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Rapid, laser-induced conversion of 20-hydroxyecdysone – A follow-up study on the products obtained

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ABSTRACT

We have recently reported the set-up of an experimental system for the laser-induced photochemical modification of bioactive substances, where two ecdysteroids, 20-hydroxyecdysone (20E) and its diacetonide derivative served as probes. As a direct continuation of our previous work, three new compounds together with five other ecdysteroid derivatives, have been identified from the novel, laser-induced photo-transformation reaction of 20E. The structures and NMR signal assignment were established by comprehensive one- and two-dimensional NMR spectroscopy supported by mass spectroscopy. Possible ways for the formation of each species is also discussed.

Similar to their parental compound, the products obtained are potentially bioactive and worthy for further investigations; due to the low yields, however, a different approach for their higher scale production is suggested.

1. Introduction

Ecdysteroids represent one of the large families of steroid hormones in Nature; these compounds play a crucial role at all stages of arthropods' development including embryogenesis, molting, egg and spermium production and diapause [1]. Interestingly, ecdysteroids can also exert various rather beneficial effects in vertebrates: a general strengthening, somewhat "Ginseng-like" activity has been attributed to them, which includes a non-hormonal anabolic action and a series of further metabolic effects usually referred to as adaptogenic [2,3]. Ecdysteroids also play a (rather unclear) role in cancer: muristerone A exerted anti-apoptotic activity on a colon carcinoma cell line *in vitro* [4], and, most recently, a strong effect of certain natural and semi-synthetic ecdysteroids was discovered on the efflux-mediated multi-drug resistance of murine leukemia cells expressing the human ABCB1 transporter [5,6]. Based on the anabolic activity and several potential (expected) health benefits, many food supplements and other products containing mainly 20-hydroxyecdysone (20E), the most common ecdysteroid of herbal origin, are available worldwide for various indications.

Ecdysteroids are significantly different from the mammalian steroid hormones, although they show some structural similarities to vitamin D, which might serve as an explanation for most (if not all) of the effects of these compounds in mammals [7]. Due to their several hydroxyl groups around the regularly side-chain bearing steroid skeleton, they are typically polar compounds, and a 7-en-6-one (α,β-enone) chromophore group is usually present in their B ring. This latter moiety confers upon these compounds a characteristic UV absorption at around $\lambda = 240–250$ nm, and allows them to be subjects of photochemical transformations. Such an approach can offer several advantages over that of the conventional chemical methods in the semi-synthetic modification of ecdysteroids – due to their typically complex hydroxylation patterns, certain selective transformations of these compounds can be challenging. On the other hand, photoreactions of α,β-enones represent a major area

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of modern photochemistry, which allows various specific, regioselective modifications that are hard or even impossible to achieve by using classical chemical strategies [8].

Unusual photochemical behavior of the enone moiety of 20E irradiated by a mercury UV-lamp over a Pyrex filter ($\lambda > 290$ nm) was discussed by Canonica et al. [9,10]. The irradiation of a 10^{-3} M water solution of 20E was performed under argon atmosphere and resulted in a full conversion after 12 h. The authors found the main photoproducts to be the Δ^{8-14} ketone (35%), 14α-deoxy-20-hydroxyecdysone (15%) and two derivatives formed by ring rearrangement (23% and 18%), while 14α-hydroperoxy-20-hydroxyecdysone was also present. The photochemical transformation of 20E in 500 mL of water with a Pyrex-filtered medium-pressure Hg lamp under inert gas (argon) was also performed by Harmatha et al. [11]. The production of additional derivatives such as 14-epi-20E and the 7–7′ homo-dimer of the Δ^{8-14} ketone was reported, whereas the monomeric Δ^{8-14} ketone could not be detected. These authors suggested differences in reactant concentration and/or the effect of oxygen present from air as possible explanations for this phenomenon. Yield of the dimer increased with the concentration of 20E; up to 19% at 1.2 mg/mL [11].

The use of lasers has some major advantages over the Hg lamp based photo-reactors due to their much higher energy and optical purity. With the use of lasers, integration of photochemistry and flow chemistry, as well as miniaturization of photoreactions with a lab-on-a-chip approach, are also possible [12].

Recently, we reported the set-up of a 266 nm laser system for the rapid photochemical transformation of bioactive substances by using 20E and its 2,3;20,22-diacetonide as first probes [13]. To our best knowledge, laser-catalyzed photoreactions of ecdysteroids have not been studied by any other groups. As a direct continuation of our previous work, here we describe the products obtained from the scale-up irradiation of 20E and discuss possible ways of their formation.

2. Experimental

2.1. General information

The starting material 20E was previously isolated from the plant *Serratula wolffii* [14]. Two milliliters of 10 mg/mL methanolic solutions of 20E were irradiated with a 266 nm laser at 15 mJ impulse energy in a 4 mL cuvette with continuous stirring, as described previously, and a total amount of 400 mg mixture of products was obtained from the repeated irradiations [13].

