Ecdysteroid Glycosides: Identification, Chromatographic Properties, and Biological Significance

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Abstract

Ecdysteroid glycosides are found in both animals and plants. The chromatographic behavior of these molecules is characteristic, as they appear much more polar than their corresponding free aglycones when analyzed by normal-phase high-performance liquid chromatography (HPLC), whereas the presence of glycosidic moieties has a very limited (if any) impact on polarity when using reversed-phase HPLC. Biological activity is greatly reduced because the presence of this bulky substituent probably impairs the interaction with ecdysteroid receptor(s). 2-Deoxy-20-hydroxyecdysone 22-O-β-D-glucopyranoside, which has been isolated from the dried aerial parts of *Silene nutans* (Caryophyllaceae), is used as a model compound to describe the rationale of ecdysteroid glycoside purification and identification.

Introduction

Ecdysteroids represent a large family of polyhydroxylated steroids found in both animals and plants (1–4). In plants, these secondary metabolites are thought to provide protection against nonadapted insect species and possibly also soil nematodes (5–7). Ecdysteroids also display a wide array of pharmacological effects in mammals, and they are present in large amounts in several plants used in traditional medicine (8,9).

Ecdysteroids have been found in many plant species belonging to the Caryophyllaceae, and the genus *Silene* consists of many species of interest in this respect (10–12). When plants contain ecdysteroids, they usually contain a complex mixture of closely related molecules (4,10,11), among which there may be a significant amount of ecdysteroid glycosides. A list of the presently known ecdysteroid glycosides is given in Table I. The *Silene* genus has in fact been the source of nearly half of the currently isolated ecdysteroid glycosides.

Ecdysteroid glycosides are not restricted to plants, as such conjugates have also been identified from insects and a nematode [in the latter case, as conjugated metabolites of exogenously applied ecdysone (37,38)]. An ecdysteroid glucosyl-transferase is also present in baculoviruses [e.g., *Autographa californica* (40)], which can disrupt ecdysteroid-related processes in infested insects.

A chemical synthesis of ecdysteroid glucosides has been described (41), making such derivatives readily available for the assessment of their biological activity (42). The purification and analysis of ecdysteroid glycosides raises specific problems, and the present aim is to describe an efficient method for the isolation and identification of such derivatives from various biological sources.

Experimental

Reference compounds used

Most ecdysteroids used in the present study were isolated by the authors' laboratories from various plant sources (*Silene otites, Silene nutans, Silene brahuica*, and *Limnanthes douglasii*) (Table I). The various glucosides of 20-hydroxyecdysone were prepared by chemical synthesis (41). Reference 26-hydroxyecdysone was isolated from *Manduca sexta* eggs.

High-performance liquid chromatographic systems

Analytical experiments described in Table II were performed with a Spectra Series high-performance liquid chromatographic (HPLC) equipment (P200 pump, UV 100 detector) (Thermo-Separations Products, Les Ulis, France). Four isocratic HPLC systems have been used in the present study. Reversed-phase HPLC used a Spherisorb 5ODS-2 column (25-cm length, 4.6-mm i.d., 5-µm particle size) (AIT Chromato, Le Mesnil le Roi, France) eluted at 1 mL/min with either 45% MeOH in water (System RP1) or 18% ACN–iPrOH (5:2 v/v) in 0.1% trifluoroacetic acid (system RP2). The normal-phase HPLC system included a Kromasil 3.5 µm (25-cm length, 4.6-mm i.d.) (AIT Chromato) eluted at 1 mL/min with either dichloromethane–isopro-panol–water (25:10:1 v/v/v, System NP1) or cyclohexane–isopropanol–water (85:40:3 v/v/v, System NP2).

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Bioassay

Ecdysteroid agonist and antagonist activities of the ecdysteroid glycosides were determined using the *Drosophila melanogaster* B_{II} cell assay, as described previously (43). None of the compounds possessed antagonist activity.

Spectroscopic methods

MS spectra were recorded on an MS 700 spectrometer (Jeol Europe, Croissy sur Seine, France) equipped with a direct inlet probe. Spectra were recorded in the chemical ionization–desorption (CI–D) mode using ammonia (or methane) as the reagent gas.

NMR spectroscopy experiments were run at 500 MHz for ^{1}H, at 300 K, on an AMX 500 spectrometer (Bruker, Wissembourg, France) equipped with a Silicon Graphics workstation. Presaturation of the solvent was used for all 1D and homonuclear 2D ^{1}H experiments (44). The sample was lyophilized twice and dissolved in D_2O. The errors in the chemical shifts were ≤ 0.01 ppm for ^{1}H and ≤ 0.2 ppm for ^{13}C. $TSPD_4$, 3-(trimethyl-silyl)-[2,2,3,3-d_4] propionic acid, and sodium salt was used as internal reference for the proton and carbon shifts.

