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BIOSYNTHETIC PREPARATION OF CARDENOLIDES FROM [1-^{14}C]ACETIC ACID BY STEM DISCS OF THE MILKWEED *ASCLEPIAS CURASSAVICA*

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Key Word Index—*Asclepias curassavica*; Asclepiadaceae; milkweed; biosynthesis; stem discs; cardenolides; calotropin; uscharidin; voruscharin.

Abstract—[^{14}C]Calotropin (11.2 μCi/mmol) and uscharidin (14.1 μCi/mmol) were biosynthesized by stem discs of *Asclepias curassavica* incubated in a medium containing [1-^{14}C]acetic acid. Relative isotope enrichment sites determined by ^{13}C NMR spectroscopy of [^{13}C]calotropin prepared by the same method were at C-23 (0.71%), C-2′ (0.28%) and C-4′ (0.21%).

INTRODUCTION

A rapid method for the preparation of labelled 5α-cardenolides from milkweeds (*Asclepias* species) would be advantageous. While cardenolides are known to be sequestered by Monarch butterflies (*Danaus plexippus*) during larval feeding on *Asclepias* species [1–3], only a few details of the disposition of these chemicals during larval feeding and tissue storage in adults are known. This is due in part to the lack of labelled cardenolides for the appropriate studies.

The only reported labelling work with 5α-cardenolides in plants was done with *Strophanthus kombè* using [4-^{14}C]5α-pregnanolone as precursor. [^{14}C]Uzarigenin, which occurs only infrequently in this plant, was formed [4]. Labelled cardenolides of the 5β-series have been biosynthesized in several cardenolide-producing plants using various labelled precursors, including [1-^{14}C]acetic acid [5], [3-^{14}C]mevalonic acid [6], [4-^{14}C]cholesterol [7–10], [4-^{14}C]pregnenolone [11, 12], [21-^{14}C]pregnenolone [13], [7α-^{3}H]progesterone and [7α-^{3}H]pregnenolone [14], [15,16-^{3}H]5β, 17αH-pregnane-3β,14β-diol-20-one [15] and [4-^{14}C]progesterone in *Digitalis lanata* [16], *S. kombè* [17] and *D. purpurea* [18]. These experiments were done primarily to demonstrate the bioconversion of precursors to cardenolides rather than to allow preparative isolation.

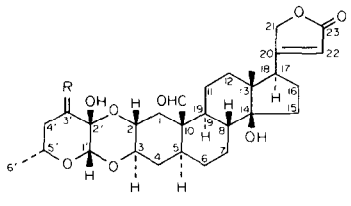
We have used *in vitro* biosynthetic techniques to prepare labelled 5α-cardenolides rather than *in vivo* methods because the former may be simpler, faster and easier to control, and larger sample sizes may be worked-up more easily. Furthermore, reported *in vitro* biosynthetic techniques [19–21] have successfully used different parts of laticiferous plants, including the leaves [14], stems [22] and latex [23, 24]. Using a modification of the technique of Groeneveld and Koning [22], we have now prepared samples of [^{14}C]calotropin and [^{14}C]uscharidin from *A. curassavica* stem discs dosed with [1-^{14}C]acetic acid. These two cardenolides are the principal cardenolide constituents of *A. curassavica* [25].

We have also used ^{13}C NMR spectrometry [26–31] to determine the enrichment sites in calotropin isolated from *in vitro* incubation of *A. curassavica* stem discs with [1-^{13}C]acetic acid.

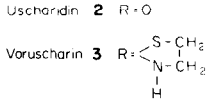
RESULTS AND DISCUSSION

Stem discs (165 g) from the first and second internodes of *A. curassavica* were incubated with 1.35 mCi [1-^{14}C]acetic acid in 0.1 M potassium phosphate buffer containing 0.5 M sucrose. The stem discs were continuously illuminated for 36 hr. At the end of this period the stem discs were harvested and extracted with ethanol. TLC and subsequent autoradiography revealed spots whose mobilities matched those of cardenolides known to be present in *A. curassavica* [25]. The extract was purified by solvent partitioning and fractionation by CC by procedures modified from Roeske *et al.* [25]. TLC combined with autoionography showed that the 3% and 4% methanol–chloroform chromatographic fractions contained radioactive cardenolides. Calotropin (**1**) was the major cardenolide present in the extract, along with smaller amounts of uscharidin (**2**). Other minor cardenolides were observed but they were not isolated. The two cardenolides were further purified by prep. TLC and the amount of total cardenolides in each TLC band was determined by a spectrophotometric assay [32].

