Isolation of a New Class of Ecdysteroid Conjugates (Glucosyl-ferulates) Using a Combination of Liquid Chromatographic Methods

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Abstract

The Polynesian medicinal fern *Microsorum membranifolium* contains very large amounts of ecdysteroids, including ecdysone, 20-hydroxyecdysone, 2-deoxy-20-hydroxyecdysone, and 2-deoxyecdysone. It also contains large amounts of unusual ecdysteroids which have been unambiguously identified by mass spectrometry and nuclear magnetic resonance. A new class of ecdysteroid conjugates (3-glucosyl-ferulates of 2-deoxyecdysone and 2-deoxy-20-hydroxyecdysone) is isolated, together with a new glycoside (2-deoxyecdysone 25-rhamnoside). The simultaneous presence of a sugar and an aromatic moiety results in a very particular chromatographic behavior of these conjugates. They behave like flavonoids and polyphenols when using the classical purification on polyamide, aimed at removing the latter from crude plant extracts, and would therefore be lost. They elute as non-polar ecdysteroids on reversed-phase high-performance liquid chromatography (RP-HPLC), whereas their behavior on normal-phase (NP) HPLC is strongly dependent on the mobile phase composition. Our data highlight the importance of selectivity in the choice of HPLC methods used for ecdysteroid separations.

Introduction

Phytoecdysteroids represent an important family of plant secondary metabolites structurally related to insect molting hormones. They are believed to protect plants against phytophagous insects (1). They are polyhydroxylated steroids and presently ca. 300 different analogues have been isolated (2), differing in the number of carbon atoms and the number/position of hydroxyl groups, which can be either free or conjugated with polar or apolar moieties (2,3). They are present in a large number of plant families within the angiosperms, gymnosperms, and ferns and even in fungi (2–4). A single species usually contains a complex cocktail of molecules (1,5).

Ecdysteroids are efficiently separated by the sequential use of differential precipitation, partitions, and various chromatographic procedures, and the complexity of ecdysteroid cocktails often requires the sequential use of two, or even three, high-performance liquid chromatography (HPLC) steps to resolve all compounds (6–9). Alternatively, optimized thin-layer chromatography (TLC) may also provide very efficient separations, as it allows a broader range of mobile phases to be used (10).

Ferns were among the first species in which phytoecdysteroids were found (11), and many species are known to contain ecdysteroids (12,13). A recent investigation of the medicinal fern *Microsorum scolopendria* has shown the presence of many different ecdysteroids, including large amounts of ecdysone and 20-hydroxyecdysone (14). Several related species also contain significant amounts of ecdysteroids (15). Among them, *Microsorum membranifolium* appeared particularly interesting, owing to the very high ecdysteroid concentrations in its fronds (15). This species thus deserved more extensive phytochemical analysis and will be used here to exemplify strategies which can be used to purify ecdysteroids. Moreover, this study has allowed the isolation of members of a novel class of the ecdysteroid family, thus showing that the diversity of structures produced by plants may be even greater than previously thought.

Material and Methods

Plants

The fronds of *Microsorum membranifolium* (R. Br.) Ching were collected in Tahiti (French Polynesia) in the district of Papenoo in April 2006 and May 2007 and were identified by Dr. Jacques Florence. A voucher specimen has been deposited at the Herbarium of the "Museum of Tahiti and its Islands".

Extraction and purification

Dried fronds (25 g) were milled and extracted with ethanol (1 L) with continuous stirring over 1 day. The extract was filtered and the filtrate evaporated to dryness. The residue was dissolved

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in methanol (100 mL), and small aliquots corresponding to 6 mg dry fronds were withdrawn for direct HPLC analysis. To the remaining solution, Celite (6 g) was added, and the mixture was evaporated to dryness, then suspended in chloroform (200 mL) and deposited at the top of a silica column (Kieselgel Merck Si60, 25 g). The column was eluted with a step-gradient of methanol in chloroform (5:95 [100 mL], then 10:90, 15:85, and 20:80 [200 mL each]). Twenty-three fractions (ca. 30 mL each) were collected and 10 µL of each were analyzed by TLC on silica F_{254} plates [chloroform–methanol (4:1) v/v]. Fractions 8 to 18 contained ecdysteroids. Fractions 8 to 11 contained essentially 2-deoxyecdysone (2dE) and 2-deoxy-20-hydroxyecdysone (2d20E), whereas fractions 12–18 contained a more complex ecdysteroid mixture. These two sets of fractions were purified by semi-preparative HPLC and, whenever necessary, by using one or two additional HPLC steps on analytical columns (see later).

HPLC methods

HPLC equipment from Thermo was used for all purification steps and for checking compound purity. Analytical reversed-phase (RP) HPLC was performed on either a Zorbax-TMS column (250 mm, 4.6 mm i.d., particle size 5 µm, from DuPont) or an ACE C_{18} column (150 mm, 4.6 mm i.d., particle size 5 µm, from A.I.T.), eluted at a flow-rate of 1 mL/min with a linear gradient (15% to 35% acetonitrile–isopropanol [5:2 v/v] in water containing 0.1% trifluoroacetic acid over 40 min) (Systems 1A and 1B, respectively). Semi-preparative separations were performed on an ACE C_{18} column (150 mm, 9.2 mm i.d.) with the same gradient, followed by 35% to 100% in 30 min at a flow-rate of 4 mL/min (System 2). Analytical NP-HPLC used a Kromasil column (250 mm, 4.6 mm i.d., particle size 3.5 µm, from A.I.T.) eluted with a flow-rate of 1 mL/min with dichloromethane–propan-2-ol–water (125:30:1.5, v/v/v) (System 3). Preparative normal-phase (NP) HPLC used a Zorbax-SIL column (250 mm, 9.4 mm i.d., particle size 5 µm, from DuPont) eluted at a flow-rate of 4 mL/min with dichloromethane–propan-2-ol–water (125:40:3, v/v/v) (System 4). Finally, another NP system using a Zorbax-SIL column (250 mm, 4.6 mm i.d., particle size 5 µm, from DuPont) eluted with a flow-rate of 1 mL/min with cyclohexane–propan-2-ol–water (100:50:3, v/v/v) (System 5) was assessed for its selective effects.

