 20E was not observed using Ag_2O/CH_3I, the tertiary 14-OH could be converted into a CH_3O group by increasing the reaction temperature (up to 60 °C) or the reagent equivalents. However, large excess or prolonged exposure to Ag_2O led to product degradation, such as dehydration and/or alteration of the 7-en-6-one chromophore. As a case in point, formation of a methylated PoA derivative with an altered chromophore (7,9(11)-dien-6-one), DaH 22-methyl ether **22**, was observed after a prolonged exposure (46 h) of PoA to Ag_2O/CH_3I at room temperature. In the search for alternative O-methylation methods suitable for chemically sensitive molecules such as ecdysteroids, we found that six equivalents each of methyl triflate and DTBMP at 25 °C promoted selective methylation at the 25-OH of 20E 2,3-acetonide (**25b**), with a reactivity sequence of 25-OH > 22-OH ⋙ 14-OH. This method represents a newly developed procedure for O-methylation of polyhydroxylated steroids. In all of our experiments, the 20-OH position remained refractory to methylation.

Drosophila B_{II} cell morphology assay

D. melanogaster B_{II} cells naturally express the EcR–USP complex and give a specific and quantitative response to EcR agonists and antagonists.[47] *O*-Alkyl ecdysteroids **1**–**23** exert agonist potencies in the B_{II} bioassay at concentrations ranging from 100 μM down to 1 nM, depending on the number and the position of the ether substituent in the molecule (Table 2). In particular, methylation of the 2-, 3-, 14- and 25-hydroxy groups of



Scheme 1. Protection/deprotection strategy for the preparation of ecdysteroid *O*-alkyl ethers. Prior to etherification (E) reactions, the 2,3- and/or 20,22-diol groups of 20E (**25**) were selectively transformed into the corresponding 20,22-phenylboronate (**25a**), 2,3-acetonide (**25b**) or 2,3;14,22-diacetonide (**25c**) groups. *Reaction conditions:* a) phenylboronic acid (PBA), anhydrous DMF, RT, 1 h; b) H_2O_2/THF, 9:1 (*v*/*v*), pH 7, RT, 2.5 h; c) 1. 2,2-dimethoxypropane (DMP), dry acetone, fused pTsOH, RT, 3 h; 2. H_2O_2/THF 9:1 (*v*/*v*), pH 7, RT, 2.5 h; d) 0.1 M aq HCl/1,4-dioxane 1:1, RT, 2.5 h; e) DMP, dry acetone, fused pTsOH, RT, 6 h; f) AcOH 70%, 1,4-dioxane, reflux, 8 h. PoA derivatives were prepared similarly.

Table 2. Potency and efficacy of O-alkylated ecdysteroids (**1**–**23**) and reference compounds (**24**–**30**) measured by the *D. melanogaster* B_{II} bioassay (B_{II}) and the *C. fumiferana* (Cf) EcR-based gene-switch assays using the wild-type (wt) CfEcR or the "VY" (E274V/V390I/Y410E) mutant CfEcR.

No.	Structure	B_{II} EC_{50} [μM]	wt-CfEcR EC_{50} [μM]	wt-CfEcR RMFI[a]	VY-CfEcR EC_{50} [μM]	VY-CfEcR RMFI[a]
1	20E 2-methyl ether	1.10	>33[b]	0.00[b]	>33[b]	0.00[b]
2	20E 3-methyl ether	0.60	>33[b]	0.00[b]	~20[b]	0.21[b]
3	20E 14-methyl ether	3.20	>33	0.00	>33	0.00
4	20E 22-methyl ether	6.3×10^{-3}	>33[b]	0.00[b]	>33[b]	0.03[b]
5	20E 22-ethyl ether	2.2×10^{-2}	4.85	0.77	0.76	1.10
6	20E 22-*n*-propyl ether	0.83	>33	0.00	~12	0.49
7	20E 22-allyl ether	0.16	>33	0.00	~20	0.08
8	20E 22-*n*-butyl ether	0.10	>33	0.01	8.99	0.29
9	20E 22-benzyl ether	2.2×10^{-2}	>33	0.00	~10	0.37
10	20E 25-methyl ether	6.0×10^{-2}	>33	0.00	~11	0.17
11	20E 2,22-dimethyl ether	0.93	>33[b]	0.00[b]	>33[b]	0.00[b]
12	20E 3,22-dimethyl ether	0.22	>33[b]	0.00[b]	>33[b]	0.00[b]
13	20E 14,22-dimethyl ether	2.50	–	–	–	–
14	20E 22,25-dimethyl ether	0.12	>33	0.00	~20	0.07
15	20E 2,3,14,22-tetra methyl ether	90	>33[b]	0.00[b]	>33[b]	0.00[b]
16	PoA 2-methyl ether	4.3×10^{-2}	~20	0.16	~3	0.54
17	PoA 14-methyl ether	6.0×10^{-2}	~20	0.16	6.60	0.76
18	PoA 22-methyl ether	2.2×10^{-2}	~2	0.58	0.7	0.58
19	PoA 2,22-dimethyl ether	3.0×10^{-2}	27.84	0.03	12.89	0.59
20	PoA 3,22-dimethyl ether	1.2×10^{-2}	~12	0.14	~2	0.47
21	PoA 14,22-dimethyl ether	0.17	>33	0.00	~15	0.21
22	DaH 22-methyl ether	0.10	13.66	0.32	1.50	0.55
23	20E 22-[(2′R/S)-2′-ethyl oxiran-2′-yl)]ether	22	–	–	–	–
24	ecdysone	1.00	>33	0.00	>33	0.00
25	20-hydroxyecdysone	7.6×10^{-3}	>33	0.00	~20	0.12
26	ponasterone A	2.6×10^{-4}	0.19	0.18	0.103	0.52
28	muristerone A	2.2×10^{-2}	7.39	0.62	1.03	0.80
29	polypodine B	1.0×10^{-3}	~12	0.21	~7	0.58
30	3,5-dimethyl-benzoic acid *N*-*tert*-butyl-*N*′-(2-ethyl-3-methoxybenzoyl)-hydrazide		reference FI (1 μM): 3796		8989	
			average background FI (DMSO control): 0.94		2.1	

[a] RMFI = relative maximum fold induction (relative to diacylhydrazine **30**); [b] Tested using the 3T3 cell line #2 (less sensitive); average background FI ~1; reference FI (1 μM) = 806 (wt-CfEcR), 1012 (VY-CfEcR)

20E reduces potency, but 20E 22-methyl ether **4** maintains 20E potency. The 22-ethyl ether analogue **5** is slightly less potent than the corresponding methyl ether analogue, while the activity difference increases with the larger alkyl groups of propyl, allyl, and butyl (**6**, **7**, **8**). However, a 22-*O*-benzyl substituent (**9**) does not significantly decrease 20E potency, a result that recalls by analogy the moderately high responsiveness of the 20E 22-benzoyl derivative.[34] On the other hand, O-alkylation of PoA at any position decreases potency in this bioassay.

Engineered EcR/RXR:USP gene switch

Capacity of the *O*-alkyl ecdysteroids to actuate gene expression was examined in a mouse 3T3 fibroblast cell line transiently transfected with the components of the ecdysteroid-inducible system. Primarily two switch versions were used: one based on the wild-type spruce budworm (Cf) EcR (wt-CfEcR-DEF) and the other based on the E274V/V390I/Y410E mutant of this receptor (VY-CfEcR-DEF) known to enhance overall sensitivity.[48,49] For high-interest steroids, confirmatory experiments were conducted with yellow fever mosquito (Aa) and fruit fly (Dm) EcRs in the same switch format. A β-chimera between human RXR and an insect USP from *Locusta migratoria* (LmUSP) was fused to VP16-AD and used as partner protein for the EcR, as this chimera was previously demonstrated to be the best combination to improve ligand sensitivity for EcR gene switches.[50]

Ecdysteroids were evaluated by both potency (EC_{50}) and efficacy. The simplest calculation of efficacy is fold induction (FI). FI of a test or reference ligand is defined as the ratio of gene expression induced by the ligand and gene expression of a DMSO control. Typically, the reference compound (nonsteroidal diacylhydrazine EcR agonist **30**) is assayed in each plate, and the FI of a test ligand is normalised to this standard as the relative maximum fold induction (RMFI). RMFI is calculated as the maximum fold induction of the test ligand relative to the maximum fold induction of **30**, each at their optimal-response concentration under the same assay conditions. Calculation and use of RMFI effectively controls for plate to plate variability in the absolute response of the luminometer. Potency calculations are not subject to this type of variation.

As summarised in Table 2, PoA **26** showed the highest inducing activity among the ecdysteroids tested (EC_{50} = 0.19 μM, RMFI = 0.18) in the wt-CfEcR switch format, while muristerone A and polypodine B were less potent (EC_{50} = 7.4 and ~12 μM, respectively), and 20E was inactive (EC_{50} > 33 μM). Potency of PoA was decreased by O-alkylation at any position, albeit PoA 22-methyl ether **18** and DaH 22-methyl ether **22** provided higher fold inductions (RMFI = ~0.6 and ~0.3, respectively) than PoA itself (RMFI = ~0.2). On the other hand, one 20E O-alkylated analogue, 20E 22-ethyl ether **5**, induced the reporter gene by 77% of diacylhydrazine **30** maximum fold induction at ~5 μM concentration.

