New Glucoside Conjugates and Other Cardenolide Glycosides from the Monarch Butterfly Reared on *Asclepias fruticosa* L.

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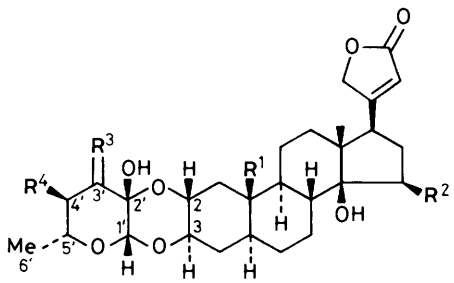
Eight cardenolide glycosides and one genin, including two new glucoside conjugates, have been isolated from adult monarch butterflies (*Danaus plexippus* L.) the larvae of which were reared on *Asclepias fruticosa* L. leaves. The glycosides calactin, calotropin (major components), calotoxin, and afroside were identified by comparison with authentic samples. The remaining compounds, after conversion into peracetates, have been shown by 400 MHz ^{1}H n.m.r. and mass spectra (and for the first two compounds by direct comparison) to be the genin calotropagenin and the glycosides frugoside, gofruside, gomphogenin 3-β-D-glucopyranoside, and calotropagenin 3-β-D-glucopyranoside. The relationship between these compounds from monarch butterflies and those previously isolated from the food plant *A. fruticosa* is discussed.

Plants of the genus *Asclepias* (of the milkweed family, Asclepiadaceae) have been used in folk medicine,[1,2] and are also responsible for livestock poisoning.[3] Their pharmacological activity is due chiefly to a group of cardenolide glycosides, which are inotropic and cytotoxic agents, causing an increase in the force of contraction of the heart[4] and inhibiting the growth of primate tumour cells.[1,5] The *Asclepias* compounds are thus similar pharmacologically to the heart drugs from *Digitalis* spp. such as digoxin.[6] However, most *Asclepias* glycosides differ from the *Digitalis* glycosides in having an *A/B-trans*-steroid skeleton and a carbohydrate moiety which, as first shown for gomphoside (**1**), is joined to the 2α- and 3β-positions of the aglycone through hemiacetal and acetal links,[7,8] and which is thus relatively stable to cleavage by acids[7] or reduction.[9]

Asclepias spp. are also notable as food plants from which some insects sequester cardiac glycosides for defence against vertebrate predators.[3,10–13] The most extensive studies on this plant–herbivore–predator interaction have been those on the sequestration of compounds by the larvae of the monarch butterfly (*Danaus plexippus* L.). Among these investigations are those in which caterpillars were fed with individual cardenolide glycosides and genins, or were reared on *Asclepias* spp.[13–17] Such studies indicate that compounds of intermediate and high polarity are stored unchanged. Examples are calactin (**2**), calotropin (**3**), humistratin (**7**), calotoxin (**4**), desglucosyrioside (**9**), and syriobioside (**8**), all with a doubly linked 6-deoxy- or 4,6-dideoxy-hexosulose; and aspecioside (**19**), with an analogous sugar but singly linked. The least polar compounds tend to be converted into more polar analogues. For example uscharidin (**6**) and labriformin (**10**), with oxo groups at position 3′ of the doubly linked carbohydrate, are metabolised to the corresponding compounds with 3′-hydroxy groups, *viz.* the 3′-epimers calactin (**2**)/calotropin (**3**), and desglucosyrioside (**9**), respectively.

Cardenolide Glycosides from Monarch Butterflies Reared on A. Fruticosa *Leaves.*—Recently the isolation of fifteen cardenolide glycosides, including eight new compounds, from the leaves of *A. fruticosa* L. (*Gomphocarpus fruticosus* R. Br.) growing near Sydney in Australia, was reported.[18] In this paper, we give the results of complementary work on the identity of eight cardenolide glycosides and a genin isolated from adult monarch butterflies reared as larvae on *A. fruticosa* leaves. Both larvae and leaves were collected from the same site as in the earlier work. The quantitative aspects of cardenolide storage from this plant are discussed in relation to earlier studies in a separate paper.[19]