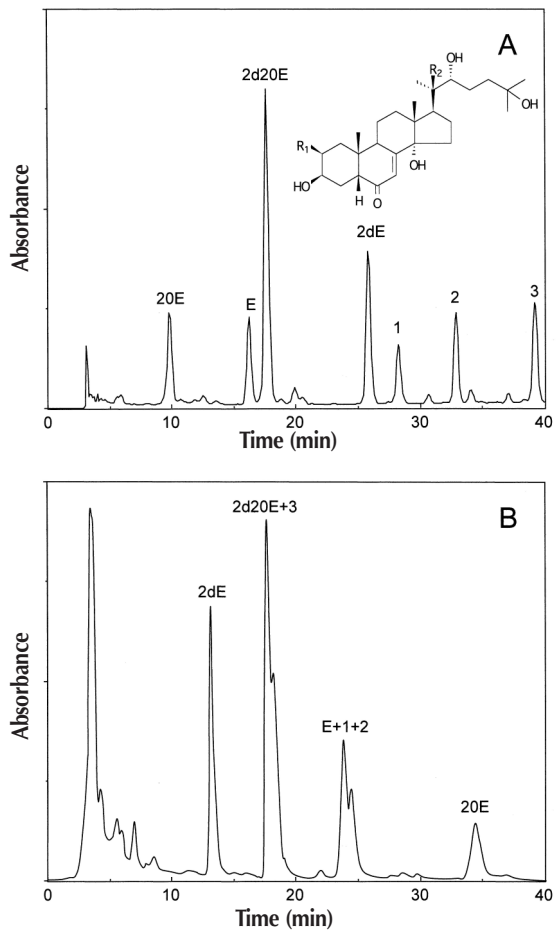

Figure 1. Analysis of a crude ethanolic extract of *M. membranifolium* fronds. RP-HPLC (System 1A) (A); NP-HPLC (System 3) (B). (2dE: R_1=R_2 =H; 2d20E: R_1=H, R_2=OH; E: R_1=OH, R_2=H; 20E: R_1=R_2=OH).

Polyamide column chromatography

Polyamide 6 for column chromatography (50–160 µm, Fluka) was suspended in EtOH–H_2O (1:3, v/v) and slurry-packed into a column (39 × 1.5 cm, 5 g) and then equilibrated with H_2O. The ecdysteroid-containing extract (100 mg dry weight) was applied in water and eluted with a linear gradient (0% to 100% EtOH over 120 min) at a flow-rate of 2 mL/min. The effluent was monitored with a diode-array detector (Agilent G1315A). Twelve fractions (20 mL each) were collected and the column was then reconditioned for further use.

Spectroscopic methods

UV spectroscopy: ecdysteroids were dissolved in absolute ethanol and UV spectra were recorded with a Varian DMS 100 spectrometer.

Mass spectrometry (MS): mass spectra were recorded on a Jeol JMS-700 spectrometer either in desorption/chemical ionization (CI/D) mode with ammonia as the reagent gas or fast-atom bombardment mode (FAB). The latter was used for fragile conjugates.

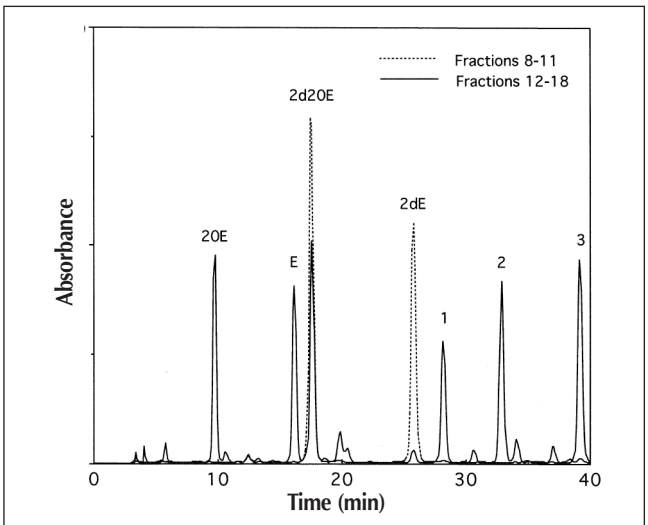

Figure 2. RP-HPLC analysis (System 1A) of fractions eluted from the low-pressure silica column.

Nuclear magnetic resonance spectroscopy (NMR): NMR spectra were obtained on a Bruker Avance 500 at 300K. The samples were lyophilized and dissolved in CD_3OD and C_5D_5N (C_5D_5N+D_2O). $TSPd_4$, 3-(trimethylsilyl) [2,2,3,3-d_4] propionic acid, sodium salt, was used as internal reference for proton and carbon shifts (δ ± 0.2 ppm). Chemical shifts are expressed in ppm. 1D ^1H and ^{13}C spectra and 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), nuclear Overhauser enhancement spectroscopy, rotating-frame NOE spectroscopy (ROESY), perfluoroguanidine-heteronuclear single-quantum correlation, and perfluoroguanidine-heteronuclear multiple-bond correlation (PFG-HMBC) NMR spectra further allowed the ^1H and ^{13}C assignments (19).