In the VY-CfEcR switch format, **26** and **18** were the best performing ecdysteroids: EC_{50} values were 0.1 μM for PoA and 0.7 μM for PoA 22-methyl ether, with RMFI values of 0.52 and 0.58, respectively. In this switch, muristerone A is relatively

weak with an EC_{50} value of 1 μM; likewise polypodine B is weak at ~7 μM. 20E was a very poor actuator (EC_{50} = 20 μM). However, the potency of 20E was substantially improved by 22-ethylation, both in terms of EC_{50} value (0.76 μM, **5**; ~20 μM, 20E) and RMFI (1.1, **5**; 0.12, 20E). Although more modestly, 20E 22-*n*-propyl (**6**), 22-*n*-butyl (**8**), 22-benzyl (**9**) and 25-methyl (**10**) ethers also enhanced the performance of their parent compound, while 20E 22-allyl ether **7** gave the same activity as 20E. Thus, in both the wild-type and VY formats of the CfEcR switch, PoA 22-methyl ether is the most potent *O*-alkyl ether and 20E 22-ethyl ether is almost equipotent.

The most promising O-alkylated ecdysteroids were further tested in gene switches of the same general format, but swapping in the wild-type AaEcR or the wild-type DmEcR for CfEcR (Table 3), and performing the assay side-by-side with VY-CfEcR and VgEcR/RXR. The resulting dose–response curves are shown in Figure 1. Remarkably, potency of PoA 22-methyl ether **18** is superior to that of muristerone A, and **18** is equally or arguably more potent than PoA in both the AaEcR- and DmEcR-based assays (EC_{50} = 0.38 nM and 66 nM, respectively). Note that, in a potency sense, the AaEcR system is more sensitive than CfEcR to both PoA 22-methyl ether and the tested standards, PoA (1.1 nM) and muristerone A (9.3 nM), but, in terms of efficacy, the AaEcR system appears to be considerably less sensitive (for example, PoA: FI = 166 at 1 μM [AaEcR]; cf. FI = ~900 at 1 μM [CfEcR]). This discrepancy is largely due to higher background levels in the "off" state in the AaEcR switch (FI = 7.6 [AaEcR] cf. 0.7 [CfEcR]), rather than a lower absolute expression of the re-

Table 3. Potency and efficacy of selected O-alkylated ecdysteroids and reference compounds measured by the *A. aegypti* (Aa) and *D. melanogaster* (Dm) EcR-based gene switch assays.

No.	Structure	AaEcR EC_{50} (μM)	AaEcR RMFI[a]	DmEcR ED50 (μM)	DmEcR RMFI[a]
7	20E 22-allyl ether	0.138	0.91	~5	0.22
8	20E 22-*n*-butyl ether	2.72	0.63	14.6	0.50
10	20E 25-methyl ether	~0.3	0.58	5.45	0.49
14	20E 22,25-dimethyl ether	0.122	1.03	1.58	0.62
18	PoA 22-methyl ether	0.00038	0.76	0.066	1.31
25	20-hydroxyecdysone	0.93	0.91	~10	0.47
26	ponasterone A	0.0011	0.67	0.113	0.64
28	muristerone A	0.0093	1.01	0.112	1.13
		reference FI (1 μM)			
	3,5-dimethyl-benzoic acid	160		494	
30	N-tert-butyl-N′-(2-ethyl-3-methoxy-benzoyl)hydrazide	average background FI (DMSO control)			
		7.7		0.76	

[a] RMFI = relative maximum fold induction (relative to diacylhydrazine **30**).

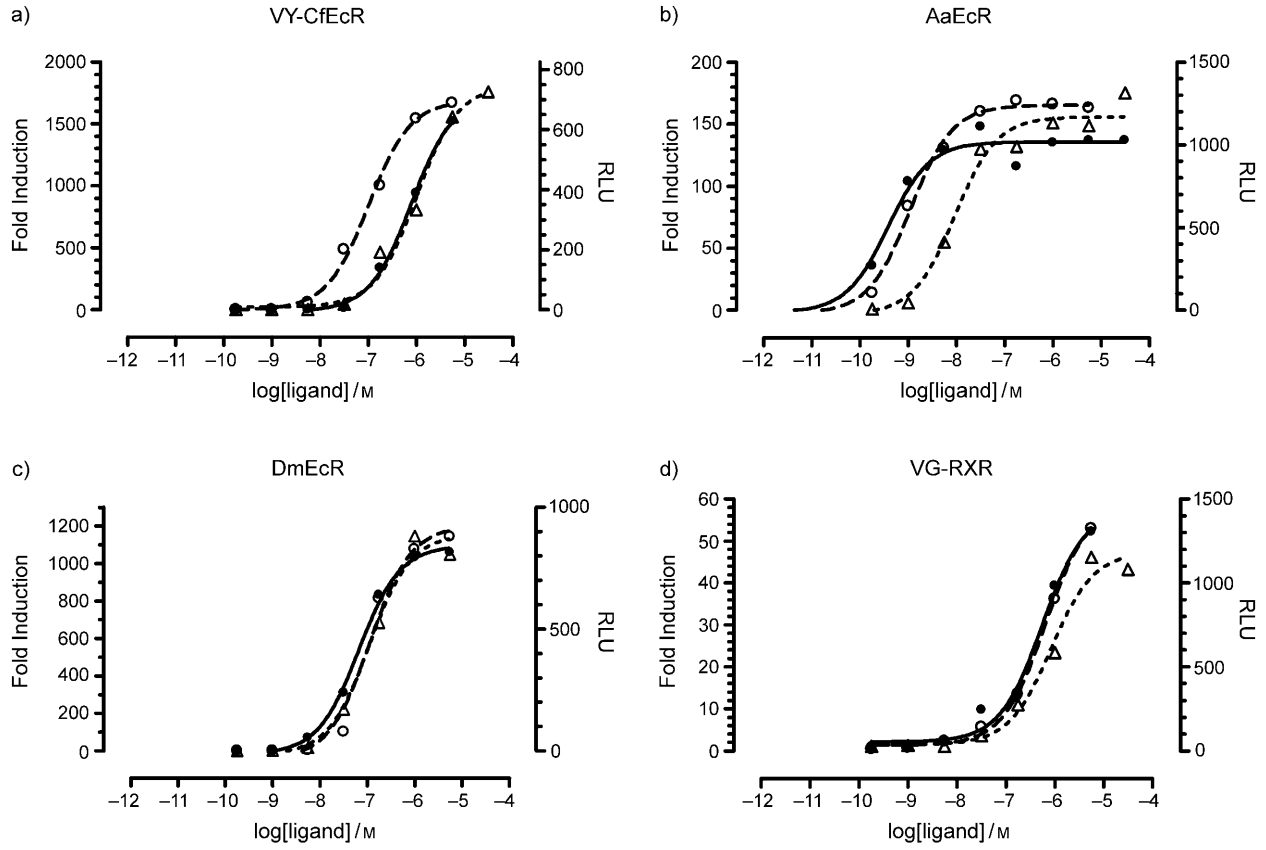


Figure 1. Comparative dose–response curves of ecdysteroid inducers PoA 22-methyl ether (22-Me PoA), PoA, and muristerone A (MuA) in gene-switch assays based on the VY-CfEcR, wild-type *A. aegypti* EcR, *D. melanogaster* EcR, and the VgEcR/RXR system (see text) in mouse 3T3 fibroblasts. The reporter gene is luciferase; fold induction relative to a DMSO standard is plotted on the left axes and absolute relative light units (RLU) are plotted on the right axes. Calculated EC_{50} values for each ligand and switch system are as follows: a) 22-Me PoA, 799 nM (●); PoA, 103 nM (○); MuA, 1030 nM (△); b) 22-Me PoA, 0.38 nM (●); PoA, 1.1 nM (○); MuA, 9.3 nM (△); c) 22-Me PoA, 66 nM (●); PoA, 113 nM (○); MuA, 112 nM (△); d) 22-Me PoA, 553 nM (●); PoA, 641 nM (○); MuA, 851 nM (△).

porter gene (RLUs = ~1046 [AaEcR] cf. 540 [CfEcR]). Relative to the AaEcR switch, the DmEcR switch is more like the CfEcR switch in terms of background transcription, and perhaps slightly less responsive in the sense of efficacy. In 3T3 cells, the previously developed VgEcR/RXR switch format[25] was induced by PoA and muristerone A at $EC_{50} = 0.64$ and 0.85 μM, respectively, and by PoA 22-methyl ether at $EC_{50} = 0.553$ μM. In comparison, the AaEcR- and DmEcR-based switches are responsive to these ligands at low (AaEcR) and mid (DmEcR) nanomolar concentrations, indicating a substantial potency improvement.

