DIMERIC ECDYSTEROID ANALOGUES AND THEIR INTERACTION WITH THE *Drosophila* ECDYSTEROID RECEPTOR

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Dedicated to Professor Antonín Holý on the occasion of his 70th birthday.

Three structurally related specific ecdysteroid derivatives, 7,7′-dimers of 14-deoxy-8(14)-ene transformed 20-hydroxyecdysone, ponasterone A and ajugasterone C, were obtained by photochemical transformation. The structures of the dimeric ecdysteroids were identified mainly by NMR spectroscopy supported by MS and IR spectroscopy. Yields of the dimerisation products were dependent on the reactant concentrations and photoreaction conditions. Inert gas atmosphere supported high yields, whereas oxygen atmosphere fully prevented the dimer formation. All the three dimers retained a rather high agonistic activity at the ecdysteroid receptor in the *Drosophila* B_{II} bioassay when compared with the relevant original ecdysteroids.

Keywords: Steroids; Ecdysteroids; 20-Hydroxyecdysone; Ponasterone A; Ajugasterone C; Photochemical transformations; *Drosophila melanogaster* B_{II} bioassay; Ecdysteroid receptor agonists; NMR spectroscopy.

Ecdysteroids interact with the ligand binding site of the insect ecdysteroid receptor mostly as agonists[1]. Only one analogue, a side-chain-modified lactone derivative, has been so far found to demonstrate antagonistic activity[2]. These activities were detected and quantified using the *Drosophila melanogaster* B_{II} cell bioassay[3]. The activities of a series of natural and chemically modified ecdysteroids prepared in our laboratory have already been compared in this bioassay[4]. The natural ecdysteroids were isolated from higher plants, mainly from *Leuzea carthamoides*[5,6], or from fungi[7,8]. The

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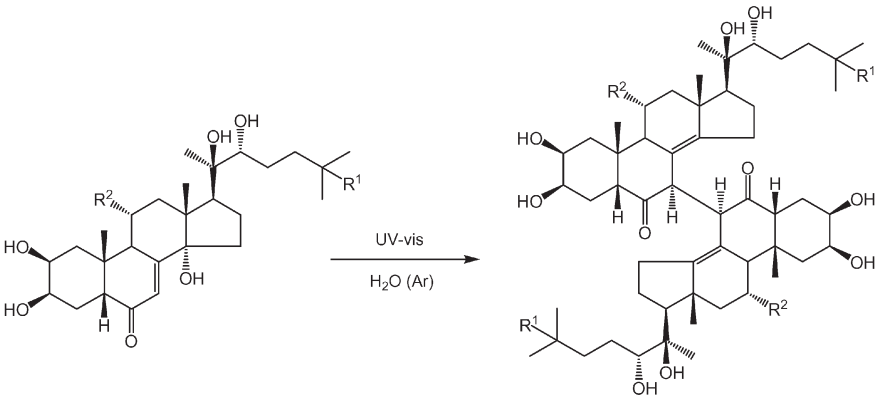
majority of structural analogues were prepared by targeted chemical transformations[9,10]. Later, a photochemically induced transformation was introduced as an alternative approach to ecdysteroid structure modifications[11]. This method was originally used for preparation of standard 20-hydroxyecdysone derivatives[12], in order to monitor whether such structural analogues could stem from the native compound as UV light-induced artefacts. Phototransformation of 20-hydroxyecdysone, as described by Canonica et al.[12,13], yielded a series of ecdysteroid deoxy-, hydroperoxy- and C/D-ring-rearranged derivatives. When the method was used in our laboratory[11], various additional analogues were obtained. The most surprising phototransformation product obtained from our experiment was a dimeric ecdysteroid[11], which was not described in the papers of Canonica et al.[12,13]. The biological activity data of the photochemically transformed ecdysteroids were used to investigate structure–activity relationships[14], and for designing further targeted structure modifications[15]. The activities in the *in vitro* bioassay generally confirmed those previously obtained by *in vivo* tests, with exception of the dimeric ecdysteroids, which have not yet been assessed *in vivo*. In order to extend this study, we prepared and tested new specific analogues and ecdysteroid dimers obtained by phototransformation of 20-hydroxyecdysone (**1**) ponasterone A (**2**) and ajugasterone C (**3**), respectively.