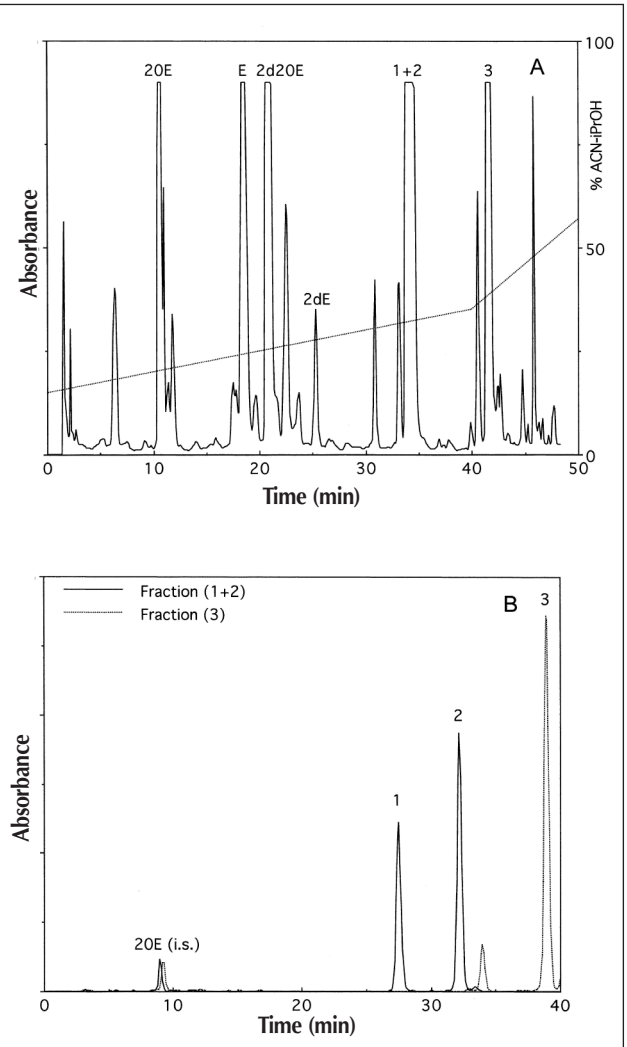

Figure 3. Purification of fraction 12–18 from low pressure silica column. RP-HPLC (System 2). Note that Compounds 1 and 2 co-elute on the ACE C18 column (A). RP-HPLC (System 1A) from fractions "1+2" and "3" collected from the previous system (20E was added as internal standard) (B).

Results

Purification of the major ecdysteroids

The analysis of small aliquots of the crude extract using System 1A and System 3 (Figures 1A and 1B) allowed the detection of many putative ecdysteroids, and determination of the concentrations of the major compounds in *M. membranifolium* dry fronds was made from the integration of UV absorbance peaks by reference to a calibration curve obtained with 20E. They gave 20E = 1%, E = 0.9%, 2d20E = 3%, and 2dE = 1.6% (i.e., a total close to 0.65% of the frond dry weight, which is among the higher values found in plants [some other samples were shown to contain more ecdysteroids, according to the season or site of collection; see ref. 15]). Such calculations suppose that peaks correspond to pure ecdysteroids, which was later shown to not be true for the '20E' peak, owing to the presence of an overlapping contaminant (thus the actual concentration is overestimated).

Silica column chromatography gave two different sets of fractions (8–11 and 12–18) as shown from HPLC analysis (System 1A; see Figure 2). The ecdysteroids in Fractions 8–11 were purified by semi-preparative NP-HPLC (System 4) to yield 2dE and 2d20E. The ecdysteroids in Fractions 12–18 could not be resolved on NP-HPLC using dichloromethane-based solvents and required the use of reversed-phase HPLC (RP-HPLC), in order to collect the four "classical" ecdysteroids (20E, E, 2d20E, and 2dE) and the three less polar major peaks (Compounds 1–3). RP-HPLC unexpectedly showed a striking difference in selectivity between the TMS and ACE columns (compare Figure 3A with Figure 2), as Compounds 1 and 2 were not resolved in the ACE-column system. The separation was thus first performed on the ACE column, then the "1 + 2" fraction was purified using System 1A (Figure 3B). Final purification of compounds 1–3 was achieved by preparative NP-HPLC using System 4. All HPLC data are reported in Table I.

Table I. HPLC Data (Retention Times in Min) for the Different Ecdysteroids Described in this Study (see M & M for the Description of the Separation Systems)

Ecdysteroid	System 1A	System 1B	System 2	System 3	System 4	System 5
20E	9.8	10.4	11.4	34.8	16.1	17.1
E	16.2	17.3	18.8	24.5	11.4	13.1
2d20E	17.6	19.5	21.3	18.2	9.1	10.5
2dE	25.8	28.8	31.3	13.7	7.0	8.5
Compound 1	28.2	32.2	34.3	24.5	11.5	12.3
Compound 2	32.9	32.9	34.3	25.2	12.0	34.3
Compound 3	39.2	40.3	42.6	19.0	9.5	26.4

Identification of Compounds 1–3

UV spectra are presented in Figure 4. Compound 1 shows a typical ecdysteroid spectrum with a maximum at 242 nm (in EtOH), but the spectra of Compounds 2 and 3 were unusual, as, in addition to a major absorbance at 240 nm, peaks were observed at 294 and 317 nm, which are indicative of the presence of an aromatic conjugating moiety (in fact, at this stage, we were not sure that these compounds were ecdysteroids at all).

The mass spectrum of Compound 1 (CI/D) showed prominent ions at *m/z* 612 $(M+NH_4)^+$, 595 $(MH)^+$, 594 $(M)^+$, 577 $(MH-H_2O)^+$, 559 $(MH-2H_2O)^+$, 448 $(M+NH_4\text{-sugar})^+$, 431 $(MH\text{-sugar})^+$, 413 $(MH\text{-sugar-}H_2O)^+$, 395 $(MH\text{-sugar-}2H_2O)^+$, and 164 $(sugar)^+$. The 164 amu value corresponds to a deoxy-hexose. These data are in agreement with a M.W. of 594 amu. NMR data are reported in Tables II and III.