Table I. Occurrence of Ecdysteroid Glycosides*

Origin	Compound	Reference
Plants		
Silene brahuica	Sileneoside A = 20E 22-gal	13
Silene brahuica	Sileneoside B = 20E 3,22-digal	14
Silene brahuica	Sileneoside C = IntA 22-gal	15
Silene brahuica	Sileneoside D = 20E 3-gal	16
Silene brahuica	Sileneoside F = Brahuisterone 3G	17
Silene brahuica	5α-Sileneoside E = 5α-2dE3G	18
Silene brahuica	Sileneoside G = 20E 3-gal 22G	19
Blechnum minus	Blechnoside A = 2dE3G	20
Silene brahuica	= Sileneoside E	21
Silene pseudotites	2dE22G	22
Silene pseudotites	2dPolypodine B 3G	22
Blechnum minus	Blechnoside B = 2dE 25G	20
Limnanthes douglasii	Limnantheoside A = 20E 3X	23
Limnanthes douglasii	Limnantheoside B = PonA 3X	23
Limnanthes alba	Limnantheoside C = 20E 3(G→3X)	24
Silene tatarica	Ecdysteroside = 20E 3-$(gal)_2$	25
Tinospora capillipes	2d20E3G	26
Silene nutans	2d20E22G	27
Xerophyllum tenax	20E2G	28
Silene otites	20E3G	29
Pfaffia iresinoides	20E25G	30
Pfaffia iresinoides	Podecdysone 25G	30
Pfaffia iresinoides	Pterosterone 24G	30
Helleborus odorus	Polypodine B 3G	31
Helleborus odorus	5α-Polypodine B 3G	31
Pteridium aquilinum	Ponasteroside A = PonA 3G	32
Cucubalus baccifer	2,22d20E 3G	33
(heteroconjugates)		
Melandrium turkestanicum	Melandrioside A = 20E22G 25Ac	34
Silene otites	20E 22Bz 25G	11
Silene brahuica	Sileneoside H = IntA 22-gal 25Ac	35
Animals		
Manduca sexta	26E22G	36
Parascaris equorum	E25G	37
Parascaris equorum	20E25G	38
Chrysolina varians	2,14,22d20E 3-sophorose	39
Viruses		
Autographa californica	E22G (baculovirus + insect)	40

* Abbreviations: acetate (Ac), benzoate (Bz), glucoside (G), galactoside (gal), xyloside (X), 20-hydroxyecdysone (20E), (5α-H)2-deoxyecdysone (5α2dE), 2-deoxyecdysone (2dE), 2-deoxypolypodine B (2dPolB), ponasterone A (PonA), 2-deoxy-20-hydroxyecdysone (2d20E), 2,22-dideoxy-20-hydroxyecdysone (2,22d20E), 26-hydroxyecdysone (26E), ecdysone (E), integristerone A (IntA), sophorose = glucopyranosyl(β1→2)glucose.

Purification of an ecdysteroid glycoside from *Silene nutans*

Plants of *Silene nutans* were collected in the area of Pradelles (Haute-Loire, France) in August, 1996. Air-dried aerial parts (750 g) were extracted with EtOH (3 × 3 L). The filtrates were combined, evaporated to dryness, and redissolved in MeOH (800 mL). An amount of 160 g of Celite 545 (Merck 1.02693.1000, particle size 0.01–0.04 mm) (Darmstadt, Germany) was added, the mixture was evaporated to dryness, then suspended in chloroform, and the slurry was poured into a column. Elution was performed with chloroform (300 mL), then with a step-gradient of MeOH in chloroform (5:95, 10:90, 20:80, and 50:50; 300 mL each). The different fractions were checked by HPLC and both chloroform and chloroform–MeOH (95:5) were selected, mixed, and evaporated to dryness in the presence of Celite (50 g). The Celite was then suspended in chloroform (200 mL) and poured onto a Si60 silicagel column (Merck 1.07734.10000, particle size 0.063–0.200 mm, 100 g). Elution was again performed with the same step-gradient of MeOH in chloroform (300 mL for each mixture, except for the 80:20 mixture, 600 mL) and 100-mL fractions were collected. Fractions 12–14 contained mainly polypodine B and 20-hydroxyecdysone, fraction 15 contained 20-hydroxyecdysone and integristerone A, and fraction 16 contained integristerone A. Fractions 17–20 contained only small amounts of more polar ecdysteroids. They were combined, then separated by normal-phase HPLC using a semipreparative (250-× 9.4-mm i.d.) Zorbax-Sil (AIT Chromato) silica column (solvent dichloromethane–isopropanol–water, 125:40:3, at a flow rate of 4 mL/min). Together with traces of the major ecdysteroids, the sample contained an unknown compound eluting between 31.4 and 36.4 min. This fraction was further purified by analytical normal-phase HPLC using the same solvent system, providing approximately 1.4 mg of pure compound U (ca. 0.002%).

Results and Discussion

HPLC behavior of ecdysteroid glycosides

The HPLC data on ecdysteroid glycosides are summarized in Table II, and an example of separation is given in Figure 1. Four isocratic systems (2 reversed-phase and 2 normal-phase) have been used in order to permit easier comparison. The two reversed-phase systems (RP1 and RP2) used methanol and acetonitrile, respectively, as organic modifiers, whereas the two normal-phase systems (NP1 and NP2) are based on dichloromethane and cyclohexane, respectively. Such a set of HPLC

systems has allowed us to take advantage of their differing selectivities (45), as will be discussed later.

Linking one sugar to any ecdysteroid molecule results in an increase in its polarity. This increase is, however, rather modest because it is equivalent to adding an extra –OH group at position 26 (Table II). This effect is more limited with reversed-phase HPLC than with normal-phase HPLC. The data from Table II can be interpreted in several ways: (*i*) for a given ecdysteroid (20E), it is possible to compare data for the same sugar (glucose) conjugated to various positions (C-2, -3, -22 or -25) of the molecule; (*ii*) for a given position, it is possible to compare the effects of conjugation with different sugars (glucose, galactose, or xylose); and, finally, (*iii*) it is possible to compare the effects of the same conjugation on different ecdysteroids.

