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Stereoselective synthesis and moulting activity of 2,3-*diepi*-20-hydroxyecdysone and 2,3-*diepi*-5α-20-hydroxyecdysone

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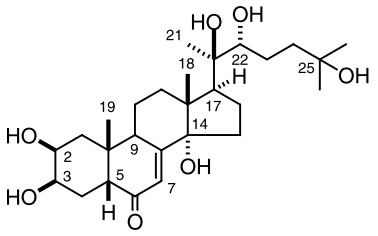
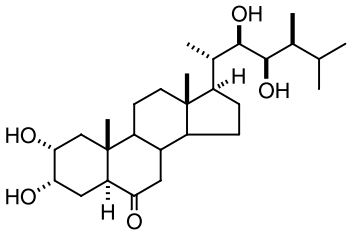
Abstract—The ecdysteroid analogues 2,3-*diepi*-20-hydroxyecdysone and 2,3-*diepi*-5α-20-hydroxyecdysone have been synthesized from the readily available ecdysteroid, 20-hydroxyecdysone, and moulting activity has been determined using the *Musca* bioassay. As expected, the 2,3-*diepi*-analogue was less active than the parent ecdysteroid, 20-hydroxyecdysone. However, the 2,3-*diepi*-5α-analogue, which was expected to be inactive in the assay, exhibited moulting activity though it was approximately 1.5-fold less active than its 5β-analogue. The activity of the 5α-analogue could possibly result from the ability of this compound to bind to the ecdysteroid receptor. Alternatively, a possible in vivo C-5 epimerization of the 2,3-*diepi*-5α-analogue to the corresponding 5β-analogue could account for its activity.

1. Introduction

Ecdysteroids are arthropod moulting hormones found in invertebrates and plant species and 20-hydroxyecdysone (**1**) is a representative of this class of compounds.[1–3] The physiological functions in invertebrates including insects are to control moulting and metamorphosis processes and are involved in the control of reproduction. Essential features contributing to high moulting activity include a *cis*-A/B ring junction, a 6-keto-7-ene system, a full sterol side chain and a free 14α-hydroxyl group.[4] The number, location and stereochemistry of the hydroxyl groups in the molecule are also responsible for the high activity of ecdysteroids. Many works indicated that the 3β-hydroxyl group is required for high activity of ecdysteroids and that the 2-hydroxyl group is not essential to such activity.[4–6] Previous works have shown that moulting activity of ecdysteroids decreased in going from the 3β-hydroxyl to the corresponding 3α-hydroxyl analogues.[4] All active ecdysteroids have a *cis*-fused A/B ring junction, whereas the 5α-epimers are inactive.[4,7] 5α-Ecdysteroid analogues, which have a *trans*-A/B ring junction, have an approximately planar ring structure while ecdysteroids (5β) are non-planar in the region of the A ring with C-2, C-3 and C-4 lying below the plane of the other rings.



1
12 2-Deoxy-5α-analogue of **1**



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It was found that the brassinosteroid castasterone (**2**), the 7,8-dihydro analogue of ecdysteroid with the 2α,3α-dihydroxyl groups and 5α-orientation, inhibited ecdysteroid activity and the effects could be explained by competitive displacement of ecdysteroids, for example, compound **1**.[8] By comparing molecular models of **1** and **2** it was found that the 3β-hydroxyl group of **1** and the 3α-hydroxyl group of **2**

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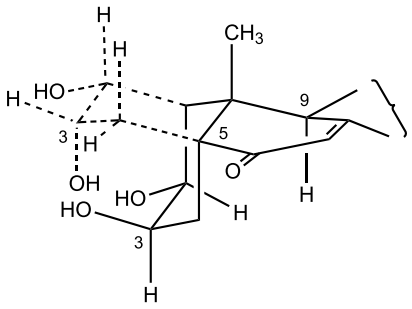
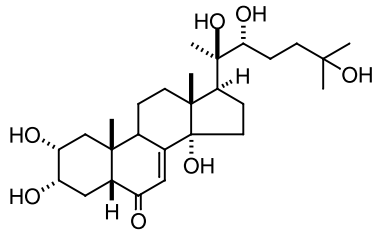


Figure 1. The A-ring geometry of the ecdysteroid **1** (solid line) and brassinosteroid **2** (broken line) and the near spatial coincidence of the 3β-hydroxyl group in **1** with the 3α-hydroxyl group in **2**. Some hydrogens are omitted in the structure for clarity.

occupied the same space (Fig. 1) and this might lead to effective competition in binding the ecdysteroid receptor of **2**. It was therefore of interest to study moulting activity of a 2,3-*diepi*-5α-ecdysteroid and, for comparison, the activity of a 2,3-*diepi*-ecdysteroid should also be evaluated. The present work deals with the stereoselective synthesis of 2,3-*diepi*-20-hydroxyecdysone (**3**) and 2,3-*diepi*-5α-20-hydroxyecdysone (**4**) from the readily available ecdysteroid **1** and evaluation of moulting activity of these two ecdysteroid analogues. Compound **4** possesses a structural framework in the A-ring region comparable to that of the brassinosteroid **2**.