Discussion

Ecdysteroids and the EcR gene switch in gene therapeutics

The EcR-based gene switch is now established as a useful and effective switch format. In its best versions, it is characterised by extremely low basal transcription, high dynamic range of induction, and highly dose-responsive ligand sensitivity. Use of the EcR gene switch has been demonstrated both in cell and tissue culture (yeast,[51] plant,[52] insect,[53] mammalian[54]) as well as in animal models (zebrafish,[55] mouse,[49,56] rat[57]), both in a simple reporter gene format, as well as in functional genomics[52,58] and actual disease models.[59,60] The most potent ligands are represented by primarily two chemotypes: the synthetic diacylhydrazines and the natural, usually plant-derived, ecdysteroids. Representatives from both groups have been used successfully with the EcR gene switch in model studies. From the perspective of bioavailability, the diacylhydrazines have class-II-type ADME characteristics (low solubility, high log *P*, high permeability) with few easily metabolised loci, while the ecdysteroids are more highly soluble, with lower log *P* and many hydroxy groups that can be readily metabolised or conjugated. Although favourable clinical data may abrogate all perceived design flaws, nevertheless, from first principles of drug design, both chemotypes leave much room for improvement in terms of physicochemical properties for use as drugs.

The numerous ecdysteroid hydroxy groups, distinctive to this steroid family, constitute both the bioavailability design problem and a potential solution. We hypothesised that methylation of one or more hydroxy groups would suppress the hydrophilicity of the molecule as a whole, and alleviate propensity for metabolism. In doing so, the modification would improve the ADME profile of the molecule, and might also conserve or even enhance potency if the hydroxy in question were not an H-bond donor. In addition, freedom to mutate EcR residues disposed toward a newly introduced methyl ether group might sustain or further enhance potency. Crystal structures of natural EcRs bound to prototypical ecdysteroids—20E, the ubiquitous arthropod moulting hormone, and its C25-deoxygenated analogue, PoA—indicate that the 2-, 3-, 14-, and 20-hydroxy groups (as well as 25-OH in 20E) participate in H-bond interactions with the receptor. Yet, the donor/acceptor nature of the H-bonds is unclear, as hydrogen atoms are below the resolution limit in usual crystallographic experiments. Thus, a systematic study of ecdysteroid structure–activity relationships toward hydroxy group alkylation would greatly illuminate ecdysteroid SAR.

Ecdysteroid synthesis: Twenty-three new semi-synthetic ecdysteroids

Twenty-three *O*-alkyl ecdysteroid analogues of 20E, PoA and DaH were synthesised (**1**–**23**). Each of the hydroxy groups of 20E and PoA could be methylated individually, with the exception of a) the 3-OH of PoA, obtained only together with 22-O-methylation, and b) the 20-OH of both ecdysteroids. Synthesis of 20E 22-methyl (**4**) and 22-ethyl (**5**) ethers[61] and isolation of 20E 25-methyl ether **10** (polypodoaurein, from the fern *Polypodium aureum* L.)[62] had been described before this study, but none of these had been subjected to gene-switch assays. In the case of compounds **4** and **5**, synthetic details were absent; in the case of **10**, analytical characterisation was lacking; we supply full synthetic details and analytical data for all twenty-three structures (Supporting Information, Sections B–D). In our hands, the most easily prepared structures were **1** and **4**, bearing an *O*-methyl group at the 2- and 22-position, respectively, and **11** bearing a double 2,22-O-methylation, which could be obtained in 40–50% yields. The most difficult structure to obtain was **17**, bearing a 14-*O*-methyl group, recovered only in 2% yield after arduous chromatography. On the other hand, the relative ease of 22-hydroxy capping made it possible to perform analogue synthesis at this position, and five additional 22-*O*-alkyl ethers were synthesised. In all of our attempts, the 20-OH of ecdysteroids resisted alkylation. While this position also proved unreactive toward earlier acetylation attempts,[63] one case of a naturally occurring 20-*O*-derivative (20E 20-benzoate) has been reported.[64] The existence of such a compound may indicate the possibility of synthesis by enzymatic means.

Cellular gene-switch assays: Parallel trends in several systems

To probe the gene-switch potency of ethers **1**–**23**, two gene switch formats were used, and a third was briefly investigated on several high-interest structures. The first was a natural one based on the *D. melanogaster* B_{II} cell line, extensively used for ecdysteroid agonist and antagonist potency quantification of plant extracts and isolated natural products. The B_{II} cell line is derived from hemocytes of a tumourous blood cell mutant (*l[2]mbn*).[65] Hemocytes contribute to insect metamorphosis by degrading those larval tissues which do not survive to adulthood. However, in the *l[2]mbn* mutant, the blood cells are no longer able to recognise, which tissue should not be degraded. Addition of ecdysteroids to B_{II} cells acts as a signal to induce phagocytosis, and the cells develop from an even layer of small cells to clumps of larger cells surrounded by clear areas. This response lend itself to turbidometric quantification. Receptor binding has been ascertained as the most important determinant for ecdysteroid B_{II} activity.[66]

The second gene-switch format is engineered by implementation of the two-hybrid concept. It uses an EcR ligand-binding domain linked to a bacterial GAL4 DNA binding-domain, which upon exposure to ligand, associates with a hybrid locust–human RXR linked to a viral VP16 activation domain. This complex in turn binds to the GAL4 response element upstream

from a luciferase reporter gene, expression of which provides a fluorescene readout. The entire switch system is expressed transiently in murine 3T3 cells. EcR-LBD variants in which the LBD sequences were derived from *Choristoneura fumiferana* (spruce budworm, CfEcR), *Aedes aegypti* (mosquito, AaEcR), and *D. melanogaster* (fruit fly, DmEcR) were used for this switch. Additionally, a triple mutant variant (E274V/V390I/Y410E) of CfEcR, dubbed VY-CfEcR[48] previously found to increase overall EcR ligand sensitivity,[49] was tested. In each case, all other components of the assay system remained identical, with the caveat that a few steroids, specified in Table 2, were tested in a somewhat less sensitive 3T3 cell line clone. The third gene-switch format used for a few compounds is the documented[25] DmEcR-derived VgEcR/RXR system previously used in murine studies in vivo, and in which a select collection of both steroids and diacylhydrazines have been examined.

SAR of ecdysteroids: 22-*O*-Alkyl ecdysteroids retain or improve the inducing activity of their parent compounds

Ecdysteroids were paired according to the presence or absence of one or more alkyl caps at given positions, and potency differences in the *Drosophila* B_{II} assay were calculated (Table 4). The 20E and PoA ether derivatives with a single ether substitution (**1**–**10**, **16**–**18**, **22**–**23**), spanning the 2-, 3-, 14-, 22- and 25-positions, permit direct derivation of a relationship between potency differences and capping of a particular OH group. On average, methylation at each of the hydroxy positions results in a decrease of potency, ranked according to the following order of depression of EC_{50} (M) log units: 14-OH (2.12), 2-OH (1.67), 25-OH (1.09), 3-OH (1.06), and 22-OH (0.35). Significantly, however, 22-methylation of five out of nine ecdysteroids, including 20E itself, results in a modest increase of potency, and an opportunity for further optimisation. Multiple methylation is generally additive in its effect.

Table 4. Ether functional group contributions to ecdysteroid activity measured in the B_{II} bioassay. Activity differences are expressed as $\Delta-\log EC_{50}$ (M) between pairs of compounds[a] that differ exclusively by the presence or absence of the indicated -OR substituent.

Functional group	$\Delta-\log EC_{50}$ [M]		Average
2β-OH→2β-OMe	−2.16 (**1**,**25**)	−2.17 (**11**,**4**)	−1.67
	−2.22 (**16**,**26**)	−0.13 (**19**,**18**)	
3β-OH→3β-OMe	−1.90 (**2**,**25**)	−1.54 (**12**,**4**)	−1.06
	0.26 (**20**,**18**)		
14α-OH→14α-OMe	−2.63 (**3**,**25**)	−2.60 (**13**,**4**)	−2.12
	−2.36 (**17**,**26**)	−0.89 (**21**,**18**)	
22*R*-OH→22*R*-OMe	0.08 (**4**,**25**)	0.07 (**11**,**1**)	−0.35
	0.44 (**12**,**2**)	0.11 (**13**,**3**)	
	−0.30 (**14**,**10**)	−1.93 (**18**,**26**)	
	−1.28 (**22**,**27**)	0.16 (**19**,**16**)	
	−0.45 (**21**,**17**)		
22*R*-OH→22*R*-OEt	−0.46 (**5**,**25**)		−0.46
22*R*-OH→22*R*-O*n*Pr	−2.04 (**6**,**25**)		−2.04
22*R*-OH→22*R*-OAllyl	−1.32 (**7**,**25**)		−1.32
22*R*-OH→22*R*-O*n*Bu	−1.82 (**8**,**25**)		−1.82
22*R*-OH→22*R*-OBn	−0.46 (**9**,**25**)		−0.46
25-OH→25-OMe	−0.90 (**10**,**25**)	−1.28 (**14**,**4**)	−1.09
2β-,22*R*-di-OH→ 2β-,22*R*-di-OMe	−2.09 (**11**,**25**)	−2.06 (**19**,**26**)	−2.08
3β-,22*R*-di-OH→ 3β-,22*R*-di-OMe	−1.46 (**12**,**25**)	−1.66 (**20**,**26**)	−1.56
14α-,22*R*-di-OH→ 14α-,22*R*-di-OMe	−2.52 (**13**,**25**)	−2.82 (**21**,**26**)	−2.67
22*R*-,25-di-OH→ 22*R*-,25-di-OMe	−1.20 (**14**,**25**)		−1.20
2β-,3β-,14α-,22*R*-tetra-OH→ 2β-,3β-,14α-,22*R*-tetra-OMe	−4.08 (**15**,**25**)		−4.08

[a] Compound numbers are given in parenthesis after the $\Delta-\log EC_{50}$ value.

