STRUCTURE OF CALOCIN

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ABSTRACT.—A new pregnane glycoside designated as calocin (**1**) has been isolated from the dried twigs of *Periploca calophylla*. Chemical and spectroscopic evidence and characterization of its hydrolysis products are consistent with the structure Δ^5-pregnene-3β,14β,20ξ-triol-(3 or 20)-β-D-canaropyranoside for calocin.

In several species of the Asclepiadaceae family, cardenolides have been reported to be present along with pregnane derivatives (1,2). In an earlier investigation of the twigs of *Periploca calophylla*, the presence of periplogenin and cymarose has been reported (3). In our recent chemical reinvestigation of the shade-dried stems of *P. calophylla*, the crude glycoside mixture was extracted by an earlier method (4,5). The resulting material was subjected to repeated chromatography on columns of alumina and silica gel, affording a small quantity of a new glycoside, $C_{27}H_{44}O_6$ (*m/e* 420, M–44), mp 243–247° $[\alpha]_D$ −60.7. The structure elucidation of this new glycoside, designated as calocin (**1**), is now reported.

RESULTS AND DISCUSSIONS

A positive reaction of calocin (**1**) with $NaIO_4$ (6) indicated the presence of a vicinal diol arrangement in the molecule. The diagnostic color tests for 2-deoxy sugar, viz., the Xanthydrol reaction (7,8) and Keller-Kiliani reaction (9), exhibited by **1** in conjunction with its molecular formula $C_{27}H_{44}O_6$ indicated it to be a pregnane (C_{21}) glycoside of a 2-deoxy hexose, commonly reported to occur in plants of Asclepiadaceae family (1,2). Its ir spectrum displayed absorption bands due to hydroxyl groups at 3430 cm^{-1}, but the absence of absorption in the carbonyl region suggested that its pregnane genin presumably contained a $-CHOH-CH_3$ chain at C–17.

The prominent fragment ion peaks at *m/e* 131 and *m/e* 289 in the ms of **1** are diagnostic of a 2,6-dideoxyhexose fragment **9** ($C_6H_{11}O_3$) and the genin fragment **8** ($C_{19}H_{29}O_2$), respectively (chart-1). Mannich hydrolysis (6) of **1** with 0.05N H_2SO_4 in 80% aq. dioxane afforded a crystalline genin 6 mp 202–205° and an amorphous reducing sugar **3**, exhibiting characteristic tests of 2-deoxy sugar. The identity of **3** with D-canarose was established by comparison on tlc and by paper chromatography. For further identification, **3** was oxidized with bromine water to a syrupy lactone **4** which with phenyl hydrazine afforded a crystalline phenyl hydrazide **5**, mp 166–68°, possessing the same properties as an authentic sample of D-canaroic acid phenyl hydrazide (10).

The genin (**6**), $C_{21}H_{34}O_3$, mp 202–205°, obtained by the mild acid hydrolysis of **1** gave a molecular ion peak in its ms at *m/e* 334 and other ions characteristic of polyhydroxy pregnanes (11,12) at *m/e* 316 ($M-H_2O$), 301 ($M-H_2O-CH_3$), 283 ($M-2H_2O-CH_3$) and 265 ($M-3H_2O-CH_3$), indicating that all the three oxygen atoms in the molecule are present as hydroxyl groups. Other significant ion peaks at *m/e* 289 (**8**, M–45), 271 ($M-45-H_2O$) and 253 ($M-45-2H_2O$) involving the loss of 45 a.m.u. are diagnostic (13,14) of a $-CHOH-CH_3$ side chain at C–17. The significant ion peaks in the lower mass region at *m/e* 137 (**13**), 119 (**13**–H_2O) and 105 (**17**) are characteristic (15) of a Δ^5-3-ol arrangement in **6** (chart 1). The inference was further supported by the ion peaks at *m/e* 231 and 213, arising by the loss of water molecules from the characteristic fragment **10** as reported by Spittler (16) for Δ^5-3-ol steroids.

