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CARDENOLIDES OF *CRYPTOSTEGIA MADAGASCARIENSIS*

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ABSTRACT.—An ethanolic extract of *Cryptostegia madagascariensis*, after chromatography, gave a number of cardenolides which were identified as oleandrigenin, 16-anhydrogitoxigenin, digitoxigenin, 16-anhydrogitoxigenin-3-rhamnoside, and 16-propionylgitoxigenin-3-rhamnoside. Along with the known cardenolides, a new glycosidic cardenolide (identified as 14,16-dianhydrogitoxigenin-3-rhamnoside) was also found in the plant.

Cryptostegia madagascariensis Bojer. (family Asclepiadaceae) is a climbing shrub with a gummy, milky sap; glossy leaves; and purple, five-lobed, funnelform flowers. Although indigenous to Madagascar, it has adapted well to the Florida climate and is commonly grown as an ornamental plant under the common name "rubber vine." *C. madagascariensis*, along with another species of the same genus (*Cryptostegia grandiflora* R. Br.), has been reported to be toxic to humans and grazing animals (1,2). Grazing a small amount of leaves of *C. madagascariensis* at the entrance to a corral and then brief, brisk exercising resulted in sudden deaths of a pony and a donkey in southern Florida (2). In Australia, similar fatalities have been reported in cattle that have eaten *C. grandiflora* in dry seasons when proper forage is scarce and then have been driven hard during herding. Feeding tests have shown horses to be very susceptible, with lethal doses varying from 0.03-0.06% of body weight. Chopped leaves in water given by stomach tube to sheep and goats have been fatal at levels of 0.026-0.038% of body weight (3). *C. madagascariensis* has been employed in Madagascar as a means of suicide and homicide, with the effects resembling those of digitalis (2).

C. grandiflora also has cytotoxic activity against a cell line derived from human nasopharyngeal carcinoma (4). The cytotoxic property of *C. grandiflora* has been attributed partly to the presence of five cardenolides—oleandrigenin, 16-propionylgitoxigenin, 16-anhydrogitoxigenin, gitoxigenin, and rhodexin B (4). In continuation of our research on plants with known biological activity, we have investigated *C. madagascariensis* for its cardenolides. In this paper, we report the isolation and chemical structure of a new glycosidic cardenolide (identified as 14,16-dianhydrogitoxigenin-3-rhamnoside), along with three genins (oleandrigenin, 16-anhydrogitoxigenin, digitoxigenin) and two rhamnosides (16-anhydrogitoxigenin-3-rhamnoside and 16-propionylgitoxigenin-3-rhamnoside), from this species.

RESULTS AND DISCUSSION

The ethanolic extract (100.3 g) of *C. madagascariensis* after partitioning gave 5.9 g combined residue (residues E and H), containing free genins and glycosides along with a number of non-cardenolides. Alumina chromatography effectively separated the genins (0.31 g) from their glycosides (0.8 g). The genins were separated from the combined fraction I by column chromatography on silica gel, followed by preparative tlc. On the basis of spectral data and by direct comparison with authentic samples, genins were identified as oleandrigenin (**1**), 16-anhydrogitoxigenin (**2**), and digitoxigenin (**3**).

Combined fraction II, upon chromatography, gave a crystalline compound (**4**, from fractions 8-26) which, when recrystallized from $CHCl_3$-MeOH, gave colorless needles,

