MINOR ECDYSTEROID COMPONENTS OF *Leuzea carthamoides*+

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Dedicated to the memory of Dr Václav Černý.

Fourteen minor ecdysteroid components were isolated and identified from the roots of *Leuzea carthamoides* (Willd.) DC. Two of them are new phytoecdysteroids: leuzeasterone (**1**) a six-member side-chain lactone, and (24*Z*)-29-hydroxy-24(28)-dehydromakisterone C (**4**) a structurally related sitostane type analogue and assumed biogenetic precursor of **1**. The next one, 5α-20-hydroxyecdysone (**6**), is a rare A/B-ring trans-annelated epimer of the most common phytoecdysteroid 20-hydroxyecdysone. Further compounds: makisterone C (**3**), 3-epi-20-hydroxyecdysone (**5**), integristerone A (**7**), integristerone B (**8**), 22-oxo-20-hydroxyecdysone (**10**), taxisterone (**11**), rubrosterone (**12**), dihydrorubrosterone (**13**) and poststerone (**14**), are new constituents of *L. carthamoides*, though already reported as compounds isolated from other natural sources. Two earlier reported minor *Leuzea* ecdysteroids: the five-membered side-chain lactone carthamosterone (**2**) and the 11-hydroxy-substituted analogue isovitexirone (**9**), are also included because they are now better characterised. Certain previously described *Leuzea* ecdysteroids were not found in our material, which may indicate geographic, seasonal or cultivar variations.

Keywords: Steroids; Ecdysteroids; Phytoecdysteroids; Lactones; 5α-20-Hydroxyecdysone; *Leuzea carthamoides*; NMR spectroscopy; Isolation; Identification.

The abundant occurrence of ecdysteroids in *Leuzea carthamoides* DC. (syn. *Rhaponticum carthamoides* (Willd.) Iljin) is interesting from several viewpoints. First is the large structure variability of all so far isolated ecdysone analogues[2–8] and second is their high content in the roots or seeds of this plant[9]. *L. carthamoides* is endemic in Siberia, but is also cultivated as a medicinal plant on a large scale in Europe. This is why *L. carthamoides* can serve as a rich source of ecdysteroids, insect moulting hormone analogues,

+ Part 59 in the series Plant Substances; Part 58 see ref.[1]

for many chemical and biological studies. Roots of this plant have been used in our laboratory as a convenient source of basic phytoecdysteroids[4], *e.g.* 20-hydroxyecdysone, polypodine B, ajugasterone C, makisterone A and some of their mono- or diacetonides. Some of them were utilised mainly for chemical transformations[10–12], phototransformations[13,14] and bioassays reflecting the affinity of ecdysteroids to the ligand-binding site of the insect ecdysteroid receptor[14–16].

Biological activities of phytoecdysteroids on the differentiation of human kerotinocytes reported some time ago[17] led to a patented design of their use in cosmetics and dermatology[18]. Further necessary experiments associated with this use required scaling up the production of 20-hydroxyecdysone and/or *Leuzea* ecdysteroid mixtures with fixed qualitative and quantitative compositions to kilogram amounts. The large-scale separations displayed many ecdysteroid-containing fractions, which have become a disposable source of several already reported major and minor *Leuzea* ecdysteroids[4] in previously unattainable quantities, as well as a rich source of certain new minor ecdysteroid constituents, undetected in the previous low-scale separations.

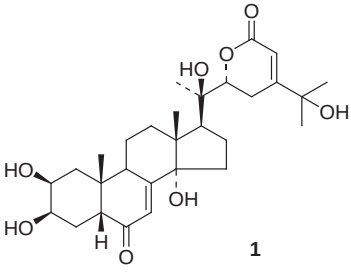
The major compounds, 20-hydroxyecdysone, polypodine B, ajugasterone C, makisterone A and 20-hydroxyecdysone mono- and diacetonides, were identified by comparing of their retention times (at RP- and NP-HPLC using Systems 1 and 2) with authentic samples (Table I) and comparing their ^{1}H NMR data with the data reported earlier[4]. All isolated and identified ecdysteroids, as well as their chemically modified analogues[10–13] were used for bioassays[14–16,19]. The new obtained minor ecdysteroids, presented in this paper, together with selected transformed analogues are scheduled[20] for ecdysteroid receptor mapping based on their interaction with the ligand-binding domain in the B_{II} bioassay[14–16]. Our results, published earlier[15], were included also into other models, using a homology modelling and docking approach[21].

The structures of minor constituents **1**–**14** were elucidated by analysis of their IR, mass, and NMR spectra (for ^{1}H and ^{13}C NMR data, see Tables II–IV). ^{1}H and ^{13}C spectra together with ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HMQC spectra were used for complete (or nearly complete) structure assignment of carbon and proton signals. Characteristic NMR data of compounds **2**, **3**, **5**, **7**–**14** (Tables II–IV) correspond with the published data of carthamosterone[3] (**2**), makisterone C[3,22] (**3**), 3-epi-20-hydroxyecdysone[23,24] (**5**), integristerone A[25,26] (**7**), integristerone B[25] (**8**), isovitexirone[4] (**9**), 22-oxo-20-hydroxyecdysone[27] (**10**), taxisterone[26,28] (**11**), rubrosterone[26,29] (**12**), dihydrorubrosterone[26,30] (**13**) and poststerone[26,31] (**14**), summarised in the Ecdysone

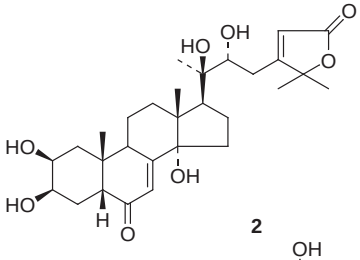
TABLE I
HPLC retention times of minor ecdysteroid compounds **1**–**14** from *L. carthamoides* under various analytical conditions compared with major ecdysteroid components