RESULTS AND DISCUSSION

Photochemical transformations were performed in a standard photoreactor under controlled reaction conditions, as described for 20-hydroxyecdysone (**1**) in our previous paper[11]. The resulting reference dimer **4**, as well as the experience gained during its photoproduction, isolation and identification, contributed to the phototransformation of two other related ecdysteroids, the 25-deoxy analogue ponasterone A (**2**) and its 11-hydroxy analogue ajugasterone C (**3**). Both compounds yielded dimeric derivatives **5** and **6**, respectively (Scheme 1). The photoreaction mixtures were analyzed, and individual compounds separated by a combination of reversed-phase and normal-phase HPLC. The retention times of the targeted dimers **4**, **5** and **6** are compared with those of the starting ecdysteroids **1**, **2** and **3** in Table I. The structures of the dimers **4**–**6** were determined mainly by NMR spectroscopy, supported by IR and MS, utilizing results of the extended and complete structure analysis of the dimeric 20-hydroxyecdysone derivative **4**, described in our previous paper[11].



1. $R^1 = OH, R^2 = H$

2. $R^1 = R^2 = H$

3. $R^1 = H, R^2 = OH$

4. $R^1 = OH, R^2 = H$

5. $R^1 = R^2 = H$

6. $R^1 = H, R^2 = OH$

SCHEME 1

TABLE I
HPLC retention times and biological activities of ecdysteroids **1**–**3** and their dimers **4**–**6**

Compound	Retention time, min			Agonist activity (EC_{50} value) M
	System 1[a]	System 2[b]	System 3[c]	
20-Hydroxyecdysone (**1**)	34.2	50.7	–	7.5×10^{-9}
Ponasterone A (**2**)	47.8	19.2	117.4	3.1×10^{-10}
Ajugasterone C (**3**)	39.2	32.3	–	3.0×10^{-8}
7-7′-*bis*-14-Deoxy-8(14)-en-20-hydroxyecdysone (**4**)	51.8	65.9	–	2.1×10^{-7}
7-7′-*bis*-14-Deoxy-8(14)-en-ponasterone A (**5**)	74.2	15.5	72.4	4.6×10^{-8}
7-7′-*bis*-14-Deoxy-8(14)-en-ajagasterone C (**6**)	67.2	25.2	–	8.2×10^{-7}

[a] System 1: Separon SGX C-18 column (7 μm, 250 × 4 mm i.d.), elution with a linear gradient of 10–70% methanol in water over 50 min, followed by isocratic elution for 30 min at a flow-rate of 0.6 ml/min. [b] System 2: Silasorb 600 column (5 μm, 250 × 4 mm i.d.), elution with n-hexane/ethanol/water (810:180:0.8, v/v/v), flow-rate 0.8 ml/min. [c] System 3: Silasorb 600 column (5 μm, 250 × 4 mm i.d.), elution with n-hexane/ethanol/water (900:99:0.5, v/v/v), flow-rate 0.8 ml/min.

The numbers of signals in both ^{1}H and ^{13}C NMR spectra of compounds **5** and **6** (Table II), corresponding to the empirical compositions $C_{27}H_{43}O_5$ and $C_{27}H_{43}O_6$, respectively, indicate the symmetry of the molecules. This means, that compounds **5** and **6** are homo-dimeric, composed of two identical parts linked at the same positions. The absence of characteristic signals for the olefinic proton H(7) and carbon C(14)–OH, the observed upfield shift of the C=O carbon at C(6) (δ 215.89 and 215.54 in dimers **5** and **6**, respectively, versus δ 206.50 and 206.66 in the starting compounds **2** and **3**), together with signals for a tetrasubstituted double bond (olefinic carbons at δ 124.51/153.00 and 123.68/152.63, respectively) and the newly appeared singlet for hydrogen H(7) at δ 3.54 indicated that the monomeric units in dimers **5** and **6** contain a C=C double bond between C(8) and C(14) and are linked at positions C(7). The ^{1}H and ^{13}C NMR data of dimers **5** and **6** (see Table II) are very similar to those we described previously[11] for the dimer of 20-hydroxyecdysone **4**, with characteristic differences only for carbon atoms and protons, which are close to the differing structure moieties (the absence of a 25-OH group in both **5** and **6**, and the presence of a 11α-OH group in **6**), already present in the corresponding starting compounds **2** and **3**. The determination of the configuration at carbon atoms C(7)/C(7′) by the analysis of a 2D-ROESY spectrum and molecular modelling is described in detail in our previous paper[11].