First, comparison of the different glucosides of 20E: on reversed-phase, the 22-glucoside is the most polar, and the elution order is 20E22G < 20E25G < 20E2G = 20E3G. On normal-phase, the cyclohexane-based solvent does not separate the different glucosides effectively, whereas the dichloromethane-based solvent allows an efficient resolution of all four molecules (i.e., 20E22G < 20E25G < 20E3G < 20E2G). Surprisingly, this sequence is similar to that obtained with reversed-phase systems.

Second, ecdysteroid glucosides and galactosides behave in a similar way, but ecdysteroid galactosides are, in most cases, slightly less polar molecules than the equivalent glucosides. Xylosides are even less polar; addition of this pentose at C-3 does not change the retention of 20E or ponasterone A on reversed-phase systems. The behavior of limnantheoside C is even more surprising. This molecule is 20E conjugated in position C-3 with a disaccharide (GX). Although the effect of the two sugars appears additive with normal-phase systems, resulting in much increased retention times, this molecule elutes after 20E3G in both reversed-phase systems.

Third, the effect of sugar conjugation is very similar when the position involved in conjugation is remote from the location of any structural difference in the aglycones. Thus, 20E22Gal/20E and IntA22Gal/IntA give similar a-values in three of the four solvent systems tested. The same is true for the 20E3X/20E and PonA3X/PonA pairs (see Table II). On the other hand, the effects are different in the case of 2-deoxy/3G or 5-hydroxy/3G, in which some interactions might be expected to occur between the conjugating moiety and the site of aglycone modification.

Isolation of 2d20E 22G from Silene nutans

Ethanolic extracts from dried aerial parts of *Silene nutans* were purified by a combination of low-pressure and HPLC steps. The latter yielded, together with several previously known ecdysteroids, a minor component (compound U; 1.4 mg), which did not correspond to any available reference compound. This compound showed a typical UV absorbance (in MeOH) with a maximum at 242.5 nm. The chemical ionization spectrum gave ions at 644 $(M+H+NH_3)^+$, 627 $(M+H)^+$, 609 $(M+H–H_2O)^+$, 591 $(M+H–2H_2O)^+$, 573 $(M+H–3H_2O)^+$, 479, 461, 447 $(M+H–hexose)^+$, 429, 411, 393, 347, 329, and 180. These data are

Table II. HPLC Behavior of Some Representative Ecdysteroids and Their Glycosides*

	RP1			RP2			NP1			NP2		
Compound	Ret (min)	*k*'	α	Ret (min)	*k*'	α	Ret (min)	*k*'	α	Ret (min)	*k*'	α
20-Hydroxyecdysone (20E)	11.1	3.1		13.9	4.1		9.6	2.6		13.0	3.8	
20E 2-glucoside	8.5	2.1	0.69	10.2	2.8	0.68	43.4	15.1	5.80	31.6	10.7	2.82
20E 3-glucoside	8.5	2.1	0.69	10.5	2.9	0.70	39.4	13.6	5.23	31.5	10.7	2.81
20E 22-glucoside	6.7	1.5	0.48	7.5	1.8	0.43	33.3	11.3	4.36	33.0	11.2	2.95
20E 25-glucoside	7.6	1.8	0.59	9.1	2.4	0.58	36.0	12.3	4.74	34.9	11.9	3.14
20E 3-galactoside (Sileneoside D)	8.8	2.3	0.73	11.0	3.1	0.75	32.4	11.0	4.23	30.2	10.2	2.68
20E 22-galactoside (Sileneoside A)	6.6	1.4	0.47	8.3	2.1	0.51	28.0	9.4	3.60	29.1	9.8	2.57
20E 3-xyloside (Limnantheoside A)	11.2	3.1	1.02	13.3	3.9	0.96	19.4	6.2	2.38	24.1	7.9	2.09
Limnantheoside C	10.0	2.7	0.87	11.4	3.2	0.79	63	22.3	8.59	56.5	19.9	5.24
Integristerone A (IntA)	7.9	1.9		8.4	2.1		10.8	3.0		15	4.6	
IntA 22-galactoside (Sileneoside C)	5.2	0.9	0.48	4.9	0.8	0.39	30.0	10.1	3.37	33.2	11.3	2.48
2-Deoxy-20-hydroxyecdysone (2d20E)	31.6	10.7		49.6	17.4		5.4	1.0		8.4	2.1	
2d20E 22-glucoside	24.0	7.9	0.74	35.5	12.1	0.70	18.8	6.0	5.96	20.3	6.5	3.09
2-Deoxyecdysone (2dE)	77.6	27.7		183.8	67.0		4.4	0.6		6.8	1.5	
2dE 3-glucoside (Sileneoside E)	37.0	12.7	0.46	72.5	25.8	0.39	15.0	4.6	7.24	18.0	5.7	3.73
Ponasterone A (25d20E)	61.1	21.6		155.1	56.4		4.7	0.7		6.3	1.3	
25d20E 3-xyloside (Limnantheoside B)	62.6	22.2	1.03	146.3	53.2	0.94	7.8	1.9	2.55	10.1	2.7	2.06
25-Deoxyecdysone	–			–			2.7			5.6	1.1	
25dE 22-glucoside	–			–			4.3			11.9	3.4	3.17
Polypodine B	10.4	2.9		12.5	3.6		8.0	2.0		12.9	3.8	
Polypodine B 3-glucoside	7.4	1.7	0.61	7.4	1.7	0.48	35.4	12.1	6.17	31.8	10.8	2.85
26-Hydroxyecdysone (26E)	12.6	3.7	1.18	18.9	6.0	1.45	14.8	4.5	1.75	18.9	6.0	1.57
20,26-Dihydroxyecdysone (20,26E)	6.5	1.4	0.45	7.0	1.6	0.57	22.6	7.4	2.88	25.8	8.6	2.24