Solvent system compositions are given in v/v%. Rotational planar chromatography (RPC) was performed on a Chromatotron equipment (Harrison Research, Palo Alto, CA, USA). Analytical and preparative reverse phase HPLC was performed by using a gradient system consisting of two Jasco PU-2080 HPLC pumps equipped with a Jasco AS2055Plus autosampler connected to a Jasco MD-2010 Plus photodiode array (PDA) detector (Jasco Co., Tokyo, Japan). For the quantitative determination of the remaining unchanged 20E within the mixture, a Zorbax SB-Aq (250 × 4.6 mm, 5 μm) column (Agilent Technologies, Santa Clara, CA, USA) was used with a solvent system of 50% aqueous MeOH at a flow rate of 1.00 mL/min detecting at $\lambda = 245$ nm, with a five-points calibration ($R^2 = 0.9999$). For isolation, a Phenomenex C18 (250 × 10 mm, 5 μm) semi-preparative column (Phenomenex, Torrance, CA, USA) was used. Compound purities were tested on an Agilent XDB-C8 (4.6 × 150 mm, 5 μm) analytical column (Agilent Technologies, Santa Clara, USA). The separation was also monitored with thin layer chromatography (TLC) on silica gel 60 F_{254} (0.25 μm; Merck, Budapest, Hungary) and 60 RP-18 $F_{254}S$ (0.25 μm; Merck, Budapest, Hungary) and the gels sprayed with vanillin sulfuric acid reagent for visualization of bands. Yields represent the isolated amounts. Low-resolution mass spectra were recorded on an API 2000 triple quadrupole tandem mass spectrometer (AB SCIEX, Foster City, CA, USA) in positive mode with an atmospheric pressure chemical ionization (APCI) ion source. High-resolution electrospray-ionization mass spectra (HRESIMS) were recorded on a Waters Micromass Q-TOF premier mass spectrometer (Waters, Milford, MA, USA).

2.2. NMR spectroscopy

^{1}H (500.1 MHz) and ^{13}C (125.6 MHz) NMR spectra were recorded at room temperature on Bruker 500 Avance III equipped with cryo probehead and on Bruker Avance 500 spectrometers. Amounts of approximately 1–5 mg of the laser derived compounds were dissolved in 0.1 mL of methanol-d_4 and transferred to 2.5 mm Bruker MATCH NMR sample tube. Chemical shifts are given on the δ-scale and are referenced to the solvent (methanol-d_4: $\delta_C = 49.1$ and $\delta_H = 3.31$ ppm). Pulse programs of all experiments (^{1}H–^{13}C, DEPTQ, DEPT-135, sel-TOCSY (mixing time: 80 ms, 120 ms), sel-ROE (300 ms), sel-NOE (350 ms, 500 ms), gradient-selected (gs) ^{1}H,^{1}H-COSY, edited gs-HSQC, gs-HMBC (optimized for 10 Hz; in case of compound **5** also 5 Hz), NOESY (350 ms, 500 ms)) were taken from the Bruker software library. For 1D measurements, 64 K data points were used to yield the FID. For 2D measurements, sweep width in F_2 was 4000 Hz; all data points ($t_2 \times t_1$) were acquired with 2 K × 256. For F_1, linear prediction was applied to enhance the resolution. Most ^{1}H assignments were accomplished using general knowledge of chemical shift dispersion with the aid of the proton–proton coupling pattern (^{1}H NMR spectra). The ^{1}H and ^{13}C NMR data of the parent 20E and that of the new compounds **2**, **5** and **7** are compiled in Table 1.

2.3. Isolation of the irradiation products

The irradiated mixture was fractionated by rotation planar chromatography (RPC) with a five step gradient of *n*-Hexane:CH_2Cl_2:MeOH (3:125:5–3:50:5) to yield fractions 1-1–5-5 representing five fractions with each solvent composition. Fraction 4-1 (25.7 mg) was purified over RP-HPLC (50% MeOH aq, 3 mL/min) to yield poststerone (**1**) (13.1 mg). Fraction 4-2 (16.1 mg) was purified by RP-HPLC (58% MeOH aq, 3 mL/min) to obtain compound **2** (1.5 mg). Fraction 4-3 (16.5 mg) was purified by RP-HPLC (50% MeOH aq, 3 mL/min) to yield 14α,15α-epoxy-14, 15-dihydrostachysterone B (**3**) (0.62 mg) and stachysterone B (**4**) (2.0 mg). The residue from fraction 4-3 (4.3 mg) was further purified with RP-HPLC (30% CH_3CN aq, 3 mL/min) to give (**5**) (1.5 mg). Fraction 5-2 (28.1 mg) was purified by RP-HPLC (21% CH_3CN aq, 3 mL/min) to obtain 14α-hydroperoxy-20-hydroxyecdysone (**6**) (5.2 mg). Fraction 5-3 (83.1 mg) was further fractionated by centrifugal partition chromatography (Armen Spot CPC 250 mL, Armen Instrument, Saint Ave, France) in descending mode with a solvent system of $CHCl_3$:MeOH:H_2O (10:7:3), and 20 mL fractions were collected. Fraction 14 (20.7 mg) was purified with RPC with a three steps gradient system of EtOAc:EtOH:H_2O (80:2:1–80:10:4), to obtain compound **7** (3.7 mg). Compound **8**, 14-epi-20-hydroxyecdysone was identified by HPLC-DAD by using authentic reference compound previously isolated from plant source [14], and the order of magnitude of its amount was estimated based on the calibration line obtained for 20E.

Each isolated compound possessed a purity of >95% by means of RP-HPLC-PDA, by using 50% aqueous MeOH at 1 mL/min of flow rate.

Table 1
^{1}H and ^{13}C chemical shifts, multiplicities and coupling constants of compounds **2**, **5** and **7** in reference to those of 20-hydroxyecdysone (20E), in ppm (MeOH-d_4).