Against the wt-CfEcR, PoA suffers loss of potency and efficacy upon hydroxy methylation. However, for the VY mutant of CfEcR, the highly potent PoA ($EC_{50}=0.10$ μM, RMFI = 0.52) loses much less potency and maintains efficacy upon 22-methylation (**18**, $EC_{50}=0.70$ μM, RMFI = 0.58) for an adequately potent and much more attractive structure from the standpoint of metabolism and permeability. This trend continues for the AaEcR and DmEcR in the same two-hybrid system in mouse 3T3 cells. With AaEcR, **18** is equal or possibly more potent ($EC_{50}=0.38$ nM) than PoA ($EC_{50}=1.1$ nM) within the confidence levels of the assay. With DmEcR, the margin is narrower, with **18** possibly stronger (**18**, $EC_{50}=66$ nM; PoA, $EC_{50}=113$ nM). In the *Drosophila*-based VgEcR/RXR system, **18** is comparable to PoA and probably more potent than muristerone A ($EC_{50}=533$ nM/RMFI = 1.5, vs. $EC_{50}=641$ nM/RMFI = 1.5 and $EC_{50}=851$ nM/RMFI = 1.3, respectively). In short, PoA can be methylated at the 22-position yielding a structure with one less hydroxy group with little loss or even improvement of activity. Moreover, since the physicochemical properties of the O-methylated structure should be superior, it may have a greater potential for therapeutic use than would PoA itself. One may draw the general inference: simple 22-*O*-alkyl (Me, Et, nPr) and 22-*O*-benzyl ecdysteroids retain or possibly improve the inducing activity of their parent compounds.

SAR conclusions: Receptor and ligand trends, H-bonding roles

In terms of EcR response trends, the B_{II} system appears to be the most sensitive to the ecdysteroids among the receptors studied. Nevertheless, low nanomolar responses were observed with the better ligands in the DmEcR and AaEcR gene switches. However, the relative efficacy responsiveness (RMFI)

of the AaEcR switch is significantly lower than CfEcR or DmEcR. Most of this apparent loss of efficacy is attributable to excessive background. Our experience has been that the "tightness" of a switch tends to be a property of the receptor, associated transcription factors, or cell system, rather than the inducing ligand. Significantly, within experimental error, PoA **26** and PoA 22-*O*-methyl ether **18** are essentially equipotent in both the AaEcR and DmEcR gene switches. Even in VY-CfEcR, **18** is almost as good as the parent PoA itself. Thus, overall, VY-CfEcR and AaEcR are the best performing engineered gene switches in this study, and the combination of PoA 22-*O*-methyl ether with either the VY-CfEcR or AaEcR could be quite effective: the former is stronger in terms of low basal activity, while the latter is more sensitive in terms of EC_{50}.

Concerning ligand SAR trends, the relative ranking of the standards PoA, 20E, muristerone A, polypodine B and ecdysone remains the same across the B_{II}, wt-CfEcR and VY-CfEcR systems, with the exception that muristerone A surpasses polypodine B in potency in the engineered systems. Generally speaking, 22-O-methylation improves or at least does not degrade potency very much. On the other hand, methylation at all other hydroxy groups suppresses potency, whereby the 14-OH is the most severe. 20E 22-*O*-methyl **4**, 20E 22-*O*-ethyl **5** and PoA 22-*O*-methyl **18** ethers are the most potent ethers overall. Compound **4** is most potent in the B_{II} assay ($EC_{50} = 6.3 \times 10^{-3}$ μM), but **18** is the most potent in the VY-CfEcR gene switch, and **5** is a close runner-up.

The SARs of the ecdysteroid ethers across several gene-switch systems and previous ecdysteroids together in the B_{II} system form a coherent picture. The sum information permits mechanistic statements regarding potency modulation owing to alkylation. If methylation maintains or enhances potency, then not only is an additional steric requirement satisfied, but a free OH is demonstrated to be nonessential. On the other hand, if methylation depletes potency, then either H-bond donation is disrupted or the steric requirement is inadequately satisfied, or both. Occasionally, supplementary data informs speculation concerning H-bonding roles. From combined data, one may infer the following:

a) The 2-OH and 3-OH are donors and/or acceptors in network with each other and the receptor. O-Methylation at either the 2- or 3-positions leads to significant potency loss. Likewise, 2-deoxy- and 3-deoxy-ecdysteroids are substantially less active.[33] In both the PoA-bound and 20E-bound HvEcR-LBD crystal structures, the 2-OH is within H-bonding distance to the guanidinium moiety of Arg383 and the backbone carbonyl group of Glu309, while the 3-OH is closer to the latter group. These residues are in H-bonding proximity to each other (the distance between the Arg383 side chain and Glu309 backbone C=O is 3.5 Å), thereby forming a network of potentially fluxional H-bonds. Both Arg383 and Glu309 residues are conserved among insect sequences[67] (cf. Arg507 and Glu435 in DmEcR); methylation would reduce H-bond network possibilities and/or introduce steric interference, thereby explaining the observed activity drop in the biological screens.

b) The 22-OH is not an obligatory H-bond donor and can be an H-bond acceptor. Potency is retained or improved by different 22-O-alkylated analogues in the B_{II} bioassay (e.g. 20E 22-*O*-methyl ether **4**), the wt-CfEcR (e.g. PoA 22-*O*-methyl ether **18**) and the VY-CfEcR (e.g. **18** and 20E 22-*O*-ethyl ether **5**) gene-switch assays versus their respective parent ecdysteroids. Compound **18** is more active than PoA also in the AaEcR, DmEcR and VgEcR gene-switch assays. Furthermore, a slight improvement in potency and/or efficacy for **4**, as compared to 20E, is observed in analogous gene-switch assays using Aa (EC_{50} = 1.04 vs. 4.38 μM), Ba[1] (EC_{50} = 3 vs. 5.35 μM), Dm (EC_{50} = 4 vs. 15 μM) and Tm[2] (EC_{50} = 7.08 vs. 12 μM) EcRs.[68] Consistent with an H-bond acceptor assignment is also the observation that steroids lacking a 22-OH group altogether are substantially depleted in potency.[33] Available crystal structures of PoA bound to *Tribolium castaneum* EcR[31] and 20E bound to HvEcR[32] indicate water-mediated H-bridges between 22-OH and a conserved Asp residue. The observations neither obligate an H-bond donor role nor preclude an H-bond acceptor role for 22-OH.

c) The 25-OH is likely an H-bond donor; however, H-bond acceptor or non-H-bonding status of the 25-position is permissible and often superior. Potency is depressed on average by 1.09 EC_{50} (M) log units in the B_{II} assay upon methylation at the 25-position. Consistent with an H-bond donor assignment, the crystal structure of 20E bound to the HvEcR reveals the 25-oxygen atom of 20E within H-bond distance to the conserved Asp504. Yet, 25-deoxy ecdysteroids, such as PoA or even 25-fluoro-20E are generally more potent than their 25-OH counterparts.[33] Also, in the engineered EcR:USP/RXR gene-switch assays, 20E 25-*O*-methyl ether **10** indicates slightly higher potencies with respect to 20E (VY-CfEcR EC_{50} = 11 vs. 20 μM; AaEcR EC_{50} = 0.3 vs. 0.93 μM; DmEcR EC_{50} = 5.4 vs. ~10 μM, Table 3). Computational studies of EcR binding of PoA and 20E have invoked differential desolvation energies to explain superior receptor affinity for PoA.[32] Subtle energy adjustments of ligand desolvation and water tally in the LBD may account for unexpected inversions in potency of ecdysteroids possessing non-H-bonding vs. H-bonding groups at the 25-position.

The inaccessibility of 20-OH ethers does not permit clarification of the 20-OH role beyond information available from ligand-receptor crystal structures. Solved PoA-bound and 20E-bound EcR crystal structures identify a tyrosine residue in H-bond proximity to the 20-OH group of both ligands. The 14-OH group remains enigmatic. Despite the presence of a 14-OH in the most potent ecdysteroids in the B_{II} assay, some 14-deoxy steroids remain quite substantially potent.[33] Yet, both 14-O-methylated derivatives of 20E and PoA drop sharply in potency (−2.63 and −2.36 EC_{50} (M) log units vs. 20E and PoA, respectively), representing the highest activity loss in both the series of 20E and PoA mono-*O*-methyl ether derivatives. It is unclear as to whether the observed potency depression is due to a steric demand or the lack of an H-bonding hydrogen.