Acetylation of **6** with acetic anhydride in pyridine afforded diacetate **7**, $C_{25}H_{38}O_5$, mp 167°, $[\alpha]_D$ −20°. The pmr spectrum of **7** in $CDCl_3$ included two 3H singlets for two acetyl groups at δ 2.02 and 2.03; two 3H singlets at δ 0.78 and 1.02

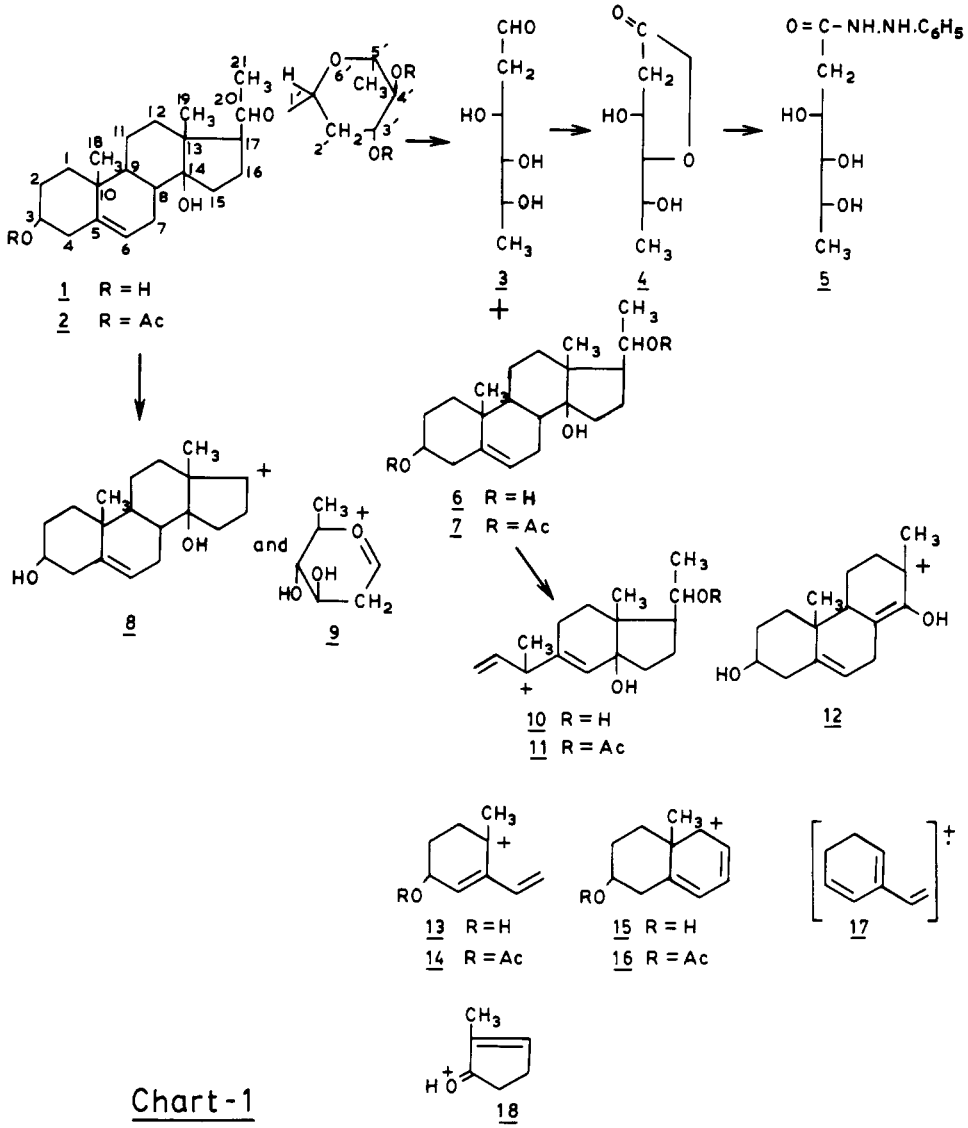

Chart-1

for two tertiary C-methyl groups and a 3H doublet ($J=7$ Hz) centered at δ 1.27 for a sec.-methyl group. A 1H singlet at δ 3.75 was assigned to one unacetylable tertiary hydroxyl group. The presence of this tertiary hydroxyl group at C–17 is precluded since **6** failed to react with $NaIO_4$. Therefore, this hydroxyl group could be present at C–14 in the β-configuration as commonly reported in pregnanes of Asclepiadaceae for which the chemical shift is reported at ca. δ 4 (17). One H in each of the two multiplets centered at δ 4.15 and 5.27 were assigned to the carbinol methine proton and vinyl proton, presumably present at C–3 and C–6, respectively. It is further presumed that the C–3 hydroxyl group is present in the commonly reported β-configuration in Δ^5-pregnenes (1,2). The 1H quartet centered at δ 5.11 ($J=7$ Hz) is evidently the carbinol methine proton at C–20. These results suggest **7** to be 3β,20ξ di-O-acetyl-14β-hydroxypregn-5-ene indicating **6** to be hitherto unreported simplest 3β,14β,20ξ trihydroxypregn-5-ene. The ms of **7** had the highest mass ion peaks at m/e 315 for M-AcOH and other significant ion peaks at m/e 298 (M–2AcOH), 280 (M–2AcOH–H_2O), 271 (M–AcOH–$CHOAcCH_3$), 265 (M–2AcOH–H_2O–CH_3) and 253 (M–2AcOH–$CHOAcCH_3$–H_2O). The fragment ion

peaks at *m/e* 213 (**11**–AcOH–H_2O), 145 (**16**–AcOH) and 119 (**14**–AcOH) further support the inference that **6** is a 3-hydroxyl-Δ^5 steroidal derivative, and the prominent ion peak at *m/e* 97 (**18**) substantiates the presence of C–14 hydroxyl group (chart 1).