The radioactive calotropin was diluted with unlabelled



Calotropin **1** R = β-H, α-OH
Uscharidin **2** R = O



Voruscharin **3** R =

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calotropin and the mixture was recrystallized to a constant specific activity of 11.21 μCi/mmol. Calculating according to Brown [33], the [^{14}C]calotropin had a dilution value of 4817, without the correction of carrier material dilution, and a percent incorporation of 0.26%.

The radioactive material chromatographing on TLC with uscharidin was diluted with a mixture of unlabelled uscharidin and calotropin. This mixture was repurified by TLC and the band corresponding to uscharidin was extracted. This material was crystallized repeatedly yielding a constant specific activity of 14.09 μCi/mmol. The [^{14}C]uscharidin had a dilution value of 3832 without the correction of carrier material dilution and a percent incorporation of 0.36%. Considering 2–4-fold carrier material dilution in the final purification step, actual percent incorporation values were correspondingly higher. Overall, the percent incorporation values are similar to the ones achieved by Groeneveld and Koning [22].

Determination of the labelled positions in calotropin was achieved by ^{13}C NMR spectroscopy using [^{13}C]calotropin (45.6 mg) which was isolated from 160 g of stem discs incubated with 500 mg [1-^{13}C]acetic acid and 2.49 mg [1-^{14}C]acetic acid. The amount of calotropin isolated per gram of fresh stem was the same as for ^{14}C dosing experiments. This indicated that increasing the acetic acid concentration by 200-fold did not adversely affect the biosynthesis of cardenolides in stem disc cultures.

The ^{13}C NMR spectra of natural abundance calotropin and voruscharin were obtained at 50.31 MHz using a pulsed Fourier transform system with proton noise decoupling (Table 1). The spectral assignments were based upon those reported for uscharidin by Cheung *et al.* [34]. Since the only structural variation between calotropin (**1**), uscharidin (**2**) and voruscharin (**3**) occurs at C-3′ in the sugar [35], aglycone chemical shifts for the three compounds are nearly identical. Major chemical shift differences are observed at C-3′ with some minor ones observed among the other sugar carbons arising from substituent effects [31]. In the ^{13}C enriched calotropin spectrum, the peak intensities of C-9 and C-13 carbons at δ48.62 and 49.52, respectively, were obliterated by several strong methanol-d_4 peaks at *ca* δ49. Since spin-lattice relaxation time (T_1) values of most carbons in the aglycone are considerably less than methanol (T_1 value of 13 sec [31]), the T_1 discriminated spectrum was used to eliminate the interference from solvent peaks. By setting T_1 to 3 sec, carbons with T_1-values greater than 3 sec, including all methanol-d_4, carbonyl and hydroxyl-substituted carbon peaks, appeared inverted whereas other peaks appeared normal or reduced in peak intensity. With this T_1 discriminated spectrum we were able to obtain individual peak intensities for C-9 and C-13.

The noise decoupled ^{13}C NMR spectra of enriched and natural abundance calotropin were then compared to determine the sites of enrichment. The peak heights were normalized to C-18, a methyl carbon located at the upfield end of the spectrum which was not likely to have been derived from C-1 of [1-^{13}C]acetic acid (Table 2). Based on a ^{13}C natural abundance of 1.1%, C-23, C-2′ and C-4′ showed ^{13}C enrichments of 0.71%, 0.28% and 0.21%, respectively.

Due to almost identical ^{13}C NMR chemical shifts of C-20 and C-23, resolution of these two peaks was not possible. Thus, the assignment of enrichment to C-23 rather than C-20 was based on reported cardenolide biosynthetic studies with [1-^{14}C]acetic acid [5]. This reasoning was supported by the fact that there were no other enrichment sites in the genin in our study, indicating that [1-^{13}C]acetic acid was incorporated primarily into the butenolide ring by one molecule of acetate adding to the C-20 position of an already existing C-21 pregnane [5, 36]. That is, the evidence indicated that labelled acetic acid precursor was incorporated into the butenolide ring relatively late in overall cardenolide biosynthesis by the *in vitro* technique employed.

