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1α,20*R*-DIHYDROXYECDYSONE FROM *AXYRIS AMARANTHOIDES*

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Key Word Index—*Axyris amaranthoides*; Chenopodiaceae; seeds; 1α,20*R*-dihydroxyecdysone [1-*epi*-integristerone A]; ecdysteroid; phytoecdysteroid.

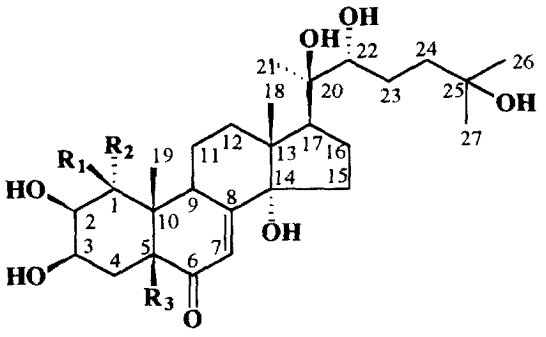
Abstract—Bioassay/RIA-directed phytochemical examination of the seeds of *Axyris amaranthoides* afforded a new ecdysteroid: 1α,20*R*-dihydroxyecdysone [1-*epi*-integristerone A], together with 20-hydroxyecdysone and polypodine B. The structure of 1α,20*R*-dihydroxyecdysone was determined unequivocally by UV, LSIMS, and a combination of 1D and 2D NMR techniques. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Axyris amaranthoides L., commonly known as "Russian pigweed", is a herbaceous annual, originally from Asia (temperate Russia to Manchuria) and introduced all over the U.S.A. [1,2]. However, this species has not been the subject of a thorough phytochemical investigation before. Immunoassay and bioassay results have demonstrated the presence of phytoecdysteroids in seed extracts of many species of the Family Chenopodiaceae [3]. *A. amaranthoides* was amongst those containing relatively high levels of ecdysteroids. As part of our continuing search for new phytoecdysteroids and also for new plant sources for phytoecdysteroids [4–15], we now report on the isolation and identification of a new phytoecdysteroid, 1α,20*R*-dihydroxyecdysone (**1**), and two known ecdysteroids, 20-hydroxyecdysone (**2**) and polypodine B (**3**), from the seeds of *A. amaranthoides*.

RESULTS AND DISCUSSION

Photo-diode-array detector-assisted HPLC analysis, guided by ecdysteroid agonist/antagonist bioassay [16] and ecdysteroid-specific RIA [17] of a methanol extract of the seeds of *A. amaranthoides* (Fig. 1) resulted in the isolation and identification of a novel phytoecdysteroid 1α,20*R*-dihydroxyecdysone (1-*epi*-integristerone A, **1**) and two known ecdysteroids: 20-hydroxyecdysone (20E; **2**) [18] and polypodine B (5,20E; **3**) [18]. Both **2** and **3** were characterized by direct comparison of their HPLC and spectroscopic characteristics with those published in the literature and with samples previously isolated in our laboratories. The novel compound **1** was characterized by spectroscopic means.



(1) R_1 = H, R_2 = OH, R_3 = H

(2) R_1 = H, R_2 = H, R_3 = H

(3) R_1 = H, R_2 = H, R_3 = OH

(4) R_1 = OH, R_2 = H, R_3 = H

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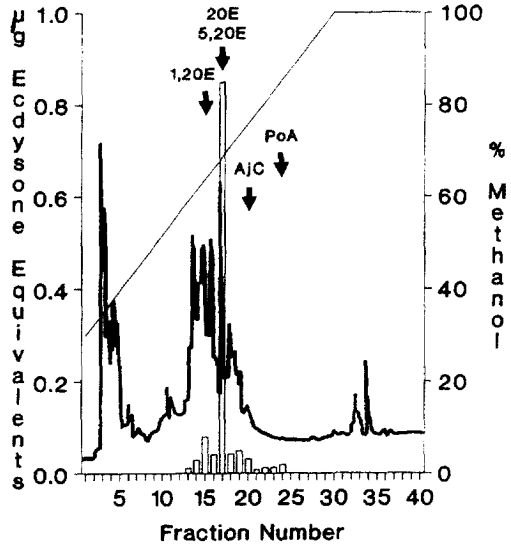
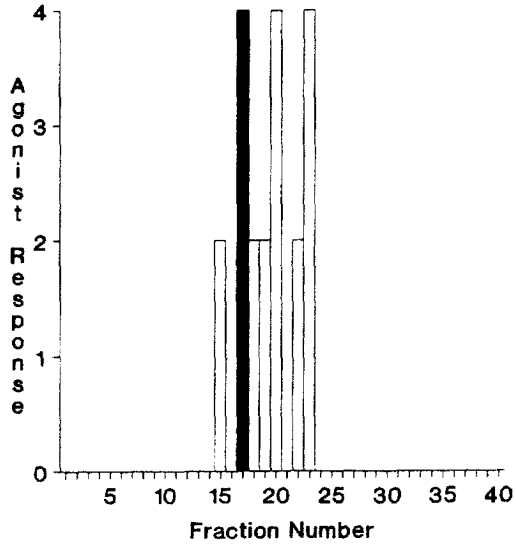