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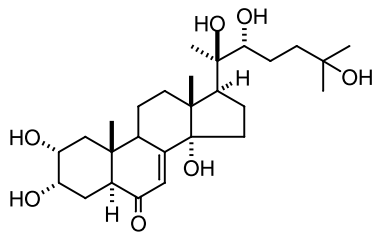

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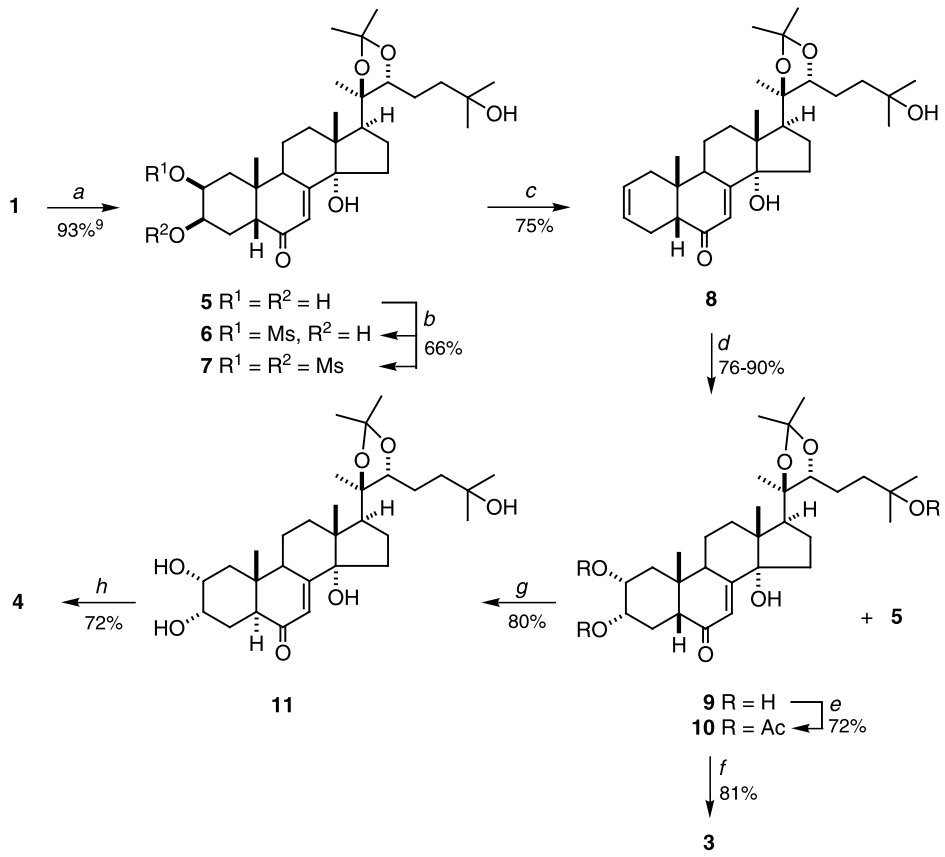
2. Results and discussion

The first ecdysteroid analogue we planned to synthesize was the 2,3-*diepi*-ecdysteroid **3**. It was then expected that this compound would be transformed into the corresponding C-5 epimer, 2,3-*diepi*-5α-ecdysteroid **4**, by base-catalyzed epimerization. Starting from **1**, the 20,22-acetonide **5** was prepared by the literature procedure.[9] Mesylation of **5** with mesyl chloride in pyridine yielded the corresponding 2-mesylate **6**[10] and 2,3-dimesylate **7** in 30 and 66% yields, respectively. The former could be recycled in the mesylation step. Prolonged reaction time gave only the dimesylate **7**, but it was usually accompanied by a less polar side product. The presence of the mesylate groups at the 2- and 3-positions in **7** was evident from a 1.13 and 1.11 ppm downfield shifts of H-2 and H-3 as compared with that of the starting acetonide **5** and the presence of two mesylate methyl signals at δ 3.10 and 3.11 in the ^{1}H NMR spectrum of **7**. Reaction of **7** with NaI and Zn in DMF at 80 °C afforded the olefin acetonide **8** in 75% yield. The ESMS exhibited a sodiated molecular ion $[M+Na]^+$ at *m/z* 509 and a pseudo-molecular ion $[M+H]^+$ at *m/z* 487, corresponding to a molecular formula of $C_{30}H_{46}O_5$. This was confirmed by positive-ion HRFABMS which exhibited a pseudo-molecular ion $[M+H]^+$at *m/z* 487.3428. The ^{1}H NMR spectrum of **8** indicated the presence of two olefinic (H-2 and H-3) signals at δ 5.52 and 5.69. The rest of the ^{1}H NMR spectroscopic data were consistent with structure (Scheme 1).