C-2′ and C-4′ of the deoxyhexosulose sugar had smaller enrichment factors that were statistically above background (Table 2). The presence of ^{13}C at these sites could be due to photosynthetic fixation of $^{13}CO_2$ produced by metabolic breakdown of [1-^{13}C]acetic acid in the incubation medium, and the subsequent redistribution of ^{13}C label. Mechanistically, label might have been expected at C-3′ and C-4′ [37] but we found that the C-2′ and C-4′

Table 1. ^{13}C NMR chemical shifts for three cardenolides from *A. curassavica*

Carbon atom	Downfield shift from TMS [ppm (δ)]		
	Uscharidin (**2**)*	Voruscharin (**3**)†	Calotropin (**1**)‡
C-1′	97.2	94.22	95.72
C-2′	—	92.32	91.25
C-3′	207.5	85.66	72.93
C-4′	45.1	44.22	38.39
C-5′	68.0	70.05	68.22
C-6′	21.5	20.42	20.98
C-1	36.0	36.00	35.92
C-2	69.5	68.53	69.03
C-3	72.1	71.24	71.95
C-4	32.0	32.31	32.01
C-5	43.5	43.37	43.42
C-6	27.7	27.47	27.61
C-7	27.5	27.30	27.40
C-8	42.3	42.48	42.25
C-9	48.7	48.58	48.62
C-10	53.1	52.66	52.85
C-11	22.0	21.84	21.90
C-12	39.4	39.31	39.28
C-13	49.6	49.29	49.52
C-14	84.5	84.96	84.50
C-15	33.3	33.06	33.25
C-16	26.9	26.73	26.83
C-17	50.8	50.47	50.67
C-18	15.6	15.56	15.59
C-19	208.1	207.02	207.85
C-20	175.5	173.98	175.18
C-21	73.9	73.37	73.77
C-22	117.7	117.90	117.63
C-23	175.5	174.33	175.18
S-CH_2	—	34.19	—
HN-CH_2	—	51.10	—
$CDCl_3$* (reference)	77.3	77.00	77.37
MeOH	—	—	49.34

*Assignments from [34].
†In $CDCl_3$ containing 1% TMS.
‡In $CDCl_3$–MeOH (9:1 v/v) containing 1% TMS.

Table 2. ^{13}C enrichment in calotropin (1)

Carbon atom	^{13}C-enriched		Natural abundance		Enrichment factor*
	Peak height (mm)	Peak height normalized to C-18	Peak height (mm)	Peak height normalized to C-18	
1	32	0.914	37	0.841	1.087
2	36	1.029	45	1.023	1.006
3	38	1.086	44	1.000	1.086
4	32	1.000	40	0.909	1.006
5	35	1.000	40	0.909	1.100
6	37	1.057	52	1.182	0.894
7	38	1.086	50	1.136	0.956
8	34	0.971	41	0.932	1.042
9	29†	0.848†	38	0.864	0.981
10	16	0.457	19	0.432	1.058
11	34	0.971	44	1.000	0.971
12	35	1.000	43	0.977	1.024
13	15†	0.424†	19	0.432	0.981
14	11	0.314	14	0.318	0.987
15	35	1.000	41	0.932	1.073
16	38	1.086	47	1.068	1.017
17	40	1.143	47	1.068	1.070
18	35	1.000	44	1.000	1.000
19	19	0.543	23	0.523	1.038
20	—	—	—	—	—
21	43	1.229	53	1.205	1.020
22	36	1.029	46	1.045	0.985
23	21	0.600	16	0.364	1.648‡
1′	40	1.143	48	1.091	1.048
2′	12	0.343	12	0.273	1.256§
3′	37	1.057	42	0.955	1.107
4′	38	1.086	40	0.909	1.195‖
5′	44	1.257	52	1.182	1.063
6′	44	1.257	54	1.227	1.024

*99% confidence interval for natural abundance sample peak enrichment factor: $1.157 > \mu > 0.889$.

†Peak height was adjusted to delete solvent (MeOH) effect by varying T_1 time to 3 sec.

‡Percentage incorporation: 0.71%.

§Percentage incorporation: 0.28%.

‖Percentage incorporation: 0.21%.

positions had the higher ^{13}C enrichment. This might arise from redistribution of label initially at C-3′ to C-2′ through one or more metabolic pathways; for example, the pentose phosphate shunt can lead to redistribution in a pentose labelled at C-2′ and C-3′ to a hexose with C-2′ and C-4′ as the labelled positions [38].

To our knowledge, this is the first report of a relatively large scale isolation of moderately radioactive cardenolides from *Asclepias* species, and only the second report of the biosynthetic preparation of a cardenolide of the 5α-series. This method of labelling cardenolides is relatively rapid and simple when compared with *in vivo* techniques. It may be advantageous in the future to scale up the experiment so as to eliminate the dilution with unlabelled carrier material. Further work may also be needed to optimize incubation time, media constituents and concentration of precursor. Nevertheless, the method as described opens up new possibilities for studying cardenolide biosynthesis in *Asclepias* species, and for obtaining labelled cardenolides for disposition studies in animals.