* The capacity factor (*k*') is defined as $(t_R - t_0)/t_0$, and the selectivity factor (α) is calculated relatively to the corresponding free ecdysteroid, and to 20E for 26E and 20,26E.

consistent with a molecular weight of 626 amu, in agreement with the empirical formula $C_{33}H_{52}O_{11}$. This conclusion was further assessed by high-resolution mass spectrometry (MS) using CI–D with methane as the reagent gas: compound U gave ions at 627.3744 ($[M+H]^+$, $C_{33}H_{53}O_{11}$ gives 627.3740) and 609.3639 ($[M+H–H_2O]^+$, $C_{33}H_{51}O_{10}$ gives 609.3643). The presence of 33 carbons suggested a hexose conjugate of an ecdysteroid genin (which are commonly C_{27} molecules).

NMR data for compound U and reference 2-deoxy-20-hydroxyecdysone (2d20E) are reported in Tables III and IV. 1D ^{1}H and ^{13}C spectra and 2D correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), heteronuclear multiple–quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC) NMR spectra allowed all of the ^{1}H and ^{13}C assignments. In the ^{1}H (Table III) and ^{13}C NMR (Table IV) spectra, signals for the protons and carbons of the steroidal ring system were identical to those of 2d20E, with the exception of the signals H-22 (δ 3.65) and C-22 (δ 89.1) in the side-chain, which were more deshielded and, thus, suggested the attachment of a hexose at C-22. The presence of a hexose moiety was evident from the peaks in the region δ 3.3–4.6 ppm. The identity of the sugar as β-D-glucopyranose was determined from the signal for the anomeric proton at δ 4.54 (δ, J = 7.8 Hz) (46) and ^{1}H–^{1}H coupling patterns observed in its ^{1}H NMR (Table III). In the ^{13}C NMR spectrum (Table IV), the presence of six additional oxygenated carbon signals was evident from the carbon resonances in the region δ 61.5–104.30 ppm. The chemical shift for C-1' (δ 105.8) supported the presence of a β-D-glucopyranose unit (44). The attachment was confirmed from the ^{1}H–^{13}C long-range coupling between the anomeric proton (H-1') and C-22 in the HMBC spectrum (47). The structure was, therefore, assigned as 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucoside (Figure 2). This compound is identical to the one independently isolated by Báthori et al. (27).

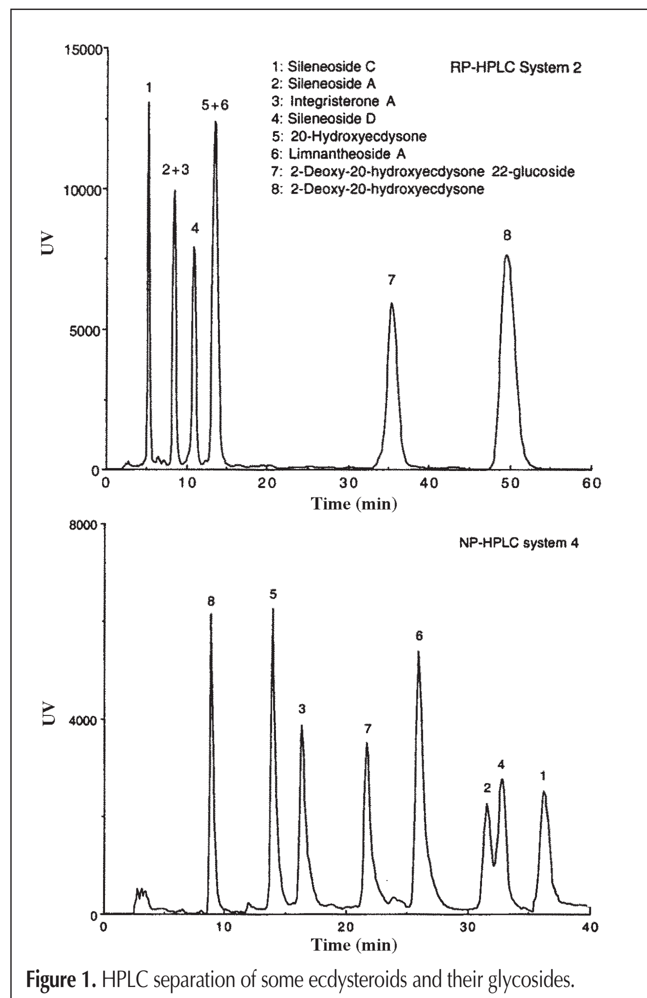

Figure 1. HPLC separation of some ecdysteroids and their glycosides.

General strategy for the identification of ecdysteroid glycosides

The identification of glycoside conjugates of ecdysteroids is obtained first from MS data that indicate whether the formula weight is compatible with the presence of hexose, pentose, or oligo-glycoside conjugates. MS using soft ionization techniques (CI–D, fast-atom bombardment, or electrospray) generates rather abundant pseudomolecular ions that provide good evidence for the addition of an hexose (+162) or a pentose (+132) when compared with the free ecdysteroid (480 if 20E). Other characteristic ions are observed that correspond to the loss of one or two water molecules, to the loss of the sugar (–180 or –150), or to the sugar itself (180 or 150).