Atom No.	20E H	C	**2**[a] H	*J* (Hz)	C	**5** H	*J* (Hz)	C	**7** H	*J* (Hz)	C
1β	1.43	37.5	1.68		37.2	1.54	dd; 13.4, 11.9	38.6	1.46		36.8
α	1.80		1.73			1.63			1.74		
2	3.84	68.8	3.68	td; 12.5, 3.0	68.5	3.89	ddd; 11.9, 4.8, 3.0	68.8	3.81	ddd; 12.5, 4.1, 2.9	68.5
3	3.95	68.6	3.88	q; ~3.0	70.1	3.98	q; ~3.0	68.6	4.04	q; ~2.9,	69.1
4β	1.76	33.0	2.03	ddd; 14.4, 4.0, 3.0	35.6	1.78	ddd; 14.0, 4.5, 3.7	32.1	2.13	ddd; 14.5, 4.4, 2.9	34.0
α	1.72		1.37			1.64			2.24		
5	2.38	51.9	2.65	dd; 12.7, 4.0	36.2	2.46	dd; 13.5, 4.5	49.6	2.42	dd; 13.6, 4.4	56.1
6	–	206.5	–		141.6	–		199.5	–		216.1
7	5.81	122.2	7.19	s	146.1	–		142.8	3.53	s	59.2
8	–	168.1	–		126.4	–		145.0	–		124.6
9	3.15	35.2	2.55		36.9	3.27	dd, 11.7, 7.2	45.8	2.99		33.6
10	–	39.4	–		37.1	–		40.1	–		41.3
11β	1.72	21.6	1.63		20.4	1.66		24.4	1.58		21.5
α	1.80		1.74			1.90			1.86		
12β	1.88	32.6	2.28		38.6	1.59		36.1	1.93		37.0
α	2.14		1.55			2.17	td; 11.5, 5.5		1.58		
13	–	48.8	–		46.7	–		50.2	–		44.0
14	–	85.3	–		161.7	–		175.5	–		153.1
15β	1.97	31.9	2.53		26.1	2.53		30.0	2.35		26.6
α	1.59		2.66			2.53			2.35		
16β	1.98	21.6	2.15		22.6	2.10		20.5	1.86		23.1
α	1.69		1.74			1.97			1.92		
17	2.39	50.6	1.69		55.8	3.13		41.9	2.37	dd; 8.0, 4.7	57.9
18	0.89	18.1	1.19	s	21.3	1.34	s	24.5	0.90	s	19.8
19	0.97	24.5	0.68	s	22.9	0.95	s	23.6	0.71	s	25.4
20	–	78.0	–		77.6	–		93.1	–		78.4
21	1.20	21.2	1.25	s	20.9	1.37	s	20.3	1.13	s	21.3
22	3.33	78.5	3.40	dd; 10.8, 1.7	78.2	3.71	dd; 10.0, 1.9	77.6	3.49	dd; 9.2, 2.7	77.6
23	1.66	27.5	1.58		27.3	1.70		26.8	1.63		27.4
	1.29		1.28			1.42			1.63		
24	1.80	42.5	1.80		42.3	1.74		42.0	1.73		41.5
	1.44		1.43			1.33			1.45		
25	–	71.4	–		71.3	–		71.4	–		71.7
26	1.19	29.1	1.17	s	28.8	1.16	s	29.0	1.19	s	29.0
27	1.20	29.8	1.20	s	29.9	1.17	s	29.9	1.21	s	30.1

[a] HC=O (δ(^{1}H): 9.40, δ(^{13}C): 196.0).

2.4. Compound characterization data

*Poststerone (**1**) [15]*

White crystalline powder (13.1 mg, 3.28%); m.p. 238–240 °C; HRESIMS $C_{21}H_{30}O_5Na$, calcd 385.1991, found 385.1996 $[M+Na]^+$; ^{1}H NMR in methanol-d_4: 0.62 (H-18), 0.96 (H-19), 1.44 (H_β-1), 1.67 (H_β-11), 1.70 (H_α-15) 1.74 (H_2-4), 1.80 (H_α-1), 1.82 (H_β-12), 1.88 (H_α-16), 1.89 (H_α-11), 2.00 (H_β-15), 2.16 (H-21), 2.23 (H_β-16), 2.33 (H_α-12), 2.39 (H-5), 3.19 (H-9), 3.33 (H-17), 3.86 (H-2), 3.97 (H-3), 5.82 (H-7); ^{13}C NMR: 17.6 (C-18), 21.7 (C-11), 22.3 (C-16), 24.5(C-19), 31.1 (C-12), 31.6(C-21), 32.2 (C-15), 32.9 (C-4), 35.2 (C-9), 37.4 (C-1), 39.3 (C-10), 48.9 (C-13), 51.9 (C-5), 60.2(C-17), 68.5 (C-3), 68.7 (C-2), 85.1 (C-14), 122.6 (C-7), 166.6 (C-8), 206.3 (C-6), 212.6 (C-20).

*(20R,22R)-2β,3β,20,22,25-hexahydroxy-5β-cholest-6, 8(14)-dien-6-carbaldehyde (**2**)*

Colorless amorphous solid (1.5 mg, 0.38%); HRESIMS: $C_{28}H_{44}O_6Na$, calcd 499.3036, found 499.3040 $[M+Na]^+$; for ^{1}H and ^{13}C NMR, see Table 1.