mp 240-242°. The ir spectrum showed the presence of an α,β-unsaturated lactone (1740 cm^{-1}) and a strong hydroxyl absorption (3450 cm^{-1}). The presence of a conjugated carbonyl was also evident from the uv spectrum (280 nm) of **4**. The 400-MHz pmr spectrum of the acetate of **4** showed a doublet [δ 0.92, 3H, J=6.9 Hz, CH_3 on a secondary carbon] and two singlets (δ 1.10 and 1.14, 3H each, CH_3 on tertiary carbons) as well as three acetoxymethyl (δ 1.96, 2.02, and 2.06) signals, along with signals integrating for almost six protons in the region of δ 4.78-5.13. The pmr data (δ 0.92, 1.96, 2.02, 2.06, and δ 4.78-5.13) indicated the possibility of a rhamnose molecule in **4**. The cardenolide nature of **4** was apparent from the characteristic pmr absorptions at δ 5.03 (2H), 5.83 (1H), which were also present in the pmr spectrum of 16-anhydrogitoxigenin. After hydrolysis (with 0.5% H_2SO_4), the aglycone **4a** gave a M^+ peak at *m/z* 354 (45%). The ms fragmentation pattern showed the loss of a H_2O (*m/z* 336) followed by a methyl (*m/z* 321) from the molecule. The base peak (*m/z* 203) resulting from the loss of ring D and the side chain also supported the cardenolide nature of **4a**, inasmuch as fragment 203 was present in every cardenolide isolated in this study. The 400-MHz pmr spectrum of **4a** had two methyl signals (δ 1.10 and 1.15, C18 and C19 methyls, respectively) and a single proton multiplet (δ 4.09) assigned to 3-αH. On the basis of the decoupling experiment, the two protons resonating at δ 5.03 (2H, AB) were assigned to 21-H, while the absorption at δ 5.81 (1H, broad singlet) was assigned to 22-H. (Irradiation at δ 5.03 collapsed the absorption at δ 5.81 to a fine singlet, while irradiation of δ 5.81 simplified the δ 5.03 peak). The two remaining absorptions at δ 6.03 (1H, dd, J=2, 1 Hz) and δ 6.72 (1H, d, J=2 Hz) were assigned to vinylic protons at C-16 and C-15, respectively. The coupling between C-16 and C-15 protons was confirmed by irradiation at δ 6.72, which simplified the absorption at δ 6.03 to a fine doublet (J=1), and irradiation at δ 6.03, which collapsed the doublet at δ 6.72 to a singlet and simplified the absorption at δ 5.03, indicating the long-range coupling between the C-21 protons and the C-16 proton. The 100-MHz pmr spectrum of **4a** was very similar to that of the 16-anhydrogitoxigenin, with the exception that the spectrum of **4a** has an additional doublet at δ 6.72. On the basis of the spectral data, **4a** was tentatively identified as 14,16-dianhydrogitoxigenin.

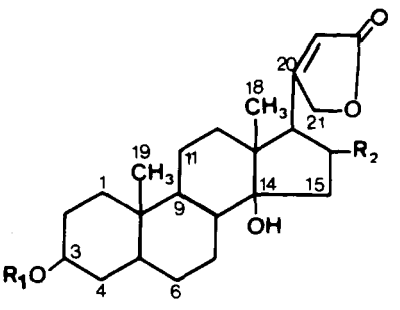
The structure of **4a** was finally secured by the conversion of 16-anhydrogitoxigenin into **4a** (see Experimental section). The sugar in **4** was identified as rhamnose by a direct gc comparison with a known standard. On the basis of its pmr data and the structure of the aglycone and sugar residues, compound **4** was thus identified as 14,16-dianhydrogitoxigenin-3-rhamnoside. In order to eliminate the possibility of **4** being an artifact generated during the isolation and purification steps, a few milligrams of **2** and **5** were dissolved in 1 ml of 6 N methanolic H_2SO_4, and the reaction mixture was allowed to stand for seven days at room temperature. No trace of 14,16-dianhydrogitoxigenin or its glycoside was detected in the reaction mixture. The possibility of the dehydration of 16-anhydrogitoxigenin to give **4** or **4a** during chromatography on alumina was eliminated by the use of silica gel 60 chromatography for the purification of **4a**. Compound **4** was also detected in the ethanolic extract of *C. madagascariensis* by tlc [silica gel 60 plates solvent system $CHCl_3$-MeOH (92.5:75 v/v)].