In the ms of **1**, structurally significant ion peaks were recorded up to *m/e* 420, which included the M–44 for it. The relatively intense peaks between *m/e* 95 and 334 were reasonably attributed to cleavages within the genin part of the molecule since they were matched by similar peaks at the same nominal masses in the ms of free calogenin (**6**). The fragment ion *m/e* 289 (**8**, M–CHO.Sugar.CH_3), presumably due to the loss of the C–17 side chain including the sugar moiety (chart 1), indicates that the sugar is presumably glycosidically linked to the C–20ξ hydroxyl group of the genin.

In the pmr spectrum of **1**, the poorly resolved double doublet, centered at δ 4.47 ($J=10$ and 2Hz), was assigned to the anomeric proton of the 2-deoxyhexose moiety. The large coupling constant ($J=10$ Hz) is typical of an axial proton of a pyranosoide 2-deoxyhexose (18), which suggests that it is present in the $^4C_1$ conformation and is linked to the genin through a β-glycosidic linkage. Assignment of the anomeric proton signal was confirmed by an irradiation experiment. Irradiation of this double doublet at 402 Hz gave a collapse of H_{eq} signals of the methylene group in the region δ 2.04–2.21. However, H_{ax} signals expected at higher field were obscured by the methylene signals of the genin part, and changes in this region after irradiation could not be properly deciphered. The two 3H singlets at δ 0.68 and 0.94 were assigned to the two tertiary C-methyl groups (C–18 and C–19), and two sets of 3H doublet centered at δ 1.27 and 1.33 ($J=6$ Hz) to two sec.-methyl groups at C–21 and C–6', indicating the 2,6-dideoxy structure of the sugar moiety and –CHOH.CH_3 side chain at C–17. The 1H multiplet at δ 5.2 was assigned to the vinyl proton at C–6, and the 1H signal at δ 4.2 and the 3H signal at δ 2.72, disappearing on shaking with D_2O, corresponded to four hydroxyl groups in the molecule. The 5H multiplet in the region 3.2–3.8 was assigned to protons at C–3, C–20, C–3', C–4', and C–5'.

Acetylation of **1** with acetic anhydride in pyridine afforded an amorphous triacetate **2**, $[\alpha]_D$ $-13.2°$, giving the characteristic acetate absorption bands in the ir at 1732 and 1230 cm^{-1} and also an unacetylable tertiary hydroxyl group absorption band at 3400 cm^{-1}. The pmr spectrum of **2** in $CDCl_3$ comprised signals for three acetyl singlets at δ 2.02, 1.97 and 1.95, two 3H singlets at δ 0.75 and 1.04 for the C–18 and C–19 tertiary C-methyl groups and two 3H doublets centered at δ 1.26 and δ 1.33 ($J=7$ Hz) for the C–6' and C–21 sec.-methyl groups. A four-line pattern at δ 3.58 ($J=7$ Hz) for the carbinol methine proton at C–20 and a 1H multiplet at δ 5.26 for the C–6 vinyl proton and another 1H multiplet in the region δ 3.65–3.90 were assigned to the proton at C–5'. A 4H composite signal spread over the region δ 4.55–4.85 was assigned to protons C–1', C–4', and C–3. Since the position of the sugar moiety in calocin could not be confirmed from the above pmr data, its structure is thus Δ^5-pregnene-3β-14β,20ξ triol (3 or 20)-β-D-canaropyranoside.

EXPERIMENTAL[1]

PLANT EXTRACTION.—Five kg of shade-dried, powdered twigs of *Periploca calophylla* was extracted with 50–95% ethanol by the earlier method (4,5) for cardiac glycosides. The combined ethanolic extract was concentrated under reduced pressure and purified by shaking with freshly prepared $Pb(OH)_2$ [obtained from 4 kg $(CH_3COO)_2$ $Pb.3H_2O$] in 50% ethanol.