	R^1	R^2	R^3	R^4
(**1**)	Me	H	β-OH, α-H	H
(**2**) Calactin	CHO	H	β-OH, α-H	H
(**3**) Calotropin	CHO	H	α-OH, β-H	H
(**4**) Calotoxin	CHO	H	β-OH, α-H	OH
(**5**) Afroside	Me	OH	β-OH, α-H	H
(**6**)	CHO	H	O	H
(**7**) (with Δ^7)	CHO	H	β-OH, α-H	H

Four cardenolide glycosides with doubly linked sugars were identified by comparison with authentic samples. They are oxidised analogues of gomphoside (**1**), *viz.* the 15β-hydroxy analogue afroside (**5**),[20] and the 19-aldehydes calactin (**2**), calotropin (**3**), and calotoxin (**4**).[18,21]

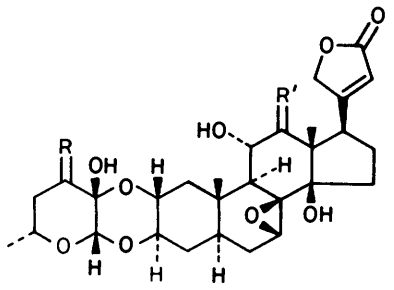
The remaining five compounds comprise one genin and four glycosides with singly linked sugars. These have been shown to be calotropagenin (**20**);[22] gofruside (**15**) and frugoside (**16**),[23] which are respectively the 3-(6-deoxy-β-D-allopyranoside)s of corotoxigenin and coroglaucigenin; and the 3-(β-D-glucopyranoside)s (**12**) and (**11**) of calotropagenin (**20**) and gomphogenin (**22**).[22] To our knowledge, the last two glycosides have not been described previously.

For purification and spectral analysis, these five compounds were converted into the peracetates under conditions such that the 14β-hydroxy group is not acetylated. In mass spectra under methane chemical ionisation (c.i.) conditions there were formed, apart from the protonated molecular ion MH^+, ions [labelled GH^+ and (*a*), respectively] diagnostic of the gross structure of the aglycone and of the carbohydrate (if present) (Table 1).

Table 1. Ions in methane chemical ionisation mass spectra (relative abundances in parentheses)[a]

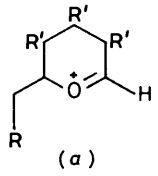
	MH^+ and derived ions		GH^+ and derived ions				(*a*) and derived ions	
Calotropagenin 2,3-diacetate (**21**)	(see under GH^+)		489 (95)		429 (100)	411 (28)		
Calotropagenin 3-glucoside penta-acetate (**14**)	777 (22)	717 (3)	447 (2)	429 (5)			331 (100)	271 (10)
Gomphogenin 3-glucoside penta-acetate (**13**)	763 (67)	703 (5)	433 (5)	415 (17)		355 (8)	331 (100)	
Gofruside triacetate (**17**)	661 (26)	601 (2)		371 (17)			273 (100)	213 (5)
Frugoside tetra-acetate (**18**)	705 (48)	645 (3)					273 (100)	213 (6)

[a] GH^+ refers to the protonated genin. Ion (*a*) derived from the carbohydrate has R = R^1 = OAc for the glucosides (**13**) and (**14**), and R = H, R^1 = OAc for the 6-deoxyallosides (**17**) and (**18**).



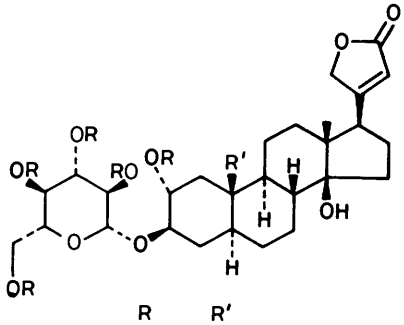
	R	R'
(**8**)	β-OH, α-H	α-OH, β-H
(**9**)	β-OH, α-H	O
(**10**)		O

Each is accompanied by ions derived by the loss of molecules of acetic acid and/or water. The mass of ion (*a*) shows the presence of four hydroxy groups in the carbohydrate of the glucosides (**11**) and (**12**), and of three in gofruside (**15**) and frugoside (**16**), which are 6-deoxyallosides.