Dihydroxylation of the olefin acetonide **8** with OsO_4 in pyridine, followed by treatment with 5% aq. $NaHSO_3$ afforded two products in high yield. The more polar product (58% yield) was identified as 20-hydroxyecdysone 20,22-acetonide (**5**) by comparison of spectroscopic (IR, ^{1}H NMR and mass spectral) data with the reported value.[9] The less polar product (32% yield) was compound **9**, the C-2 and C-3 epimer of **5**. That the ecdysteroid **5** was the major product was expected, since dihydroxylation of **8** took place on the less-hindered β-face. The ^{1}H and ^{13}C NMR spectroscopic signals of **9** were assigned from 2D (COSY, HMQC, HMBC) and DEPT techniques and were found to be much different from those of **5**. A striking difference was the unusual downfield H-9 signal of **9** (in C_5D_5N) at δ 4.70, whereas that of **5** appeared at δ 3.54. A close proximity of H-9 and the 2α-hydroxyl group resulted in a large downfield shift of the former signal. A marked upfield shift of the H-5 signal of **9** at δ 2.37, as compared with that of **5** at δ 2.99, was also noted. The strong steric interaction between the 2α-hydroxyl group and H-9 might lead to facile C-5 epimerization of **9**. The structure of this minor dihydroxylation product could possibly, therefore, be **9** or its C-5 epimer of **9**. To prove whether the stereochemistry at C-5 was in the β-orientation, a NOE experiment was performed. The key experiment was to irradiate the 19-Me signal to see if enhancement of the H-5 signal would occur.[11] The result, however, was not conclusive since the H-5 signal was obscured by other signals. Compound **9** was therefore subjected to acetylation to the corresponding 2,3,25-triacetate acetonide **10**. Assignments of the NMR spectroscopic signals of **10** were achieved by 2D NMR techniques. Irradiation at the 19-Me frequency of **10** resulted in NOE enhancement of the H-5 signal at δ 2.20, whereas irradiation at the H-9 frequency caused enhancement of the 2α-acetoxyl signal at δ 1.98. To confirm that the position of the H-5 resonance was at δ 2.20, the H-3 resonance was irradiated and enhancement of this proton signal was also observed. The results indicated that H-5 of compound **10** was in the β-orientation. It was thus concluded that compound **9** was a 5β-ecdysteroid as shown. Acetonide deprotection of **9** with 70% AcOH in the presence of the phase transfer catalyst benzyltrimethylammonium chloride afforded 2,3-*diepi*-20-hydroxyecdysone (**3**) in 81% yield.

In order to increase the ratio of the product **9**:**5**, asymmetric dihydroxylation[12] was investigated. The chiral ligands used were those of the dihydroquinidine (DHQD) series, that is,



Scheme 1. Synthesis of ecdysteroid analogues **3** and **4**. Reagents and conditions: (a) CH_3COCH_3, *p*-TsOH; (b) MsCl, pyridine; (c) NaI, Zn, DMF, 80 °C; (d) OsO_4, ligand, solvent (see text); (e) Ac_2O, pyridine; (f) 70% AcOH, $PhCH_2NMe_3^+Cl^-$; (g) 2% Na_2CO_3, MeOH; (h) 70% AcOH, $PhCH_2NMe_3^+Cl^-$.

dihydroquinidine 4-methyl-2-quinolyl ether (DHQD-MQE), dihydroquinidine 9-phenanthryl ether (DHQD-PE) and dihydroquinidine 1,4-phthalazinediyl diether $(DHQD)_2$-PHAL), and the dihydroquinine (DHQ) series, that is, DHQ-MQE, DHQ-PE and $(DHQ)_2$-PHAL (Table 1). The best **9**:**5** product ratio was 4:1, the chiral ligand of which was $(DHQD)_2$-PHAL. In this case the yield of **9** was raised to 68%. The overall yield of **3** from the starting ecdysteroid **1** was 25%, based on the utilization of the chiral ligand $(DHQD)_2$-PHAL in the dihydroxylation step.