Compound 1 was identified on the basis of the following evidence. ^1H and ^{13}C spectra present no significant difference of chemical shifts for the steroid nucleus as compared to 2-deoxyecdysone. Thus, this product bears only modifications to this side-chain. ^1H NMR spectra show six methyl signals (4 singlets and 2 doublets). The presence of a sugar is straightforward, because one observes additional peaks in the region of hydrogen bound to oxygenated carbons (3.2–4.95 ppm), and in the ^{13}C NMR spectrum for the corresponding carbon signals (60–100

Table II. ^1H-NMR Data (Solvent CD_3OD, Unless Otherwise Stated, T = 300K; δHCD_2OD) = 3.31 ppm, ref. $TSPD_4$*

1H	2d20E	2dE	Compound 1	Compound 1 $C_5D_5N + \varepsilon D_2O$	Compound 2	Compound 3
1-Ha	1.47	1.47	1.46	1.25	1.46	1.46
1-He	1.61	1.61	1.64	1.32	1.76	1.76
2-Ha	1.64	1.64	1.66	1.73	1.87	1.87
2-He	1.80	1.80	1.82	1.83	1.94	1.94
3-He	3.99 ($w_{1/2}$ = 15)	3.98 m ($w_{1/2}$ = 20)	3.98 m ($w_{1/2}$ = 23)	4.13 m ($w_{1/2}$ = 10)	5.18 (br, $w_{1/2}$ = 13)	5.16 m ($w_{1/2}$ = 14)
4-Ha	1.57	1.57	1.57	1.93	1.77	1.77
4-He	1.81	1.81	1.79	1.93	1.87	1.87
5-H	2.42 (d,d, 12.5, 4.1)	2.42 (d,d ,12.1, 4.2)	2.42 (d,d, 12.3, 4)	2.96 m, br ($w_{1/2}$ = 32)	2.40 (d,d ,12.3, 4)	2.41 (d,d, 12.6, 4.2)
7-H	5.81 (d, 2.4)	5.81 (d, 2.6)	5.81 (d, br, 2.4)	6.15 (d, 2.2)	5.84 (d, 2.4)	5.85 (d, 2.0)
9-Ha	3.21 m ($w_{1/2}$ = 25)	3.20 m ($w_{1/2}$ = 26)	3.22 m ($w_{1/2}$ = 26)	3.49 m, br ($w_{1/2}$ = 30)	3.27	3.27 m ($w_{1/2}$ = 26)
11-Ha	1.63	1.63	1.64	1.59	1.68	1.67
11-He	1.76	1.76	1.74	1.76	1.76	1.78
12-Ha	2.12 (dt, 13.8, 4.8)	2.09	2.09 (m)	2.51	2.15 (d,t,13.8,4.8)	2.13 (d, t, 13.4, 5)
12-He	1.86	1.76	1.77	1.84	1.88	1.78
15-H	1.98	1.98	1.98	1.95	2.00	1.98
15-H?	1.57	1.60	1.64	1.95	1.63	1.62
16-H?*	1.98	1.98	1.96	2.26	2.00	1.98
16-H?*	1.74	1.51	1.51	1.60	1.76	1.51
17-H	2.41 (t, 8)	2.03	2.03	2.53	2.42 (t, 9.9)	2.06
20-H	–	1.75	1.76	2.14	–	1.77
22-Hb	3.33 (d, 11)	3.60 (dbr, 11)	3.59 (d, br, 10.6)	4.04 (d,d, 10, 3)	3.35	3.61 (d, br, 10.1)
23-Ha	1.30	1.33	1.31	1.74	1.31	1.33
23-Hb	1.65	1.54	1.58	1.91	1.69	1.55
24-Ha	1.80	1.78	1.84	1.81	1.81	1.79
24-Hb	1.45	1.42	1.50	2.28	1.46	1.42
18-Me	0.89 (s)	0.731 (s)	0.73 (s)	0.71 (s)	0.907 (s)	0.745 (s)
19-Me	0.968 (s)	0.968 (s)	0.97 (s)	1.03 (s)	0.97 (s)	1.01 (s)
21-Me	1.193 (s)	0.945 (d, 6.6)	0.94 (d, 6.6)	1.30 (d, 6.4)	1.203 (s)	0.957 (d, 6.6)
26-Me	1.193 (s)	1.195 (s)	1.23 (s)	1.25 (s)	1.197 (s)	1.200 (s)
27-Me	1.205 (s)	1.204 (s)	1.25 (s)	1.29 (s)	1.211 (s)	1.210 (s)
H-1′	–	–	4.95 (dbr, 1.5) Y	5.54 (dbr, 1.3)	4.98 (d, 7.3)	4.98 (d, 7.3)
H-2′	–	–	3.68 m ABXY	4.42 (dd, br, $w_{1/2}$ = 6.5)	3.53 (d,d, 7.5, 9)	3.53 (d,d, 7.5, 9)
H-3′	–	–	3.69 m ABXY	4.49 (d, d, 9.2, 3.3)	3.48 (t, 9)	3.48 (t, 9)
H-4′	–	–	3.34 (t*, 9.3) X	4.24 (t, 9.4)	3.43 (d,d, 9.5, 8.6)	3.43 (d,d, 9.5, 8.6)
H-5′	–	–	3.77 (d,q, 9.3, 6.3)	4.38 (d,q, 9.3,6.4)	3.45 (d,d,d, 9.5, 5.2, 2.2)	3.45 (d,d,d, 9.5, 5.2, 2.2)
H-6′		–	–	–	3.70 (d,d, 12, 5)	3.70 (d,d, 12, 5)
H-6″		–	–	–	3.89 (d,d, 12.2, 1.8)	3.89 (d,d, 12.2, 1.8)
6′-Me	–	–	1.235 (d, 6.3)	1.62 (d, 6.4)	–	–
H-2f	–	–	–	–	7.31 s ($w_{1/2}$ = 3)	7.31 s ($w_{1/2}$ = 3)
H-5f	–	–	–	–	7.19 AA′	7.19 AA′
H-6f	–	–	–	–	7.19 AA′	7.19 AA′
H-7f	–	–	–	–	7.66 (d, 16)	7.66 (d, 15.8)
H-8f	–	–	–	–	6.50 (d, 16)	6.50 (d, 15.8)
MeO-3f	–	–	–	–	3.92 (s)	3.92 (s)

* Multiplicity of signals: s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet; br = broad signal; $w_{1/2}$ = width at half-height in Hertz; δ in ppm; * assignments could be reversed; t* = deceptively simple triplet.

ppm). Moreover, the methyl doublet at δ = 1.24 ppm could be correlated by means of 2D COSY and TOCSY experiments with one proton signal of the sugar, in agreement with a deoxy glycoside as a substituent of the side-chain of 2-deoxyecdysone.