Differences in the water solubility of the ecdysteroids **1**–**3** led us to performing the photoreaction at rather low ecdysteroid concentrations (0.6–0.8 mmol/l). We already knew from our previous observation[11] that the reaction yielded only 3% of dimer **4**, when the 20-hydroxyecdysone was added at a low concentration (0.83 mmol/l). Ajugasterone C (**3**) did not dissolve completely at this concentration, but after dissolving during the reaction, it yielded 4.5% of dimer **5**, besides 12% of unreacted ajugasterone C (**3**). Ponasterone A (**2**) is even less soluble in water. Therefore, the photoreaction was performed at a concentration of 0.65 mmol/l (where a part of the reactant still remained in suspension). All the reactant did not dissolve completely, even in a prolonged reaction time. Thus, ponasterone A (**2**) yielded only 2.5% of dimer **5**, with up to 32.5% of unreacted **2** remaining in the reaction mixture. A higher solubility, but a different reaction product profile, was obtained by adding organic solvent to water (tetrahydrofuran/water, 1:1 v/v). These results, as well as effects of the presence of oxygen in the reaction mixture instead of using inert argon protection, will be published later in another context. The presence of oxygen had a greater influence on the reaction product composition than the reactant concentration. However, in connection with this report, it is relevant only that the dimer

TABLE II
^{1}H and ^{13}C NMR chemical shifts of compounds **4**–**6** in CD_3OD

Proton	**4**[a]	**5**	**6**	Carbon	**4**[a]	**5**	**6**
1α	1.75	1.75	2.02	1	36.88	36.69	38.15
1β	1.47	1.46	1.48	2	68.37	68.41	68.74
2α	3.81	3.80	4.12	3	69.05	69.05	69.19
3α	4.04	4.03	4.03	4	33.88	33.97	33.95
4α	2.24	2.24	2.20	5	56.03	56.03	56.18
4β	2.13	2.12	2.13	6	215.99	215.89	215.54
5	2.42	2.42	2.43	7	59.07	59.09	58.77
7	3.53	3.54	3.54	8	124.54	124.51	123.68
9	2.99	3.00	2.83	9	33.49	33.52	43.30
11α	1.87	1.87	–	10	41.39	41.30	41.02
11β	1.60	1.60	4.03	11	21.42	21.43	67.50
12α	1.92	1.95	2.18	12	36.70	37.32	48.14
12β	1.59	1.53	1.57	13	43.95	44.27	45.54
15α	2.38	2.37	2.43	14	152.97	153.00	152.63
15β	2.31	2.32	2.32	15	26.54	26.71	26.70
16α	1.92	1.89	1.99	16	23.04	22.94	23.19
16β	1.85	1.89	1.89	17	57.79	57.81	57.60
17	2.37	2.24	2.18	18	19.68	19.69	19.88
18	0.90	0.94	0.96	19	25.33	25.36	27.10
19	0.70	0.71	0.75	20	78.33	78.31	78.18
21	1.13	1.12	1.15	21	21.24	21.26	21.39
22	3.48	3.45	3.46	22	77.52	77.30	77.46
23a	1.60	1.50	1.49	23	27.36	37.32	36.96
23b	1.68	1.60	1.58	24	41.25	30.53	30.54
24a	1.46	1.30	1.28	25	71.58	29.35	29.23
24b	1.75	1.45	1.43	26	28.95	22.81	22.88
25	–	1.57	1.58	27	29.93	23.54	23.66
26	1.19	0.91	0.92				
27	1.21	0.93	0.93				

[a] Data from lit.[11]

formation was not detected in the presence of oxygen during the photoreaction. This corresponds with the observation that dimeric steroids with C–C linked B rings, when subjected to photooxidation under fluorescent light, were transformed to their monomeric units[16].