To effect C-5 epimerization, compound **9** was treated with 2% Na_2CO_3 in MeOH and, as expected, epimerization occurred more readily than the 2β,3β-dihydroxyl analogue[7] (e.g., compound **5**) to give the corresponding 5α-analogue **11** in 80% yield. The *trans*-A/B ring fusion of **11** was evident from a large upfield shift (1.47 ppm) of H-9 in going from **9** to **11**. A downfield shift (0.65 ppm) of H-5 signal of **11** indicated the proximity of this proton and the 3α-hydroxyl group. The H-5 proton of **11** was further confirmed to be in the α-orientation by NOE experiments. Thus irradiation at the H-5 frequency did not cause enhancement of the 19-Me signal at δ 0.96, whereas irradiation at the H-9 frequency resulted in enhancement of the H-5 signal at δ 3.02. Compound **11** was subjected to deacetonation to give 2,3-*diepi*-5α-20-hydroxyecdysone (**4**) in 72% yield, the spectroscopic data of which were consistent with the structure. The overall yields of **4** from the starting ecdysteroid **1** was 18%, or 58% from compound **9**.

Table 1. Asymmetric dihydroxylation of compound **8**

Entry	Ligand	Ratio of products **5**:**9**	Yield (%)
1	DHQD-MQE	5:6	77
2	DHQD-PE	7:3	83
3	$(DHQD)_2$-PHAL	1:4	85
4	DHQ-MQE	7:3	76
5	DHQ-PE	3:1	80
6	$(DHQ)_2$-PHAL	5:4	84

The ratio of the ligand:OsO_4: compound **8** was 3:3:1. *tert*-BuOH–THF–H_2O (7:4:1) was used as a solvent.

2.1. Biological activity

The *Musca* bioassay[13] has been used to evaluate moulting activity of ecdysteroid and their analogues in our study. As expected for a 3α-hydroxy ecdysteroid, compound **3** which is the 2α,3α-analogue of the parent ecdysteroid **1** was much less active; it was 30-fold less active than compound **1**. Surprisingly, compound **4**, the 5α-analogue of compound **3** and was expected to be inactive,[4] was active in the assay. It was 42-fold less active than compound **1**. The activity of **4**, though it was approximately 1.5-fold less active than that of **3**, deserved special attention. One possible explanation for the activity of **4** was that it could bind to the ecdysteroid receptor by analogy to that which occurred in castasterone (**2**). Unlike **2**, compound **4** possesses a 6-keto-7-ene system, the essential structural requirement for moulting activity. The α-nature of the 2- and 3-hydroxyl groups resulted in effective binding to the receptor and this could possibly

compensate the *trans*-A/B ring junction of **4**. An alternative explanation was that compound **4** could undergo in vivo C-5 epimerization to compound **3**. The unfavorable steric interaction of the 2α-hydroxyl group and H-9 in **3** could, in part, be compensated for by the absence of 1,3-diaxial interaction between 19-Me and the 2β- and 4β-H (see Fig. 1). One supported example was the activity of compound **12**, a 2-deoxy-5α-ecdysteroid analogue, which exhibited low moulting activity in the *Musca* assay.[14] Whether the first or second hypothesis was more likely could not be judged from the existing data.

3. Experimental

3.1. General experimental procedures

Melting points were determined on an Electrothermal melting point apparatus and were uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer. FAB and ES mass spectra were measured on a Finnigan MAT 90 and a Bruker Esquire-LC instruments. Column chromatography and TLC were carried out using Merck silica gel 60 (<0.063 mm) and precoated silica gel 60 F_{254} plates, respectively. Spots on TLC were visualized under UV light and by spraying with anisaldehyde–H_2SO_4 reagent followed by heating.

3.1.1. 20-Hydroxyecdysone 20,22-acetonide 2,3-dimesylate (7). Compound **5**[9] (1.4 g, 2.69 mmol) was dissolved in pyridine (4 mL) and the mixture stirred at 0–5 °C for 10 min, then mesyl chloride (1.5 mL, 19.30 mmol) was added. The reaction mixture was left to stir at 0–5 °C for 1 h and at ambient temperature for another 1 h. Water was added and the mixture extracted with $CHCl_3$ (4×25 mL). The combined chloroform extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The residue was subjected to column chromatography (silica gel 50 g) using $CHCl_3$–MeOH, with gradually increasing concentration of MeOH, to give 20-hydroxyecdysone 20,22-acetonide 2,3-dimesylate (**7**) (1.2 g, 66%) eluted by $CHCl_3$–MeOH (98.5:1.5) and 20-hydroxyecdysone 20,22-acetonide 2-mesylate (**6**) (490 mg, 30%), eluted by $CHCl_3$–MeOH (96:4).