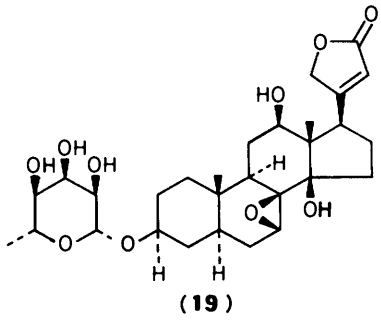


(*a*)

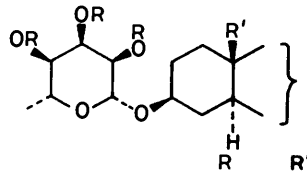
In the following discussion on the ^1H n.m.r. data of the acetylated compounds, features of the aglycone and carbohydrate signals are covered separately. All have the following common signals in the 400 MHz n.m.r. spectrum (Table 2):



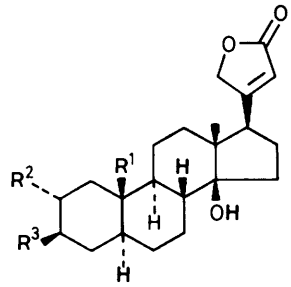
	R	R'
(**11**)	H	Me
(**12**)	H	CHO
(**13**)	Ac	Me
(**14**)	Ac	CHO



(**19**)



		R	R'
(**15**)	Gofruside	H	CHO
(**16**)	Frugoside	H	CH_2OH
(**17**)		Ac	CHO
(**18**)		Ac	CH_2OAc



	R^1	R^2	R^3
(**20**)	CHO	OH	OH
(**21**)	CHO	OAc	OAc
(**22**)	Me	OH	OH
(**23**)	Me	H	OH

Table 2. 400 MHz ^{1}H N.m.r. data, (half-height-widths in Hz in parentheses)[a]

Proton	Calotropagenin 2,3-diacetate (**21**)	Calotropagenin 3-(β-D-glucopyranoside) 2,2′,3′,4′,5′,6′-penta-acetate (**14**)	Gomphogenin 3-(β-D-glucopyranoside) 2,2′,3′,4′,5′,6′-penta-acetate (**13**)	Gofruside 2′,3′,4′-tri-acetate (**17**)	Frugoside 2′,3′,4′,19-tetra-acetate (**18**)	Frugoside (**6**) (from *Calotropis procera*)[25]	Aspecioside[b] (**19**) (from *A. speciosa*)
1′-H		4.59 d	4.62 d	*ca.* 4.8 m	4.86 d	4.72 d	4.67 d
2′-H		4.87 t	4.94 m[f]		4.81 dd[f]	3.42 ddd[e]	3.36 dd
3′-H		5.17 t	5.20 t	5.59 t	5.61 t	4.24 m	4.12 t
4′-H		5.10 t	5.09 t	4.66 dd	4.67 dd	3.30 dt[e]	3.24 dd
5′-H		*ca.* 3.7 m[f]	*ca.* 3.7 m[f]	3.92 dq	3.94 m	3.70 m[f]	3.67 dq
6′-H		*ca.* 4.1 m[f]	*ca.* 4.1 m	1.20 d	1.21 d	1.32 d	1.27 d
1-H$_\beta$	2.69 dd	2.67 dd		2.44 dt			
2-H$_\beta$	4.97 m[f](25)	4.82 m[f]	*ca.* 4.95 m[f]				
3-H$_\alpha$	4.84 m[f](25)	*ca.* 3.6 m[f]	*ca.* 3.6 m[f]	3.65 dt	3.66 dt	3.70 m[f]	
17-H$_\alpha$[c]	2.77 dd	2.76 dd	2.77 dd	2.76 dd	2.78 dd	2.78 dd	
18-H	0.82	*f*	*f*	0.82	0.87	0.92	
19-H	10.04	9.99	*f*	9.96	4.10 d	3.79 dd[e]	
					4.34 d	3.90 dd[e]	
21-H[d]	4.79 dd	4.79 dd	4.79 dd	4.78 dd	4.80 dd[f]	4.80 dd	
	4.94 dd	4.93 dd	4.96 dd	4.94 dd	4.96 dd	4.98 dd	
22-H[d]	5.88	5.88	5.88	5.88	5.88	5.88	
OAc	2.01, 2.02	2.01, 2.02, 2.02, 2.03, 2.08	2.01, 2.03, 2.04, 2.05, 2.08	2.01, 2.01, 2.14	2.01, 2.02, 2.05 2.15		