Compound	Retention time, min		
	system 1[a]	system 2[b]	system 3[c]
Leuzeasterone (**1**)	35.6	66.0	
Carthamosterone (**2**)	34.0	61.4	54.3
Makisterone C (**3**)	34.2	32.2	23.8
(24*Z*)-29-Hydroxy-24(28)-dehydromakisterone C (**4**)	33.6	74.6	129.4
3-Epi-20-hydroxyecdysone (**5**)	36.2	44.7	95.2
5-α-20-Hydroxyecdysone (**6**)	32.5	58.2	55.7
Integristerone A (**7**)	31.0	78.5	
Integristerone B (**8**)	28.8		
Isovitexirone (**9**)	35.4	36.5	39.0
22-Oxo-20-hydroxyecdysone (**10**)	39.9	37.5	
Taxisterone (**11**)	42.7	40.9	56.4
Rubrosterone (**12**)	27.9	30.7	23.2
Dihydrorubrosterone (**13**)	23.7	44.9	33.9
Poststerone (**14**)	35.4	30.9	22.1
20-Hydroxyecdysone (20E)[d]	34.2	50.7	75.4
Polypodine B[d]	33.5	47.9	62.0
Ajugasterone C[d]	39.2	32.3	33.2
Makisterone A[d]	37.7	44.9	52.7
20-Hydroxyecdysone-2,3-monoacetonide[d]	48.6	16.7	
20-Hydroxyecdysone-20,22-monoacetonide[d]	55.6	20.9	
20-Hydroxyecdysone-2,3;20,22-diacetonide[d]	69.9	7.7	

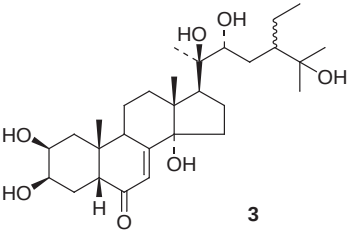
[a] *System 1*: Separon SGX C-18 column (5 μm, 250 mm × 4 mm i.d.) eluted with linear gradient of 10–70% methanol in water over 50 min at flow rate 0.6 ml/min. [b] *System 2*: Silasorb 600 column (5 μm, 250 mm × 4 mm i.d.) eluted with hexane–ethanol–water (812 : 180 : 8) at flow rate 0.8 ml/min. [c] *System 3*: Silasorb 600 column (5 μm, 250 mm × 4 mm i.d.) eluted with diethyl ether–acetonitrile–water (880 : 102 : 18) at 0.8 ml/min. [d] Major ecdysteroids from *L. carthamoides*[4] used as authentic standards for HPLC analyses.



1



2



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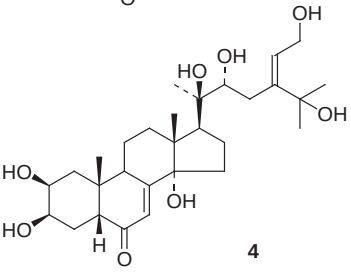

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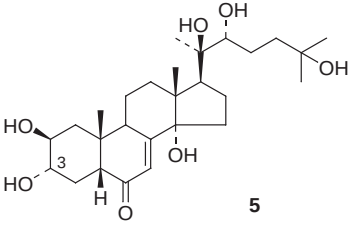

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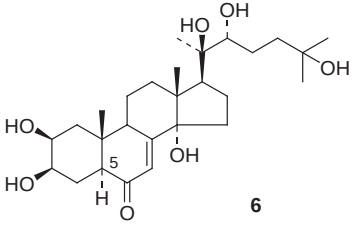
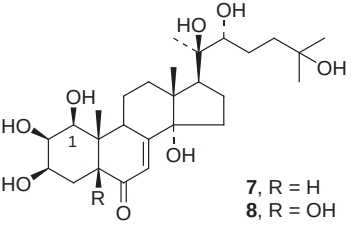
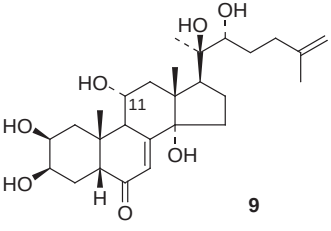


6



7, R = H
8, R = OH



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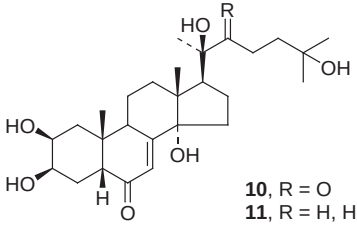
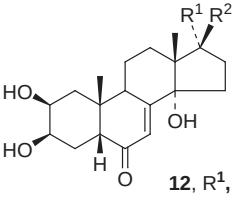


10, R = O
11, R = H, H



12, R^1, R^2 = O
13, R^1 = H, R^2 = OH
14, R^1 = H, R^2 = $COCH_3$

TABLE II
^{1}H NMR chemical shifts of ecdysteroids **1–14** in CD_3OD

Proton	**1**	**2**	**3**	**4**[a]	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1α	~1.79	1.80 dd	1.79 dd	1.80 dd	1.09 dd	1.54 dd	3.82	3.90 bd	2.58 dd	[b]	1.79 dd	1.80 dd	1.80 dd	1.80 dd
1β	1.44	1.44 dd	1.43 dd	1.43 dd	2.10 dd	2.09 dd	–	–	1.36 dd	[b]	1.42 dd	1.45 dd	1.44 dd	1.44 dd
2	3.84 ddd	3.83 ddd	3.84 ddd	3.84 ddd	3.64 ddd	3.96 dq	3.87	4.00 t	4.00 ddd	3.84 ddd	3.84 ddd	3.83 ddd	3.83 ddd	3.84 ddd
3	3.95 bq	3.95 bq	3.95 q	3.95 bq	3.35 ddd	3.58 ddd	4.04	4.12 bq	3.94 bq	3.95 bq	3.95 q	3.96 bq	3.95 bq	3.96 bq
4α	~1.73	~1.73	1.74	~1.73	1.57	1.90 m	1.80	2.14 dd	1.77	[b]	1.75	~1.74	~1.73	1.75
4β	~1.70	~1.70	1.70	~1.73	1.75	1.75 q	1.75	1.90 dd	1.68	[b]	1.70	~1.71	~1.68	1.70
5	2.39 dd	2.39 dd	2.38 dd	2.39 dd	2.40 dd	2.38 dd	2.61 dd	–	2.32 dd	2.39 dd	2.38 dd	2.43 dd	2.39 dd	2.39 dd
7	5.82 d	5.82 d	5.81 d	5.81 d	5.82 d	5.84 d	5.80 d	5.88 d	5.79 d	5.80 d	5.81 d	5.91 d	5.78 d	5.82 d
9	3.17	3.15 ddd	3.16	3.16 ddd	3.18 ddd	2.72 ddd	3.08 bt	3.08 ddd	3.14 dd	3.17	3.15 ddd	3.18 ddd	3.15 ddd	3.19 ddd
11α	~1.82	1.83	1.80	~1.82	1.82	~1.77	~1.79	~1.82	–	[b]	1.78	1.91	1.85	1.88
11β	~1.71	1.72	1.71	~1.70	1.70	~1.66	~1.70	~1.75	4.09 ddd	[b]	1.68	1.66	1.66	1.68
12α	2.21	2.15	2.13 dt	2.15 dt	2.14	2.11 dt	2.11	2.10	2.20 dd	2.24 dt	2.12 dt	2.13	2.06	2.33 dt
12β	~1.83	1.90	1.88	1.88	1.88	1.84 dd	1.87	1.86	2.14 dd	[b]	1.84	1.58	1.60	1.82
15α	~2.00	1.99	1.96	1.96	1.96	1.95	~2.00	~1.98	1.95	[b]	1.95	2.03	2.08	2.00
15β	~1.64	1.62	1.61	1.61	1.61	1.58	~1.60	~1.59	1.56	[b]	1.61	2.30	1.61	1.68
16α	~1.77	1.74	1.76	1.83	1.74	1.71	~1.73	~1.74	1.70	[b]	1.71	2.37	2.27	2.25
16β	~2.01	2.06	2.03	2.02	1.99	1.97	~1.97	~1.98	1.95	[b]	1.92	2.51	1.58	1.88
17	2.49 dd	2.36 dd	2.41 dd	2.42 dd	2.40	2.37 m	2.39 dd	2.38 dd	2.40	2.68 t	2.34 dd	–	4.31 dd	3.33 dd
18	0.90 s	0.91 s	0.90 s	0.90 s	0.89 s	0.88 s	0.90 s	0.91 s	0.86 s	0.87 s	0.86 s	0.88 s	0.71 s	0.62 s