[1] *Ba,* Bemisia argentifolii *(silverleaf whitefly).*
[2] *Tm,* Tenebrio molitor *(yellow meal worm).*

ADME

Several ADME properties—aqueous solubility, log *P* (Mlog *P*), blood–brain barrier (BBB) permeation, Caco-2 cell permeability, and human serum albumin (HSA) binding—were calculated for illustrative ecdysteroid alkyl ethers and their parent compounds to look for trends and assess the relative effects of alkylation within the series. The results are shown in Table 5.

Solubility

Generally, calculated aqueous solubility increases with the number of hydroxy groups (e.g. muristerone A > 20E > PoA); correspondingly, hydroxy group capping generally decreases solubility, ostensibly owing to fewer H-bonds. Noteworthy exceptions are the 20E 22-alkyl ethers. For example, solubilities of steroids **5** (20E 22-*O*-ethyl ether) and **7** (20E 22-*O*-allyl ether) are slightly higher than their parent compound with a free 20,22-diol. One explanation is intramolecular H-bonding of the 20,22-diol of 20E with consequent diminished solubilising intermolecular H-bonding, as compared to the 22-alkyl analogues, which can participate only in the 20-OH donor/22-OH acceptor sense and are therefore under more thermodynamic constraint to H-bond with the solvent. In like manner, 22-OH/25-OH intramolecular H-bonding effects may also be significant. Methylation at O-22 of 20E disrupts the intramolecular H-bond in the 22-OH donor/25-OH acceptor sense, and therefore depression of water solubility of 20E 22-*O*-methyl ethers versus 20E is less than that of PoA 22-*O*-methyl ethers versus PoA, which lack a 25-OH and hence cannot form this intramolecular H-bond.

The relative order for calculated solubility of PoA and 20E, but not the absolute value for 20E, is consistent with experimentally obtained values. Muristerone A solubility is probably considerably higher than the lower limit reported. The predicted relative order is more important than the absolute values. Internal H-bonding effects among the 20, 22, and 25 hydroxy groups may be idiosyncratic for 20E. As concerns the diacylhydrazine standard **30**, there are ~3 orders of magnitude difference between the calculated (3.6 mg mL^{-1}) and observed aqueous solubility (6.2 μg mL^{-1}). Experimentally, diacylhydrazines are highly crystalline materials. Perhaps the solubility discrepancy reveals a physical behaviour of the solid state unaccounted for by the MI-QSAR model.

Mlog P *values*

Like aqueous solubility, calculated Mlog *P* values trend positively with alkylation. Again, 22-alkylation is an exception; alkylation at this position can actually lower Mlog *P*, for the same intramolecular bonding reasons invoked for aqueous solubility trends. Mlog *P* overestimates experimental values for 20E and PoA, which could be indicative of the compounds associating with one another in the aqueous phase.

Blood–brain barrier partition

A measure of the ability of a molecule to cross the BBB is the logarithm of the BBB partition coefficient, log(BB), which is equal to $\log(C_{brain}/C_{blood})$, where C_{brain} is the concentration of the compound in the brain and C_{blood} is the concentration of the compound in the blood. According to published experimental BBB partition data, log(BB) values > 0.3 are associated with compounds that are readily distributed to the brain, whereas log(BB) values < −1 indicate molecules, which poorly distribute to the brain.[69] The ADME estimates suggest that 20E, PoA, and muristerone A moderately distribute into the brain ($-0.89 < \log(BB)_{calcd} < -0.35$). On the other hand, by calculation, *O*-alkyl ether ecdysteroids show an increased ability to cross the BBB, particularly PoA 2-methyl ether **16** ($\log(BB)_{calcd} = 0.16$) and PoA 22-methyl ether **18** ($\log(BB)_{calcd} = 0.23$). The positive $\log(BB)_{calcd}$ values of these latter compounds suggest that certain ecdysteroidal gene-switch actuators may penetrate the blood–brain barrier, and be suitable as gene switch actuators in the central nervous system.

Table 5. Calculated 1-octanol–water partition coefficient, blood–brain barrier penetration, Caco-2 cell penetration, human serum albumin (HSA) binding, and aqueous solubility for a set of *O*-alkyl ecdysteroids, nonalkylated analogues, and diacylhydrazine **30**.

No.	Compound	Mlog *P*	BBB log (BBB)	Caco-2 [cm s^{-1}]	HSA binding K_a [M]	H_2O solubility [mg mL^{-1}]
4	20E 22-methyl ether	0.98	−0.48	16.3×10^{-6}	2.3×10^{-4}	0.540
5	20E 22-ethyl ether	0.81	−0.61	13.9×10^{-6}	2.1×10^{-4}	0.922
7	20E 22-allyl ether	1.26	−0.31	20.4×10^{-6}	2.5×10^{-4}	0.856
10	20E 25-methyl ether	1.38	−0.77	17.3×10^{-6}	2.3×10^{-4}	0.510
14	20E 22.25-dimethyl ether	1.52	−0.55	24.1×10^{-6}	2.7×10^{-4}	0.293
16	PoA 2-methyl ether	1.88	0.16	20.0×10^{-6}	3.4×10^{-4}	0.137
18	PoA 22-methyl ether	2.10	0.23	26.5×10^{-6}	3.1×10^{-4}	0.052
20	PoA 3.22-dimethyl ether	2.31	−0.47	29.1×10^{-6}	3.8×10^{-4}	0.083
25	20-hydroxyecdysone	1.25[a]	−0.89	16.3×10^{-6}	2.7×10^{-4}	0.755[b]
26	ponasterone A	2.19[a]	−0.35	19.0×10^{-6}	2.5×10^{-4}	0.270[b]
28	muristerone A	0.69	−0.69	12.7×10^{-6}	2.2×10^{-4}	1.031[b]
30	3,5-Dimethyl-benzoic acid *N*-*tert*-butyl-*N*′-(2-ethyl-3-methoxy-benzoyl)hydrazide	2.24[a]	−0.45	20.9×10^{-6}	19.3×10^{-4}	3.590[b]

[a] Experimental log *D* values: 20-hydroxyecdysone, 0.01; ponasterone A, 1.95; diacylhydrazine **30**, 3.4. [b] Experimental H_2O solubility values: 20-hydroxyecdysone, 6.7 mg mL^{-1}; ponasterone A, 0.18 mg mL^{-1}; muristerone A, > 2.9 mg mL^{-1}; diacylhydrazine **30**, 6.2 μg mL^{-1}.

Permeability

Calculated Caco-2 cell permeation[70] coefficients ($P_{Caco\text{-}2}$) increase progressively from muristerone A to 20E to PoA, in parallel with an increase in molecular lipophilicity (Mlog *P* values) and a decrease in aqueous solubility. Notably, according to the MI-QSAR model,[71] PoA *O*-alkyl

ether derivatives **16**, **18** and **20** show equal or higher $P_{Caco\text{-}2}$ values ($20–29\times10^{-6}$ cm s^{-1}) than the parent molecule PoA (19×10^{-6} cm s^{-1}), and 20E *O*-alkyl ether derivatives **4**, **5**, **7**, **10** and **14** also permeate Caco-2 cells equally or more readily ($14–24\times10^{-6}$ cm s^{-1}) than the parent compound 20E (16.3×10^{-6} cm s^{-1}). These calculated values suggest a potential improvement in the oral bioavailability of the ecdysteroid ether derivatives.

Plasma protein binding

HSA binding affinity is a crucial pharmacokinetic property for drug discovery and development. HSA binding allows solubilisation of hydrophobic molecules in the circulatory system. The binding strength of a compound to serum albumin is one of the main factors determining the distribution of the compound to target tissues and, therefore, its bioavailability. Ecdysteroids show similar calculated HSA binding affinities, ranging from 2.1×10^{-4} to 3.8×10^{-4} (K_a values). The lowest HSA binding compound in the series is 20E 22-ethyl ether **5**, which also has the lowest Mlog*P* value of the ethers in the set and the highest calculated aqueous solubility. The highest HSA binding compound is PoA **3**, 22-dimethyl ether **20**, which also has the highest Mlog*P* value of the ethers in the set and is in the lower range of calculated aqueous solubility for ecdysteroids. Thus, there is a general correlation among calculated ecdysteroid HSA binding, calculated compound hydrophobicity, and calculated aqueous solubility.

Metabolism and excretion

The estimated half-life for 20E in human is 9 h.[28] Known metabolites in mice, rats and humans include products of dehydroxylation, reduction of the B-ring, epimerisation at C-3 and 20,22-diol cleavage.[26] From first principles, as well as precedent examples of alkylation as a means of capping and thus enhancing metabolic stability,[42–44] ecdysteroid alkyl ethers should be more resilient than the corresponding free hydroxy groups toward dehydroxylation, oxidative cleavage, and conjugation reactions, as O-dealkylation steps would have to occur first.