The biological activities of the relatively large dimeric ecdysteroid analogues **4**–**6** were compared with those of their native precursors **1**–**3** in the B_{II} bioassay, in which potency reflects the affinity of binding to the ligand-binding site of the *Drosophilla melanogaster* ecdysteroid receptor[1,3]. We have already compared[12] the activity of the dimeric 20-hydroxyecdysone derivative **4** with its structurally related, but considerably smaller, monomeric analogues. The results led to the correction of some former general presumptions concerning ecdysteroid structure–activity relationships, concerning in particular the influence of the C(14)–OH configuration and the corresponding C/D-ring anelation, as well as unusual double bond location. We can now compare additional data obtained from the B_{II} bioassay for the structurally transformed dimeric ecdysteroids **5** and **6** in relation to dimer **4** and to their native, structurally rather different precursors (Table I).

All the compared compounds possessed agonistic activity; none of them showed any antagonistic activity. The relative potencies of the agonist activity of dimeric ecdysteroids **4**–**6** (with EC_{50} values in the range 10^{-8}–10^{-7} M) are unexpectedly high. They are only two orders of magnitude lower than the activities of the very active parent ecdysteroids **1**–**3**, and are significantly higher than the activities of some structurally-related monomers, e.g. podecdysone B ($EC_{50} = 1.2 \times 10^{-5}$ M)[11]. This finding should be considered in the following context: (i) Dimers are considerably bulkier than monomeric ecdysteroids and one might expect this to prevent interaction with the receptor binding site. However, other large molecules, such as oligostilbenes, can also interact with this binding site[17,18]. (ii) If the dimers can accommodate themselves at the binding site, then dimerisation effectively doubles the concentration of ecdysteroid moieties and may facilitate the reloading of the binding site after dissociation of the ligand–receptor complex. If the association rate constant (k_a) is enhanced while the dissociation rate constant (k_d) is not significantly affected, then the dissociation constant ($K_d = k_d/k_a$) is lower (i.e. the affinity increases). (iii) The dimer might interact simultaneously with the two receptor–proteins (EcR/USP complex), both of which are members of the nuclear receptor family. Simultaneous ecdysteroid interaction with EcR and USP (the natural ligand for USP is currently unknown) might account for the high activity of the dimers. The rather high stability of the dimers[11] and low activity of structurally related monomers[14], expected as products of possible degradation,

do not support the opinion that the activity could depend on the reverse monomer formation at the ligand binding site.

The less bulky 20-hydroxyecdysone glucosides[9] have lower activity[4] by two orders of magnitude (EC_{50} = 10^{-6}–10^{-5} M) than the dimers (Table I). The glucosides are substituted in positions 2, 3, 22 or 25, which are critical for the ecdysteroid activity according to the pharmacophore hypothesis[15], whereas dimerisation, creating links through C(7), leaves these positions free, but affects the region between rings B, C and D, where molecular modelling does not indicate essential roles for the C(6)=O and 14α-OH in receptor interactions[19].

Our experiments have proven that the specific dimerisation of ecdysteroids induced by UV light (under inert gas conditions) is not an exclusive photoreaction occurring only with 20-hydroxyecdysone; it is more general, independent of the number and location of hydroxyl groups in the ecdysteroid molecule. The unexpectedly high biological activity in terms of the interaction with the ecdysteroid receptor is evidently also more general.

EXPERIMENTAL

NMR spectra were measured on a Varian Unity-500 and Bruker Avance-500 instrument (^{1}H at 500 MHz, ^{13}C at 125.7 MHz) in CD_3OD. Chemical shifts in CD_3OD were referenced to the solvent signal at 3.31 ppm (^{1}H) and 49.00 ppm (^{13}C). Homonuclear 2D-COSY and 2D-ROESY spectra were used for structure assignment of protons. ^{13}C APT spectra and heterocorrelated 2D-HSQC and 2D-HMBC spectra were combined to assign signals of all carbon atoms. Mass spectra were recorded on a ZAB-EQ spectrometer with fast atom bombardment (FAB) ionization using a glycerol–thioglycerol mixture as matrix. Infrared spectra (ν_{max} in cm^{-1}) were recorded on a Bruker IPS-88 instrument using KBr pellets. Melting points were determined on a Boetius apparatus and are uncorrected.