EXPERIMENTAL

Mps are uncorr. IR were taken in KBr. ^1H NMR spectra were taken in $CDCl_3$ plus TMS (1%) as an int. standard with a 90 MHz NMR spectrometer. CC used Biosil A Si gel, 100–200 mesh, with a slight head pressure. The spectrometric assay was done as previously reported [32, 39]. TLC was carried out on Si gel G activated for 3 hr at 100° with solvent system I, EtOAc–MeOH (97:3) and II, $CHCl_3$–MeOH–$HCONH_2$ (90:6:1). Plates were developed twice in I and ×4 in II, then sprayed with a 4% soln of 2,2′,4,4′-tetranitrodiphenyl in toluene, followed by a 1% soln of KOH in 50% aq. MeOH. The prep. TLC plate was 2 mm in thickness and a sample streaker was used to load the compounds. For prep. TLC, solvent system I was used exclusively. Radioactive bands were scraped off and extracted with 10% MeOH–$CHCl_3$ (3 × 50 ml) by shaking for 20 min.

Radioactivity on the TLC plates was located by autoionography and by autoradiography. The autoionography was done with a β-camera (Berthold) with CO_2, 200 ml/min, and P-10 (10% CH_4–Ar), 2 l/min, as purging gases. The exposure was for

10 min at 1760 V. The autoradiogram was developed for at least 2 weeks using X-ray film (Kodak X-omat, XR-5 film). Radioactivity in soln was counted with a scintillation counter in 15 ml of scintillant (5 g/l. PPO, 100 mg/l. POPOP in toluene). All vials were internally standardized using [^{14}C]toluene. Residual radioactivity in extracted stem discs was counted by oxidation with a sample oxidizer. $^{14}CO_2$ was absorbed in 4 ml carbamate-2. Oxasol 306 (16 ml) was added and the vials were counted on the liquid scintillation counter. No attempt was made to identify EtOH unextractable radioactivity in the stem discs.

The ^{13}C NMR spectra were determined on a Nicolet NT-200 Fourier transform spectrometer operated at 50.31 MHz. The spectral width was δ200.17 and the flip angle was 62.30° (pulse length, 18.00 μsec) at a pulse interval of 1.008 sec. Each sample (46.5 mg) was dissolved in $CDCl_3$–MeOH-d_4 (9:1, 3 ml) with the solvent deuterium providing the lock signal. TMS (1%) was used as int. standard. The shifts are estimated to be accurate to δ0.01. Repetitive accumulations (*ca* 30 000) with 32 768 data points were used for assignments. Unenriched calotropin was isolated from *A. curassavica* leaves and stems [40] and voruscharin was isolated from the latex of *A. curassavica* [35].

Plant material. A. curassavica L. seeds were germinated at room temp. in a vermiculite bed. The seedlings were transferred to pots (15.24 cm diameter) containing U.C. soil mixture [41] and grown for 4–6 months in a greenhouse located at the Department of Environmental Horticulture, UCD, Davis, California. All plants were fully matured and blooming.

Incubation. The first and second internodes of fresh stems were sliced into discs of 100 μm thickness using a sliding microtome. Fresh stem discs (165 g, *ca* 20 g/dish) were transferred to 50 × 100 mm crystallizing dishes each containing 40 ml of a 1:1 mixture of 0.1 M K_2HPO_4 buffer adjusted to pH 7.5, 0.5 M sucrose and 180–200 μCi [1-^{14}C]NaOAc (54 mCi/mmol). These dishes were each covered with a watch glass and sealed with silicone vacuum grease. Dishes were incubated at room temp. with illumination by two 150 W GE plant Growlites placed 30 cm above the dishes. Illumination was continued for 36 hr with occasional gentle shaking of the dishes. Also, fresh stem discs (160 g) were incubated with 2.49 mg [1-^{14}C]HOAc (Na salt, 54 mCi/mmol) and 500 mg [1-^{13}C]HOAc (99% ^{13}C) under the same conditions as described above.