*14α,15α-epoxy-14, 15-dihydrostachysterone B (**3**) [16]*

Colorless amorphous solid (0.62 mg, 0.16%); HRESIMS: $C_{27}H_{42}O_7Na$, calcd 501.2828, found 501.2831 $[M+Na]^+$; ^{1}H and ^{13}C NMR data are identical with those we published earlier [16].

*Stachysterone B (**4**)*

Colorless amorphous solid (2 mg, 0.5%); HRESIMS $C_{27}H_{42}O_6$ Na, calcd 485.2879, found: 485.2886 $[M+Na]^+$; ^{1}H and ^{13}C NMR data are identical with those we published earlier [16].

*Compound **5***

Colorless amorphous solid (1.5 mg, 0.38%); APCI-MS: 477 ($M+H^+$), 459 ($M+H^+-H_2O$), 441 ($M+H^+-2H_2O$); for ^{1}H and ^{13}C NMR, see Table 1.

*14α-hydroperoxy-20-hydroxyecdysone (**6**) [11]*

Colorless amorphous solid (5.2 mg, 1.3%); HRESIMS $C_{27}H_{44}O_8Na$, calcd 519.2934, found 519.2931 $[M+Na]^+$; ^{1}H and ^{13}C NMR data are identical with those published earlier [11].

*(20R,22R)-2β,3β,7α,20,22,25-hexahydroxy-5β-cholest-8,14-en-6-one (**7**)*

Colorless amorphous solid (3.7 mg, 0.93%); HRESIMS $C_{27}H_{44}O_7Na$, calcd 503.2985, found 503.2970; for ^{1}H and ^{13}C NMR, see Table 1.

3. Results and discussion

As previously reported [13], laser irradiation of 20E resulted in a complex mixture of photoproducts within 15 min at 2 mg/mL and 6.5 mJ impulse energy, whereas an increase of the concentration to 10 mg/mL led to the need for significantly stronger (15 mJ) and longer (30 min) irradiation, further increase in concentration drastically decreased the efficiency of photo-transformation. In order to obtain a reasonable amount of starting material applicable for isolation and structure elucidation of the products, the scale-up was performed by repeated irradiations. In this manner, a total amount of 400 mg irradiated material could be obtained. However, the continuous effort in optimizing the scale-up process, despite its monitoring by TLC, resulted in a combined irradiated material that still

contained a significant amount of unchanged 20E. This remaining quantity of unchanged 20E was determined by HPLC-DAD (λ = 245 nm) to be 14%.

The irradiated mixture was subjected to purification by rotational planar chromatography (RPC) followed by RP-HPLC (compounds **1**–**6**), or by centrifugal partition chromatography (CPC) and a final purification step by RP-HPLC (compound **7**). Beyond those isolated compounds, a minor product, 14-epi-20-hydroxyecdysone (**8**) was identified by HPLC-DAD. By means of the calibration data for 20E, the amount of this compound in the irradiated mixture was *ca.* 0.3%.

The structures and NMR signals of the isolated products were assigned by comprehensive one- and two-dimensional NMR methods using widely accepted strategies [17,18]. Structures of 20E and compounds **1**–**8** are shown in Fig. 1.

The ^{1}H and ^{13}C NMR spectra of **1** clearly referred to the splitting of the C_{22}–C_{27} side-chain moiety, and the hydrogen atoms of the new $CH_3C{=}O$ group (2.16s) exhibited a strong HMBC cross-peak with the C-17 carbon atom (60.2 ppm) proving the connection of this group to C-17, *i.e.* compound **1** is identical to poststerone [19]. In order to achieve the complete ^{1}H and ^{13}C NMR signal assignment in methanol-d_4, HSQC, HMBC, NOESY and COSY spectra were utilized.

In the case of compound **2**, the molecular formula of $C_{28}H_{44}O_6$ was established by means of HRMS, *i.e.* the molecule consists of one more carbon and one less oxygen atom than the parent 20E. The most characteristic changes in the NMR spectra, as compared to that of 20E, indicated the disappearance of the O═C—CH═C group in the B ring and the presence of a new HC═O group (9.40s/196.0 ppm). To explore the position of the new formyl group, the HMBC and HSQC correlations were utilized. The HMBC cross-peaks of the H_3-19 atoms of the angular methyl group (0.68 ppm) indicated the HC-5 and HC-9 methine carbons, whereas the HSQC correlations gave the corresponding ^{1}H chemical shifts, 36.2/2.65 ppm and 36.9/2.55 ppm, respectively. In addition, the H_3-18/C-14 response (1.19/161.7) proved the splitting of the parent OH group and the presence of a quaternary carbon atom in this position. Lastly, the HMBC responses of the formyl H atom (9.40) to C-5, to C-6 (141.6) and C-7 (146.1) proved that the formyl group is attached to C-6. The H-7 olefinic hydrogen atom (7.19 ppm) correlated with C-5, C-8, C-9 and C-14 carbon atoms in the HMBC spectrum, verifying the presence of a conjugated $\Delta^{6,7;8,14}$-diene-moiety. The H_α-9/H_α-2 and H-19/H_β-5 correlations in the NOESY spectrum of **2** established the *cis* type junction of the A/B rings. We have previously reported an analog structure without the formyl moiety, namely the 2β,3β,20*R*,22*R*,25-pentahydroxy-5β-cholest-6, 8 (14)-dien isolated from the roots of *S. wolffii* [16].