The nature and attachment of this glycoside could be determined following a general strategy for the identification of ecdysteroid glycosides (17). A 25-glycosidic link is established owing to the observation of a HMBC correlation H1'-25C (δ = 77.9 ppm), the large ^{13}C change (ca. 7 ppm) of the chemical shift observed for 25°C in respect to the corresponding chemical shift for the non-conjugated ecdysteroid, and from ROESY correlation observed for 26 and 27 methyl signals with the H5' proton signal of the sugar at δ = 4.38 ppm. The identity of the sugar was elucidated by a careful examination of ^1H–^1H coupling patterns observed in ^1H NMR (from the standard ^1H spectrum and selective homodecoupling spectra of the sugar proton signals) and ROE correlations observed in ROESY experiments. This study was more easily carried out in wet $C_5D_5N+D_2O$ (a method used

Table III. ^{13}C-NMR Data [Solvent CD3OD, T= 300K, δ ($^{13}CD_3OD$) = 49.0 ppm]

^{13}C	Multiplicity	2d20E	2dE	Compound 1	Compound 1 $C_5D_5N + εD_2O$	Compound 2	Compound 3
C-1	CH_2	29.3	29.9	29.3	29.3	30.1	30.1
C-2	CH_2	28.7	28.7	28.9	28.4	26.3	26.3
C-3	CH	65.1	65.3	65.2	63.5	69.4	69.4
C-4	CH_2	33.0	33.0	32.8	32.8	30.3	30.3
C-5	CH	52.2	52.3	52.1	51.2	53.0	53.1
C-6	C	206.3	*	206.3	*	206.1	205.6
C-7	CH	121.6	121.5	121.5	120.6	121.7	121.5
C-8	C	*	*	168.8	*	*	*
C-9	CH	37.4	37.4	37.5	36.4	37.3	37.4
C-10	C	37.4	37.4	37.5	36.4	37.3	37.4
C-11	CH_2	21.6	21.6	21.8	20.8	21.4	21.5
C-12	CH_2	32.5	32.1	32.4	31.1	32.5	32.0
C-13	C	49.2	48.6	48.2	48.2	49.0	48.2
C-14	C	85.2	84.8	84.9	83.6	85.4	85.4
C-15	CH_2	31.4	31.6	31.5	30.8	31.7	31.6
C-16	CH_2	21.4	26.9	27.0	26.0	21.4	26.9
C-17	CH	50.3	48.8	49.1	48.1	50.5	48.6
C-18	CH_3	17.8	16.0	15.9	15.3	17.9	15.9
C-19	CH_3	24.1	24.2	24.2	23.8	24.2	24.2
C-20	C	77.4	43.3 CH	43.3 CH	42.0 CH	77.7	43.3 CH
C-21	CH_3	20.8	13.0	13.0	13.0	20.8	13.0
C-22	CH	78.2	75.0	75.3	73.7	78.3	75.0
C-23	CH_2	27.2	25.2	24.8	24.4	27.3	25.2
C-24	CH_2	42.2	42.0	40.5	39.9	42.2	42.2
C-25	C	71.1	71.1	77.9	76.5	71.1	71.1
C 26	CH_3	28.6	28.9	26.4	26.3	29.0	29.0
C-27	CH_3	29.4	29.1	26.7	25.6	29.3	29.3
C-1′	CH	–	–	97.1	95.0	102.1	102.1
C-2′	CH	–	–	73.8	73.0	74.9	74.9
C-3′	CH	–	–	72.6	72.0	77.6	77.6
C-4′	CH	–	–	74.2	73.6	71.3	71.3
C-5′	CH	–	–	69.7	68.9	78.1	78.1
C-6′	–	–	–	17.7 CH_3	18.1 CH_3	62.41 CH_2	62.2 CH_2
C-1″	–	–	–	–	–	130.4	130.4
CH-2″	–	–	–	–	–	112.3	112.3
C-3″ C	–	–	–	–	–	150.7	150.7
C-4″ C	–	–	–	–	–	149.5	149.5
CH-5″	–	–	–	–	–	117.1	117.1
CH-6″	–	–	–	–	–	123.4	123.4
CH-7″	–	–	–	–	–	145.8	145.8
CH-8″	–	–	–	–	–	117.5	117.5
C-9″ C	–	–	–	–	–	168.0	168.0
CH_3-O	–	–	–	–	–	56.7	56.7

* signal not detected (too low concentration of the sample).

to exchange hydrogens of –OH groups to deuterium atoms) leading under these conditions to a well-resolved quasi first-order spectrum for the ^{1}H signal of the sugar moiety.

This compound presents only large coupling constants (8–9 Hz) for H3', H'4', H5' in agreement with ^{3}J H_{axial}–H_{axial} coupling constant and consequently with an axial position of these protons. On the other hand, one observes small ^{3}J coupling constants between H3'-H'2' (3.3 Hz) and H1'–H'2' (1.3 Hz), in agreement with an equatorial position for both H1' and H'2'.

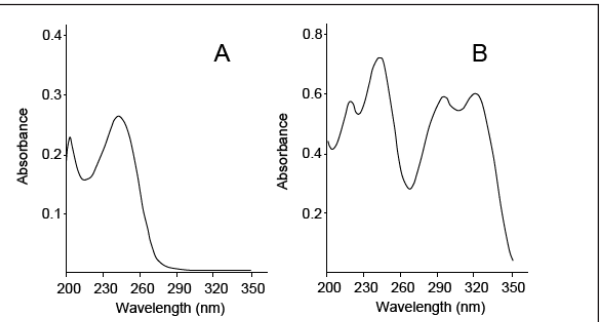

Figure 4. UV spectra in EtOH of Compounds 1 (A) and 2 or 3 (B).