Toxicology

Ecdysteroids are not endogenous products of mammalian metabolism and appear to exert a range of beneficial pharmacological effects in mammals including humans.[26] The apparently benign presence of ecdysteroids in the human diet as well as the long-lasting use of several ecdysteroid plant sources in traditional medicines, bodes well for more scrutinising safety studies of these compounds. Applicability of ecdysteroids as actuators for therapeutic gene programmes is a completely different matter; further pharmacokinetic and pharmacodynamic studies would have to be undertaken.

The prognosis from ADME calculations is that ecdysteroid *O*-alkyl ethers should have a more favourable profile than the nonalkylated counterparts. An overall balance is achieved through alkylation: properties in excess (i.e., water solubility and hydrophilicity) are modulated in order to enhance properties that are deficient (absorption, tissue permeation, metabolic stability). Biological data measured in natural and engineered cell systems (*Drosophila* B_{II} and *Choristoneura* EcR gene-switch data), in context with steroid standards, in best cases indicates conservation or even improvement of bioactivity. Thus, hydroxy group capping may be an effective way to improve physicochemical properties of ecdysteroids.

Prospects: Semi-synthetic ecdysteroids as drugs

Are semi-synthetic ecdysteroids a realistic chemotype for gene-switch actuators in a therapeutic setting? Gene-switch controlled gene therapy has passed the stage of proof-of-concept. Animal models for both non-EcR switches and EcR-based switches alike provide ample precedent.[1,3,25,72,73] The next steps pertain to practicalities; this study addresses the pragmatic issue of the inducer drug delivery. The alkylation strategy applied here toward modulating ADME properties has successful precedent in other natural product-derived drugs, such as erythromycin/clarithromycin. We expect that it could be relevant here as well.

For engineered gene switches, even after the physicochemical properties of ligands have been optimised by alkylation or a related modification, the switch may be improved through mutation in order to match the receptor to a ligand with desirable ADME properties. Yet other questions will need clarification. It is unknown if methylated ecdysteroids adversely modify the apparently salutary intrinsic pharmacology of ecdysteroids, and a suitably efficient and scalable preparation of semi-synthetic steroids would need to be developed.

Conclusions

EcR gene switches are potential effective enhancements for cell and gene therapy. However, natural ecdysteroids have unoptimised ADME properties as gene-switch ligands to be used as drugs in vivo. On the idea that one might improve the bioavailability characteristics through alkylation of free hydroxy groups, twenty-three new alkylated ecdysteroids were synthesised and tested for potency and efficacy in gene-switch assays in mouse cells. Examination of the SAR of the steroid ethers revealed information regarding the specific H-bonding roles of individual hydroxy groups. Relying on MI-QSAR, a trend in calculated ADME values toward moderately improved properties (lower solubility, higher Caco-2 cell permeation and higher blood–brain barrier partition) was observed with respect to the nonalkylated counterparts, in best cases without compromising potency. 22-O-Alkylation is the favoured modification. This study represents the first rational and systematic approach to semi-synthetic ecdysteroids with a view toward drug development. The strategy of alkylation of the natural steroid hormones points the way to improved ecdysteroidal actuators for switch-activated gene therapy. In this context, development of scalable syntheses for the most promising compounds and thorough pharmacological evaluations represent future challenges.

Experimental Section

Chemistry

Materials and general protocols: PoA was supplied by Prof. René Lafont, Université Pierre et Marie Curie, Paris. 20E was supplied by Dr. V. Volodin, Institute of Biology, Russian Academy of Sciences, Syktyvkar, Russia. For solubility and log *D* measurements, PoA was purchased from Axxora/Alexis Corp., while 20E and muristerone A were obtained from Sigma–Aldrich Inc. Other reagents and solvents were purchased from Fisher Scientific and Sigma–Aldrich; deuterated solvents for NMR analysis were purchased from Goss Scientific Instruments Ltd (Great Baddow, UK). Dry acetone and CH_2Cl_2 were distilled before use. Water for HPLC was deionised to a degree of purity of 17 Ω. All other HPLC solvents were degassed immediately prior to use by filtration under suction through 0.45 μm (for aqueous solutions) or 0.5 μm (for organic solutions) Waters Millipore® filters. Anhydrous reaction conditions were achieved by flame-drying Schlenk reaction tubes under vacuum and introduction of a nitrogen or argon atmosphere before the reagents. Cannulae were used to transfer liquids. Ecdysteroids were freeze-dried before use. Silver oxide reactions were protected from light with an aluminium foil covering.

Reactions were monitored by HPLC interfaced with a diode-array detector (DAD) on a Gilson 170 system (Anachem Limited, Luton, UK), using a Sphereclone ODS2 column (5 μm, 150×4.60 mm; Phenomenex, Macclesfield, UK), subjected to a linear gradient from 30% to 100% methanol in water over 25 min, followed by 10 min at isocratic 100% methanol, at a flow-rate of 1 mL min^{-1}. Chromatographic monitoring was at wavelengths (λ) of 242 nm and 300 nm. Equal volumes of reaction mixture were taken out at regular time intervals, the samples quenched with methanol, centrifuged and the supernatants filtered through a Minisart® 0.20 mm filter (Sartorius, Epsom, UK). The filtrates were concentrated under reduced pressure, made up to 30% methanol in water (*v*/*v*) to the minimum volume required for dissolution, and injected.

Separation of individual ecdysteroid ethers in the crude reaction mixtures was carried out by development of suitable HPLC systems, which involved one or more of the following methods.[74] (A) semi-preparative C_{18}-HPLC (Phenomenex Sphereclone ODS2; 250× 10 mm, 5 μm) at a flow-rate of 2 mL min^{-1}; (B) preparative C_{18}-HPLC (Phenomenex Sphereclone ODS2; 250×21.20 mm, 5 μm, flow-rate = 5 mL min^{-1}). (C_1/C_2) semi-preparative silica column (Kinesis Zorbax Sil; 250×9.4 mm, 5 μm, flow-rate = 2 mL min^{-1}), eluted isocratically with CH_2Cl_2/2-PrOH/H_2O 160:30:1.5 (C_1) or 125:30:2.0 (C_2), (*v*/*v*/*v*).

Compound purity was HPLC-verified using two different reversed-phase columns (Phenomenex Sphereclone C_{18} and C_6, 5 μm, 150× 4.60 mm) and one normal-phase column (Kinesis-GRACE Apex II Diol, 5 μm, 150×4.60 mm), and is expressed as percent total peak area at λ = 242 nm, for all compounds except **22**, (λ_{max} = 299 nm), for which λ = 300 nm was used.

Product quantification was carried out by UV spectroscopy on a Shimadzu UV-2401PC (Shimadzu GB, Milton Keynes, UK) for compounds containing either the 14α-hydroxy-7-en-6-one moiety (λ_{max} = 242 nm, molar extinction coefficient ε = 12400 L mol^{-1} cm^{-1})[64] or the 14α-hydroxy-7,9(11)-dien-6-one moiety (λ_{max} = 299 nm, ε = 14190 L mol^{-1} cm^{-1}). Concentrations were calculated according to the Lambert–Beer equation.

One-dimensional (^{1}H and ^{13}C) and two-dimensional (^{1}H–^{1}H COSY, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C HMQC and ^{1}H–^{13}C HMBC) NMR spectra were recorded either on a automated Bruker ACF-300 spectrometer or on a Bruker AVANCE DRX-400 spectrometer. Samples were dissolved in [D_4]MeOH containing tetramethylsilane (TMS) as an internal standard. ^{13}C spectra were calibrated with the middle signal of the methanol heptet at 49.00 ppm. ^{1}H and ^{13}C chemical shifts (δ) are expressed in parts per million (ppm). Coupling constant (*J*) and width at half-height ($w_{1/2}$) values are reported in Hertz (Hz). High-resolution mass spectrometry was performed in either the chemical ionisation mode (CIMS) or the positive-ion fast atom bombardment mode (FABMS). CIMS was recorded on a Micromass GCT spectrometer equipped with a direct inlet probe or on a Jeol 700 spectrometer equipped with a direct inlet probe, using in both cases CH_4 as reagent gas, methanol as solvent, source temperature of 200 °C and a probe temperature of 500–650 °C. FABMS was also recorded on the Jeol 700 spectrometer, using Xe as reagent gas, source temperature at 30 °C and "Magic Bullet" (a 4:1 mixture of 1,4-dithio-l-threitol and 1,4-dithioerythreitol) as matrix.

Synthesis of ecdysteroids with *O*-alkyl ether substitutions at the 2-OH, 3-OH, 14-OH and/or 22-OH (1–9, 11–13, 15–23): Prior to etherification, the 2,3- and/or 20,22-diol groups of the starting ecdysteroid were selectively protected by transformation into the corresponding 20,22-phenylboronate, 2,3-acetonide or 2,3;20,22-diacetonide analogue (Scheme 1; see Supporting Information, Section A, for synthesis details). Synthetic procedures for ecdysteroid ethers **1** and **2** are described below as illustrative examples of O-alkylation at the 2-, 3-, 14- and 22-positions (details of synthesis of the remaining analogues are reported in the Supporting Information, Section B).