TABLE II
(*Continued*)

Proton	**1**	**2**	**3**	**4**[a]	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
19	0.97 s	0.97 s	0.97 s	0.97 s	0.96 s	1.02 s	0.91 s	1.14 s	1.05 s	0.97 s	0.96 s	0.99 s	0.98 s	0.96 s
21	1.35 s	1.27 s	1.20 s	1.22 s	1.21 s	1.19 s	1.19 s	1.19 s	1.20 s	1.40 s	1.28 s	–	–	–
22	4.23 dd	3.71 dd	3.42 dd	3.55 dd	3.33 dd	3.34 dd	3.32 dd	3.32 dd	3.35 dd	–	1.38–1.52	–	–	–
23a	2.62 dd	2.58 ddd	1.55	2.31	1.66	1.66	1.66	1.65	1.68	[b]	1.38–1.52	–	–	–
23b	2.40 ddd	2.28 ddd	1.40	2.16	1.29	1.28	~1.29	1.27	1.32	[b]	1.38–1.52	–	–	–
24a	–	–	1.15	–	1.80	1.78	~1.78	1.80	2.27	[b]	1.38–1.52	–	–	–
24b	–	–	–	–	1.43	1.43	1.43	1.43	2.06	[b]	1.38–1.52	–	–	–
26	1.40 s	1.49 s	1.22 s	1.42 s	1.20 s	1.20 s	1.20 s	1.20 s	4.72 um 4.58 um	1.20 s	1.19 s	–	–	–
27	1.38 s	1.49 s	1.11 s	1.36 s	1.19 s	1.19 s	1.19 s	1.19 s	1.74 bs	1.20 s	1.19 s	–	–	–
28	6.02 d	5.93 t	1.58 1.48	5.43 bt	–	–	–	–	–	–	–	–	–	–
29	–	–	1.02 t	4.31 dd 4.28 dd	–	–	–	–	–	–	–	–	–	–

[a] At 50 °C. [b] Position of these signals was not determined.

TABLE III
Proton coupling constants of ecdysteroids **1**–**14** in CD_3OD

Coupled protons	**1**[a]	**2**[b]	**3**[c]	**4**[d,j]	**5**	**6**	**7**	**8**	**9**[e]	**10**	**11**	**12**[f]	**13**[g]	**14**[h]
1α,1β	13.3	13.3	~13.0	13.2	13.0	14.4	–	–	13.0	[i]	13.3	13.4	13.6	13.5
1α,2	~4.5	4.2	4.5	4.0	4.4	3.3	~3.1	~3.5	4.2	4.5	4.4	4.5	4.4	4.5
1β,2	12.2	12.0	12.2	12.0	11.6	3.2	–	–	11.7	11.5	12.0	12.3	12.4	12.2
2,3	~3.0	3.3	~3.0	~3.0	8.8	4.6	~3.1	~4.0	3.2	~3.0	3.2	3.2	3.3	3.0
3,4α	~3.0	~3.0	~3.0	~3.0	11.2	3.2	4.8	~3.4	~3.0	~3.0	~3.0	~3.0	~3.0	~3.0
3,4β	~3.0	~3.0	~3.0	~3.0	4.7	~12.0	3.5	~2.6	~3.0	~3.0	~3.0	~3.0	~3.0	~3.0
4α,4β	[i]	[i]	[i]	[i]	13.0	13.2	13.4	15.0	~13.5	[i]	[i]	[i]	[i]	[i]
4α,5	13.0	12.7	12.7	12.3	13.0	4.5	12.0	–	~13.2	11.2	12.6	12.0	12.6	12.6
4β,5	4.2	4.6	4.6	4.8	4.5	~12.0	4.6	–	4.0	6.0	4.6	5.5	4.8	4.8
7,9	2.6	2.7	2.6	2.6	2.6	2.7	2.6	2.5	2.5	2.5	2.6	2.7	2.5	2.6
9,11α	~11.0	11.0	11.4	11.5	11.9	~11.0	[i]	~11.0	–	[i]	11.3	11.8	11.3	11.6
9,11β	~7.0	7.2	~7.0	7.4	6.8	7.4	[i]	~7.0	8.8	[i]	7.2	7.0	7.2	7.2
22,23a	13.1	10.6	11.0	10.4	~11.0	~11.0	11.0	~10.0	10.7	–	[i]	–	–	–
22,23b	3.2	1.6	1.8	2.3	~2.0	1.7	1.8	1.6	1.2	–	[i]	–	–	–
23a,23b	17.8	17.2	[i]	13.7	[i]	[i]	[i]	~14.0	[i]	–	[i]	–	–	–

Additional coupling constants: [a] J(23a,28) = 2.6; [b] J(23a,28) = J(23b,28) = 1.6; [c] J(28,29) = 7.4; [d] J(23a,28)= J(23b,28) ~ 0.8, J(28,29a) = J(28,29b) = 6.2, J(29a,29b) = 14.0; [e] J(11,12α) = 10.5, J(11,12β) = 6.2, J(12α,12β) = 12.2; [f] J(11α,11β) = 13.5, J(11α,12α) = 5.0, J(11α,12β) = 2.1, J(11β,12α) = 13.4, J(11β,12β) = 5.0, J(12α,12β) = 13.0, J(15α,15β) = 12.4, J(15α,16α) = 8.5, J(15α,16β) = 2.0, J(15β,16α) = 9.1, J(15β,16β) = 9.7, J(16α,16β) = 18.7; [g] J(16α,16β) = 14.0, J(17,16α) = 9.3, J(17,16β) = 6.5; [h] J(17,16α) = 9.6, J(17,16β) = 8.0. [i] The J-value was not determined. [j] At 50 °C.