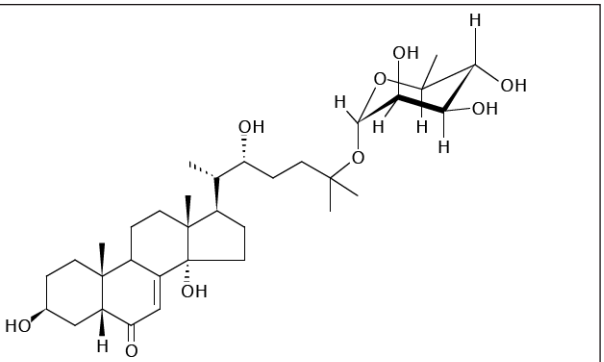

Figure 5. Structure of Compound 1.

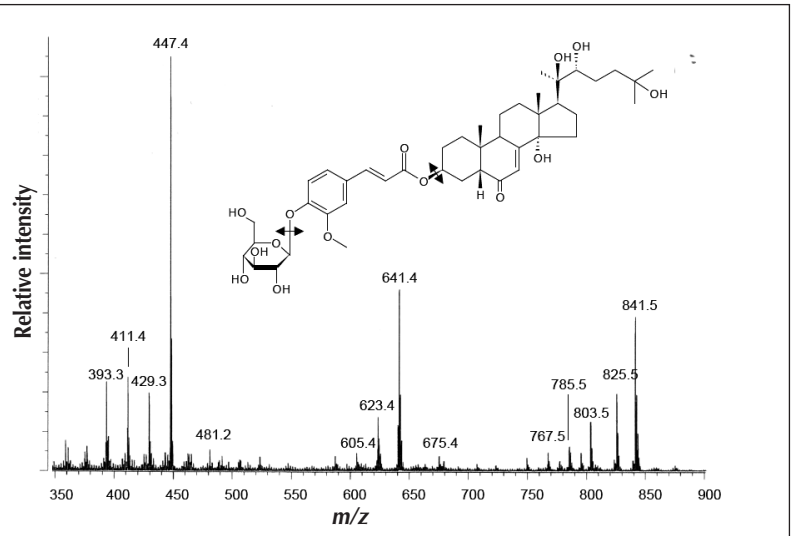

Figure 6. FAB mass spectrum of Compound 2.

Moreover, in a 2D-ROESY experiment, one observes a strong roe for H1'-H'2', a small roe for H1'-H'4', and no roe for H1'-H'3'or H1'-H'5', all consistent with an equatorial position for H1'. All these elements are in accordance with a α-rhamnopyranose (6-deoxy α-mannopyranose) sugar. As this compound was obtained from a plant, an L-configuration is the most probable for the α-rhamnopyranoside. So, we propose that this new ecdysteroid is 2-deoxyecdysone 25-α-L-rhamnopyranoside (6-deoxy-α-L-mannopyranoside) (Figure 5).

The mass spectrum of Compound 2 (FAB^+; Figure 6) gave ions at *m/z* 841 $(M+K)^+$, 825 $(M+Na)^+$, 803 $(M+H)^+$, 785 $(M+H–H_2O)^+$, 767 $(M+H–2H_2O)^+$, 749 $(M+H–3H_2O)^+$, 641 $(M+H–sugar)^+$, 623 $(MH–sugar–H_2O)^+$, 605 $(MH–sugar–2H_2O)^+$, 587 $(MH–sugar–3H_2O)^+$, 447 (ecdysteroid core+ $H–H_2O$), 429, 411, 393, 345, 329, 327, 309, and 241. These data are consistent with an M.W. of 802 amu. The mass spectrum of Compound 3 was very close to that of Compound 2, showing a similar pattern of ions, but shifted by 16 amu, with major ions at *m/z* 825 $(M+K)^+$, 809 $(M+Na)^+$, 787 $(M+H)^+$, 769 $(M+H–H_2O)^+$, 751 $(M+H–2H_2O)^+$, 625 $(M+H–sugar)^+$, 607 $(MH–sugar–H_2O)^+$, 431 (ecdysteroid core+$H–H_2O$), 413, 395, and 377, consistent with a M.W. of 786 amu.

Initial examination of ^{1}H NMR spectra of these two compounds showed that they present ecdysteroid aglycones conjugated with identical aromatic and sugar moieties. Inspection of the ^{1}H NMR of the ecdysteroid cores of these molecules shows five singlet methyl signals for Compound 3 and four singlets and one methyl doublet for Compound 2. This is in agreement with 20-hydroxy- and 20-deoxy-derivatives, respectively. Moreover, after assignment of ^{1}H or ^{13}C signals by means of 1D and 2D experiments, these two compounds do not show significant changes in their ^{1}H or ^{13}C chemical shifts for the signals of the side-chain or of rings B, C, and D of the ecdysteroid core. However, for the A-ring, one observes the typical features of 2-deoxy compounds (lack of H-2 in the >CHOH zone, broadening of $H3_{eq}$, see 18) and of 3-esterified derivatives (β = 5.17 ppm sb, $w_{1/2}$ =14 Hz). Finally, examination of the ^{1}H and ^{13}C spectral data of the conjugated moiety led to the identification of the aromatic moiety as a ferulate and the sugar moiety as a β–D glucoside as follows:

(*i*) The sugar presents one oxymethylene and five oxymethine groups in agreement with a hexose sugar; this hexose presents an ^{1}H anomeric NMR signal H-1' at δ = 4.98 ppm in agreement with a 1'-glycosidic link with the rest of the molecule. On the other hand, the large ^{3}J coupling (doublet, 7.3 Hz) is in accordance with an H_{axial}–H_{axial} coupling constant and consequently with an axial position of both H1'and H'2' protons. ^{1}H selective homonuclear decoupling experiments for H1', H6' and H6'' show that the ^{3}J coupling constants of H2'–H3', H3'–H4' and H4'–H5' are large and therefore H3', H4', and H5' are in axial positions. 2D ROESY experiments present ROE correlation in agreement with this conclusion. So, the sugar moiety is a β–D glucoside.