[a] Chemical shifts in p.p.m. from $SiMe_4$ in $CDCl_3$ except for aspecioside (**19**). Symbols d, t, q, and m refer to doublet, *apparent* triplet, quartet, and multiplet, respectively. Most coupling constants are given in Table 3. Decoupling experiments are denoted by braces linking signals. [b] Data from ref. 24 for a solution in 10:1 v/v $CDCl_3$–CD_3OD; carbohydrate signals only given. [c] $J_{16,17\alpha}$ 5.5 and 9 Hz. [d] $J_{21,21}$ 18 Hz: $J_{21,22}$ 1.5 Hz. [e] Extra splitting due to coupling with OH was removed when CD_3OD was added. [f] Masked by water or other signals.

Table 3. ^{1}H–^{1}H Coupling constants (Hz) of carbohydrate and ring *A* protons[a]

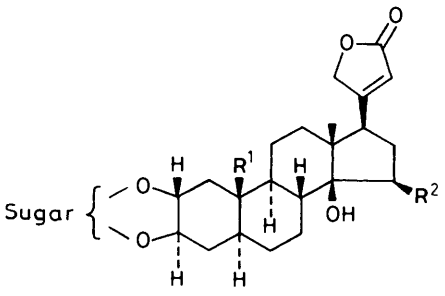
Protons	Genin (**21**)	Peracetyl-β-glucopyranosides (**14**)	Peracetyl-β-glucopyranosides (**13**)	Peracetyl-(6-deoxy-β-allopyranosides) (**17**)	Peracetyl-(6-deoxy-β-allopyranosides) (**18**)	6-Deoxy-β-allopyranosides: Frugoside (**16**)[c]	6-Deoxy-β-allopyranosides: Aspecioside (**19**)[b]
1′,2′		8	8	*e*	8	8	8
2′,3′		*ca.* 9[d]	*ca.* 9.5[d]	3	3	3	3
3′,4′		*ca.* 9.5[d]	*ca.* 9.5[d]	3	3	3	3
4′,5′		*ca.* 10[d]	*ca.* 9.5[d]	10	10	9.5	9.5
5′,6′				6	6	6	6
1α,1β	13	13		13.5			
1β,2β	5	5		*ca.* 4			
2β,3α				10			
3α,4α				5.5			
3α,4β				10			*ca.* 12.5
19,19					12.5	12	

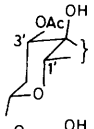
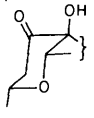
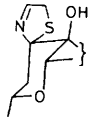
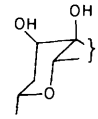
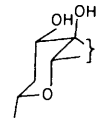
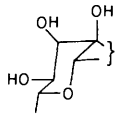
[a] Data refer to $CDCl_3$ as solvent except for aspecioside (**19**). [b] Data from ref. 24 for a solution in 10:1 v/v $CDCl_3$–CD_3OD. [c] From *Calotropis procera*.[25] [d] Approximate first-order analysis since $\Delta\nu/J < 5$ for 3′-H and 4′-H. [e] Coupling constants not measured as spectrum is not first-order.

acetates at δ 2.01—2.15; butenolide 22-H at δ 5.88, and 21-H near δ 4.79 and 4.94; and in the allylic region, 17-H$_\alpha$ near δ 2.77 (doublet of doublets with *J* 5.5 and 9 Hz). Characteristic of the three 19-aldehydes is a singlet for 19-H near δ 10.0 and another doublet of doublets in the δ 2.4—2.7 region due to an equatorial 1-H$_\beta$ (*J* 5 and 13 Hz) which is deshielded by the 19-carbonyl group.[18] This deshielding effect is not seen in gomphogenin 3-glucoside 2,2′,3′,4′,6′-penta-acetate (**13**) nor in frugoside 2′,3′,4′,19-tetra-acetate (**18**), with, respectively, methyl and CH_2OAc (AB quartet at δ 4.22) at C-10. Calotropagenin 2,3-diacetate (**21**) and the two glucoside penta-acetates (**14**) and (**13**) all have an acetoxy function at C-2, and a multiplet at δ 4.8—5.0 is assigned to a proton on this carbon atom. For the former two compounds the relationship of this proton to the deshielded 1-H$_\beta$ was demonstrated by mutual decoupling experiments. The 3-H$_\alpha$ signals of the four acetylated 3-glycosides are found near δ 3.6. For acetylated gofruside and frugoside, (**17**) and (**18**), the observed multiplicity of this signal (Table 2) is consistent with the proton at position 3 being axial. In calotropagenin 2,3-diacetate (**21**) the protons at positions 2 and 3 are *trans*-diaxial and give rise to signals with half-height-widths of 25 Hz. The foregoing n.m.r. data show that for all four glycosides (**11**), (**12**), (**15**), and (**16**), the carbohydrate is attached to position 3 of the steroid aglycone.