TABLE IV

^{13}C NMR data of ecdysteroids **1–14** in CD_3OD

Carbon	1	2	3	4[e]	5	6	7	8	9	10	11	12	13	14
1	37.35	37.33	37.37	37.54	43.03	43.86	76.43	76.06	39.89	37.37	37.37	37.35	37.40	37.37
2	68.70	38.70	68.71	68.78	72.11	70.30	70.98	69.05	69.21	68.70	68.72	68.65	68.68	68.70
3	68.51	68.44	68.53	68.60	75.36	72.68	68.50	70.01	68.55	68.51	68.53	68.44	68.48	68.48
4	32.82	32.82	32.87	32.84	33.65	24.71	43.80	37.57	33.28	32.74	32.86	32.88	32.87	32.86
5	51.78	51.75	51.80	51.85	57.47	55.29	46.79	80.36	52.77	51.80	51.80	51.99	51.88	51.80
6	206.40	206.35	206.50	206.40	204.65	203.00	205.57	202.33	206.66	206.36	206.51	205.92	206.48	206.26
7	122.34	122.29	122.12	122.20	121.99	123.57	122.17	120.37	122.72	122.19	122.10	122.44	121.59	122.52
8	168.22	167.56	168.04	167.83	168.12	166.23	167.19	166.63	165.71	167.58	168.14	164.69	166.90	166.53
9	35.05	35.06	35.12	35.24	35.97	48.26	35.65	39.71	42.92	35.11	35.05	35.80	35.46	35.15
10	39.30	39.26	39.27	39.31	39.61	39.23	[c]	47.81	39.06	39.28	39.26	39.34	39.35	39.24
11	21.49[a]	21.52	21.54[a]	21.61	21.50	21.41	21.91	22.63	69.50	21.51[a]	21.51	20.69	21.34	21.60
12	32.42	32.46	32.51	32.56	32.48	32.42	32.54	32.61	43.76	32.48	32.38	24.97	31.48	32.09
13	~49.0[d]	~49.0[d]	~49.0[d]	~49.0[d]	~49.0[d]	~49.0[d]	~49.0[d]	48.46	~49.0[d]	~49.0[d]	48.07	54.09	48.27	~49.0[d]
14	85.30	85.28	85.20	85.33	85.10	85.02	85.10	84.97	84.40	85.14	85.54	80.47	83.37	85.00
15	31.78	31.83	31.80	31.81	31.74	31.86	31.80	31.79	31.81	31.81	31.58	29.12	29.05[a]	31.07
16	21.54[a]	21.63	21.62[a]	21.61	21.50	21.67	21.40	21.40	21.46	21.69[a]	21.96	34.00	29.74[a]	22.18
17	50.52	50.43	50.43	50.57	50.55	50.59	50.58	50.48	50.26	51.64	53.36	220.22	79.24	60.16
18	18.20	18.02	18.06	18.01	18.03	17.98	18.02	18.02	18.86	17.91	18.12	17.60	15.84	17.50
19	24.38	24.41	24.40	24.38	23.88	15.72	20.01	13.95	24.61	24.39	24.39	24.58	24.45	24.40
20	76.46	77.41	78.04	77.86	77.93	77.94	77.89	77.87	77.65	82.02	75.99			212.49
21	21.42	21.08	20.96	21.00	21.04	21.02	21.05	21.02	20.97	25.37	26.46			31.51
22	84.90	75.49	77.22	78.20	78.44	78.44	78.43	78.42	77.05	217.29	45.89[a]			
23	26.45	30.65	33.06	38.01	27.35	27.34	27.36	27.34	30.84	32.86	20.10			
24	169.78[b]	174.91	50.30	147.18	42.40	42.41	42.38	42.37	36.21	38.14	45.50[a]			
25	72.65	89.74	74.14	73.94	71.31	71.31	71.29	71.31	146.87	70.68	71.48			
26	28.38	25.02	29.09	30.24	29.69	29.67	29.70	29.72	110.75	29.21	29.33			
27	27.77	24.87	25.97	30.94	28.96	28.96	28.97	28.94	22.70	29.21	29.10			
28	113.16	115.46	25.61	128.78										
29	167.50[b]	178.79	14.35	60.46										

[a,b] Signals with same symbols may be interchanged. [c] Signal was not detected. [d] Signal overlapped by solvent. [e] At 50 °C.

Handbook[32]. Compounds **5**, **10**–**14** are new in *L. carthamoides*, although their occurrence was already reported in other unrelated plants or animals (see ref.[32]). Compound **3** was only recently reported as a plant constituent[24]. Integristerones A and B (**7**, **8**) were found in the related *Rhaponticum integrifolium*[25]. NMR data of those compounds were in earlier reports mostly incomplete, therefore we publish here their currently completed data (Tables II–IV). There were completed also the NMR data of carthamosterone (**2**), makisterone C (**3**) and isovitexirone (**9**), previously isolated from *L. carthamoides*[3,4], but only now obtained in amounts sufficient for their full characterisation (Tables II–IV).

Two new ecdysteroid analogues **1** and **4** were found among the so far isolated minor constituents. Compound **1** (leuzeasterone), with the composition $C_{29}H_{42}O_8$ (HR-MS), exhibited in addition to the typical 7-ene-6-one IR absorption band (1 653 cm^{-1}) also a lactone carbonyl band (1 705 cm^{-1}). The ^{13}C NMR spectrum of compound **1** confirmed 29 carbon atoms in the molecule. Chemical shifts of all carbons of the steroid skeleton fit very well the data of 20-hydroxyecdysone[4] indicating structure modification in the side chain only. Molecular formula $C_{29}H_{42}O_8$ corresponds to nine unsaturations, three of them located in the side chain. Both ^1H and ^{13}C NMR spectra confirmed the presence of three methyl groups on tertiary carbons (in the side chain), which according to ^1H chemical shifts (δ 1.35, 1.38 and 1.40) have to be of the >C(OR)–Me type (apparently Me groups 21, 26 and 27). The presence of three sp^2 carbons (two quaternary at δ 169.78, 167.50 and one =CH– at δ 113.16) indicates the presence of six-membered unsaturated lactone ring closed in position C(22). This is confirmed in ^1H NMR spectra by observation of the >C(22)H–C(23)H_2– system (δ 4.23 dd, J = 3.2 and 13.1 Hz; 2.62 dd, J = 17.8 and 3.2 Hz; 2.40 ddd, J = 17.8, 13.1 and 2.6 Hz) and of the olefin proton =C(28)H– as doublet at δ 6.02 with allylic coupling J = 2.6 Hz to one of the –C(23)H_2– protons (at δ 2.40). The complete structural assignment of protons and carbons in compound **1** is given in Tables II–IV.