The presence of a sugar can be rapidly confirmed thanks to the examination of ^{1}H and ^{13}C NMR data in which one observes additional peaks in the region of hydrogen bound to oxygenated carbons (3.3–5.5 ppm) and, in the ^{13}C NMR spectrum, signals for the corresponding carbons (50–110 ppm). Furthermore, the number of oxymethine and oxymethylene groups can be estimated, and this indicates the nature of the sugar (hexose, pentose, or oligoglucoside)

1D ^{1}H and ^{13}C spectra and 2D COSY, TOCSY, pulsed-field gradient (PFG)-enhanced heteronuclear single-quantum coherence, and PFG-enhanced HMBC NMR spectra allow for all of the ^{1}H and ^{13}C assignments. With a 500-MHz spectrometer, this can be achieved with only 100 µg of pure compound. These data normally allow one to assign the identity of the ecdysteroid aglycone (47,48). The position of attachment of the glycoside moiety can then be located.

(1) By comparison of the ^{1}H and ^{13}C chemical shifts of the glycoside conjugate of ecdysteroid with respect to the corresponding chemical shifts of the nonconjugated ecdysteroid aglycone, one observes small variation (~ 0.1–0.5 ppm) for the chemical shifts of the ^{1}H signal of the oxymethine group of the ecdysteroid engaged in the glycoside link and of the neighboring protons. The assignment is more straightforward from the large variation (~ 5–12 ppm) of the chemical shifts of the ^{13}C signal corresponding to the oxymethine (or oxymethylene) groups.

(2) However, the glycoside link is unambiguously established from 2D PFG-HMBC (thanks to the ^{1}H –^{13}C long-range [^{3}J] coupling), where one can observe a ^{1}H–^{13}C correlation between the ^{1}H signal of the oxymethine group of the sugar (generally the anomeric proton H1') and the carbon of the ecdysteroid where the sugar is bound. Reciprocally, if the sugar is linked to the ecdysteroid via an oxymethine group, one observes ^{1}H–^{13}C correlation between ^{1}H signal of the oxymethine group of the ecdysteroid and the carbon of the sugar where the ecdysteroid is linked (Figure 3).

(3) The position involved in glycoside conjugation can also be assigned/confirmed through nuclear Overhauser effect (nOe)

experiments, which reveal spatial proximity between protons of the sugar and the aglycone part. However, as 2D PFG-HMBC is unambiguous (see previous), these data are more useful for molecular modelling and 3D-structure determination of the compound.

Once the structure of the ecdysteroid nucleus and the position of the glycoside link is established, the identity of the sugar can be worked out by careful examination of ^{1}H–^{1}H coupling patterns observed in ^{1}H NMR. As ^{3}J H_{axial}–H_{axial} coupling constants (7–8 Hz) are large with respect to ^{3}J H_{axial}–$H_{equatorial}$ and ^{3}J $H_{equatorial}$–$H_{equatorial}$ coupling constants (3–4 Hz), the axial or equatorial position of H1', H2', H3'–H4', and H5' can be determined from the values of whole ^{3}J H–H coupling constants of the sugar. If necessary, nOe experiments allow the determination of the relative spatial proximity to provide additional evidence.

First, α- or β-configurations are determined for glucoside, galactoside and xyloside moieties (Figure 4) by observation of the ^{3}J H1'–H2' coupling constant, as H2' is axial for these sugars in the pyranoside form. For a compound with an α-configuration, H1' is equatorial, leading to a small $H1'_{equatorial}$–$H2'_{axial}$ coupling constant (3–4 Hz), but for β-configuration, H1' is axial, leading to a large $H1'_{axial}$–$H2'_{axial}$ coupling constant (7–8 Hz). This assignment can also be confirmed because of the ^{1}H and 13C chemical shifts of the anomeric ^{1}H and 13C, which are relatively different for α- or β-configurations of the glucosides. These values can be compared with reference compounds as α- or β-methyl glycosides for which data could be found in the literature (44) or data base. A nOe experiment could also be useful as the anomeric