Fraction 27-72 from the rechromatography of the combined fraction II gave a crystalline compound, mp 251-256° (**5**). Hydrolysis of **5** gave an aglycone identified as 16-anhydrogitoxigenin on the basis of its spectral properties. The structure of the aglycone was confirmed by a direct comparison with an authentic sample of 16-anhydrogitoxigenin. The sugar of **5** was proven to be rhamnose. On the basis of biogenic considerations, **5** was thus identified as 16-anhydrogitoxigenin-3-rhamnoside.

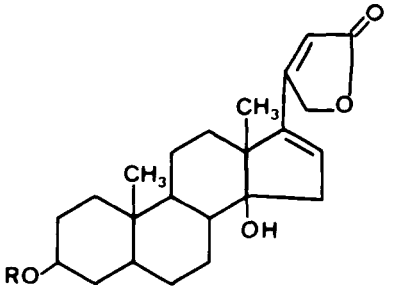
Fraction 73-91 from the rechromatography of the combined fraction II gave a crystalline compound (**6**). The aglycone of **6** was identified as 16-propionylgitoxigenin on

the basis of its spectral data and by a direct comparison with an authentic sample of 16-propionylgitoxigenin. The sugar proved to be rhamnose. On the basis of the biogenic considerations and 80-MHz pmr data, **6** was confirmed as 16-propionylgitoxigenin-3-rhamnoside.

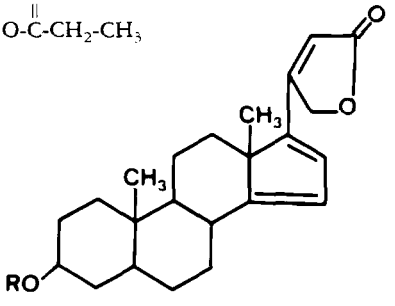
Trace amounts of the free aglycones (14,16-dianhydrogitoxigenin, 16-propionylgitoxigenin, and 16-anhydrogitoxigenin) of **4**, **5**, and **6** were also present in the crude extract (tlc).