A



B

Fig. 1. RP-HPLC separation of the methanolic seed extract of *Axyris amaranthoides*. A portion of the extract (1 μg ecdysone equivalents with the DBL-1 antiserum) was separated on a C_{18} column (25 cm × 4.6 mm i.d.; 5 μm particle size) with a gradient of methanol in water (given in panel A). The eluate was monitored at 242 nm (panel A). Fractions of 1-minute duration were collected and aliquots assessed by RIA (panel A) and in the agonist version of the B_{II} bioassay (panel B; open columns and solid columns: 20 and 2 μL aliquots, respectively). The arrows in panel A indicate the elution times of reference ecdysteroids: 1,20E (integristerone A), 20E (20-hydroxyecdysone), 5,20E (polypodine B), AjC (ajugasterone C) and PoA (ponasterone A).

The positive responses in the agonist bioassay and with both ecdysteroid-specific antisera, and UV absorption spectrum readily identified compound **1** as an ecdysteroid. The LSIMS (-ve ion mode) of compound **1** revealed the molecular mass 496, compatible with the empirical formula $C_{27}H_{44}O_8$. In its ^{1}H NMR and ^{13}C PENDANT NMR [19] spectra (Table 1), ^{1}H and ^{13}C signals were comparable with those of 20E (**2**) with the notable exceptions of a signal (δ_H 4.31, δ_C 76.2), suggesting the presence of an extra oxymethine, and of a methyl signal (Me-19), which was much more deshielded (δ_H 1.44 for **1** vs 1.06 for **2**), indicating the presence of this extra hydroxyl group in its close proximity. Thus, there were two possibilities: the extra hydroxyl was on C-11 or on C-1. In the COSY45 spectrum, the H-7 (δ_H 6.30) showed long-range zig–zag coupling (characteristic for ecdysteroids) with H-9 (δ_H 3.60) which was coupled to two methylene protons (H_2-11, δ_H 1.76, 1.89), and in turn coupled to the protons of another methylene group (H_2-12, δ_H 2.60, 2.00). Moreover, another chain of ^{1}H–^{1}H correlation, H-1 (δ_H 4.31) ↔ H-2 (δ_H 4.29) ↔ H-3 (δ_H 4.30) ↔ H_2-4 (δ_H 1.85, 2.10) ↔ H-5 (δ_H 3.30) located this extra hydroxyl at C-1. In the ^{1}H–^{13}C HMBC spectrum (Table 2) a ^3J correlation from Me-19 (δ_H 1.44) to C-1 (δ_C 76.2) confirmed this location. A ^{1}H–^{1}H NOESY spectrum (Fig. 2) revealed the nOe interactions among Me-19, H-1 and H-5 protons, and thus confirmed that both H-1 and H-5, like Me-19 were at the β-face of the molecule. H-2 showed nOe to H-3 and H-9, but not to H-1, and thus established their relative stereochemistries as α. The ^{1}H NMR spectrum taken in pyridine-d_5 did not give good separation for the signals arising from H-1, H-2 and H-3. A ^{1}H NMR and a COSY45 spectrum were also taken in CD_3OD (Table 1) which revealed well-separated signals for H-1 (δ_H 3.82), H-2 (δ_H 3.87) and H-3 (δ_H 4.30), and a series of nOe difference spectra (in CD_3OD): irradiation of the signals for H-1, H-2, H-3, H-5, H-9, Me-18 and Me-19 were also conducted to verify the nOe relationships found in the NOESY spectrum. It is worth emphasizing that if H-1 were in the α-orientation, it would show nOe interactions to H-2 and H_2-11, but no such interactions were observed. Detailed examination of COSY-45, HMQC, HMBC and NOESY unequivocally unveiled the structure of **1** as 1α,20*R*-dihydroxyecdysone. It is noteworthy that the 1β-epimer of **1**, integristerone A (**4**), was reported previously from *Rhaponticum integrifolium* [20] and *Serratula xeranthemoides* [21]. However, since the reported ^{1}H NMR data differ considerably from those of **1** and there is no report of ^{13}C data for **4**, the ^{1}H and ^{13}C data of **1** are compared to those of 20E (**2**) [18] in Table 1. 1α,20*R*-Dihydroxyecdysone co-chromatographs with a sample of integristerone A on C_{18}