Compound **6**. Spectroscopic (^{1}H NMR and mass spectral) data were identical to those reported in literature.[10]

Compound **7**. Amorphous; ν_{max} 3436, 2971, 1660, 1449, 1354, 1176, 1106, 1024, 909 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.77 (s, 3H, 18-Me), 1.03 (br s, 3H, 19-Me), 1.14 (s, 3H, 21-Me), 1.22 (s, 3H, 26-Me), 1.23 (s, 3H, 27-Me), 1.31, 1.39 (each s, 2×3H, acetonide Me), 2.22 (dd, *J*=9.1, 7.9 Hz, 1H, H-17), 2.98 (m, 1H, H-9), 3.10, 3.11 (each s, 2×3H, 2-OMs, 3-OMs), 3.63 (dd, *J*=9.4, 2.4 Hz, 1H, H-22), 4.94 (m, 1H, H-2), 5.12 (br s, 1H, H-3), 5.87 (d, *J*=2.1 Hz, 1H, H-7); ESMS *m/z* (% rel. intensity) 699 $[M+Na]^+$(100)]; HRFABMS (positive ion mode) *m/z* 677.3045 $[M+H]^+$ (calcd for $C_{32}H_{52}O_{11}S_2-H$, 677.3029).

3.1.2. 2,3-Didehydro-2,3-dideoxy-20-hydroxyecdysone 20,22-acetonide (8). Compound **7** (960 mg, 1.42 mmol) was dissolved in DMF (5 mL) and NaI (820 mg, 5.47 mmol) and zinc dust (196 mg, 3 mmol) was added. The reaction mixture was left to stir at 80 °C for 3 days. Water was added and the mixture was extracted with $CHCl_3$. The combined $CHCl_3$ layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to dryness. The product was purified by column chromatography (silica gel 30 g) using $CHCl_3$–MeOH with gradually increasing concentration of MeOH to give compound **8** (520 mg, 75%), eluted by $CHCl_3$–MeOH (99:1).

Compound **8**. Colorless needles (from $CHCl_3$–MeOH), mp 117–119 °C; ν_{max} 3426, 2971, 1659, 1443, 1376, 1213, 1200 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.75 (s, 3H, 18-Me), 1.02 (s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.20 (s, 3H, 26-Me), 1.21 (s, 3H, 27-Me), 1.29, 1.38 (each s, 2×3H, acetonide Me), 2.22 (t, *J*=ca 9 Hz, 1H, H-17), 2.85 (br m, 1H, H-9), 3.63 (br d, *J*=7.8 Hz, 1H, H-22), 5.50 and 5.67 (each br d, *J*=10 Hz, 2×1H, H-2 and H-3), 5.76 (d, *J*=1.3 Hz, 1H, H-7); ESMS (positive ion mode) *m/z* (% rel. intensity) 995 $[2M+Na]^+$ (25), 509 $[M+Na]^+$ (100), 487 $[M+H]^+$ (17); HRFABMS (positive ion mode) *m/z* 487.3428 $[M+H]^+$ (calcd for $C_{30}H_{46}O_5+H$, 487.3423).

3.1.3. Reaction of compound 8 with osmium tetroxide. Synthesis of 2,3-*diepi*-20-hydroxyecdysone 20,22-acetonide (9) and 20-hydroxyecdysone 20,22-acetonide (5). To a solution of compound **8** (134 mg, 0.275 mmol) in pyridine (0.8 mL) was added OsO_4 in pyridine (0.55 mL, prepared by dissolving 500 mg of OsO_4 in 3 mL of pyridine and the amount used was equivalent to 92 mg or 0.36 mmol of OsO_4). The mixture was stirred for 5 min and 5% $NaHSO_3$ (2 mL) was added. Stirring was continued for 15 min and the mixture was extracted with EtOAc (3×15 mL). The combined organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The crude mixture (150 mg) was subjected to column chromatography (silica gel 25 g), using $CHCl_3$–MeOH as eluting solvent, with increasing amount of MeOH. Fractions eluted by $CHCl_3$–MeOH (95:5) afforded 46 mg (32%) of 2,3-*diepi*-20-hydroxyecdysone 20,22-acetonide (**9**). The second fraction (83 mg, 58%) eluted by $CHCl_3$–MeOH (94:6), was identified as 20-hydroxyecdysone 20,22-acetonide (**5**).