Table III. Chemical Shifts (^{1}H) of Some Ecdysteroids*

Proton	2d20E	2d20E 22-β-D-glucopyranoside	20-Hydroxy-ecdysone	Sileneoside A (20E 22α-gal)	Sileneoside D (20E 3α-gal)
1ax	1.39	1.39	1.38	1.38 *t* (13)	1.51 *t* (12.5)
1eq	1.70	1.70	1.88	1.88	1.94
2ax	1.67†	1.67†	3.99 m ($w_{1/2}$ = 22)	3.99 m ($w_{1/2}$ = 22)	4.03 *m*
2eq	1.85†	1.85†	–	–	–
3eq	4.11 *m* ($w_{1/2}$ = 23)	4.12 *m* ($w_{1/2}$ = 24)	4.07 *m* ($w_{1/2}$ = 8)	4.07 *m* ($w_{1/2}$ = 8)	4.05 *m*
4ax	1.62	1.62	1.75	1.75	1.74†
4eq	1.75	1.77	1.75	1.75	1.95†
5	2.40 *dd* (12, 2)	2.41 *dd* (12, 2.5)	2.36 *t*‡	2.36 *t*‡	2.37 *dd* (13.7, 1.8)
7	5.97 *d* (1.8)	5.97 *d* (2.2)	5.97 *d* (2.5)	5.97 *d* (2.3)	5.97 *d* (2)
9ax	3.16 *m* ($w_{1/2}$ = 26)	3.17 *m* ($w_{1/2}$ = 26)	3.11 *m* ($w_{1/2}$ = 22)	3.11 *m* ($w_{1/2}$ = 22)	3.10 *m* ($w_{1/2}$ = 22)
11ax	1.67	1.67	1.73	1.73	1.73
11eq	1.78	1.78	1.86	1.87	1.86
12ax	1.96	1.97	1.95	1.98	1.97
12eq	1.70	1.70	1.75	1.75	1.75
15a†	2.07 *m*	2.08 *m*	2.05	2.05	2.05
15b†	1.65	1.65	1.65	1.66	1.65
16a†	1.90	1.94	1.90	1.92	1.87
16b†	1.80	1.79	1.80	1.78	1.80
17	2.34 *t* (9.3)	2.29 *t* (9.5)	2.34 *m*	2.28 *t* (9)	2.32 *t* (9)
22	3.44 *d* (10)	3.65 *d,br* (7)	3.43 *d* (10)	3.44 *d* (8.7)	3.43 *d* (10)
23a	1.33	1.55	1.33	1.49	1.32 *m*
23b	1.67	1.75	1.65	1.70	1.65
24a	1.80	1.97	1.80	1.85	1.78
24b	1.51 *dt* (3.6,12.5)	1.55	1.51 *dt* (12.8, 3.4)	1.54 *dt* (12.3, 2.7)	1.49
18-Me	0.87 *s*	0.88 *s*	0.87 *s*	0.87 *s*	0.87 *s*
19-Me	0.98 *s*	0.98 *s*	1.00 *s*	1.00 *s*	1.00 *s*
21-Me	1.24 *s*	1.29 *s*	1.24 *s*	1.326 *s*	1.24 *s*
26-Me	1.23 *s*	1.24 *s*	1.23 *s*	1.219 *s*	1.22 *s*
27-Me	1.24 *s*	1.25 *s*	1.24 *s*	1.232 *s*	1.23 *s*
1′	—	4.54 *d* (7.8)	—	5.11 *d* (3.8)	5.14 *d* (3.8)
2′	—	3.38 *dd* (9, 7.8)	—	3.89 *dd* (10.5, 3.6)	3.83 *dd* (10.4, 3.9)
3′	—	3.52 *t* (9)	—	3.93 *dd* (10.5, 2.8)	3.95 *dd* (10.4, 3.3)
4′	—	3.46 *m*	—	4.03 *m* ($w_{1/2}$ = 6)	4.04 *m* ($w_{1/2}$ = 6)
5′	—	3.46 *m*	—	4.10 *t* (6.5)	4.15 *m*
6′	—	3.93 *d* (12.5)	—	3.77 *dd* (11.5, 6)	3.76 *dd* (11.8, 7.6) Sys. AB
6″	—	3.76 *dd* (12.5, 5)	—	3.72 *dd* (11.5, 7.1)	3.79 *dd* (12, 5.4) Sys. AB

* Solutions in D_2O referenced to TSP-d_4. Multiplicity of signals: singlet (*s*), doublet (*d*), triplet (t), multiplet (*m*), broad signal (*br*), width at half-height in Hertz ($w_{1/2}$), and δ in ppm.
† Assignments could be reversed.
‡ Triplet-like signal (4ax and 4eq isochronous).

proton H1'(axial) is on the opposite face of the sugar for β-glycosides with respect to H6'–H6". This leads to small nOes with these protons and a strong one with $H5'_{axial}$, which is on the same face.

Second, distinction between glucosides and galactosides can be achieved by examination of the ^3J H3'–H4' coupling constant corresponding to a large $H3'_{axial}$–$H4'_{axial}$ coupling constant (7–8 Hz) for glucosides and a small $H3'_{axial}$–$H4'_{equatorial}$ coupling constant (3–4 Hz) for galactosides.

Biological activity of ecdysteroid glycosides in insect assays

Table V summarizes the biological activities of several ecdysteroid glycosides (glucosides, galactosides, and xylosides) in the *Drosophila melanogaster* B_{II} cell bioassay for ecdysteroid agonists and antagonists. The activities of each of the glycosides is signficantly lower than that of the corresponding free ecdysteroid. Because the possibility cannot be completely excluded that ecdysteroid glycosides may undergo a partial hydrolysis during the course of the bioassay, the activities determined for the conjugates should be regarded as maximal activities.

Table IV. Chemical Shifts (^{13}C) of Some Ecdysteroids

Carbon	Multiplicity	2d20E	2d20E 22β-D glucopyranoside	20-Hydroxy-ecdysone	Sileneoside A 20E 22α-gal	Sileneoside D 20E 3-α-gal
1	CH_2	30.6	30.8	37.9	37.8	39.1
2	CH_2 or CH	29.6	29.5	69.8	69.9	70.3
3	CH	67.2	67.2	69.7	69.7	79.9
4	CH_2	34.2	34.2	33.8	33.7	33.5
5	CH	53.8	53.8	52.9	53.1	54.1
6	C	*	*	210.8	211.0	211.0
7	CH	123.3	123.3	123.6	123.7	123.7
8	C	*	*	*	*	171.1
9	CH	38.9	39.1	36.4	36 .3	36.5
10	C	38.9	39.1	40.6	40.6	40.5
11	CH_2	22.5	22.7	22.5	22.4	22.4
12	CH_2	33.4	33.9	33.5	33.4	33.1
13	C	49.8	50.5	49.8	50.3	50.1
14	C	87.6	87.9	87.6	87.7	87.7
15	CH_2	32.6	32.6	32.7	32.6	32.8
16	CH_2	22.6	22.6	22.5	22.4	22.4
17	CH	51.7	52.2	51.7	51.7	51.7
18	CH_3	19.5	19.5	19.5	19.5	19.5
19	CH_3	25.6	25.6	25.7	25.5	25.5
20	CH	80.7	79.9	80.9	82.1	80.6
21	CH_3	22.1	23.4	22.1	23.2	22.0
22	CH	79.8	90.6	79.9	92.0	79.9
23	CH_2	28.3	26.8	28.5	28.3	28.4
24	CH_2	42.9	42.3	43.1	43.0	43.1
25	C	74.0	74.2	74.1	74.3	74.6
26	CH_3	29.8	30.0	29.8	30.2	30.0
27	CH_3	30.7	30.8	30.8	30.9	30.6
1'	CH	—	105.8	—	104.4	103.7
2'	CH	—	76.1	—	72.0	71.7
3'	CH	—	78.4	—	72.0	72.1
4'	CH	—	72.3	—	71.8	72.0
5'	CH_2	—	78.5	—	73.8	74.3
6'	CH_2	—	63.0	—	63.3	63.8

* Signal not detected (concentration of the sample too low); solutions in D_2O, referenced to TSP-d_4.