Table 1. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data of **1** and **2** (coupling constant *J*, Hz in parentheses)

Carbon No.	δ_H				δ_C	
	1[a]	**1**[b]	**2**[a,c]	**2**[b,c]	**1**[a]	**2**[a,c]
1	4.31 *bs*	3.82 *bs*($w_{1/2}$ 6.8)	1.91	1.43	76.2	38.1
			2.14	1.78		
2	4.29 *m*	3.87 *bt*	4.17 *m*	3.83 *m*	68.1	68.3
3	4.30 *m*	4.03 *m*	4.21 *m*	3.94 *m*	70.5	68.2
4	1.85	1.70	1.80	1.65	33.0	32.5
	2.10	1.80	2.01	1.75		
5	3.30	2.60 *dd* (13, 4)	3.01	2.38 *dd* (13, 4)	46.4	51.5
6					ND	203.6
7	6.30 *d* (2.5)	5.82 *d* (2.3)	6.25 *d* (2.5)	5.85 *d* (2.5)	121.6	121.8
8					ND	166.1
9	3.60 *m*	3.07 *m*	3.58 *m*	3.09 *m*	35.0	34.7
10					42.0	38.8
11	1.76	1.70	1.71	1.65	21.4	21.3
	1.89	1.80	1.88	1.78		
12	2.60 *dd* (13, 5)	2.14 *dd* (13, 5)	2.58 *dd* (13, 5)	2.13 *dd* (13, 5)	32.0	32.2
	2.00	1.82	1.95	1.85		
13					47.0	48.3
14					84.2	84.4
15	2.20	2.01	2.14	2.00	31.5	31.9
	1.90	1.57	1.89	1.55		
16	2.46	1.95	2.44	1.95	21.5	21.6
	2.10	1.76	2.08	1.75		
17	3.01 *t* (8)	2.38 *t* (8)	3.00	2.39 *m*	50.1	50.3
18 (Me)	1.25 *s*	0.90 *s*	1.21 *s*	0.89 *s*	17.8	18.0
19 (Me)	1.44 *s*	1.08 *s*	1.06 *s*	0.96 *s*	20.3	24.6
20					76.8	77.1
21 (Me)	1.59 *s*	1.19 *s*	1.58 *s*	1.18 *s*	21.6	21.8
22	3.87 *m*	3.33	3.87 *m*	3.33 *dd* (11, 2)	77.5	77.8
23	1.84	1.32	1.85	1.30	27.5	27.6
	2.14	1.65	2.14	1.65		
24	2.30	1.75	2.28	1.75	42.6	42.6
	1.85	1.43	1.81	1.45		
25					69.5	69.9
26 (Me)	1.38 *s*	1.20 *s*	1.36 *s*	1.19 *s*	30.0	30.1
27 (Me)	1.38 *s*	1.20 *s*	1.36 *s*	1.20 *s*	30.1	30.2

ND: Could not be detected either from ^{13}C PENDANT or from HMBC experiments.
[a]Spectra taken in pyridine-d_5.
[b]Spectra taken in CD_3OD.
[c]Published data from literature [18].