Compound **5**. Colorless needles (from acetone–hexane), mp 221–223 °C (lit.[9] 222–224 °C); ν_{max} 3423, 2974, 1649, 1454, 1377, 1216, 1170, 1103, 1057, 1001, 877 cm^{-1}; ^{1}H NMR (400 MHz, C_5D_5N) δ 1.00, 1.03 (each s, 2×3H, 18-Me, 19-Me), 1.32 (s, 3H, acetonide Me),[a] 1.34 (s, 3H, 26-Me), 1.35 (s, 3H, 27-Me), 1.44 (s, 3H, acetonide Me), 1.53 (s, 3H, 21-Me),[a] 2.75 (t, *J*=8.6 Hz, 1H, H-17), 2.99 (dd, *J*=13.1, 3.5 Hz, 1H, H-5), 3.54 (m, 1H, H-9), 3.93 (dd, *J*=9.6, 2.4 Hz, 1H, H-22), 4.16 (m, 1H, H-2), 4.22 (br s, 1H, H-3), 6.24 (d, *J*=2.1 Hz, 1H, H-7), ('a' stands for the assignments may be reversed for signals with the same superscript); ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 3H, 18-Me), 0.93 (s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.18 (s, 3H, 26-Me),[b] 1.26 (s, 3H, 27-Me),[b] 1.29,[b] 1.38 (each s, 2×3H, acetonide Me), 2.38 (dd, *J*=12.9, 4.4 Hz, 1H, H-5), 2.95 (m, 1H, H-9), 3.59 (m, 1H, H-22), 3.81 (m, 1H, H-2), 4.01 (br s, 1H, H-3), 5.81 (d, *J*=1.8 Hz, 1H, H-7), ('b' stands for the assignments may be reversed for signals with the same superscript); ESMS (positive ion mode) *m/z* (% rel. intensity) 543 $[M+Na]^+$ (100); HRFABMS (negative ion

mode) *m/z* 519.3322 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3321).

Compound **9**. Amorphous; ν_{max} 3418, 2970, 1654, 1458, 1384, 1258, 1200, 1173, 1075, 1002, 927, 867 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.99 (br s, 3H, 19-Me), 1.03 (s, 3H, 18-Me), 1.30 (s, 3H, 21-Me), 1.37 (s, 2×3H, 26-Me, 27-Me), 1.45, 1.53 (each s, 2×3H, acetonide Me), 2.37 (obscured signal, 1H, H-5), 2.77 (t, *J*=8.3 Hz, 1H, H-17), 3.92 (obscured signal, 1H, H-3), 3.94 (dd, *J*=9.3, 2.8 Hz, 1H, H-22), 4.33 (br s, $W_{1/2}$=12 Hz, 1H, H-2), 4.70 (br, 1H, H-9), 6.22 (d, *J*=2.3 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 17.3 (C-18), 21.3 (C-16), 22.1 (C-11), 22.4 (C-21), 24.2 (C-19), 24.4 (C-23), 27.2 (acetonide Me), 29.4 (acetonide Me), 29.7 (C-4), 29.8 (C-26), 30.1 (C-27), 31.6 (C-12), 31.8 (C-15), 36.2 (C-9), 36.6 (C-10), 39.9 (C-1), 42.1 (C-24), 47.9 (C-13), 49.9 (C-17), 57.7 (C-5), 69.2 (C-25), 70.4 (C-2), 71.5 (C-3), 82.5 (C-22), 84.0 (C-14), 85.1 (C-20), 106.9 (acetonide C), 121.1 (C-7), 167.8 (C-8), 202.1 (C-6); HRFABMS (negative ion mode) *m/z* 519.3324 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3321).

3.2. Asymmetric dihydroxylation of 8 with OsO_4 and chiral ligands

General procedure. To a solution of 0.03 mmol of a chiral ligand in *tert*-BuOH–THF–H_2O (7:4:1, 0.6 mL) was added a THF solution of OsO_4 (14 μL, 0.03 mmol. The solution was prepared by dissolving 500 mg of OsO_4 in 9 mL of THF.) and the mixture stirred for 3 min. A solution of the olefin acetonide **8** (5 mg, 0.01 mmol) in *tert*-BuOH–THF–H_2O (7:4:1, 0.5 mL) was then added and stirring continued for 5 min. The ratio of the ligand, OsO_4 and olefin acetonide was 3:3:1. A 5% solution of $NaHSO_3$ (10 mL) was added and stirring continued for another 10 min. The mixture was extracted with EtOAc (4×20 mL); the combined organic phase was evaporated and the residue was chromatographed to separate compounds **5** and **9** from the ligand. Since the two products could easily be separated from each other by column chromatography, the **5**:**9** ratio for each ligand was determined from the isolated products **5** and **9**. The results are shown in Table 1.

3.2.1. Acetylation of compound 9. A mixture of compound **9** (9 mg, 0.017 mmol), Ac_2O (0.1 mL, 1.05 mmol) and pyridine (0.7 mL) was stirred for 6 h. The reaction mixture was worked up in the usual manner and the product purified by column chromatography to give 2,3-*diepi*-20-hydroxyecdysone 2,3,25-triacetate (**10**) (8 mg, 72%).