Phototransformations were performed in a 500-ml original Hanau Photoreactor, equipped with a magnetic turbine pump for forced circulation, with a jacket for water stream cooling, and with an immersed Pyrex-filtered medium-pressure broad-band (250–600 nm) Hg lamp. The reaction was performed in water solution (500 ml) circulating in the reactor under inert gas (argon) and at controlled temperature (25 °C). The reaction time was adjusted according to the results of RP-HPLC monitoring of samples taken and analyzed every 30 min until the conversion of the reactant reached a steady state. The reaction mixture was transferred to a flask, and water was evaporated to give a dry residue.

The residue was analyzed by RP-HPLC (reversed-phase) using a 4 × 250 mm column packed with 7 μm size Separon SGX C-18 (Tessek, Prague). Elution was performed with solvent System 1 (as indicated in Table I). The residue was later fractionated by preparative RP-HPLC using a 26 × 600 mm column packed with the same stationary phase and eluted with the same mobile phase at the same gradient over 300 min and at a flow rate of 5 ml/min. Collected fractions were monitored by NP-HPLC (normal-phase) using a 4 × 250 mm column packed with 5 μm size Silasorb 600 (Lachema, Brno) and a mobile phase (System 2 in Table I). Fractions containing dimeric ecdysteroid analogues were further separated and puri-

fied by NP-HPLC using a 12 × 500 mm column packed with the same stationary phase and eluted with the same mobile phase at a flow rate of 3 ml/min. For analysis of the dimeric ponasterone A derivative **5** System 3 (Table I) was more effective. Characteristic HPLC data recorded under analytical conditions are summarized in Table I.

20-Hydroxyecdysone (**1**) and ajugasterone C (**3**) were obtained in sufficient quantity and high quality (over 98% purity) from roots of *Leuzea carthamoides*[6]. Ponasterone A (**2**) was prepared from 20-hydroxyecdysone (**1**) by the following four-step procedure: *1*. Conversion of 20-hydroxyecdysone into its 2,3,22-triacetate and purification of the product by LC. *2*. Dehydration of 20-hydroxyecdysone-2,3,22-triacetate with $POCl_3$ in pyridine, which produced a mixture of Δ^{24} and Δ^{25} derivatives. The reaction mixture was purified by LC without separation of the two derivatives. *3*. Deprotection with potassium carbonate in aqueous methanol giving a mixture of $\Delta^{24/25}$ derivatives (stachysterone C and its isomer). *4*. Reduction of the mixture from step *3* with hydrogen using 10% Pd/C catalyst. This procedure, based on previously known original transformations[20–22], represents upgraded straightforward modification suitable for large-scale preparation. The resulting ponasterone A was purified by preparative RP-HPLC and identified by ^{1}H and ^{13}C NMR and by comparison with an authentic standard by NP-HPLC (System 3, Table I).

General Procedure for Phototransformation of Ecdysteroids **1**–**3**

The photoreactor (see above) was filled with water (500 ml), and the reactant was added in one portion. The mixture was stirred by forced circulation under a continuous flow of argon. After dissolving the reactant (in the case of **1**) and further argon rinsing (1 h), the UV lamp was switched on, and the reaction was performed. The reaction mixture was transferred to a flask, and water was evaporated. The residue was fractionated by preparative RP-HPLC (using System 1 adapted for preparative separations). The main constituent of the photoreaction product (dimeric ecdysteroid) was separated from the major fraction as described above.

The phototransformation reaction was performed in the same way with ponasterone A (**2**) and ajugasterone C (**3**), with the only difference that both reactants at the same concentration did not dissolve completely. Thus, these reactions were performed partly in suspension. The dimeric compounds were obtained from reaction mixtures in various yields (varied from 2.5 to 4.5%).