Table 2. ^{1}H–^{13}C-HMQC direct correlation (^1J) and ^{1}H–^{13}C-HMBC long-range correlation (^2J and ^3J) in **1**

Proton	δ^{13}C		
	^1J	^2J	^3J
H-1	76.2 (C-1)		
H-2	71.0 (C-2)		
H-3	68.0 (C-3)		
H_2-4	33.0 (C-4)		
H-5	46.4 (C-5)		76.2 (C-1), 70.5 (C-3)
H-7	122.6 (C-7)		35.0 (C-9), 84.2 (C-14)
H-9	35.0 (C-9)		
H_2-11	21.4 (C-11)		
H_2-12	32.0 (C-12)		
H_2-15	31.5 (C-15)		
H_2-16	21.5 (C-16)		
H-17	50.1 (C-17)	47.0 (C-13)	17.8 (C-18), 21.6 (C-21)
H-22	77.5 (C-22)		
H_2-23	27.5 (C-23)		
H_2-24	42.6 (C-24)	69.5 (C-25)	30.0 (C-26), 30.1 (C-27)
Me-18	17.8 (C-18)	47.0 (C-13)	32.0 (C-12), 50.1 (C-17), 84.2 (C-14)
Me-19	20.3 (C-19)	42.0 (C-10)	76.2 (C-1), 46.4 (C-5), 35.0 (C-9)
Me-21	21.6 (C-21)	76.8 (C-20)	50.1 (C-17), 77.5 (C-22)
Me-26	30.0 (C-26)	69.5 (C-25)	30.1 (C-27), 42.6 (C-24)
Me-27	30.1 (C-27)	69.5 (C-25)	30.0 (C-26), 42.6 (C-24)

Spectra obtained in pyridine-d_5.

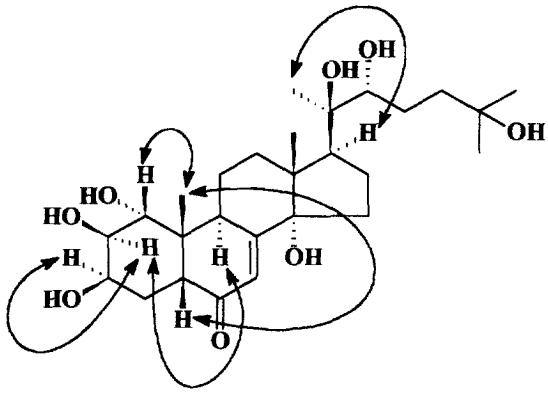


Fig. 2. Key nOe interactions observed in ^1H–^1H NOESY and a series of nOe diff. spectra.

and C_6 RP-HPLC eluted with methanol–water mixtures and DIOL NP-HPLC eluted with methanol–methylene dichloride mixtures.

It is apparent from radioimmunoassay and bioassay results (Fig. 1) that other, minor ecdysteroids which are less polar than 20E are present in the seed extract of *A. amaranthoides*. However, as is apparent from the HPLC chromatogram, the amounts of these were too low for identification. On the basis of their retention times on RP-HPLC and relative biological activities, one can speculate that these minor components are ajugasterone C, ponasterone A and their respective 1-hydroxy derivatives. Enzymic hydrolysis of HPLC fractions followed by RIA provided no evidence for the presence of hydrolyzable ecdysteroid conjugates in the seed extract.

The presence of phytoecdysteroids has earlier been detected in seed extracts of many species of the family Chenopodiaceae, including *A. amaranthoides* [3]. The distribution of immunoassay-positive ecdysteroids throughout the plant in *A. amaranthoides* has also been determined [22], where it was found that highest levels are present in the roots. In the few species of chenopods where the identities of the ecdysteroids have been determined, 20-hydroxyecdysone is the predominant component and a further hydroxylated molecule (polypodine B or 20,26-dihydroxyecdysone) is the other major phytoecdysteroid [23–28]. The seeds of *A. amaranthoides* are unusual in that a significant amount of 1α,20*R*-dihydroxyecdysone is also present in addition to 20E and 5,20E. The concentration of 1α,20*R*-dihydroxyecdysone giving a 50% response in the *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonists [16] is 2.5×10^{-7} M (*cf.* 7.5×10^{-9} M for 20-hydroxyecdysone and 1.0×10^{-9} M for polypodine B). 1α,20*R*-Dihydroxyecdysone has an equivalent biological activity in this bioassay to integristerone A ($ED_{50} = 1.8 \times 10^{-7}$ M). In view of the identical chromatographic behaviours and biological activities of these samples, it is possible that what was previously identified as integristerone A is, in fact, its 1α-epimer.