PonA 3G was previously found to be highly active in the in vivo *Sarcophaga* bioassay (49,50), but this may be a consequence of extensive hydrolysis of the conjugating moiety to release free ponasterone A. Sileneosides A (20E22gal), C (IntA22gal), and E (2dE3gal) were biologically inactive when injected into *Dermestes vulpinus*, *Galleria mellonella*, and *Sarcophaga bullata*. Furthermore, a range of 20E glucosides was predominantly inactive in these species after injection or topical application (51). Sileneosides A and C possess low, but quantitatable activity in the B_{II} bioassay, whereas sileneoside E (blechnoside A) was inactive in this assay (Table V).

Within the 20E glucoside series, it is possible to consider the effect of the location of the glucosidic moiety (42) whereby activity decreases in the following order: 20E (1) >> 20E25G (1133-fold less active than 20E) > 20E3G (1733-fold less active than 20E) > 20E22G (6266-fold less active than 20E) > 20E2G (26666-fold less active than 20E). All the glucoside derivatives are considerably less active than 20E.

From a comparison of the activities of 20E3G and 20E3X, it would appear that xylosides are somewhat more active than the corresponding glucosides, which may be a consequence of the reduced bulk or polarity of the xyloside moiety vis-à-vis a glucoside moiety. However, the similar activities of limnantheoside A (20E3X) and limnantheoside C (20E3G[1-3]X) would indicate that bulk extending out from C-3 of the steroid is not a restricting factor in the interaction with the ecdysteroid receptor. The presence of a xylose moiety at C-3 of ponasterone A depresses the activity much more (4839-fold) than an equivalent group at C-3 of 20-hydroxyecdysone (213-fold), indicating that spatial constraints affect higher affinity interactions more extensively, which accords with the suggestion that the ligand-binding cavity of the ecdysteroid receptor can, to some extent, change its conformation to accommodate the ligand (52). Thus, although there is excellent complementarity between poA and the binding cavity, the addition of a 3-xylose moiety disturbs this close complementarity extensively. Because the fit of 20E is not as good as that of poA, there is more scope to accommodate a glycose moiety on 20E such that, although it does still reduce activity, the extent of the reduction is not as great as for the poA/poA3X pair. Similarly, the biological activity of 2d20E22G is only 4.5-fold lower than that of the moderately active 2d20E. In the case of the poor ligand, 2dE, the addition of a glucose unit hardly affects the biological activity at all. In fact, 2dE25G has a slightly greater activity, suggesting that, when the fit of the genin is poor, addition of a glucose unit may actually slightly improve the interaction (Table V).

Extensive quantitative structure-activity relationships (QSAR) studies (53,54) and x-ray crystallographic studies of the ecdysone receptor ligand- binding domains (52) have started to shed

light on the role of each of the hydroxyl groups in an ecdysteroid, such as 20E as hydrogen bond donors or acceptors and the spatial constraints around several positions of the steroid. A hydroxyl at C-25 is detrimental to receptor affinity, and it is known that synthetic ecdysteroids with extended side-chains retain biological activity (55); therefore, it is not surprising that the C-25 monoglucoside retains the greatest activity amongst the various 20E monoglucosides. Its much lower activity, relative to 20E, is probably a consequence of the side-chain needing to fit into a nonpolar cylinder within the ligand-binding pocket. 4D-QSAR (54) indicates that the hydroxyl groups at C-2 and C-22 should be H-bond acceptors, which, while not being prevented by glycosylation, could be hindered especially if one takes into account the bulky nature of the glycose unit. 4D-QSAR predicts restricted space around the C-2 hydroxyl, which is in accord with the particularly low activity of 20E2G.

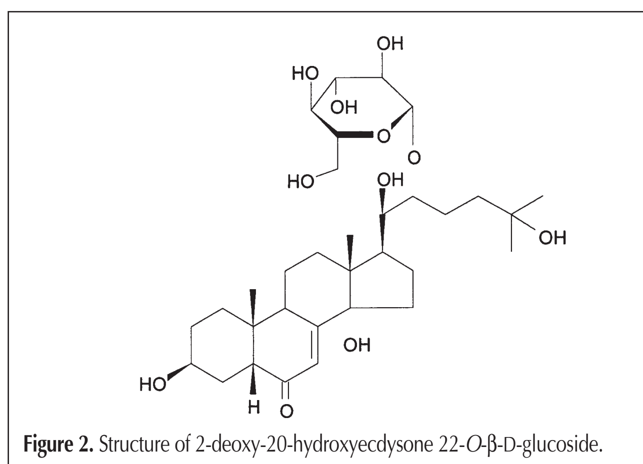

Figure 2. Structure of 2-deoxy-20-hydroxyecdysone 22-*O*-β-D-glucoside.