(*ii*) The linkage of this β–D glucoside was

deduced from 2D HMBC experiments as one observes a correlation from H1' to a quaternary carbon of the aromatic part of the molecule. This shows that the glucoside is linked with the aromatic moiety, which is itself linked to the ecdysteroid core moiety by an ester bond (see earlier). This is confirmed from a ROE correlation observed for 5-H (δ = 2.41ppm) with H-9f (δ = 6.50 ppm). No correlation could be observed from 2D HMBC experiment for the broad 3-H, as this signal has unfavorable relaxation properties.

(*iii*) The aromatic moiety presents ^{1}H and ^{13}C spectral data of an ester group linked to a double bond bearing two ethylenic protons in *trans*-configuration (unambiguously established from the value of their large [15.8 Hz] ^{3}J coupling constant). Again, from 2D HMBC experiments, this double bond could be linked to a phenyl ring bearing three protons, a methoxy group, and the glucosidic link with the sugar (see earlier). The singlet aromatic proton at δ = 7.31 ppm presents ROE correlations with the singlet methyl at δ = 3.92 ppm of the methoxy group and with the two ethylenic protons (Figure 7). All these elements, together with MS data, are in agreement with a ferulate structure for this aromatic group.

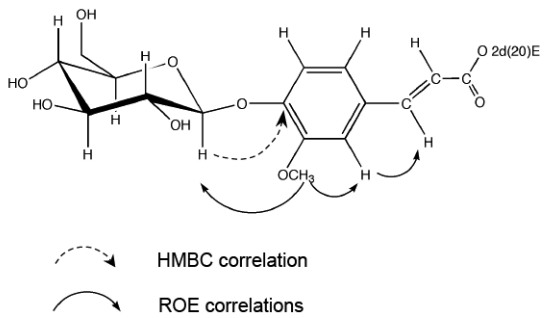

Figure 7. Structures of Compounds 2 and 3.

In conclusion, Compound 2 corresponds to 2-deoxy 20-hydroxy-ecdysone 3-[4-(1-β-D glucopyranosyl)]-ferulate. Similar experiments have led to the identification of Compound 3 as 2-deoxyecdysone 3-[4-(1-β-D glucopyranosyl)]-ferulate.

Polyamide column fractionation

Crude plant extracts contain a wide array of polyphenols/flavonoids which absorb UV and are eluted within the same range of solvent strength as ecdysteroids, especially in RP-HPLC. As a consequence, polyamide column chromatography is often used to remove them at an early stage of the purification sequence, and this approach has been efficiently used in the purification of phytoecdysteroids (19–21). Given the structures of the unusual conjugates (Compounds 2 and 3), it was of interest to determine their behavior during this step. Preliminary experiments indeed showed that (owing to the presence of the ferulate moiety?) these conjugates elute much later