Compound **4** with the composition $C_{29}H_{46}O_8$ (HR-MS) manifests also in the ^{13}C NMR spectrum 29 carbon atoms. Nearly identical chemical shifts of all carbon atoms of steroid skeleton with 20-hydroxyecdysone[4] suggest a structure modification of the side chain. Two additional carbons of the side chain form the =CH–CH_2OH fragment (^1H NMR: =CH– at δ 5.43 t, J = 6.2 Hz; –CH_2OH at δ 4.31 dd, J = 14.0 and 6.2 Hz and δ 4.28 dd, J = 14.0 and 6.2 Hz. ^{13}C NMR: =CH– at δ 128.78 and –CH_2OH at δ 60.46), which has to be located in position 24 in the modified side chain and is confirmed by

allylic couplings (J = 0.8 Hz) between C(23)H_2 protons and the olefinic proton H-28. The *Z*-configuration of the double bond C(24)=C(28) has been determined from a NOESY spectrum of compound **4** on the basis of the observed NOEs between H-28 and H-23a, H-23b, H-22 and between C(29)H_2 and methyl protons H-26, H-27. The complete structure assignment of protons and carbons in compound **4** is given in Tables II–IV. This compound is apparently biogenetically related to makisterone C (**3**). Therefore, we suggest its name (24*Z*)-29-hydroxy-24(28)-dehydromakisterone C (**4**), although it is in fact a 25-hydroxy analogue of an earlier reported *L. carthamoides* seed constituent with a complex name derived from amarasterone B (ref.[6]).

Compound **6** was already reported[33,34], but without complete structure characteristics. This is why the structure analysis of this compound is now presented. Compound **6** with composition $C_{27}H_{44}O_7$ (HR-MS) shows 27 carbon atoms in the ^{13}C NMR spectrum and all structure fragments with very similar chemical shifts to those of 20-hydroxyecdysone[4] for all carbon atoms except those of rings A and B. The significant upfield shift of methyl carbon C-19 (δ 15.72 *vs* 24.38 in 20-hydroxyecdysone), large differences in chemical shifts of other carbon atoms and protons in rings A and B, and a

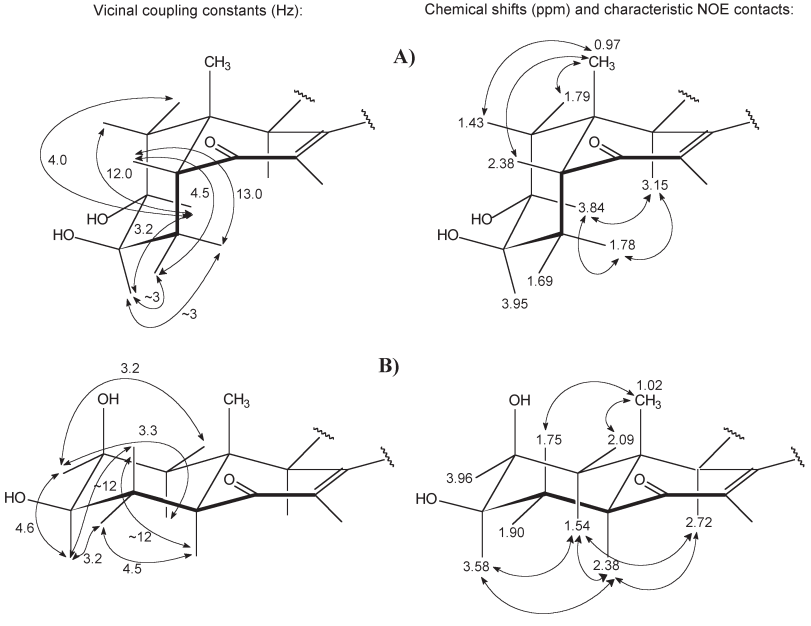


FIG. 1

Vicinal coupling constants, chemical shifts and characteristic NOE contacts of protons in ring A and B (indicated with arrows): in 20-hydroxyecdysone A) and in its 5α-epimer **6** B)

different coupling pattern of protons in ring A, suggested the trans-annelation of rings A and B. This has been confirmed by the observed NOEs of H-5 with H-1α, H-3 and H-9, corresponding to the 5αH configuration, *i.e.* to the structure of 5-epi-20-hydroxyecdysone. Figure 1 compares vicinal coupling constants, chemical shifts and characteristic NOE contacts observed in 20-hydroxyecdysone and its 5α-epimer. The complete structure assignment of protons and carbons in the 5α-20-hydroxyecdysone (**6**) is given in Tables II–IV. The ^{1}H NMR spectrum of compound **6** in pyridine-d_5 showed chemical shifts of methyl protons identical with those described for 5-epiecdysterone by Hikino and Takemoto[33] (including characteristic downfield shift of 19-methyl signal to δ 1.41 in comparison with δ 1.06 in 20-hydroxyecdysone). It should be noticed that this difference is much smaller in methanol-d_4 (δ 0.97 *vs* 1.02).

Certain previously described *Leuzea* ecdysteroids were not found in our material, which indicates geographic, seasonal or cultivar variations. However, our crude fractions contain still more minor compounds possessing characteristic ecdysteroid properties. Their purification and structure elucidation is in progress.

EXPERIMENTAL

Infrared spectra (wavenumbers in cm^{-1}) were recorded on a Bruker IPS-88 instrument using KBr pellets. Circular dichroism (CD) was recorded on a Jobin Yvon CD6 instrument in methanol. Optical rotations were measured at 20 °C on a Rudolph Research Analytical Autopol IV polarimeter in methanol ($[\alpha]_D^{20}$ values are given in 10^{-1} deg cm^2/g). NMR spectra were measured on a Varian UNITY-500 spectrometer (^{1}H at 500 MHz; ^{13}C at 125.7 MHz) in CD_3OD. Chemical shifts (in ppm, δ-scale) in CD_3OD were referenced to the solvent signal at 3.31 (^{1}H) and 49.00 (^{13}C). Homonuclear 2D-COSY and 2D-ROESY spectra were used for structure assignment of protons. Carbon-13 APT spectra and heterocorrelated 2D-HMQC spectra were combined to assign all carbons. Mass spectra were recorded on a ZAB-EQ spectrometer with fast atom bombardment (FAB) ionisation using a glycerol–thioglycerol mixture as a matrix.