The structures and relative stereochemistry of the carbohydrate moieties in the four acetylated glycosides (**13**), (**14**), (**17**), and (**18**) were shown by mutual decouplings which interlink all the vicinal protons in the sugar including those on C-6′ (see Table 2). For the acetylated 3-glucosides (**13**) and (**14**) of gomphogenin and calotropagenin, the uniformly large vicinal coupling constants amongst protons 1′–5′ as shown in Table 3 are consistent with a pyranoside with all substituents equatorial. In particular the 8 Hz *trans*-diaxial coupling between the anomeric proton (1′-H) and the adjacent 2′-H establishes

Table 4. Cardenolides with doubly linked sugars isolated from aerial parts of *A. fruticosa*[18] and, in square brackets, from monarch butterflies reared on the leaves, listed in approximate order of increasing polarity (down, and left to right)



Sugar	R^1 = Me, R^2 = H	R^1 = Me, R^2 = OH	R^1 = CHO, R^2 = H
	3′-*epi*-Gomphoside 3′-acetate[a]	3′-*epi*-Afroside 3′-acetate[a]	Asclepin
	3′-Didehydro-gomphoside	3′-Didehydro-afroside	Uscharidin
	19-Deoxyuscharin		Uscharin[a]
	Gomphoside	Afroside[b] [afroside]	Calactin [calactin]
	3′-*epi*-Gomphoside	3′-*epi*-Afroside	[Calotropin]
	4′β-Hydroxy-gomphoside		[Calotoxin]

[a] Major components of leaves.[18] [b] Major components of stems.[18]

that the sugar moiety is a β-glucoside. Likewise for gofruside 2′,3′,4′-triacetate (**17**) and frugoside 2′,3′.4′.19-tetra-acetate (**18**), the magnitude of the vicinal coupling constants given in Table 3 shows that the substituents at positions 2′,4′, and 5′ of the pyranoside are equatorial, while that at 3′ is axial. Evidence that the anomeric proton is also axial comes from examination of the spectrum of frugoside (**16**)* wherein an 8 Hz coupling between the protons at positions 1′ and 2′ is observed, as for aspecioside (**19**) (Table 3). The same carbohydrate, 6-deoxy-β-allopyranoside, is thus present in aspecioside (**19**) from monarch butterflies reared on *A. speciosa* and *A. syriaca*,[17,24] and in frugoside (**16**) and gofruside (**15**) from those reared on *A. fruticosa*.

* The spectrum was obtained by using a sample of frugoside isolated from *Calotropis procera* seeds,[25] which was related to frugoside from monarch butterflies (see Experimental section) *via* the tetra-acetate.

The two β-glucosides (**11**) and (**12**) from the monarch butterfly have the D absolute configuration at position 5′, since they were cleaved by β-D-glucosidase to gomphogenin (**22**) and calotropagenin (**20**),[22] identified by h.p.l.c. and t.l.c. The same conclusion for all four β-glycosides (**11**), (**12**), (**15**), and (**16**) comes from biogenetic considerations in conjunction with Klyne's rule.[26] Calotropagenin 2,3-diacetate (**21**) and frugoside tetra-acetate (**18**) were also identified by direct comparison.