1 R_1=H, R_2=O-C-CH_3 (O)
3 R_1=R_2=H
6 R_1=Rhamnose, R_2=O-C-CH_2-CH_3 (O)



2 R=H
5 R=Rhamnose



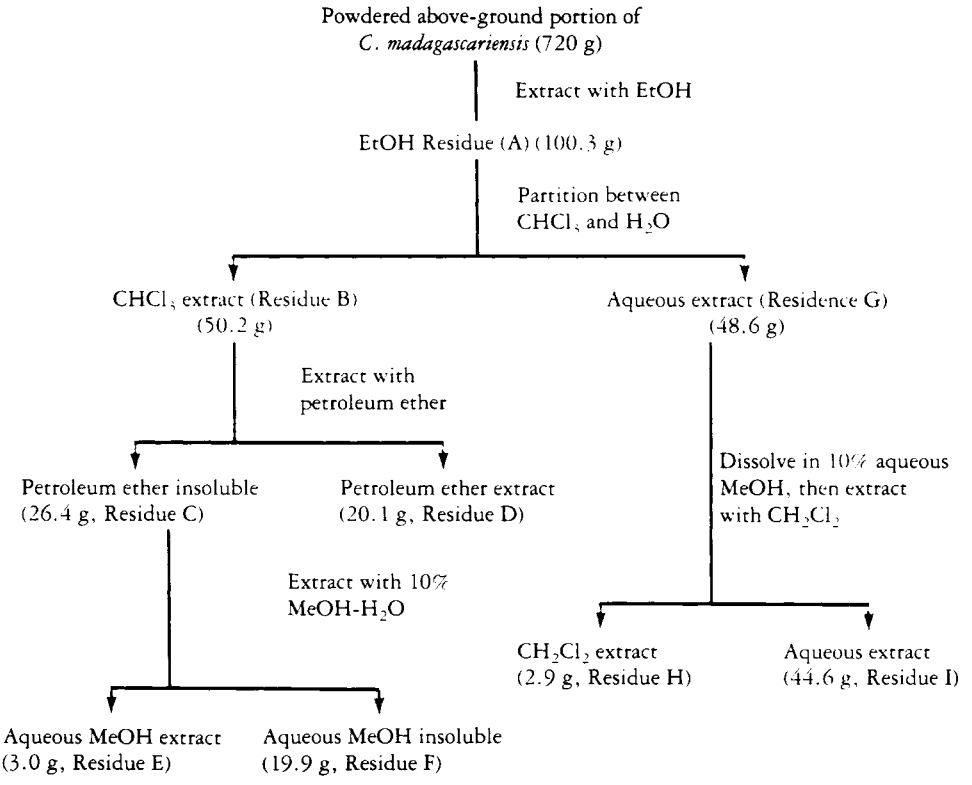
4 R=Rhamnose
4a R=H

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined on a Fisher-Johns mp block and are uncorrected. Spectral data were obtained on the following instruments: ir, Perkin-Elmer, model 283; pmr, Varian FT-80A, Varian XL 100, and Brüker WH-400; ms, Hewlett-Packard HP 5930A gc-ms equipped with a HP-5933 data system, direct inlet at 70 eV; uv, Perkin-Elmer, model 200. Adsorbents for cc and tlc (neutral alumina, AG 7, 100-200 mesh; silica gel 60, 230-400 mesh), and silica gel 60 precoated tlc plates (250 μ thickness) were purchased from Bio Rad Laboratories and E. Merck and Co. Column chromatography was carried out at 40-50 psi, using a FMI pump to deliver the solvent. Tri Sil Z was purchased from Pierce Chemical Co. Tlc solvent system: $CHCl_3$-MeOH (92.5:7.5 v/v).

PLANT MATERIAL.—Leaves and branches of *C. madagascariensis* were collected around the Miami, Florida, area in August 1979 and in July 1982. Voucher specimens are deposited in the Department of Medicinal Chemistry and Pharmacognosy Herbarium, University of Houston, Houston, Texas.

EXTRACTION AND PURIFICATION OF THE CARDENOLIDES.—The initial extraction and separation of the extract into fractions containing cardenolides (as genins and glycosides) was accomplished by a modification of the method reported by Doskotch, *et al.* (4), and is shown in Scheme 1. Residues E and H were combined, and the combination (28 g) was chromatographed on an alumina grade II column (4.5×48 cm), which was eluted with $CHCl_3$ (3 liters), 1% MeOH in $CHCl_3$ (2 liters), 10% MeOH in $CHCl_3$ (4 liters), and 50% MeOH in $CHCl_3$ (8 liters). Fractions of 500-ml volume were collected. Fractions 3-7 and 30-34 gave intense purple spots with Kedde's reagent (8) and were combined to yield combined fractions I



SCHEME 1. Flow diagram for fractionation of an alcoholic extract of *C. madagascariensis*.

and II, respectively. The combined fraction I showed the presence of three genins with Kedde's reagent, while II had three Kedde-positive spots.

Combined fraction I was rechromatographed on a silica gel 60 column [1.5×60 cm, eluting solvent linear gradient of Me_2CO (0-35%) in hexane]. Fractions of 250-ml volume were collected and combined on the basis of their tlc patterns. Fractons 15-18 gave Kedde-positive spots. Major cardenolides (**1**, **2**, **3**) were then purified by preparative tlc.

Combined fraction II upon rechromatography on a silica gel 60 column [1.5×60 cm; eluting solvent, linear gradient of MeOH (10-30%) in $CHCl_3$, fractions of 10-ml volume] gave three groups of fractions (8-24, 29-70, and 74-91), which gave Kedde-positive spots.