(20*R*,20′*R*,22*R*,22′*R*)-2β,2′β,3β,3′β,20,20′,22,22′,25,25′-Decahydroxy-[7αH,7′αH-bi(5β-cholest-8(14)-ene)]-6,6′-dione, i.e. 7-7′-*bis*-14-Deoxy-8(14)-ene-20-hydroxyecdysone (**4**)

20-Hydroxyecdysone (**1**; 500 mg) was added to the photoreactor filled with water (500 ml). After dissolving the reactant (10 min), the photoreaction was performed for 4 h. The reaction mixture, after evaporation of water, was fractionated by RP-HPLC (using modified System 1) yielding 13 fractions. Compound **4** (87 mg, 17%) was obtained directly from fraction 11 after solvent evaporation and crystallization from methanol–water. M.p. 171–173 °C. IR: 3485, 3290 (O–H), 1695 (C=O), 1059 (C–O). UV (nm, MeOH): 210 (4.06), 242 (3.69), 310 (2.78). CD (MeOH): $\Delta\varepsilon_{209}$ +55.0, $\Delta\varepsilon_{237}$ +4.5, $\Delta\varepsilon_{259}$ –12.2, $\Delta\varepsilon_{299}$ –1.8. FAB-MS, *m/z* (%): 949 [M + Na] (11), 927 [M + H] (6), 891 (8), 873 (10), 464 [1/2 M + 1] (100), 429 (55). Composition $C_{54}H_{86}O_{12}$ (M = 926) determined by HR FAB-MS: 949.6111 [M + Na], for $C_{54}H_{86}O_{12}Na$ calculated 949.6017. For ^{1}H and ^{13}C NMR data, see Table II.

(20*R*,20′*R*,22*R*,22′*R*)-2β,2′β,3β,3′β,20,20′,22,22′-Octohydroxy-[7αH,7′αH-bi(5β-cholest-8(14)-ene)]-6,6′-dione, i.e. 7-7′-*bis*-14-Deoxy-8(14)-ene-ponasterone A (**5**)

Ponasterone A (**2**; 150 mg) was added to the photoreactor filled with water (500 ml). The mixture was stirred under a continuous flow of argon for more than 1 h. The reactant did not dissolve completely. The photoreaction was performed in suspension for 4 h. Owing to small conversion of ponasterone A and low yield of the dimer, the reaction was repeated 3 times. The combined reaction mixtures (450 mg), after evaporation of water, were fractionated by RP-HPLC (using adapted System 1). Compound **5** (11 mg, 2.5%) was obtained in addition to unchanged ponasterone A (145 mg, 32.5%) from the relevant fractions. Compound **5** was purified by NP-HPLC (using adapted System 3) giving a white solid. IR: 3440 (O–H), 1698 (C=O), 1060 (C–O). FAB-MS, *m/z* (%): 893 [M – H] (57), 447 [1/2 M] (27), 255 (34), 153 (100), 113 (33). Composition $C_{54}H_{86}O_{10}$ (M = 894) determined by HR FAB-MS: 895.632213 [M + H], for $C_{54}H_{87}O_{10}$ calculated 895.629925. For ^{1}H and ^{13}C NMR data, see Table II.

(20*R*,20′*R*,22*R*,22′*R*)-2β,2′β,3β,3′β,11α,11′α,20,20′,22,22′-Decahydroxy-[7αH,7′αH-bi(5β-cholest-8(14)-ene)]-6,6′-dione, i.e. 7-7′-*bis*-14-Deoxy-8(14)-en-ajugasterone C (**6**)

Ajugasterone C (**3**; 200 mg) was added to the photoreactor filled with water (500 ml). The mixture was stirred under a continuous flow of argon for more than 1 h. The reactant did not dissolve completely. The photoreaction was initiated in suspension. The reactant gradually dissolved during the reaction over 8 h. The reaction mixture, after evaporation of water, was fractionated by RP-HPLC (using adapted System 1). Compound **6** (9 mg, 4.5%) was obtained in addition to unchanged ajugasterone C (25 mg, 12.5%) from the relevant fractions. Compound **6** was purified by NP-HPLC (using adapted System 2) giving a white solid. IR: 3418 (O–H), 1698 (C=O), 1054 and 1023 (C–O). FAB-MS, *m/z* (%): 949 [M + Na] (22), 926 [M] (2), 486 (57), 464 [1/2 M + 1] (31), 413 (41), 231 (29), 149 (82), 57 (100). Composition $C_{54}H_{86}O_{12}$ (M = 926) determined by HR FAB-MS: 949.599062 [M + Na], for $C_{54}H_{86}O_{12}Na$ calculated 949.601699. For ^{1}H and ^{13}C NMR data, see Table II.

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