EXPERIMENTAL

UV: MeOH; NMR: on a Bruker AVANCE DRX400 instrument using standard Bruker microprograms. Chemical shifts are expressed in ppm; LSIMS (-ve ion mode); glycerol matrix using a Cs^+ primary ion beam on a VG Quattro triple quadrupole mass spectrometer (VG Biotech, Altrincham); Sep-Pak Vac 35cc (10 g) C_{18} cartridge (Waters) were used for initial fractionation of extract; HPLC: (a) semipreparative — Gilson model 806 HPLC coupled with Gilson UV–Visible detector, (b) analytical — Gilson model 811 HPLC coupled with Gilson 160 diode array detector and using Gilson Unipoint computer program. RP, NP, RP-semiprep. RP-anal. and NP-semiprep. stand, respectively, for reversed-phase, normal-phase, semipreparative C_6 column, Spherisorb 5 ODS-2 analytical C_{18} column and Apex II Diol 5 μm (Jones Chromatography) semipreparative column throughout this text. Chromatographic separations were monitored at 242 nm.

Radioimmunoassay

RIA was performed according to the procedure described previously [17] using the ecdysteroid-specific antisera DBL-1 and Black, which were donated by Professor J. Koolman (University of Marburg, FRG). The cross-reactivities of these antisera for a number of phytoecdysteroids are given elsewhere [29].

Bioassay

The biological activities (ecdysteroid agonist or antagonist) of extracts and HPLC fractions were determined with a microplate-based bioassay using the *Drosophila melanogaster* B_{II} cell line [16].

Enzymic hydrolysis

The possible presence of ecdysteroid conjugates was assessed by hydrolysis of HPLC fractions with *Helix pomatia* hydrolases (Sigma: Type H1, 10 mg/mL) in 0.1 M acetate buffer (pH 5.4; 200 μL) containing 5% EtOH for 2 days at 37°C. The incubation was stopped by the addition of EtOH (1 mL) and the levels of RIA-positive material were determined and compared to the levels prior to hydrolysis.

Plant material

Seeds of *A. amaranthoides* were purchased from B and T World Seeds, Paguignan, 34210 Olonzac, France. A voucher specimen has been retained at

the Department of Biological Sciences, University of Exeter, U.K.

Extraction

Ground seeds (19.8 g) were extracted five times (5 × 24 h) with 5 × 150 mL MeOH at 55°C with constant stirring using a magnetic stirrer. Extracts were combined and made in to 70% aq. methanolic solution. After being defatted with *n*-hexane the extract was concentrated using a rotary evaporator at a maximum temperature of 45°C.

Isolation of compounds

Sep-Pak fractionation of the concentrated extract (redissolved in 10% aq. MeOH) using MeOH–H_2O step gradient, followed by bioassay/RIA revealed the presence of ecdysteroids in the 60% MeOH–H_2O fraction which was then subjected to HPLC using an RP-semiprep. column (isocratic elution with 40% MeOH–H_2O, 2 mL/min) to yield eighty one-minute fractions. While 20E and 5,20E were eluted as a mixture in the fractions 16–20, compound **1** was found in fractions 14–15. Further NP-HPLC analyses (NP-semiprep column, isocratic elution with 6% MeOH in CH_2Cl_2, 2 mL/min) of the mixture of 20E and 5,20E resulted in the isolation of those two compounds in pure form (2.1 mg and 0.6 mg, respectively). Compound **1** (0.7 mg) was further purified on a C_6 semipreparative column eluted with 35% MeOH in water, 2 mL/min (R_t 21 min.; 20E = 24.2 min).

1α,20R-dihydroxyecdysone (**1**). Amorphous. UV λ_{max} nm (log ε): 240.6 (4.00). ^1H and ^{13}C NMR (Table 1). LSIMS: *m/z* 495 $[M-H]^-$ (negative ion mode).

20-Hydroxyecdysone. Amorphous. HPLC, UV, ^1H NMR and ^{13}C NMR data as reported [18].

Polypodine B. Amorphous. HPLC, UV, ^1H NMR and ^{13}C NMR data as reported [18].

NOTE ADDED IN PROOF

NMR studies performed on a sample of integristerone A isolated from *Silene nutans* (Caryophyllaceae) confirm that it possesses a 1β-hydroxyl group (J.-P. Girault, personal communication).

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