Ecdysteroid concentrates were prepared in co-operation with Mediplant Co. (Modra, Slovakia) and Galena Co. (Opava, Czech Republic) using a pilot plant modification of the extraction and separation methods reported earlier[4]. Roots of *Leuzea carthamoides* (1 000 kg) were purchased from several agricultural producers. Separations were performed by liquid–liquid extractions, large-scale column chromatography and crystallisation processes. Besides the main product, 20-hydroxyecdysone (1 kg, 95% purity), several crude fractions of ecdysteroids were obtained. Qualitative and quantitative compositions of these fractions, monitored by TLC and HPLC methods[10,35] were various. One selected crude fraction (40 g) served us as a source for isolation of the reported minor ecdysteroids. This fraction is characterised by the content of its major ecdysteroids, ajugasterone C (3.6%), makisterone A + polypodine B (3.5%) and 20-hydroxyecdysone (29.2%), as recorded by HPLC (in System 2). Single ecdysteroids were separated and purified by combination of LH-20 gel-CC (A), RP-HPLC (B) and NP-HPLC (C). *System A*: Sephadex LH-20 separation on a column (80 ×

900 mm), linear gradient 20–80% methanol in water during 1 100 min (18 h), flow rate 6 ml/min and UV detection at 244 nm. The crude ecdysteroid fraction (40 g) was separated in four doses (10 g each) supplying 29 fractions: 1A–29A (250 ml each). Analysis of fractions A was performed by RP-HPLC in System 1 (Table I). *System B*: RP-HPLC separation on a column Separon SGX C18 (26 × 600 mm), linear gradient 18–80% methanol in water during 300 min, flow rate 5 ml/min and UV detection at 244 nm. Selected fractions A were separated in this system yielding fractions B collected according to the HPLC records. Analysis of fractions B was performed by NP-HPLC in System 2 (Table I). *System C*: NP-HPLC on a column Silasorb 600, 5 μ (12.5 × 500 mm), isocratic conditions eluted with Systems 2 or 3 (see Table I), but at a flow rate of 3 ml/min, UV detection at 244 nm. Some separations of fractions B and final purification of compounds were performed in this system.

Structure elucidation and identification of isolated compounds was performed by analysis of their ^{1}H and ^{13}C NMR spectra, supported by IR and MS spectral data. ^{1}H and ^{13}C NMR spectral data are summarised in Tables II–IV. Characteristic HPLC data recorded under analytic conditions are given in Table I.

Leuzeasterone (**1**)

Compound **1** (11 mg) was obtained from fraction 12A (1.65 g) separated by RP-HPLC in *System B*. Fraction 13B (278 mg) was further separated in *System C* with a mobile phase of the System 2 (Table I). $[\alpha]_D^{20}$ +51.6 (*c* 0.41, MeOH). CD (MeOH), Δε [nm]: +7.41 [206], –1.00 [237], +1.29 [267], +1.29 [329]. IR (KBr): 3 340, 1 705, 1 653. FAB-MS, *m/z* (rel.%): 519 (19), 501 (79), 485 (15), 465 (7), 347 (9), 331 (11), 329 (11), 303 (75), 276 (22), 185 (100). HR-MS, *m/z*: 519.3046 $[M + H]^+$, for $C_{29}H_{43}O_8$ required 519.2958.

Carthamosterone (**2**)

Compound **2** (54 mg) was obtained from combined fractions 13A (1.21 g), 14A (0.72 g) and 15A (0.62 g), each separated by RP-HPLC in *System B*. Fractions 11B/13A, 10B/14A and 9B/15A (148 mg) containing compound **2** were further separated in *System C* with mobile phase of the System 3 (Table I). IR (KBr): 3 400, 1 730, 1 652. FAB-MS, *m/z* (rel.%): 519 (57), 501 (59), 483 (74), 432 (5), 363 (32), 355 (9), 345 (17), 329 (13), 319 (9), 303 (100), 291 (17), 263 (76), 257 (30), 250 (57). HR-MS, *m/z*: 519.2637 $[M + H]^+$, for $C_{29}H_{43}O_8$ required 519.2958.

Makisterone C (**3**)

Compound **3** (23 mg) was obtained from fractions 19B of 12A and 17B of 13A (105 mg) after repeated chromatography in *System C* using mobile phases of Systems 2 and 3 (Table I). IR (KBr): 3 410, 1 654 . FAB-MS, *m/z* (rel.%): 531 (41) $[M + Na]^+$, 509 (41) $[M + H]^+$, 492 (45), 491 (50), 473 (89), 455 (62), 445 (44), 437 (27), 427 (28), 363 (33), 347 (73), 329 (88), 313 (46), 303 (98), 301 (100). HR-MS, *m/z*: 509.3416 $[M + H]^+$, for $C_{29}H_{49}O_7$ required 509.3478.

(24*Z*)-29-Hydroxy-24(28)-dehydromakisterone C (**4**)

Compound **4** (10 mg) was obtained from fraction 11B of 12A (49 mg) after repeated chromatography in *System C* with the mobile phase of System 2 (Table I). $[\alpha]_D^{20}$ +32.5 (*c* 0.24, MeOH). CD (MeOH), Δε [nm]: +2.90 [216], –3.86 [252], +1.53 [329]. IR (KBr): 3 405, 1 653.

FAB-MS, *m/z* (rel.%): 545 (20) $[M + Na]^+$, 523 (26) $[M + H]^+$, 505 (20), 487 (17), 480 (34), 469 (15), 465 (21), 451 (9), 433 (15), 417 (13), 405 (12), 392 (20), 387 (17), 373 (31), 365 (15), 363 (13), 351 (55), 350 (58), 341 (100), 334 (51), 327 (50), 319 (20). HR-MS, *m/z*: 523.3177 $[M + H]^+$, for $C_{29}H_{47}O_8$ required 523.3271.

3-Epi-20-hydroxyecdysone (**5**)

Compound **5** (14 mg) was obtained from fraction 14B of 12A (87 mg) after repeated chromatography in *System C* with the mobile phase of System 2 (Table I). IR (KBr): 3 410, 1 653. FAB-MS, *m/z* (rel.%): 503 (85) $[M + Na]^+$, 481 (21) $[M + H]^+$, 463 (34), 445 (100), 427 (98), 409 (36). HR-MS, *m/z*: 479.2971 $[M - H]^+$, for $C_{27}H_{43}O_7$ required 479.3009.