[1]All the melting points were determined on a Boetius micro melting point apparatus and are uncorrected. Optical rotations were measured in a l-dm tube with a Jasco-Dip 180 automatic polarimeter. The ir spectra were recorded with a Perkin-Elmer IR-177 spectrophotometer and the pmr spectra with a 90-MHz Perkin-Elmer R-32 spectrometer and with a CFT-20 spectrometer in solution in $CDCl_3$ (unless otherwise mentioned), with Me_4Si as the internal standard. Mass spectra were recorded with a JEOL High Resolution JMS-300 mass spectrometer. The adsorbent for tlc was Silica Gel G (BDH), and for column chromatography Silica Gel for columns (BDH), which were developed by Duncan's method (19).

The filtrate was again concentrated (1 liter) and successively extracted with petroleum ether, ether, chloroform, chloroform-ethanol (4:1) and chloroform-ethanol (3:2). On evaporation, the following residues were obtained: from petroleum ether, 2.5 g; ether, 1 g; chloroform 20 g; (4:1) chloroform-ethanol, 7 g; and (3:2) chloroform-ethanol, 2.5 g, respectively. The last four extracts were rich in glycosides (xanthydrol test positive).

Ether and chloroform extracts found to contain identical constituents by tlc were combined (21 g) and chromatographed on alumina (400 g). Fraction of 250-ml were collected. Repeated chromatography of fractions 43–47 over silica gel, eluted with chloroform-methanol (96:4), afforded calocin (**1**), which crystallized from acetone-petroleum ether as colorless needles, mp 243–247°; $[\alpha]_D$ −60.7°, (c=0.3, methanol). It gave positive tests by the Xanthydrol and Keller-Kiliani reaction. It showed ν max (KBr) cm^{-1}: 3460–3400, 1368, 1063, 1028 and 850, pmr data δ 5.2 (m, 1H, H-6), 4.47 (dd, 1H, J=10 and 2 Hz, H-1'), 4.2 (1H, OH), 3.2–3.8 (m, 5H, H-3, H-20, H-3', H-4' and H-5'), 2.72 (m, 3H, 3 OH), 2.42–2.21 (m, 1H, H-2'e) 1.27 and 1.33 (2d, 3H each, J=6 Hz, 6'-CH_3 and 21-CH_3), 0.68 and 0.94 (2s, 3H each, 18-CH_3 and 19-CH_3); and ms: *m/e* 420 (0.1%, M-44), 402 (0.03), 333 (0.3), 316 (0.5), 299 (1), 289 (4.3), 281 (0.4), 271 (3.7), 253 (2.3), 231 (0.4), 227 (0.4), 213 (1), 203 (0.2), 197 (0.4), 173 (0.4), 159 (1.1), 145 (1.1), 131 (100), 119 (0.8), 113 (6.6), 105 (1), 81 (1.1), 73 (1.6), 59 (1.2) and 43 (0.07). Anal. Calcd. for $C_{27}H_{44}O_6$: C, 69.83. H. 948, Found: C, 69.49; H, 9.32.

PERIODATE OXIDATION OF CALOCIN (**1**).—To a solution of calocin (2 mg) in methanol (0.2 ml) was added a solution of sodium metaperiodate (6 mg) in water (0.1 ml), and the mixture was kept at room temperature for 4 hours, diluted with water (0.4 ml), and evaporated under reduced pressure. The residue showed complete consumption of calocin (tlc).

TRI-*O*-ACETYL CALOCIN (**2**).—A solution of **1** (15 mg) in pyridine (0.3 ml) and acetic anhydride (0.3 ml) was kept for 48 hours at room temperature. The pyridine and the excess of acetic anhydride were then removed under reduced pressure. The viscous residue was taken up in chloroform and washed in sequence with 2N HCl, 2N Na_2CO_3 solution, and water. It was then dried over Na_2SO_4, filtered, and evaporated; **2** was obtained as an amorphous residue (15 mg), which failed to crystallize. It showed ν max (KBr) cm^{-1}: 3400–3500, 1732, 1460, 1360, 1230, 1040 and 660; pmr: δ 5.26 (m, 1H, H-6), 4.55–4.85 (m, 4H, H-1', H-3', H-4' and H-3), 3.65–3.90 (m, 1H, H-5'), 3.58 (q, 1H, J=7 Hz, H-20), 1.26 and 1.33 (2d, 3H each, J=7 Hz, 6'-CH_3 and 21-CH_3), 0.75 and 1.04 (2s, 3H each, 18-CH_3 and 19-CH_3).