Compound **10**. Amorphous; ν_{max} 3482, 2977, 1744, 1666, 1458, 1370, 1246, 1168, 1137, 1106, 928, 874 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.76 (s, 3H, 18-Me), 0.94 (br s, 3H, 19-Me), 1.12 (s, 3H, 21-Me), 1.29, 1.38 (each s, 2×3H, acetonide Me), 1.43 (s, 3H, 26-Me), 1.45 (s, 3H, 27-Me), 1.97 (s, 2×3H, 3-OAc, 25-OAc), 1.98 (s, 3H, 2-OAc), 2.20 (partially obscured signal, 1H, H-5), 3.59 (dd, *J*=ca 9, 2.5 Hz, 1H, H-22), 3.75 (br, $W_{1/2}$=21 Hz, 1H, H-9), 4.88 (br, $W_{1/2}$=20 Hz, 1H, H-3), 5.26 (br s, $W_{1/2}$=13 Hz 1H, H-2), 5.86 (d, *J*=1.9 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 17.1 (C-18), 20.9 (acetate Me), 21.1 (2×C, acetate Me), 21.9 (C-21), 23.2 (C-16), 23.6 (C-19), 25.7 (C-26), 26.1 (C-27), 26.8 (acetonide Me), 28.9 (acetonide Me), 30.9, 31.9 (C-12, C-15), 35.0 (C-9), 36.1 (C-10), 36.7 (C-1), 38.5 (C-24), 47.2 (C-13), 49.0 (C-17), 55.8 (C-5), 69.3, 71.0 (C-2, C-3), 81.4 (C-22), 82.0 (C-25), 84.0 (C-20), 84.9 (C-14), 106.8 (acetonide C), 120.9 (C-7), 165.8 (C-8), 170.0 (2×C, acetate CO), 170.5 (acetate CO), 200.6 (C-6); HRFABMS (positive ion mode) *m/z* 647.3787 $[M+H]^+$. (calcd for $C_{36}H_{54}O_{10}+H$, 647.3787).

3.2.2. Acetonide deprotection of compound 9. Compound **9** (36 mg, 0.069 mmol) was dissolved in 70% AcOH (0.7 mL, excess) and benzyltrimethylammonium chloride (25 mg, 0.135 mmol) was added. The reaction mixture was left to stir for 4 h; water was then added and the mixture extracted with *n*-BuOH (3×15 mL). The combined organic layer was washed with water; the solvent was removed by co-evaporation with water under reduced pressure. The crude product was purified by column chromatography using $CHCl_3$–MeOH as eluting solvent to afford 2,3-*diepi*-20-hydroxyecdysone (**3**) (27 mg, 81%).

Compound **3**. Colorless needles (from MeOH–EtOAc), mp 204–206 °C; ν_{max} 3420, 2965, 1654, 1383, 1065, 929 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 1.00 (s, 3H, 19-Me), 1.22 (s, 3H, 18-Me), 1.38 (s, 2×3H, 26-Me, 27-Me), 1.54 (s, 3H, 21-Me), 2.40 (obscured signal, 1H, H-5), 3.00 (t, *J*=8.9 Hz, 1H, H-17), 3.87 (br d, *J*=8.7 Hz, 1H, H-22), 3.90 (obscured signal, 1H, H-3), 4.34 (br s, $W_{1/2}$=8.5 Hz, 1H, H-2), 4.76 (br m, 1H, H-9), 6.22 (d, *J*=2.6 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 18.0 (C-18), 21.4 (C-16), 21.5 (C-11), 21.6 (C-21), 24.2 (C-19), 27.5 (C-23), 29.8 (C-4), 29.9 (C-26), 30.3 (C-27), 31.8 (C-12), 32.1 (C-15), 36.3 (C-9), 36.7 (C-10), 39.9 (C-1), 42.7 (C-24), 48.2 (C-13), 50.1 (C-17), 57.8 (C-5), 69.6 (C-25), 70.5 (C-2), 71.6 (C-3), 76.9 (C-20), 77.5 (C-22), 84.1 (C-14), 121.0 (C-7), 168.4 (C-8), 202.2 (C-6); HRFABMS (negative ion mode) *m/z* 479.3001 $[M-H]^-$. (calcd for $C_{27}H_{44}O_7-H$, 479.3008).

3.2.3. Epimerization of compound 9. A mixture of compound **9** (35 mg, 0.067 mmol) in MeOH (0.8 mL) and 2% Na_2CO_3 (0.2 mL, 0.038 mmol) was stirred at ambient temperature for 5 h and water was then added. The solution was extracted with *n*-BuOH (3×10 mL); the combined butanol layer was washed with water and the solvent removed by co-evaporation with water. The product was purified by column chromatography to afford 2,3-*diepi*-5α-20-hydroxyecdysone 20,22-acetonide (**11**) (28 mg, 80%).