5-Epi-20-hydroxyecdysone (**6**)

Compound **6** (4 mg) was obtained from fraction 11B of 12A (49 mg) after repeated chromatography in *System C* with the mobile phase of System 2 (Table I). $[\alpha]_D^{20}$ +56.2 (*c* 0.13, MeOH). CD (MeOH), $\Delta\varepsilon$ [nm]: –11.10 [248], +4.10 [327] (for 20-hydroxyecdysone: –5.25 [252], +2.34 [328]). IR (KBr): 3 422, 1 651. FAB-MS, *m/z* (rel.%): 503 (5) $[M + Na]^+$, 481 (100) $[M + H]^+$, 463 (51), 445 (84), 427 (38), 363 (12), 347 (24), 329 (28), 301 (21), 277 (39), 257 (49), 215 (46). HR-MS, *m/z*: 481.2829 $[M + H]^+$, for $C_{27}H_{45}O_7$ required 481.3165.

Integristerone A (**7**)

Compound **7** (31 mg) was obtained from fraction 8A (430 mg) separated by RP-HPLC in *System B*. Fraction 2B/8A (59 mg) was further separated in *System C* with a mobile phase of the System 2 (Table I). IR (KBr): 3 355, 1 646. FAB-MS, *m/z* (rel.%): 497 (21) $[M + H]^+$, 480 (50), 479 (54), 461 (97), 443 (59), 407 (25), 387 (40), 379 (27), 363 (31), 345 (43), 319 (44), 317 (45), 299 (38), 255 (42), 243 (45), 227 (61), 211 (100). HR-MS, *m/z*: 479.2941 $[M + H - H_2O]^+$, for $C_{27}H_{43}O_7$ required 479.3009.

Integristerone B (**8**)

Compound **8** (125 mg) was obtained from fraction 6A and 7A (1.38 g) directly after rechromatography in *System B*. Characteristic HPLC data recorded under analytic conditions are summarised in Table I. IR (KBr): 3 484, 1 671. FAB-MS, *m/z* (rel.%): 535 (5) $[M + Na]^+$, 513 (11) $[M + H]^+$, 495 (2), 477 (2), 459 (1), 201 (6), 185 (13), 149 (17), 135 (14), 125 (16), 109 (22), 99 (42), 93 (68), 81 (59), 69 (100). HR-MS, *m/z*: 513.3090 $[M + H]^+$, for $C_{27}H_{45}O_9$ required 513.3064.

Isovitexirone (**9**)

Compound **9** (66 mg) was obtained from combined fractions 16B of 12A, 14B of 13A and 12B of 14A (total 233 mg) by repeated HPLC in *System C* using mobile phase of System 3 (Table I). IR (KBr): 3 391, 1 653, 1 581. FAB-MS, *m/z* (rel.%): 501 (56) $[M + Na]^+$, 479 (65) $[M + H]^+$, 461 (100), 443 (22), 278 (56). HR-MS, *m/z*: 479.2993 $[M + H]^+$, for $C_{27}H_{43}O_7$ required 479.3009.

22-Oxo-20-hydroxyecdysone (**10**)

Compound **10** (30 mg) was obtained from combined fractions 18B of 12A and 16B of 13A and 13B of 14A (total 146 mg) by repeated HPLC in *System C* using mobile phase of System 2 (Table I). IR (KBr): 3 402, 1 702, 1 653. CD (MeOH), Δε [nm]: +2.25 [218], –2.63 [257], –0.98 sh [285], +1.01 [328]. FAB-MS, *m/z* (rel.%): 501 (6) $[M + Na]^+$, 479 (32) $[M + H]^+$, 461 (100), 443 (38), 425 (10), 413 (5), 411 (4), 405 (6), 363 (18), 355 (6), 345 (6), 329 (12), 327 (6), 303 (11), 301 (12), 250 (57). HR-MS, *m/z*: 479.2937 $[M + H]^+$, for $C_{27}H_{43}O_7$ required 479.3009.

Taxisterone (**11**)

Compound **11** (23 mg) was obtained from combined fractions 19B of 12A and 17B of 13A (total 105 mg) by repeated HPLC in *System C* using mobile phase of System 2 (Table I). IR (KBr): 3 392, 3 318, 1 639. FAB-MS, *m/z* (rel.%): 487 (2) $[M + Na]^+$, 465 (6) $[M + H]^+$, 447 (11), 429 (12), 411 (10), 397 (3), 371 (3), 355 (5), 303 (10), 301 (12), 263 (6), 249 (10), 239 (8), 227 (9), 213 (11), 181 (16), 165 (19), 145 (21), 128 (27), 109 (48), 91 (65), 69 (100). HR-MS, *m/z*: 465.3202 $[M + H]^+$, for $C_{27}H_{45}O_6$ required 465.3216.

Rubrosterone (**12**)

Compound **12** (12 mg) was obtained from combined fractions 9B of 12A, 8B of 13A and 7B of 14A (total 68 mg) by repeated HPLC in *System C* using mobile phase of System 2 (Table I). IR (KBr): 3 537, 3 036, 1 747, 1 649. FAB-MS, *m/z* (rel.%): 357 (3) $[M + Na]^+$, 335 (18) $[M + H]^+$, 317 (6), 299 (4), 229 (5), 198 (9), 181 (18), 167 (14), 149 (18), 131 (26), 115 (56), 91 (91), 69 (60), 55 (100). HR-MS, *m/z*: 335.1916 $[M + H]^+$, for $C_{19}H_{27}O_5$ required 335.1859.

Dihydrorubrosterone (**13**)

Compound **13** (10 mg) was obtained from combined fractions 6B of 12A and 5B of 14A (total 55 mg) by repeated HPLC in *System C* using mobile phase of System 2 (Table I). IR (KBr): 3 365, 1 646. FAB-MS, *m/z* (rel.%): 337 (11) $[M + H]^+$, 319 (5) $[M + H - H_2O]^+$, 301 (4) $[M + H - 2\ H_2O]^+$. HR-MS, *m/z*: 337.2005 $[M + H]^+$, for $C_{19}H_{29}O_5$ required 337.2015.