20-hydroxyecdysone 2-methyl ether (1) and 20-hydroxyecdysone 3-methyl ether (2): Ag_2O (116.0 mg, 10 equiv) was added to a stirred solution of freshly prepared 20E 20,22-phenylboronate (25a; 30 mg, 53 μmol) in DMF (2 mL) at room temperature under anhydrous conditions. CH_3I (258 μL, 44.7 equiv) was added in four portions during the course of the reaction, and additional Ag_2O (10 equiv) was added after 4 h. The reaction was monitored by HPLC-DAD. After 7.5 h, ethyl acetate (25 mL) was added and the mixture was filtered through a Celite® (BDH Chemical Ltd., Poole, UK) pad over a sintered-glass filter funnel of porosity 4 (Weiss–Gallenkamp, UK). The filter was washed with additional ethyl acetate (150 mL) and the solvents evaporated in vacuo. The crude reaction mixture was pre-purified by solid-phase extraction using a Sep-Pak® Vac 35 cc C_{18}-10 g cartridge (Waters, Elstree, UK). The phenylboronate group was then removed by dissolving the products in a 9:1 (*v*/*v*) mixture of THF and H_2O_2 (100 volumes, pre-neutralised with NaOH 0.1 N) and stirring at room temperature and neutral pH for 2.5 h, following by dilution with H_2O, evaporation of THF and solid-phase extraction. The crude products were purified by semi-preparative C_{18}-HPLC (Phenomenex Sphereclone ODS2, 250× 10 mm, 5 μm, flow-rate = 2 mL min^{-1}, at 242 nm) with isocratic 1:1 CH_3OH/H_2O, wherein **2** eluted after 20 min (6 mg, 25%; purity > 99%) and **1** after 23 min (13 mg, 50%; purity > 99%).

20-hydroxyecdysone 25-methyl ether (10) and 22,25-dimethyl ether (14): DTBMP (88.8 mg, 6 equiv) and methyl triflate (47 μL, 6 equiv) were added to a solution of 20E 2,3-acetonide (**25b**; 37.5 mg, 72.1 μmol) in dry CH_2Cl_2 (3 mL). The mixture was stirred at room temperature under anhydrous conditions. After 55 h, the methyl triflate was removed under vacuum and the residue was treated with a 1:1 (*v*/*v*) mixture of 0.1 M HCl and 1,4-dioxane. The methylated steroids were purified by preparative C_{18}-HPLC (Phenomenex Sphereclone ODS2, 250×21.20 mm, 5 μm, flow-rate = 5 mL min^{-1}) using isocratic 60% CH_3OH/H_2O. Yield: 11.15 mg (31%; purity > 99%) 10 and 5.63 mg (15%; purity > 99%) 14.

For each steroid prepared, the starting ecdysteroid, purification method, purity, isolated yield, and diagnostic NMR signals follow. Full $^{1}H/^{13}C$ NMR assignments and HRMS data for compounds **1**–**23** are available in the Supporting Information, Sections C and D, respectively.

20E 2-methyl ether (1): Starting ecdysteroid, yield and purity data: see above. ^{1}H NMR (400 MHz) $\delta = 3.47$ (m, $w_{1/2} = 23$: 2α-H), 4.19 (m, $w_{1/2} = 8$: 3β-H), 3.39 ppm (s: 2β-OCH_3).

20E 3-methyl ether (2): Starting ecdysteroid, yield and purity data: see above. ^{1}H NMR (400 MHz) $\delta = 3.85$ (m, $w_{1/2} = 23$: 2α-H), 3.52 (m, $w_{1/2} = 8$: 3β-H), 3.40 ppm (s: 3β-OCH_3).

20E 14-methyl ether (3): Starting ecdysteroid: **25c**. Purified by HPLC Method A (50% CH_3OH). Yield = 13%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 2.78$ (m, $w_{1/2} = 20$: 9α-H), 2.97 ppm (s: 14α-OCH_3).

20E 22-methyl ether (4): Starting ecdysteroid: **25b**. Purified by HPLC Method A (58% CH_3OH). Yield = 48%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 2.95$ (dd, $J = 9$, 2: 22-H), 3.50 ppm (s: 22-OCH_3).

20E 22-ethyl ether (5): Starting ecdysteroid: **25b**. Purified by HPLC Method A (70% CH_3OH) followed by Method C2. Yield = 20%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 3.07$ (dd, $J = 9$, 2: 22-H), 3.75/3.61 (dq, $J = 9$, 7: 28-Ha/b), 1.19 ppm (t, $J = 7$: 29-CH_3).

20E 22-*n*-propyl ether (6): Starting ecdysteroid: **25b**. Purified by HPLC Method A (70% CH_3OH). Yield = 23%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 3.05$ (dd, $J = 9$, 2: 22-H), 3.66/3.51 (dq, $J = 9$, 7: 28-Ha/b), 0.94 ppm (t, $J = 7$: 30-CH_3).

20E 22-allyl ether (7): Starting ecdysteroid: **25b**. Purified by HPLC Method B (70% CH_3OH). Yield = 38%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 3.12$ (dd, $J = 9$, 2: 22-H), 4.26/4.09 (dd, $J = 13$, 5: 28-Ha/b), 5.97 (s: 29-H), 5.26 (dd, $J = 17$, 2: 30-Ha), 5.11 ppm (dd, $J = 10$, 2: 30-Hb).

20E 22-*n*-butyl ether (8): Starting ecdysteroid: **25b**. Purified by HPLC Method B (75% CH_3OH). Yield = 30%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 3.03$ (dd, $J = 9$, 2: 22-H), 3.70/3.54 (dq, $J = 9$, 7: 28-Ha/b), 0.94 ppm (t, $J = 7$: 31-CH_3).

20E 22-benzyl ether (9): Starting ecdysteroid: **25b**. Purified by HPLC Method B (70% CH_3OH). Yield = 9%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 3.26$ (dd, $J = 9$, 2: 22-H), 1.29 (s: 21-Me), 4.82/4.59 (d, $J = 11$: 22-OCH_2-Ph), 7.28 (Ph-*meta*, 2H), 7.33 (Ph-*ortho*, 2H), 7.40 ppm (Ph-*para*, 2H).

20E 25-methyl ether (10): Starting ecdysteroid, yield and purity data: see above. ^{1}H NMR (300 MHz) $\delta = 3.33$ (dd, $J = 9$, 2: 22-H), 3.19 ppm (s: 25-OCH_3).

20E 2,22-dimethyl ether (11): Starting ecdysteroid: **25**. Purified by HPLC Method A (60% CH_3OH). Yield = 40%. Purity > 98%. ^{1}H NMR (400 MHz) $\delta = 3.48$ (m, $w_{1/2} = 23$: 2α-H), 4.18 (m, $w_{1/2} = 8$: 3β-H), 3.39 (s: 2β-OCH_3), 3.50 ppm (s: 22-OCH_3).

20E 3,22-dimethyl ether (12): Starting ecdysteroid: **25**. Purified by HPLC Method A (60% CH_3OH). Yield = 25%. Purity > 98%. ^{1}H NMR (400 MHz) $\delta = 3.83$ (m, $w_{1/2} = 23$: 2α-H), 3.52 (m, $w_{1/2} = 8$: 3β-H), 3.40 (s: 3β-OCH_3), 3.50 ppm (s: 22-OCH_3).

20E 14,22-dimethyl ether (13): Starting ecdysteroid: **25b**. Purified by HPLC Method A (60% CH_3OH) followed by Method C1. Yield = 6%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 2.76$ (m, $w_{1/2} = 20$: 9α-H), 2.97 (s: 14α-OCH_3), 3.50 ppm (s: 22-OCH_3).

20E 22,25-dimethyl ether (14): Starting ecdysteroid, yield and purity data: see above. ^{1}H NMR (300 MHz) $\delta = 2.95$ (dd, $J = 9$, 2: 22-H), 3.50 (s: 22-OCH_3), 3.19 ppm (s: 25-OCH_3).

20E 2,3,14,22-tetramethyl ether (15): Starting ecdysteroid: **25**. Purified by HPLC Method A (70% CH_3OH). Yield = 30%. Purity > 99%. ^{1}H NMR (400 MHz) $\delta = 3.39$ (s: 2-OCH_3), 3.40 (s: 3-OCH_3), 2.97 (s: 14-OCH_3), 3.50 ppm (s: 22-OCH_3).

PoA 2-methyl ether (16): Starting ecdysteroid: **26**. Purified by HPLC Method B (75% CH_3OH) followed by Method A (55% CH_3OH). Yield = 6%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 3.47$ (m, $w_{1/2} = 23$: 2α-H), 4.18 (m, $w_{1/2} = 8$: 3β-H), 3.39 ppm (s: 2β-OCH_3).

PoA 14-methyl ether (17): Starting ecdysteroid: **26**. Purified by HPLC Method B (75% CH_3OH) followed by Method A (65% CH_3OH). Yield = 2%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 2.76$ (m, $w_{1/2} = 20$: 9α-H), 2.98 ppm (s: 14α-OCH_3).