The HRMS measurement of **3** indicated the molecular formula of $C_{27}H_{42}O_7$, *i.e.* the splitting of two hydrogens from the parent 20E. Moreover, the ^{1}H and ^{13}C NMR chemical shifts perfectly agreed with those previously obtained for 14α,15α-epoxy-14, 15-dihydrostachysterone B [16].

The ^{1}H and ^{13}C NMR spectra of compound **4** confirmed that this compound is identical with stachysterone B [16].

The APCI-MS spectrum of compound **5** showed the $[M+H]^+$ peak at *m/z* 477, and, even though HRMS data could not be obtained due to decomposition of compound **5**, the yielded APCI-MS spectrum of this product corresponds to the molecular formula of $C_{27}H_{40}O_7$, *i.e.* a molecule containing four less hydrogen atom than the parental 20E. In agreement with this, twenty-seven ^{13}C signals were discernible in the DEPTQ spectrum indicating the presence of five methyl, eight methylene, six methyne groups furthermore four quaternary sp^3 and two quaternary sp^2 carbon atoms, one conjugated C═O (δ 199.5) and one new O—C═O (δ 175.5) moieties. Integration of the signals in the ^{1}H NMR spectrum obtained in methanol-d_4 summed up to 37 H atoms. Considering the identical δ C-2, C-3 and C-25 chemical shifts of compound **5** and 20E**,** respectively, all of these should have HO– substituents, and thus compound **5** consists of 40 hydrogen atoms. The number of double bond equivalents in compound **5** increased to eight; therefore this

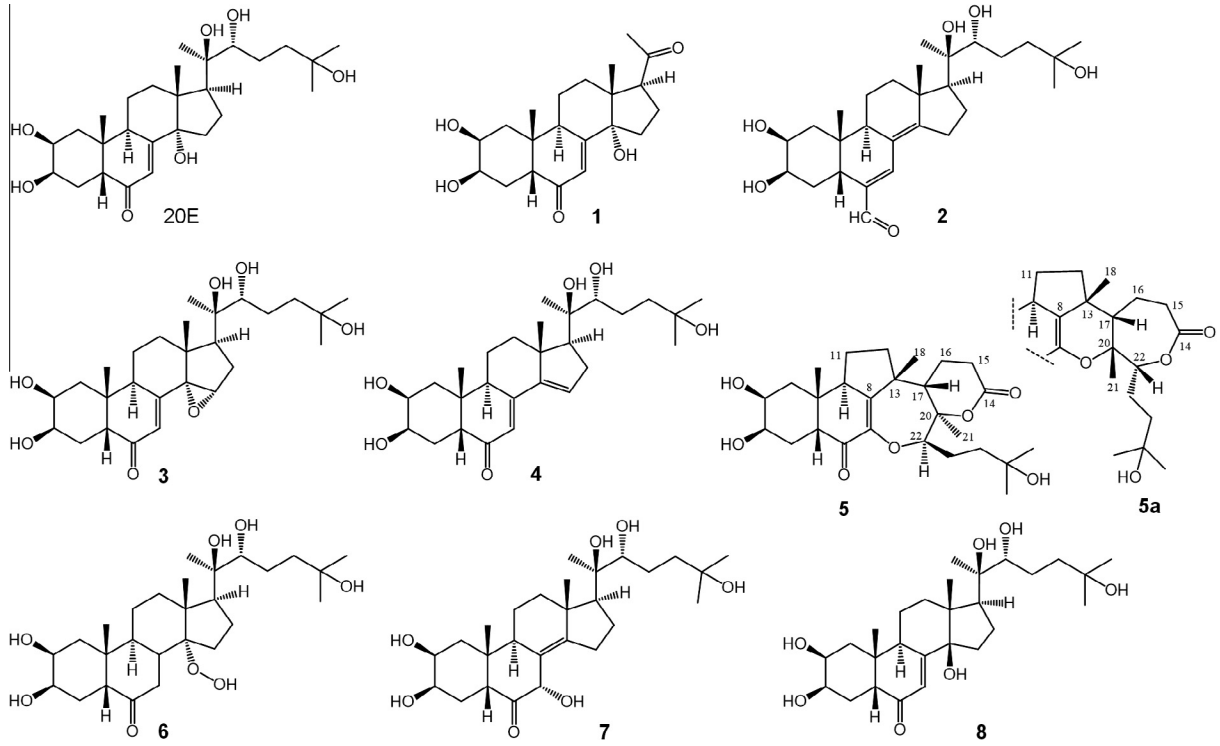

Fig. 1. Structures of 20E and the compounds isolated (**1**–**7**) or detected (**8**) after laser irradiation. **5a** represents an alternative structure for compound **5** which was ruled out by means of 2D and a set of selective 1D NOESY experiments.