Extraction and fractionation. Stem discs were harvested and extracted in a Soxhlet extractor with 95% EtOH (2 × 800 ml) for 24 hr. Incubation media were combined with the extract. The extract was subjected to partitioning with, first, petrol (2 × 800 ml), and then CH_2Cl_2 (3 × 800 ml). The CH_2Cl_2 phase was concd to *ca* 800 ml and washed serially with 800 ml each of 0.1 N HCl, satd aq. $NaHCO_3$ and H_2O. The CH_2Cl_2 layer was then dried over Na_2SO_4 and concd to *ca* 20 ml. The CH_2Cl_2 phase was fractionated by CC using 30 g Si gel. Elution was with $CHCl_3$ (100 ml), increments of 1–5% MeOH in $CHCl_3$, then 10% MeOH–$CHCl_3$ and finally 100% MeOH. Each fraction was analysed for radioactivity and for individual cardenolide contents: 1% MeOH–$CHCl_3$, 3.73 μCi; 2% MeOH–$CHCl_3$, 3.22 μCi; 3% MeOH–$CHCl_3$, 12 15 μCi; 4% MeOH–$CHCl_3$, 5.90 μCi; 5% MeOH–$CHCl_3$, 3.11 μCi; 10% MeOH–$CHCl_3$, 3.54 μCi; and 100% MeOH, 3.67 μCi.

Isolation of [^{14}C]*calotropin.* The 4% MeOH–$CHCl_3$ chromatographic fraction was purified by prep. TLC. The developed plate was divided into two bands according to radioactivity and cardenolide content, located at 4.5–9.2 cm above the origin (band C) and 11.2–14.0 cm above the origin (band D). Extracts of both bands were analysed for total cardenolide content by spectrophotometric assay, referencing to a standard curve generated with 3.0×10^{-5}–15×10^{-5} M solns of digitoxin. Band D contained neither cardenolide nor appreciable radioactivity. Band C extract (43.7 mg calotropin, 3.324 μCi) was diluted with an equal amount of calotropin and the mixture taken-up in a minimal vol. (1–2 ml) of 10% MeOH–CH_2Cl_2. While the soln was chilled in ice, Et_2O was added dropwise until the soln remained cloudy. A ppt was allowed to form and the procedure was repeated again until no more ppt formed. The mother liquor from each preceding crystallization was concd and calotropin was crystallized once more. The ppt was collected by vacuum filtration and washed with cold Et_2O. Recrystallizations were carried out by this method until the sp. act. became constant. [^{14}C]Calotropin (27.0 mg) had 46 800 dpm/mg and its mp was 202–205° from MeOH–CH_2Cl_2–Et_2O (lit. for solvent free calotropin, mp 221° [42]). The IR spectrum was identical to that reported for calotropin [43].

Isolation of [^{14}C]*uscharidin.* The 3% MeOH–$CHCl_3$ chromatographic fraction was purified by prep. TLC. The developed plate was divided into band A (origin–1.2 cm) and band B (5.8–10.2 cm) according to radioactivity and cardenolide content. Extracts of both bands were analysed for total cardenolide content by spectrophotometric assay, referenced to the same digitoxin standard curve used for [^{14}C]calotropin. Band A (2.76 μCi) did not contain detectable amounts of cardenolide. Band B (11.7 mg uscharidin, 4.909 μCi) was diluted with 90 mg of an uscharidin–calotropin mixture as the carrier material. This mixture was purified by prep. TLC once again, and the band corresponding to uscharidin (5.0–7.6 cm above the origin) was extracted. This extract (35.1 mg, 1.466 μCi) was dissolved in 1–2 ml CH_2Cl_2–Et_2O (1:1), chilled in ice and Et_2O was added slowly until the soln turned cloudy. 1–2 drops of CH_2Cl_2 were added to the soln until clear and then it was very slowly concd by evaporation. The resulting ppt was filtered and washed with cold Et_2O or Et_2O–CH_2Cl_2 (2:1). [^{14}C]Uscharidin (24.0 mg) had 58 900 dpm/mg and mp 267–279° (CH_2Cl_2–Et_2O) (lit. for solvent free uscharidin, mp 290° [42, 44]. The ^{1}H NMR spectrum of [^{14}C]uscharidin was identical to that reported for uscharidin [45].

Isolation of [^{14}C/^{13}C]*calotropin.* The 4% and 5% MeOH–$CHCl_3$ CC fractions from stem discs incubated with [1-^{13}C]HOAc and [1-^{14}C]HOAc were combined and purified by prep. TLC. The developed plate was divided according to radioactivity into bands A (4.7–6.7 cm), B (6.4–7.6 cm) and C (7.6–9.2 cm). The extract from band A (*ca* 79 mg dry wt; 2500 dpm/mg) was determined to contain 79.95 mg of digitoxin equivalents of calotropin by spectrophotometry and TLC. The extract was crystallized for calotropin as described earlier but without any carrier dilutions. After a single crystallization 46.5 mg of chromatographically pure calotropin (1133 dpm/mg) was collected. Physical characterization of calotropin was done as described earlier.

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