Hydrolyses were carried out by refluxing (40 min) a known volume of a methanolic solution of the glycoside with an equal volume of H_2SO_4 (0.5 N), followed by the addition of an equal volume of H_2O. After removing the MeOH, the mixture was refluxed for an additional 10 min. The cooled reaction mixture was extracted with $CHCl_3$, and the $CHCl_3$ layer was washed with H_2O, 5% $NaHCO_3$, followed by H_2O again, and dried over anhydrous Na_2SO_4. The aqueous layer was neutralized with $BaCO_3$, filtered, and lyophilized to give sugar(s). The residue ($\simeq$1 mg) was mixed with 0.5 ml of Tri Sil Z, and the mixture was heated for 10 min in a 60° H_2O bath. The silylated sugar(s) was analyzed by gc (2.5% SE-30, temperature 150°, N_2 flow 30 ml/min).

OLEANDRIGENIN (**1**) (15.8 mg).—Isolated from fraction 16 (column chromatography of combined fraction I) by preparative tlc [solvent system $CHCl_3$-MeOH (92.5:7.5 v/v)], and crystallized from MeOH, mp 225-228°. A direct comparison [ir, pmr (100 MHz), ms, and mixed mp] of the isolated compound with an authentic sample of oleandrigenin showed the two to be indistinguishable.

16-ANHYDROGITOXIGENIN (**2**) (32.0 mg).—Purified by preparative tlc from fraction 17. Crystallization with MeOH gave colorless needles, mp 240-245°. Compound **2** was identified as 16-anhydrogitoxigenin on the basis of the comparison of pmr (100 MHz), ms, ir, uv, and mixed mp of the free and acetylated products (mp 198°) of **2** with an authentic sample of 16-anhydrogitoxigenin.

DIGITOXIGENIN (**3**) (14 mg).—Column fraction 18 upon concentration gave long needles, which, after separation, were recrystallized with $CHCl_3$-MeOH to afford colorless needles, mp 246-250°. A direct

comparison [ms, pmr (80 MHz), ir, mixed mp of the free and acetylated products] with an authentic sample of digitoxigenin showed the two to be indistinguishable.

14,16-DIANHYDROGITOXIGENIN-3-RHAMNOSIDE (**4**) (5.4 mg).—Fractions 8-26 (chromatography of the combined fraction II) upon concentration gave a crystalline compound that was recrystallized from $CHCl_3$-MeOH to give colorless needles mp 240-242°; ir (KBr) ν max 3450, 2940, 1740, 1620 cm^{-1}; uv λ max (MeOH) 220 and 281 nm; 400 MHz pmr (acetate, $CDCl_3$) δ 0.92 (3H, d, J=6.9 Hz), 1.10 (3H, s), 1.14 (3H, s), 1.96 (3H, s), 2.02 (3H, s), 2.06 (3H, s), 4.78-5.13 ($\simeq$6H, m), 5.03 (2H, ddd, J=1.9, 1.2, 1 Hz), 5.83 (1H, bs), 6.02 (1H, narrow t, J=2.0 Hz), and 6.72 (H, d, J=2.2 Hz).

Hydrolysis of **4** with 0.5 N H_2SO_4 followed by standard work-up resulted in the separation of the aglycone and sugar(s) from the organic and aqueous layers, respectively. The aglycone was recrystallized with Me_2CO to give colorless needles, mp 175-177°, ms 354 (M^+, 100%), 336 (25), 321 (14), 228 (26), 189 (11), 175 (10), 147 (23); pmr (400 MHz, $CDCl_3$) δ 1.10 (3H, s), 1.15 (3H, s), 4.09 (1H, m), 5.03 (2H, ddd, J=1.92, 1.2, 1 Hz), 5.81 (1H, broad singlet), 6.03 (1H, dd, J=2,1 Hz), and 6.72 (1H, d, J=2.0 Hz).