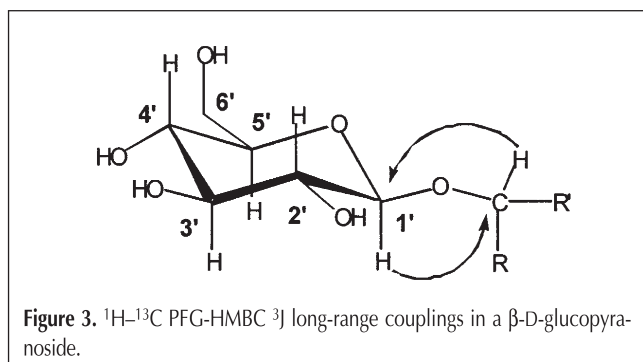

Figure 3. ^{1}H–^{13}C PFG-HMBC ^{3}J long-range couplings in a β-D-glucopyranoside.

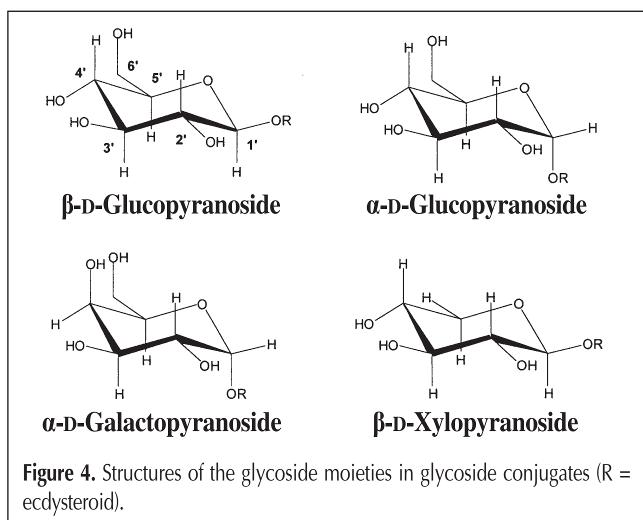

Figure 4. Structures of the glycoside moieties in glycoside conjugates (R = ecdysteroid).

Significance of ecdysteroid glycosides in insects

Glycoside conjugation seems a minor pathway by comparison with conjugation with phosphates or fatty acids. In *Manduca sexta* embryos, a glucose conjugate of 26-hydroxyecdysone (26E) accumulates during the second half of embryonic development at the expense of free 26E and its phosphate conjugate (55,56), and the significance of this pattern is presently unclear. Surprisingly, glycoside conjugation has not been described for other species—with the exception of an early work on *Calliphora erythrocephala* (57) showing a rapid conjugation by the fat-body of 20E and ponasterone A into conjugates tentatively identified as 3-glucosides. More recently, glycoside formation was only documented in insects infested by baculoviruses (58).

Table V. Biological Activities of Some Ecdysteroid Glycosides and Their Parent Free Ecdysteroids*

Compound	BII cell assay EC50 value (M)
20-Hydroxyecdysone (20E)	7.5×10^{-9}
20E 2G	2.0×10^{-4}
20E 3G	1.3×10^{-5}
20E 3gal (Sileneoside D)	3.0×10^{-5}
20E 3X (Limnantheoside A)	1.6×10^{-6}
Limnantheoside C	1.3×10^{-6}
20E 22G	4.7×10^{-5}
20E 22gal (Sileneoside A)	4.1×10^{-5}
20E 25G	8.5×10^{-6}
2d20E	6.6×10^{-7}
2d20E22G	3.0×10^{-6}
Ponasterone A	3.1×10^{-10}
Ponasterone A 3X	1.5×10^{-6}
2-Deoxyecdysone (2dE)	2.0×10^{-5}
2dE 3gal (Sileneoside E)	Inactive
2dE 22G	2.0×10^{-5}
2dE 25G	7.3×10^{-6}
Integristerone A	2.0×10^{-7}
Sileneoside C (IntA22gal)	1.0×10^{-4}

* EC50 = efficient concentration giving half-maximal response.

Table VI. Number of Each Category of Glycosides in Plants

Sugar position	Glucose	Galactose	Xylose	Total
2	1	–	–	1
3	9	4	3	16
22	4	4	–	8
24	1	–	–	1
25	4	–	–	4
Total	19	8	3	30

The significance of ecdysteroid glycosides in plants

Up to now, many sugar derivatives of ecdysteroids have been isolated, most of them from plants of the *Silene* genus. The majority are conjugates of the major ecdysteroid (i.e., 20-hydroxyecdysone), although glycosides of integristerone A and ponasterone A have also been described (Table I). In the present study, the aglycone moiety of Compound U was identified as 2d20E. Therefore, glycosylation is probably not very substrate-specific. As shown in Table VI, most glycosides isolated so far from plants are glucosides, followed by galactosides. Conjugation preferentially involves carbon 3 and then 22. With the exception of *Silene brahuica*, glycosides seem to be minor components of the ecdysteroid cocktail. They possibly represent a means to sequester ecdysteroids in cell vacuoles, or alternatively a way to transport them between plant organs (59), but the substantiation of such hypotheses requires additional experiments.

Conclusion

Are ecdysteroid glycosides important for insect–plant relationships? It is already known that ecdysteroids can be detected by taste receptors (60–62) and therefore deter insects, but up to now only free ecdysteroids have been tested in such systems. It would be of interest to test ecdysteroid glycosides alone or in combination with the free genins in order to determine their biological activity. Finally, it cannot be excluded that these molecules can display cytotoxic and haemolytic properties, as described for the polyhydroxysteroid glycosides from starfish (63).

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