compound contains one more ring than the parent 20E. To facilitate the understanding of the different rearrangements of 20E during its photo-irradiation resulting structure **5**, we applied the specific atomic numbering of the parental compound to compound **5** (see Fig. 1). For the ^{1}H and ^{13}C signal assignment, we first identified the five singlets in the ^{1}H NMR spectrum. The identification of the geminal H_3C-26 (1.16/29.0) and H_3C-27 (1.17/29.9) groups is straightforward owing to their mutual HMBC correlation. The HMBC responses of these methyl groups assigned the quaternary C-25 (71.4) and also the H_2C-24 methylene (42.0). In addition, the 3.71/42.0, 3.71/26.8 and 3.71/20.3 cross-peaks revealed the assignment of HC-22 (3.71/77.6), H_2C-23 (1.71; 1.42/26.8) and the third angular methyl H_3C-21 (1.37/20.3). By utilizing the HMBC correlations of H_3-21, the quaternary C-20 (93.1) and HC-17 (3.13/41.9) were assigned, and the H-17/H_3C-18 cross-peak (3.13/24.5) revealed the assignment of the fourth angular methyl group. The HMBC correlations of H_3-19 0.95/49.6 and 0.95/45.8, respectively, assigned the HC-5 and HC-9 methines. For their unambiguous differentiation selective one-dimensional TOCSY experiments were utilized. Irradiation of the well-separated H-3 signal (3.98q, $J \sim 2.5$ Hz) revealed the 7-membered spin system of the A-ring, whereas excitation of the signal at 3.27 gave the 5-membered spin system of H-9, H_2-11 and H_2-12. A similar experiment, the irradiation of H-17 (3.13dd, *J*: 8.0; 4.7 Hz) assigned the 5-membered spin system of H-17, H_2-16 and H_2-15 hydrogen atoms. To achieve an unequivocal assignment of C-14, the HMBC correlations of the H_2-15 and the H_2-16 hydrogens were utilized. Their responses to the signal at δ 175.5 ppm revealed that the original OH substituted quaternary sp^3 C atom turned into a lactone O=C—O carbon atom.

Despite the absence of the characteristic H-7 olefin signal, the δ 199.5 ppm chemical shift of C-6 strongly indicated the presence of a conjugated $\Delta^{7,8}$-6-one moiety in the B ring. The HMBC correlations of the H-5 hydrogen to the O=C-6 and to the quaternary =C-7 atoms (δ 142.8 ppm), in addition with the H-9/C-7 (3.27/142.8) and H-9/C-8 (3.27/145.0) cross-peaks, completed the signal assignment of the B ring. The C-7 signal exhibits $\Delta\delta$ 20.6 ppm paramagnetic, whereas C-8 $\Delta\delta$ 23.1 ppm diamagnetic shift in comparison with the corresponding values measured for the parental 20E, revealing that the substituent on the =C-7 atom should be oxygen.

Surprisingly, a strong correlation between the H_3-18 methyl hydrogens and the =C-8 carbon atom was also detected, providing evidence of a contraction in the C-ring. In the course of the photochemical transformation of 20E, a similar rearrangement was reported by Canonica et al. [9,10]. They explained the contraction by assuming that the initially produced O-6, C-8 diradical would undergo a homolytic cleavage of the 13–14 bond, and that the recombination of C-13 and C-8 radicals led to the five-member C-ring. In our case (due to the high distances between possible members of the new lactone ring), the homolytic cleavage of the 8–14 bond also had to be taken into account. Following such a cleavage, C-14 can reasonably form two possible lactone rings: either with the 20-OH resulting a six-member lactone, or with 22-OH forming a seven-member lactone ring. *A priori* both structures are feasible; in the case of compound **5**, an H-22/C-7 correlation; and in the case of **5a**, an H-22/C-14 HMBC correlation should be expected. However, probably due to spatial reasons, neither of these correlations could be detected even in the case where the HMBC measurement was optimized to 5 Hz long-range couplings instead of 10 Hz. In the course of building-up the fourth and fifth rings, the configuration around the C-20 and C-22 chirality centers should remain unchanged. Therefore, in structure **5**, both H_3C-20 and H-22 necessarily exist in the α position, whereas in case of **5a** they are in a β position. To choose between the two alternative structures and to elucidate the configuration of the stereogenic centers, and to determine the α or β position of the methylene hydrogen atoms, a two-dimensional and a set of selective one-dimensional NOESY experiments were performed, irradiating H-9, H_3-18, H_3-19 and H_3-21, respectively in the latter cases. The NOESY results were in accordance with structure **5** and ruled out **5a**. The schematic stereo-structure of **5** with the atomic numbering is depicted in Fig. 2A; the arrows indicate the detected characteristic spatial proximities, whereas the structure of Fig. 2B shows the refined stereochemistry obtained by PM3 semi-empirical calculation [20]. For a better visualization of the steric arrangement of compound **5**, the C(23)–C(27) side chain is replaced with R.

The HRMS measurement of **6** yielded molecular formula $C_{27}H_{44}O_8$. Therefore, one oxygen atom is incorporated into the parental 20E. The ^{1}H and ^{13}C NMR chemical shifts corresponded to those previously published by Harmatha et al. for 14α-hydroperoxy-20-hydroxyecdysone [11], a known photoproduct of 20E. The presence of the peroxy group in position 14 of compound **6** is straightforward considering the characteristic ($\delta\Delta \sim 12$ ppm) paramagnetic shift of C-14 to δ 96.6 ppm.