The structure of **4** was secured by the conversion of 16-anhydrogitoxigenin to 14,16-dianhydrogitoxigenin by the method of Jacobs and Gustus (9). 16-Anhydrogitoxigenin (**2**, 15 mg) was dissolved in 0.2 ml of MeOH, and 0.5 ml of 2% HCl was added to the solution. The mixture was refluxed for 15 min, and a colorless solid deposited upon cooling and dilution. Crystallization with MeOH gave colorless needles, mp 175°; ms (354, M^+); ir (CCl_4) 2940, 1745, 1620 cm^{-1}; pmr (80 MHz, $CDCl_3$) 1.10 (3H, s), 1.15 (3H, s), 4.07 (1H, m), 5.04 (2H, d, J=2.0 Hz), 5.81 (1H, bs), 6.04 (1H, dd, J=2, 1 Hz), 6.71 (1H, d, J=2.0 Hz). A direct comparison (ms fragmentation pattern, 80 MHz pmr, ir, and mp) of the natural 14,16-dianhydrogitoxigenin with the synthetic one showed the two to be indistinguishable.

The aqueous layer of the hydrolysis work-up was found to contain rhamnose (determined by co-gc of the TMS-product and TMS-ester of an authentic sample). Compound **4** was thus identified as 3β-rhamnosyl-5β-carda-14,16,20(22)trienolide.

16-ANHYDROGITOXIGENIN-3-RHAMNOSIDE (**5**) (30.7 mg).—Fractions 27-72 were combined and concentrated to yield a crystalline compound, which, upon recrystallization with MeOH, afforded colorless crystals, mp 251-256°; pmr (80 MHz, acetate $CDCl_3$) δ: 0.92 (3H, d, J=6.8 Hz), 0.91 (3H, s), 1.22 (3H, s), 2.05 (3H, s), 2.06 (3H, s), 2.15 (3H, s), 2.16 (3H, s), 2.48 (2H, dd, J=22 Hz and 3.8 Hz), 4.01 (1H, m), 4.85-5.23 (6-7H, m), 5.9 (1H, distorted t), and 6.03 (1H, t, J=2.0 Hz).

Hydrolysis of **5** with 0.5 N H_2SO_4, followed by extraction with $CHCl_3$, gave a colorless crystalline aglycone mp 240-245°. The aglycone was identified as 16-anhydrogitoxigenin on the basis of its ir, uv, 100-MHz pmr, and ms data. No depression of mp was observed in a mixed mp determination with an authentic sample of 16-anhydrogitoxigenin. The sugar was identified as rhamnose.

16-PROPIONYLGITOXIGENIN-3-RHAMNOSIDE (**6**) (9 mg).—Fractions 73-91 upon evaporation gave a crystalline compound (**6**), which, after recrystallization with MeOH, gave colorless needles, mp 210-212°, pmr (80 MHz acetate, $CDCl_3$) δ 0.91 (3H, d, J=6.7 Hz), 0.96 (3H, s), 0.98 (3H, s), 1.07 (3H, t, J=7.5 Hz), 2.00 (3H, s), 2.06 (6H, s), 2.09 (3H, s), 2.27 (2H, q, J=7.2 Hz), 3.30 (1H, d, J=8.5 Hz), 4.79 (1H, m), 5.08 (2H, distorted t), and 5.99 (1H, narrow t).

Hydrolysis followed by work-up gave an aglycone that was recrystallized with C_6H_6-$CHCl_3$ to afford colorless needles, mp 205-207°; ms 446 (M^+), 390, 372, 354, 336, 203 (100%), 147, 127, and 109; pmr 80 MHz ($CDCl_3$) δ 0.96 (3H, s), 0.98 (3H, s), 1.05 (3H, t, J=7.5 Hz), 2.25 (2H, q, J=7.5 Hz), 3.29 (1H, d, J=8.5 Hz), 4.75 (1H, m), 5.05 (2H, distorted t), and 5.95 (1H, narrow t). A comparison (uv, ir, pmr, ms, and mixed mp) of the free and acetylated products of the aglycone with an authentic sample of 16-propionylgitoxigenin showed them to be identical. The sugar was identified as rhamnose.

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