Compound **11**. Amorphous; ν_{max} 3422, 2971, 2942, 1664, 1458, 1375, 1219, 1175, 1105, 1054, 1001, 905, 868 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.95 (s, 3H, 19-Me), 0.99 (s, 3H, 18-Me), 1.30 (s, 3H, 21-Me), 1.35 (s, 2×3H, 26-Me, 27-Me), 1.43, 1.52 (each s, 2×3H, acetonide Me), 2.74 (t, *J*=ca 8 Hz, 1H, H-17), 3.02 (br d, *J*=11.1 Hz, 1H, H-5), 3.23 (m, 1H, H-9), 3.93 (br d, *J*=8.5 Hz, 1H, H-22), 4.02 (m, $W_{1/2}$=21 Hz, 1H, H-2), 4.42 (br s, $W_{1/2}$=8 Hz, 1H, H-3), 6.16 (br s, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 13.6 (C-19), 17.3 (C-18), 20.8 (C-16), 22.0 (C-11), 22.4 (C-21), 24.4 (C-23), 27.2 (acetonide Me), 28.2 (C-4), 29.5 (acetonide Me), 29.9 (C-26), 30.1 (C-27), 31.5 (C-12), 31.6 (C-15), 40.3 (C-10), 41.2 (C-1), 42.2 (C-24), 46.7 (C-9), 47.6 (C-13), 48.7 (C-5), 49.9 (C-17), 68.3 (C-2), 69.1 (C-3), 69.3 (C-25), 82.5 (C-22), 83.9 (C-14), 85.1 (C-20), 106.9 (acetonide C), 123.3 (C-7), 164.4 (C-8), 201.7 (C-6);

HRFABMS (negative ion mode) *m/z* 519.3313 $[M-H]^-$. (calcd for $C_{30}H_{48}O_7-H$, 519.3322).

3.2.4. Acetonide deprotection of compound 11. Compound **11** (15 mg, 0.029 mmol) was subjected to acetonide deprotection in the same manner as described for the preparation of **3** from **9**. The product was purified by column chromatography to afford 2,3-*diepi*-5α-20-hydroxyecdysone (**4**) (10 mg, 72%).

Compound **4**. Amorphous; ν_{max} 3415, 2925, 1660, 1384, 1062 cm^{-1}; ^1H NMR (400 MHz, C_5D_5N) δ 0.84 (s, 3H, 19-Me), 1.21 (s, 3H, 18-Me), 1.38 (s, 2×3H, 26-Me, 27-Me), 1.58 (s, 3H, 21-Me), 2.99 (t, J=9.2 Hz, 1H, H-17), 3.03 (dd, J=12.4, 3.6 Hz, 1H, H-5), 3.28 (m, 1H, H-9), 3.89 (br d, J=8.9 Hz, 1H, H-22), 4.04 (m, $W_{1/2}$=20 Hz, 1H, H-2), 4.44 (br s, $W_{1/2}$=9 Hz, 1H, H-3), 6.18 (d, J=2.4 Hz, 1H, H-7); ^{13}C NMR (100 MHz, C_5D_5N) δ 13.6 (C-19), 17.9 (C-18), 20.9 (C-16),[a] 21.4 (C-11),[a] 21.7 (C-21), 27.4 (C-23),[b] 28.3 (C-4),[b] 29.9 (C-26), 30.2 (C-27), 31.8 (C-12, C-15), 40.3 (C-10), 41.3 (C-1), 42.7 (C-24), 46.7 (C-9), 47.9 (C-13), 48.7 (C-5), 50.1 (C-17), 68.4 (C-2), 69.1 (C-3), 69.6 (C-25), 76.9 (C-20), 79.8 (C-22), 84.0 (C-14), 123.3 (C-7), 164.9 (C-8), 201.8 (C-6), ('a and b' stand for assignments may be reversed for signals with the same superscript); HRFABMS (negative ion mode) *m/z* 479.3018 $[M-H]^-$. (calcd for $C_{27}H_{44}O_7-H$, 479.3008).

3.5. Moulting bioassay

Compounds **3** and **4** were subjected to the *Musca* bioassay, using *Musca domestica* larvae.[13] The purity of the ecdysteroid and their analogues was checked by reversed-phase HPLC. The bioassay results were scored[15] and EC_{50}, the molar concentration of each steroid required to effect puparium formation of 50% effectiveness, of each compound was determined by plotting concentrations against % effectiveness of puparium formation.[16] The EC_{50} values of compounds **3** and **4** were 5.0×10^{-4} and 7.0×10^{-4} M, respectively, whereas that of the reference compound **1** was 1.65×10^{-5} M.

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