Despite the unchanged molecular formula, $C_{27}H_{44}O_7$ of compound **7** as compared to that of 20E, significant differences could be observed in the ^{13}C and ^{1}H NMR spectra. A comparison of the chemical shifts with those of 20E strongly suggested the shift of the $\Delta^{7,8}$ double bond to the $\Delta^{8,14}$ position. The observed 10 ppm paramagnetic shift of δ C(6)=O to 216.1, together with the absence of the characteristic signals of =CH in position 7, revealed that this carbon atom evolved to sp^3 CH (δ H-7 3.53s; δ C-7 59.2). These ^{1}H and ^{13}C chemical shifts also indicated the presence of one –OH group attached to C-7. Considering the strong NOE contacts of the protons H_3-19–H-7 and also to H-5 (2.42dd) hydrogen atoms detected in the selective one-dimensional NOESY spectrum, the β position of these atoms is straightforward, *i.e.* the 7-OH group is in α position and the *cis* A/B ring-junction remained unchanged. Both of the two quaternary sp^2 carbon signals at δ 124.6 and δ 153.1 exhibited strong responses to the H-9 and H-7 signals in the HMBC spectrum, respectively, and their differentiation provided the H_3-18/C-14 (0.90/153.1) cross-peak. The detailed NMR analysis combining two-dimensional ^{1}H,^{1}H-COSY, -NOESY, -ROESY, edited gs-HSQC, gs-HMBC and one-dimensional sel-ROESY and DEPTQ measurements allowed the complete structural assignment of all hydrogen and carbon signals (see Table 1), and confirmed the suggested structure for compound **7**. It should also be noted, that the 7-deoxy analog of **7** has been reported by Canonica et al. [9], and their NMR chemical shifts correlate well with our data.

Unsurprisingly, our results show fundamental differences as compared to the previous studies on the photo-transformation of 20E due to highly different experimental conditions. First of all, the powerful laser impacted the solution at much higher energy than previously used [9–11], and the wavelength was also different: in our case an optically pure, 266 nm UV–light was used, in contrast with the Pyrex-filtered (>300 nm) UV–light provided by Hg lamps. Moreover, in our case, methanol was used as a solvent, which is a better solvent for 20E than water and allows to reach higher concentrations of starting material (and products). Last but not least, our experimental set-up made it a challenge to remove oxygen from the system and to provide an inert atmosphere, therefore our conditions must have been more oxidative than those of the previous studies.

Compound **1** was formed by an oxidative side chain cleavage between C-20 and C-22, which, due to the large distance from the B-ring chromophore, should be the result of rather inter- than intra-molecular processes. At the employed wavelength and energy, direct photolysis of methanol (which could result in a large amount of strong oxidizing species) is unlikely [21]. Oxygen or other radicals (*i.e.* OH radical originated from C-14, see below) provided by neighboring 20E molecules can however give a reasonable explanation for the formation of **1**. In the case of all other

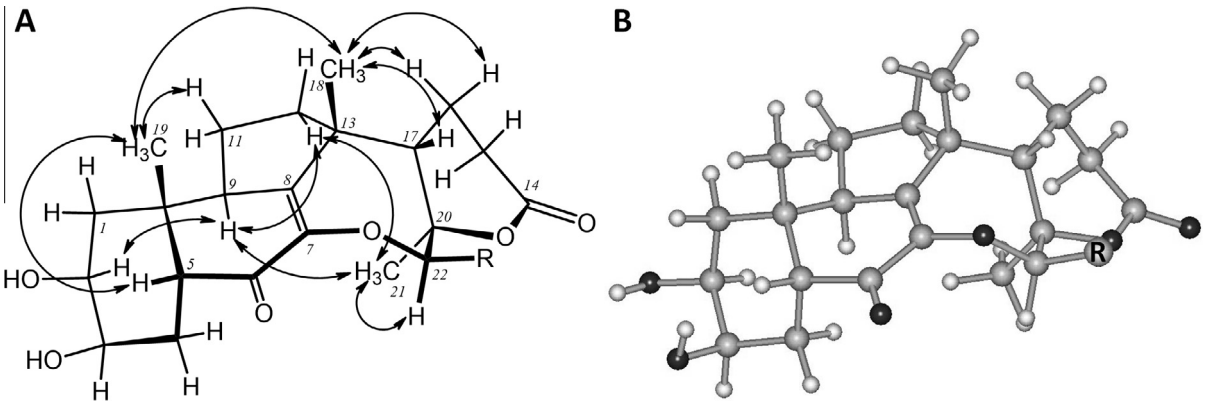

Fig. 2. Stereo-structure and characteristic NOE steric proximities (see arrows) of compound **5** (A) and its refined structure by means of PM3 semi-empirical calculations (B). The C(23)–C(27) side chain is replaced with *R* in both structures.

compounds, rearrangements of the O-6, 8-C bi-radical can be recognized. Compound **2** is particularly interesting in this regard due to the formation of a carbon–carbon bond with methanol, possibly following the homolytic cleavage of the 14-OH. The leaving OH radical can directly couple to the same 20E molecule's remaining radical delocalized between the 6-carbonyl and C-14 [9], leading to the formation of **7** or **8**, oxidize another 20E molecule (to form compound **1**, for example), or react with the solvent methanol. Oxidation of methanol by OH radicals has previously been studied under atmospheric conditions [22], and the primary product CH_2OH radical seems to be the most likely species to couple to C-6 forming compound **2** with the elimination of a water molecule.

Compound **5** represents a novel photo-rearrangement of ecdysteroids. Although closely related 13(14 → 8)-abeo-steroids were previously obtained upon UV irradiation of 20E [9], the conserved $\Delta^{7,8}$ double bond of our product suggests a different chemical route than that found before [9,10]. As a most likely intermediate, formation of an aldehyde is suggested at C-14, following the homolytic cleavage of the 8–14 bond. The oxidative environment can serve as a reasonable explanation for the subsequent lactone ring formation, while the tetrahydro-oxepine ring closure would possibly require a secondary photoreaction.