PoA 22-methyl ether (18): Starting ecdysteroid: **26a**. Purified by HPLC Method A (65% CH_3OH). Yield = 6%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 2.95$ (dd, $J = 9$, 2: 22-H), 3.50 ppm (s: 22-OCH_3).

PoA 2,22-dimethyl ether (19): Starting ecdysteroid: **26**. Purified by HPLC Method B (75% CH_3OH). Yield = 16%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 3.46$ (m, $w_{1/2} = 23$: 2α-H), 4.17 (m, $w_{1/2} = 8$: 3β-H), 2.95 (dd, $J = 9$, 2: 22-H), 3.39 (s: 2β-OCH_3), 3.50 ppm (s: 22-OCH_3).

PoA 3,22-dimethyl ether (20): Starting ecdysteroid: **26**. Purified by HPLC Method B (75% CH_3OH) followed by Method A (65% CH_3OH). Yield = 7%. Purity > 98%. ^{1}H NMR (300 MHz) $\delta = 3.84$ (m, $w_{1/2} = 23$: 2α-H), 3.52 (m, $w_{1/2} = 8$: 3β-H), 2.95 (dd, $J = 9$, 2: 22-H), 3.40 (s: 3β-OCH_3), 3.50 ppm (s: 22-OCH_3).

PoA 14,22-dimethyl ether (21): Starting ecdysteroid: **26a**. Purified by HPLC Method B (75% CH_3OH). Yield = 8%. Purity > 98%. ^{1}H NMR (300 MHz) $\delta = 2.76$ (m, $w_{1/2} = 20$: 9α-H), 2.93 (dd, $J = 9$, 2: 22-H), 2.98 (s: 14α-OCH_3), 3.50 ppm (s: 22-OCH_3).

Dacryhainansterone 22-methyl ether (22): Starting ecdysteroid: **26a**. Purified by HPLC Method B (75% CH_3OH) followed by Method A (65% CH_3OH). Yield = 3%. Purity > 98%. ^{1}H NMR (300 MHz) $\delta = 5.74$ (br s: 7-H), 6.27 (m, $w_{1/2} = 11$: 11-H), 2.70 (br d, $J = 18$: 12-Hax), 1.10 (s, 19-CH_3), 2.96 (dd, $J = 9$, 2: 22-H), 3.50 (s: 22-OCH_3), 0.92 (d, $J = 6.5$: 26-CH_3), 0.91 ppm (d, $J = 6.5$: 27-CH_3).

20E 22-[(2′*R*/*S*)-2′-ethyloxiran-2′-yl)] ether (23): Starting ecdysteroid: **25b**. Purified by HPLC Method B (75% CH_3OH) followed by Method B (70% CH_3OH). Mixture of diastereomers not separable under any of the HPLC conditions cited above. Yield = 11%. Purity > 99%. ^{1}H NMR (300 MHz) $\delta = 3.73$ (dd, $J = 9$, 2: 22-H), 3.49/3.39 (d, $J = 12$: 29-Ha/b), 0.93 ppm (t, $J = 7$: 31-CH_3). ^{13}C NMR (75 MHz) $\delta =$ 110.25 ppm (O–C–O group).

Biology

Cellular gene-switch assays: *Drosophila* B_{II} cell morphology: The *D. melanogaster* B_{II} cell line bioassay was used to test the agonist activity of potential EcR ligands. Assays were performed in quadruplicate, according to the reported procedure.[47,66] In brief, stock solutions (10^{-3} M to 10^{-10} M) in CH_3OH were prepared for each of the test compounds. Aliquots (20 μL) of each dilution were transferred to wells of a microtitre plate and solvent was evaporated. Cell suspension (200 μL) at approximately 2×10^5 cells per mL medium was added to each well and the covered plate was incubated in a humid environment at 25 °C for 7 days. Cellular response is measured turbidometrically (405 nm) as a function of steroid concentration.

Cellular gene-switch assays: Engineered EcR:USP/RXR systems:[75] Cellular gene-switch assays were performed by transfecting the following constructs in mouse embryonic fibroblast cells (NIH3T3). The wild-type D-, E-, and F-domains from a) *C. fumiferana* EcR (CfEcR-DEF),[76] b) *C. fumiferana* EcR with a E274V/V390I/Y410E mutation (VY-CfEcR-DEF),[48] c) *A. aegypti* EcR (AaEcR-DEF),[77] and d) *D. melanogaster* EcR (DmEcR-DEF)[78] were fused to a GAL4-DBD and placed under the control of the CMV promoter in a pBIND vector (Promega Corporation, Madison, WI, USA). A chimeric RXR from Homo sapiens RXRβ and Locusta migratoria RXR fused to VP16-AD and under the control of an SV40e promoter has previously described.[79] The inducible luciferase reporter plasmid, pFRLuc, (Stratagene Cloning Systems, La Jolla, CA, USA) contains five copies of the GAL4 response element and a synthetic minimal promoter. The VgEcR/RXR gene switch system, which employs a hybrid EcR bearing a VP16 activation domain and a 3-residue mutated DBD that recognises an asymmetric EcR and glucocorticoid receptor response element,[21,25] was obtained from Invitrogen Inc. (Carlsbad, CA, USA), and employed in an analogous manner by transient transfection in NIH3T3 cells using pIND-luciferase vector.

NIH3T3 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum, both obtained from Mediatech, Inc., Manassas, VA (USA). Cells were planted in a 96-well plate at a density of 2500 cells per well in 50 μL of growth medium. The following day cells were first treated with 35 μL of serum-free DMEM containing dimethyl sulfoxide (DMSO; control) or a DMSO solution containing ligand. Cells were then transfected with 15 μL of serum-free DMEM containing 0.04 μg of EcR construct, 0.04 μg of RXR construct, and 0.16 μg of luciferase reporter construct per well, using SuperFect transfection reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. Ligands were tested at 8 doses from 0.01–33 μM and the final DMSO concentration was 0.33% in both control and treatment wells. After a 48-hour post-treatment and transfection incubation, the cells were assayed for luciferase activity using the Bright-GloTM Luciferase Assay System (Promega Corporation, Madison, WI, USA) following the manufacturer's instructions. Assays were performed minimally in duplicate and definitive assays as many as six times. Data was fitted to a sigmoidal dose–response curve.

Physicochemical measurements

Estimation of ADME properties: The 1-octanol–water partition coefficients (M log *P*)[80] of ecdysteroid *O*-alkyl ether analogues were determined by scaling against previously calculated[34] M log *P* values of nonether ecdysteroids. Estimations of the Caco-2 cell permeation coefficients and the blood–brain barrier partition coefficients, were made using established membrane-interaction QSAR (MI-QSAR) models.[71] MI-QSAR analysis includes, in the descriptor pool used in the development of a QSAR model, properties and features explicitly derived from the simulation of the transport of each of the solutes (small organic compounds) comprising the training set through a model membrane assembly composed of phospholipids, in this case dimyristoylphosphatidylcholine (DMPC) molecules. A detailed description of the MI-QSAR paradigm can be found elsewhere.[81,82] Estimates of ecdysteroid binding to human serum albumin (HSA) were obtained using 3D-FEFF-QSAR analysis.[83] This approach calculates the free energy, ΔG, of the binding of an ecdysteroid ligand to HSA using a scaled QSAR model as a scoring function. K_a values were derived using $\Delta G = -RT\ln(K_a)$; $K_a = (1/K_b)$, where K_b is the binding affinity of the molecule to HSA, under the assumption that binding occurs exclusively to HSA, a binary complex is formed, and an excess of HSA ([HSA] = 0.6 mm) is present as compared to the concentration of the ligand. Aqueous solubility of the ecdysteroid *O*-alkyl ether analogues were determined using the AMSOL method and software.[84]

Log *D* measurements: log *D* was measured by Absorption Systems, Inc. PoA and 20E were measured at 100 μM in equal volumes of pH 7.4 buffer and water-saturated 1-octanol in a 1.5 mL shake flask system in duplicate using testosterone standard. Each shake flask was agitated for 60 min at room temperature, then allowed to stand for 1 hour at room temperature. Serial dilutions of the organic and aqueous layers were prepared and concentrations of test compound at each dilution were determined using a generic LC–MS–MS method with a minimum four-point calibration curve.

Water solubility measurements: Water solubility was measured by Robertson-Microlit, Inc. Samples of saturated solutions of PoA, 20E, muristerone A, and diacylhydrazine **30** were dissolved in HPLC-grade water, stirred at 25 °C for 1, 5, and 10 days, and then filtered using a 0.45-μm filter to obtain a clear solution. For each substance, UV absorbance was measured at the maxima of 249, 248, 239, and 219 nm, respectively, diluting if necessary. Absorbance was compared to that of a reference standard at the same absorbance maximum for a 1–2% solution of the same steroid in CH_3OH, allowing for up to 10 nm maxima shift due to solvent effect.

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Keywords: structure–activity relationships · drug design · ecdysteroid · gene switch · gene therapy

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