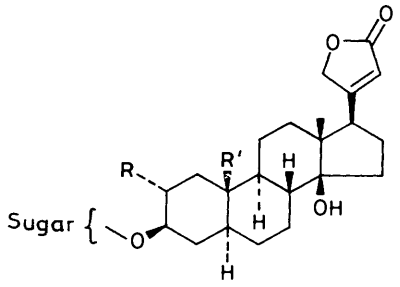
Storage and Transformation of Cardenolines in Monarch Butterflies.—Earlier studies have shown that cardenolines from *Asclepias* spp. are selectively concentrated in the monarch butterfly, with storage reaching a capacity when there is a high cardenolide content in the plant.[15,19] The present work may have some bearing on the processes involved. In Tables 4 and 5 are listed the seventeen compounds isolated from *A. fruticosa*[18,23] and, in square brackets the nine from the monarch butterfly. With regard to glycosides with doubly linked sugars (Table 4), the less polar cardenolide glycosides with α-acetoxy, oxo, or thiazoline groups at position 3′ (shown near the top of Table 4) are not stored as such in the butterfly. Results of earlier feeding experiments, as summarised in the introduction, suggest the possibility of conversion into the corresponding 3′α- or 3′β-alcohols (which are more polar). Among the 'non-polar' cardenolide glycosides, those with a 10-methyl group (see the two left-hand columns in Table 4) constitute a substantial proportion of cardenolide glycosides isolated from the plant.[18] Nevertheless, of the four possible 3′-alcohols (gomphoside, afroside, and their 3′-epimers) only afroside was found in the butterfly (Table 4), and as a very minor component. In contrast, the 3′ epimeric alcohols with the 19-aldehyde group, *viz.* calactin and calotropin (right-hand column in Table 4) are the predominant cardenolide glycosides in the butterfly, accompanied by calotoxin (with an additional hydroxy group at position 4′). It is possible that in the monarch butterfly metabolic transformations other than those noted in the earlier work are taking place (see later).

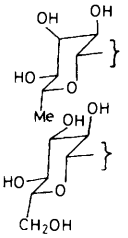
Of the glycosides with singly linked sugars which are found in the monarch butterfly, the 3-(6-deoxy-β-D-alloside)s gofruside (**15**) and frugoside (**16**) were found in the seeds of *A. fruticosa* by Reichstein *et al.*[23] (Table 5) but were not isolated from the aerial parts.[18] It is possible that trace quantities present in the leaves were sequestered and concentrated by the larvae.

The determination of the structures of gomphogenin and calotropagenin 3-β-D-glucosides (**11**) and (**12**) in this work represents, to our knowledge, the first full documentation of cardenolide 3-β-D-glucosides from insects. The latter glucoside is accompanied in the butterfly by the corresponding genin, calotropagenin (**20**) (Table 5). These occurrences suggest a metabolic pathway in the monarch butterfly for cardenolide glycosides with doubly linked sugars that are found in its food plant, such as calotropin and 3′-*epi*-gomphoside (Table 4). This postulated process involves cleavage of the doubly linked sugar to give the genins, which are then conjugated to form the β-D-glucosides. Glucoside formation is a general pathway for the metabolism of foreign compounds in insects.[27] More specifically, the major metabolites stored in monarch butterfly larvae dosed[14] with uzarigenin (**23**) and digitoxigenin (**23**; 5β-H) have been shown by enzymic[3] and mass spectral analysis[28] to be β-D-glucosides.

Some cardenolides, in common with many secondary plant products,[29] occur in the plant as glucosides.[30] However *Asclepias* cardenolide glycosides tend to have at least one other sugar group attached between the genin and glucose, the only known exception being uzarigenin 3-β-D-glucoside.[3,31] The occurrence in the monarch butterfly of the glucosides of calotropagenin and gomphogenin is unlikely to be the result of direct sequestration and storage. Rather it is, we propose, a

Table 5. Cardenolide genins and glycosides with singly linked sugar isolated from *A. fruticosa* and, in square brackets, from monarch butterflies reared on the leaves



Sugar	R^1 = Me, R = H	R^1 = CHO, R = H	R^1 = CH_2OH, R = H	R^1 = Me, R = OH	R^1 = CHO, R = OH
(absent)	Uzarigenin[a]				[Calotropagenin]
		Gofruside[b] [gofruside]	Frugoside[b] [frugoside]		
				[Gomphogenin glucoside]	[Calotropagenin glucoside]

[a] From aerial parts.[18] [b] From seeds.[23]

result of the insect's ability to synthesize these conjugates from the respective genins which are derived mainly from cardenolides with doubly linked sugars.