The 14-hydroperoxi derivative **6** can be formed by the previously suggested 14α-oxyl radical of 20E [9] and an OH radical; this mechanism is also supported by the fact that low yields of this compound were also found to appear in both previous studies, despite the removal of the residual oxygen from their systems [9,11]. Suggested mechanism for the formation of the three newly reported compounds, **2**, **5** and **7**, is presented in Fig. 3.

Based on our observations on the chromatographic fingerprints, there seems to be low chance for the presence of further major products that would have been neglected during the systematic isolation procedure. On the other hand, the low isolated yields can only partially be explained with chromatographic overlapping: a very large number of minor compounds were observed, certainly accounting for a significant fraction of the total amount of the irradiated mixture.

4. Summary

The applied 266 nm laser irradiation of 20-hydroxyecdysone provided a rather complex mixture of various photo-derived products within a short period of time, clearly showing how powerful

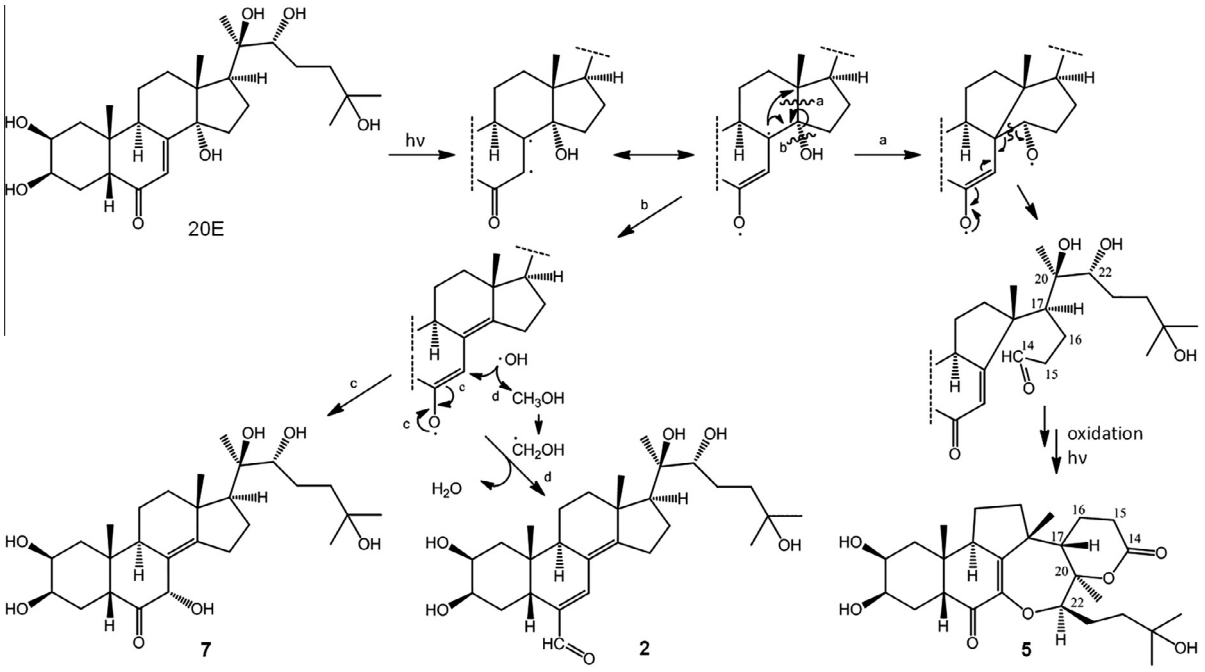

Fig. 3. Suggested mechanism for the formation of compounds **2**, **5** and **7**.

such a laser can be in the transformation of ecdysteroids. Unique, new steroid derivatives (compounds **2** and **7**) were obtained. In the case of compound **2**, a novel, unexpected carbon–carbon coupling reaction took place between the ecdysteroid radical and methanol. Performing this reaction with conventional chemical methods is very difficult if not impossible. A very interesting, new, supposedly secondary photo-product was formed by a ring rearrangement followed by the formation of a lactone and a tetrahydro-oxepine ring (compound **5**). Other isolated or detected compounds (**1**, **3**,**4**, **6** and **8**) confirmed that the same products found previously with Pyrex-filtered Hg-lamps can also be obtained in much shorter time by using a laser. Based on the bioactivity of the parental 20E, all obtained derivatives are of interest for testing by various bioassays.

On the other hand, the complexity of the mixture made the isolation a challenging task with low yields of products, and it appears that a different experimental set-up would be needed for a more convenient and more selective photo-transformation of 20E and related compounds. The presence of oxygen (which could not be excluded with the actual set-up) might have played a significant role in the formation of some products. Dimensions of the cuvette might have decreased some benefits coming from the homogenous UV light: for example, despite the continuous stirring, 20E molecules at the irradiated side had higher chances for undergoing multi-photon processes than those of the opposite side. In addition to these, a longer irradiation time could probably lead to several secondary, tertiary, etc. photoproducts.

All the above outlined difficulties could readily be overcome by using a small flow-cuvette. Experimental conditions (irradiation energy, flow rate and concentration) for such a set-up could be optimized by coupling a photodiode array detector and/or a tandem mass spectrometer to the system for on-line monitoring the products, which would provide much higher flexibility and allow a simple, time-dependent scale-up; development of such a system is among our future objectives.

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Appendix A. Supplementary data

Key one- and two-dimensional NMR spectra and HPLC-UV chromatograms for compounds **2**, **5** and **7** are available as supporting information. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.steroids.2014.07.016.

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