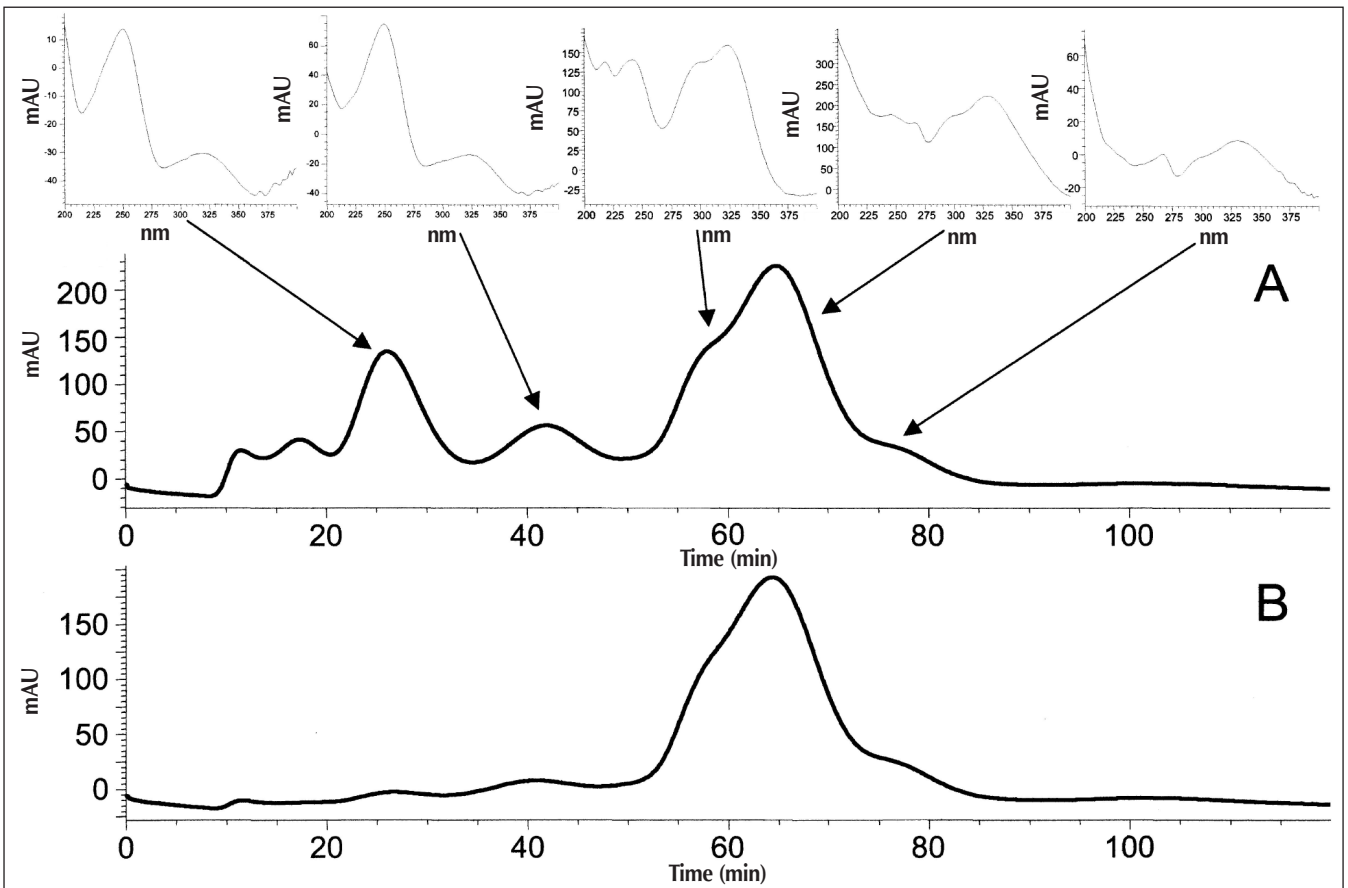

Figure 8. Polyamide column chromatographic separation of a *M. membranifolium* extract. UV monitoring at 245nm (A); UV monitoring at 320 nm (B). Inserts show the UV spectra of representative fractions.

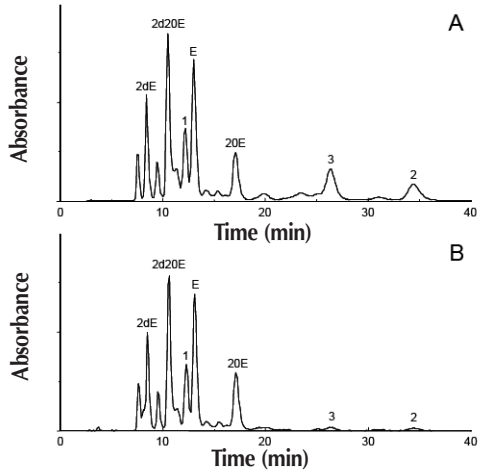

Figure 9. NP-HPLC (System 5) of (A) the crude *M. membranifolium* extract (compare with Figure 1) and (B) of an aliquot of the Fraction 15–20 min from the polyamide column.

than "classical" ecdysteroids, and were lost during this clean-up process. Continuous monitoring of the column effluent (Figure 8) showed that the bulk ecdysteroids eluted in early fractions (20–30 and 40–50 min), whereas Compounds 2 and 3 eluted much later (50–60 min), as evidenced by the UV spectra of the effluent and confirmed by RP-HPLC analysis (data not shown). In fact, the polyamide column behaved like a low-resolution RP system and, among the free ecdysteroids, 20-hydroxyecdysone eluted first and 2-deoxyecdysone last.

Quest for a selective NP-HPLC system

As previously shown (Figure 1B), NP-HPLC proved rather inefficient for the purification of ecdysteroids from *M. membranifolium*. However, it appeared of interest to search for an NP system, because it is always preferable to have a combination of several HPLC systems to resolve complex ecdysteroid mixtures (22). For that reason, we tried other solvent systems and obtained rather unexpected results when using a cyclohexane–isopropanol–water mixture (System 5). With this system, Compound 1 eluted much later than 2-deoxyecdysone, as is usual for sugar conjugates (17), but surprisingly Compounds 2 and 3 behaved like very polar ecdysteroids, and they eluted much later than 20E (Figure 9). Such a system gave a very different pattern to that obtained with the dichloromethane-based mobile-phase (Figure 1B) and may prove suitable to purify minor components (Ho et al., in press).

Discussion

The fronds of *Microsorum membranifolium* contain a complex cocktail of ecdysteroids. We showed previously that they contain large amounts of 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, ecdysone, and 20-hydroxyecdysone, together with smaller amounts of inokosterone, makisterone A, and makisterone C (15). We did not pay attention to the compounds less polar (on RP-HPLC) than 2-deoxyecdysone, and in addition we used polyamide chromatography during the purification sequence. In the present experiments, we removed the polyamide step and also considered the low-polarity UV-absorbing peaks, and this led to the isolation of three new ecdysteroids. These new ecdysteroids display an unusual chromatographic behaviour. The most efficient separations were obtained with a Zorbax-TMS column (system 1A) and a Zorbax-SIL column (System 5). The biggest surprise came from the contrast between the two NP systems; selective effects of solvents are well-established (22), but in the present case they are particularly dramatic.

A second general comment concerns the use of polyamide as a pre-purification step (15,19–21). This is indeed a very efficient procedure, but we must now be aware that it can result in the loss of some conjugates. Compounds 2 and 3 are structurally related to 20E conjugates (20E 2-cinnamate and 20E 3-*p*-coumarate) previously isolated from another fern, *Dacrydium intermedium* (23,24). Cinnamic, coumaric, ferulic, and isoferulic acids are widespread in the plant kingdom, so we may expect that in those species producing ecdysteroids, conjugates incorporating these moieties may be present. In *M. membranifolium*, these conjugates were found only for 2-deoxy-ecdysteroids and involve the 3β-OH. Ferulate esters (as well as glycosides or acyl-glycosides) of sterols have already been found in many plant species (e.g., 25, 26), but we did not find data for the existence of glucosyl-ferulates of sterols, although the presence of such a sterol ester class should be investigated, at least in this fern.

The isolation of a rhamnoside conjugate is also an original finding. Many ecdysteroid glycosides have been isolated (2,17), but so far no 6-deoxy-sugar derivative was described. We previously showed (17) that on RP-HPLC the presence of a hexose results in a moderate increase of polarity, and that of a pentose does not change polarity at all. Here we show that the presence of a 6-deoxyhexose results in a decrease of polarity! On the other hand, whatever the sugar, conjugation with a sugar always results in a large increase of the retention time on NP-HPLC (17).

Finally, we have focused here on three major compounds, but many minor ones clearly belonging to the ecdysteroids are presently under investigation. We will particularly investigate whether glycosyl-ferulate conjugates exist only for 2-deoxyecdysteroids, whether ferulate esters (without the glucose moiety) are also present and, when sufficient amounts are available, we shall investigate the biological activity of these original molecules.

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