The foregoing metabolic pathway in the monarch butterfly has some parallel in mammals. In rats dosed with gomphoside (**1**), a minor metabolite excreted was gomphogenin (**22**), formed by stepwise cleavage of the doubly linked 4,6-dideoxyhexosulose. The major metabolite was gomphoside 3′-β-D-glucuronide, formed by direct conjugation of glucuronic acid to the sugar moiety of gomphoside.[32]

Experimental

Cardenolide glycosides and a genin isolated in sub-milligram quantities from the monarch butterfly were characterised, in some cases after acetylation, by chemical ionisation mass spectra (Table 1) and 400 MHz ^{1}H n.m.r. spectra (Tables 2 and 3). The former were obtained using a Finnigan 3200E GC mass spectrometer and associated Finnigan 6110 data system, with methane at 1 Torr as the reagent gas. 400 MHz N.m.r. spectra were run on a Bruker WM 400 spectrometer. T.l.c. separations were carried out preparatively on 0.25 mm thick silica layers on 10 × 20 cm (width) plates, unless otherwise stated. Plates were pre-washed by development with 1% ammonia in diethyl ether. Preparative high-pressure liquid chromatography (h.p.l.c.) was performed by using either a reverse phase semi-preparative column (HPLC Technology Ltd; 10 μm Spherisorb) or a normal phase preparative column (Whatman Magnum 9; 10/50 Partisil) connected to an Altex 100 pump and a JASCO UVIDEC-1OO-III variable wavelength detector set at 217 nm. Light petroleum refers to the fraction of b.p. 40—70 °C. All evaporations of organic liquids were carried out under reduced pressure, or under nitrogen.

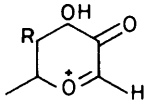
Rearing of Danaus plexippus *on* Asclepias fruticosa *Leaves.*—Fifth instar larvae of *D. plexippus* and leaves of *A. fruticosa* were collected at the same site (near Wisemans Ferry, New South Wales) as used for the collection of *A. fruticosa* for the isolation of cardenolide glycosides from the plant.[18] The larvae were reared on *A. fruticosa* leaves at 26 °C and on a light/dark cycle of 16 h/8 h. Within 24 h of emergence the adults were frozen and dried overnight at 60 °C.

Isolation of Cardenolides from Monarch Butterflies.—One hundred and twenty-four dried adult *D. plexippus* were ground, and the resulting fine powder (23.4 g) was extracted at 50 °C with light petroleum (100 ml; then 2 × 50 ml), then at 70 °C for 0.5 h first with 75% ethanol (3 × 100 ml) and finally with 95% ethanol (3 × 100 ml). The combined ethanolic extracts were evaporated and the residue was chromatographed on t.l.c. grade Merck 60H silica gel (100 g) dry-packed under suction in a Buchner funnel to form a bed 7.5 cm diam. × 3.5 cm[18] with elution under suction using successively increasing proportions of ethyl acetate in light petroleum (210 ml), ethyl acetate (80 ml), increasing proportions of methanol in ethyl acetate (480 ml), and finally methanol (120 ml).

Cardenolide glycosides with a doubly linked sugar were present in fractions eluted with 6—9% v/v methanol in ethyl acetate. These were separated on six pre-washed t.l.c. plates, developed twice with ethyl acetate, into three fractions, two of which were shown by t.l.c. and h.p.l.c. comparison with samples from *A. curassavica*[14] and *Calotropis procera*,[18] respectively, to be calotropin (**3**) (R_F 0.33) and calotoxin (**4**) (R_F 0.21). The least polar t.l.c. fraction was separated on a reverse-phase semi-preparative h.p.l.c. column with elution by 50% aqueous methanol at a flow rate of 4.0 ml min^{-1} to separate calotropin (**3**) (R_V 59 ml) and afroside (**5**) (R_V 62 ml) from calactin (**2**) (R_V 94 ml), the major component of this t.l.c. fraction. The same conditions were used to purify calotoxin (R_V 42 ml) (see before). A normal-phase preparative h.p.l.c. column was used to separate calotropin (R_V 58 ml) from afroside (R_V 42 ml), the mobile phase being 1 : 2 v/v propan-2-ol–hexane at a flow rate of

1.5 ml min^{-1}. Calactin (**2**) and afroside (**5**) were identified by t.l.c. and h.p.l.c. comparison with authentic samples from *A. fruticosa*.[18]

Methane chemical ionisation mass spectral data of the aforementioned glycosides are as follows (GH^+ refers to the protonated genin): calactin (**2**) and calotropin (**3**), *m/z* 533 (0.1%, MH^+), 515 (0.1), 405 (2, GH^+), 387 (1), 369 (0.2), 129 [100, (*b*)], and 111 (7); calotoxin (**4**), *m/z* 549 (0.1, MH^+), 531 (0.4), 405 (0.4, GH^+), 387 (0.2), 145 [90, (*b*)], 129 (40), and 127 (100); afroside (**5**), *m/z* 535 (0.4, MH^+), 517 (0.2), 407 (3, GH^+), 389 (0.5), 371 (1), 353 (0.3), 129 [100, (*b*)], and 111 (5).