MILD ACID HYDROLYSIS OF CALOCIN (**1**).—To a solution of crystalline **1** (20 mg) in 80% aqueous 1,4-dioxane (1 ml) was added 0.1N H_2SO_4 (1 ml), and the solution was warmed for 30 minutes at 50°, then concentrated under reduced pressure to remove dioxane. The aqueous portion was repeatedly extracted with chloroform-methanol (9:1), and the organic layer was washed in sequence with water, 2N Na_2CO_3, and again with water, dried over Na_2SO_4, and evaporated to a genin, calogenin (**6**), as an amorphous residue (14 mg), which crystallized from methanol-ether as colorless prisms (12 mg), mp 203–205°.

The aqueous hydrolyzate was neutralized with freshly prepared $BaCO_3$, filtered, and concentrated under reduced pressure. It was further purified by molecular distillation at both 100 and 120° and 0.01 mm pressure; the sugar **3** was obtained as a colorless thick syrup, which failed to crystallize. It gave a pink coloration in the xanthydrol test and underwent positive $NaIO_4$-oxidation. Its tlc and pc comparison showed identical mobilities to D-canarose.

CALOGENIN (**6**).—Calogenin gave the following ms *m/e* 334 (0.2%), 316 (0.4), 289 (4.4), 271 (6), 253 (4.3), 213 (2.5), 203 (0.2), 197 (0.7), 171 (0.9), 159 (1.9), 145 (2.2), 133 (2), 119 (1.5), 105 (2.2), 97 (0.6), 95 (1.5), 91 (2.2), 81 (2.4), 69 (1.5), 55 (2) and 43 (3). Anal. Calcd. for $C_{21}H_{34}O_3$: C, 75.44; H, 10.18. Found: C, 75.40; H, 10.15.

PERIODATE OXIDATION OF CALOGENIN (**6**).—To a solution of calogenin (2 mg) in methanol (0.2 ml) was added a solution of sodium metaperiodate (6 mg) in water (0.1 ml). The mixture was kept at room temperature for 4 hrs, diluted with water (0.4 ml), and evaporated under reduced pressure. The residue showed no reaction of calogenin (tlc).

DI-*O*-ACETYL CALOGENIN (**7**).—Crystalline **6** (10 mg) dissolved in anhydrous pyridine (0.2 ml) was mixed with acetic anhydride (0.2 ml), and the mixture was kept for 48 hrs at room temperature. After the usual work-up of the reaction mixture as for **2**, it afforded the acetylated product **7** (10 mg), which crystallized from methanol-ether, mp 167°; pmr: 5.27 (m, 1H, H-6), 5.11 (q, 1H, J=7 Hz, H-20), 4.15 (m, 1H, H-3), 3.75 (s, 1H, OH), 2.02 and 2.03 (2s, 3H each 2Ac), 0.78 and 1.02 (2s, 3H each, 19-CH_3 and 18-CH_3), and 1.27 (d, 3H J=7 Hz, 21-CH_3); ms: *m/e* 358 (2%, M-60), 298 (1.6), 280 (2.9), 271 (1), 265 (1.4), 253 (2.3), 226 (2.3), 171 (0.7), 15.8 (1.2), 145 (1.8), 133 (1.2), 119 (1.3), 105 (2.2), 97 (0.7), 95 (1.3), 91 (1.2), 81 (3.1), 67 (1.7), 55 (5.2) and 4.3 (11).

Anal. Calcd. for $C_{25}H_{38}O_5$: C, 71.77; H, 9.09. Found: C, 71.75 H, 9.06.

CANAROIC ACID LACTONE (**4**).—A solution of **3** (6 mg) in water (0.1 ml) was mixed with bromine (1 μl) and shaken in a stoppered flask in the dark for 24 hrs at room temperature. Excess bromine was then removed under reduced pressure; the acidic mixture was neutralized with freshly precipitated Ag_2CO_3 and filtered. H_2S was passed through the filtrate to remove Ag^+ ions, and the suspension was again filtered. The filtrate, again evaporated to dryness under reduced pressure, yielded the lactone as a dark-brown syrup (3 mg). Comparison by tlc showed a mobility identical to an authentic preparation of D-canaroic acid lactone, visualized with NH_2OH–$FeCl_3$ (20) spray reagent.

PHENYLHYDRAZIDE (**5**) OF LACTONE (**4**).—A solution of the lactone (3 mg) in absolute ethanol (0.04 ml) was mixed with freshly distilled phenylhydrazine (0.04 ml), and the mixture was

heated for 30 min at 100°. The viscous reaction mixture was cooled and repeatedly triturated with absolute ether to remove the excess phenylhydrazine; the phenyl-hydrazide **5** was obtained as a brown powder (2 mg) which crystallized from methanol-ether as colorless needles (1.5 mg), mp 166–68°, (canaroic acid phenylhydrazide, mp 165–167°).

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