Poststerone (**14**)

Compound **14** (11 mg) was obtained from fraction 17B of 12A (210 mg) by repeated HPLC in *System C* using mobile phase of System 2 (Table I). IR (KBr): 3 400, 1 711, 1 644. FAB-MS, *m/z* (rel.%): 363 (100) $[M + H]^+$, 345 (38), 331 (12), 329 (7), 327 (7), 303 (12), 301 (10), 269 (10), 239 (10), 227 (12), 215 (17), 199 (15), 173 (26), 159 (25), 147 (27), 131 (25), 121 (36), 101 (62). HR-MS, *m/z*: 363.2165 $[M + H]^+$, for $C_{21}H_{31}O_5$ required 363.2172.

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REFERENCES

1. Vokáč K., Buděšínský M., Harmatha J., Kohoutová J.: *Phytochemistry* **1998**, *49*, 2109.
2. Baltaev U. A., Abubakirov N. K.: *Khim. Prir. Soedin.* **1987**, 681.
3. Girault J.-P., Lafont R., Varga E., Hajdu Zs., Herke I., Szendrei K.: *Phytochemistry* **1988**, *27*, 737.
4. Píš J., Buděšínský M., Vokáč K., Laudová V., Harmatha J.: *Phytochemistry* **1994**, *37*, 707.
5. a) Baltaev U. A.: *Khim. Prir. Soedin.* **1991**, 806; b) Baltaev U. A.: *Khim. Prir. Soedin.* **1992**, 231; c) Baltaev U. A.: *Phytochemistry* **1995**, *38*, 799.
6. Baltaev U. A., Dinan L., Girault J.-P., Lafont R.: *Phytochemistry* **1997**, *46*, 103.
7. a) Ramazanov N. S., Maksimov E. C., Saatov Z., Mamatkhanov A. U., Abdullaev N. D.: *Khim. Prir. Soedin.* **1997**, 392; b) Ramazanov N. S., Maksimov E. C., Saatov Z., Mamatkhanov A. U., Abdullaev N. D.: *Khim. Prir. Soedin.* **1997**, 395.
8. a) Borovikova E. B., Shangaraeva G. S., Baltaev U. A.: *Chem. Nat. Comp.* **1999**, *35*, 182; b) Borovikova E. B., Shangaraeva G. S., Baltaev U. A.: *Chem. Nat. Comp.* **1999**, *35*, 184.
9. Lafont R., Bouthier A., Wilson I. D. in: *Insect Chemical Ecology* (I. Hrdý, Ed.), p. 197. Academia, Prague and SPB Academic Publishing bv, The Hague 1991.
10. Píš J., Harmatha J.: *J. Chromatogr.* **1992**, *596*, 271.
11. Píš J., Hykl J., Buděšínský M., Harmatha J.: *Collect. Czech. Chem. Commun.* **1992**, *58*, 612.
12. Píš J., Hykl J., Buděšínský M., Harmatha J.: *Tetrahedron* **1994**, *50*, 9679.
13. Harmatha J., Buděšínský M., Vokáč K.: *Steroids* **2002**, *67*, 127.
14. Harmatha J., Dinan L., Lafont R.: *Insect Biochem. Mol. Biol.* **2002**, *32*, 181.
15. Harmatha J., Dinan L.: *Arch. Insect Biochem. Physiol.* **1997**, *35*, 219.
16. Dinan L., Hormann R. E., Fujimoto T.: *J. Comput.-Aided Mol. Des.* **1999**, *13*, 185.
17. Detmar M., Dumas M. Bonté F., Meybeck A., Orfanos C. E.: *Eur. J. Dermatol.* **1994**, *4*, 558.
18. Meybeck A., Bonté F., Redziniak G.: PCT/FR93/00819, 1994, WO 94/04132.
19. Kosař K., Opletal L., Vokáč K., Harmatha J., Sovová M., Čeřovský J., Krátký F., Dvořák J.: *Pharmazie* **1997**, *5*, 406.
20. Harmatha J., Dinan L.: *Ecdysone 2002 – XVth Int. Ecdysone Workshop, Kolymbyri, Crete, Greece, June 30, 2002*; Book of Abstracts.
21. Wurtz J.-M., Guillot B., Fagart J., Moras D., Tietjen K., Schindler M.: *Protein Sci.* **2000**, *9*, 1073.
22. Imai S., Hori M., Fujioka S., Murata E., Goto M., Nakanishi K.: *Tetrahedron Lett.* **1968**, 3883.
23. Thompson M. J., Kaplanis J. N., Robbins W. E., Dutky S. R., Nigg H. N.: *Steroids* **1974**, *24*, 359.
24. Odinokov V. N., Galyautdinov I. V., Nedopekin D. V., Khalilov L. M., Shashkov A. S., Kachala V. V., Dinan L., Lafont R.: *Insect Biochem. Mol. Biol.* **2002**, *32*, 161.
25. a) Baltaev U. A., Gorovits M. B., Abdullaev N. D., Rashkes J. V., Jagudaev M. R., Abubakirov N. K.: *Khim. Prir. Soedin.* **1977**, 813; b) Baltaev U. A., Gorovits M. B., Abdullaev N. D., Rashkes J. V., Jagudaev M. R., Abubakirov N. K.: *Khim. Prir. Soedin.* **1978**, 457.
26. Girault J.-P., Bathori M., Varga E., Szendrei K., Lafont R.: *J. Nat. Prod.* **1990**, *53*, 279.
27. Rudel D., Bathori M., Gharbi J., Girault J.-P., Racz I., Melis K., Szendrei K., Lafont R.: *Planta Med.* **1992**, *58*, 358.
28. Nakano K., Nohara T., Tominatsu T., Nishikawa M.: *Phytochemistry* **1982**, *21*, 2749.

29. Takemoto T., Hikino Y., Hikino H.: *Tetrahedron Lett.* **1968**, 3053.
30. Bathori M., Girault J.-P., Mathe I., Lafont R.: *Biomed. Chromatogr.* **2000**, *14*, 464.
31. Hikino H., Okuyama T., Konno C., Takemoto T.: *Chem. Pharm. Bull.* **1975**, *23*, 125.
32. Lafont R., Wilson I. D. (Eds): *The Ecdysone Handbook*, 2nd ed. The Chromatography Society, Nottingham 1996.
33. Hikino H., Takemoto T.: *Naturwissenschaften* **1972**, *59*, 91.
34. Saatov Z., Gorovits M. B., Abdullaev N. D., Abubakirov N. K.: *Khim. Prir. Soedin.* **1987**, 678.
35. Lafont R., Blais C., Harmatha J., Wilson I. D. in: *Encyclopedia of Separation Science* (I. D. Wilson, E. R. Adlard, M. Cooke and C. F. Poole, Eds), Vol. III, p. 2631. Academic Press, London 2000.