(*b*)

Calotropagenin (**20**) and cardenolide glycosides with singly linked sugar were present in fractions from the silica gel column eluted with 12—100% methanol in ethyl acetate. These fractions were combined and rechromatographed on 60H silica gel (33 g in a 4.5 × 4.0 cm bed). Further analysis (see later) showed that the rechromatographed fractions which were eluted with 9—15% methanol in ethyl acetate contained calotropagenin (**20**) and the 6-deoxyallosides gofruside (**15**) and frugoside (**16**); fractions elated with 18—27% methanol in ethyl acetate contained primarily the glucosides (**11**) and (**12**) of gomphogenin and calotropagenin.

Separation of the More Polar Cardenolides and Conversion into Acetates.—The combined 9—15% methanol fractions (see before) were separated on three t.l.c. plates (20 × 20 cm; 0.5 mm thick), developed twice with 10% methanol in ethyl acetate, into bands containing calotropagenin (**20**) (R_F 0.43), gofruside (**15**) (R_F 0.35), and frugoside (**16**) (R_F 0.20). The material eluted from each band was individually acetylated by treatment with acetic anhydride in pyridine (0.05 ml each) at room temperature overnight. After evaporation of the reagents each acetylated sample was chromatographed on two t.l.c. plates developed twice in ethyl acetate. Final purification of gofruside 2′,3′,4′-triacetate (**17**) was achieved by futher t.l.c. Preparative h.p.l.c. on a normal-phase column using 3:8 v/v propan-2-ol–hexane at a flow rate of 1.5 ml min^{-1} was used to purify calotropagenin 2,3-diacetate (**21**) and frugoside 2′,3′,4′,19-tetra-acetate (**18**). These two acetates ran concurrently (on t.l.c. and h.p.l.c.) with the corresponding acetylated samples derived respectively from authentic calotropagenin provided by Professor T. Reichstein, and from frugoside isolated[25] from *Calotropis procera* seeds; frugoside from *Calotropis* was identical with an authentic sample provided by Professor Reichstein.[23]

The most polar fractions from the silica column (see before) were combined and separated on two t.l.c. plates, developed twice in 1:4 v/v methanol–ethyl acetate, into frugoside (**16**) and the 3-β-D-glucosides (**11**) and (**12**) of gomphogenin and calotropagenin. The latter were separately acetylated as already described and the acetylated products individually purified by chromatography on two t.l.c. plates, developed twice with ethyl acetate, and finally on a single plate, developed four times in 1:1:50 v/v formamide–methanol–chloroform, giving *gomphogenin* 3-β-D-*glucopyranoside* 2,2′3′,4′,6′-*penta-acetate* (**13**) and *calotropagenin* 3-β-D-*glucopyranoside* 2,2′,3′,4′,6′-*penta-acetate* (**14**).

Cleavage of Glucoside Conjugates by β-D-*Glucosidase.*—A portion of the t.l.c. fraction containing principally the glucoside (**12**) of calotropagenin but also the glucoside (**11**) of gomphogenin was treated at 37 °C with 60 U of β-glucosidase (β-D-glucoside glucohydrolase: type II from almonds; Sigma Chemicals) in 0.1M sodium acetate–acetic acid buffer (1 ml) at pH 5 for 5 h. The sample was extracted with dichloromethane (4 × 2 ml) after the addition of ammonium sulphate (0.5 g). The extract was dried (Na_2SO_4) and concentrated. T.l.c. comparison of the extract (developed twice with 10% methanol in ethyl acetate) with authentic samples of calotropagenin (**20**) from Professor T. Reichstein and of gomphogenin (**22**) derived from gomphoside[7] showed that the glucosides (**11**) and (**12**) were completely converted into the corresponding genins. The identity of the genins was confirmed by h.p.l.c.

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