CH_3). Anal. $C_{16}H_{15}N_3O_2$: C, H, N.

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Supplementary Material Available: Tables I and II of crystal data, final positional parameters, and thermal parameters (2 pages). Ordering information is given on any current masthead page.

Structure of Humistratin: A Novel Cardenolide from the Sandhill Milkweed *Asclepias humistrata*[1]

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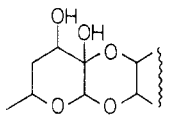
Humistratin (**5**), a new Δ^7-cardenolide monoglycoside of an ecological interest, was isolated from the leaves and stems of the sandhill milkweed *Asclepias humistrata* Walt. (Asclepiadaceae). Its structure and stereochemistry were elucidated by IR, EIMS, CIMS, ^1H NMR, ^{13}C NMR, and X-ray crystallography. Its hypothetical sugar, 4,6-dideoxy-β-D-*glycero*-D-*glycero*-2-hexosulopyranose, is doubly linked at 1 and 2, through acetal (glycosidic) and hemiketal bonds, to positions 3β and 2α, respectively, of its hypothetical genin, 2α,3β,14-trihydroxy-19-oxo-5α,14β-carda-7,20(22)-dienolide, to form a dioxane ring with the resultant chiral center at C(2) of the sugar moiety *S*.

Cardenolides constitute one of several groups of plant secondary compounds that are sequestered by phytophagous insects for defense against predation.[2] Most members of the milkweed genus *Asclepias* (Asclepiadaceae) produce these cardioactive steroids at varying concentrations.[3] In the southeastern region of the United States, the sandhill milkweed *Asclepias humistrata* is one of the most common milkweeds,[4] serving as an abundant food source for several insect herbivores,[5] including larvae of the Monarch butterfly *Danaus plexippus*.[6] When larvae are reared on leaves of this milkweed, the resultant butterflies contain high levels of cardenolides (ca. 0.4% dry weight expressed as digitoxin equivalents)[7] and are highly emetic to the blue jay *Cyanocitta cristata bromia*.[8] However, no investigation has been undertaken on the chemical structure of any individual cardenolide stored in those butterflies or originally present in their food plant. Preliminary TLC analysis[5] has shown that humistratin, the most concentrated cardenolide in the leaves of *A. humistrata*, is the one stored at the highest level in *A. humistrata* fed Monarch butterflies. We report here the isolation and structural elucidation of humistratin.

The scheme of isolation was based on that employed by Reichstein et al.[9] The hot aqueous methanol extract of leaves and stems of *A. humistrata* was partitioned between water and organic solvents of increasing polarity. The chloroform partition layer, into which the major portion of humistratin had been transferred from the aqueous suspension, was further fractionated by column chromatography to give needle crystals of humistratin in a yield of 0.018%.

Humistratin gave a strong positive osazone reaction for methylreductinic acid, and its electron-impact mass spectrum had two prominent peaks at m/z 128 and 113, assignable to methylreductinic acid ($C_6H_8O_3$) and its demethylation product, respectively. These facts are considered[10,11] to constitute strong evidence that the cardenolide glycoside may contain a 4,6-dideoxyhexosulose moiety which is doubly conjugated to the aglycon, thereby producing a dioxane ring, as depicted in A. Such a doubly



A

linked 4,6-dideoxyhexosulose, first formulated by Coombe and Watson[12] for the sugar moiety in gomphoside, has also

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(2) Review (a) M. Rothschild, *Symp. R. Entomol. Soc. Lond.*, **6**, 59 (1972); (b) S. S. Duffey, *Annu. Rev. Entomol.*, **25**, 447 (1980).
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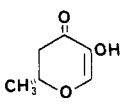
(10) F. Brüschweiler, K. Stöckel, and T. Reichstein, *Helv. Chim. Acta*, **52**, 2276 (1969).
(11) P. Brown, J. von Euw, T. Reichstein, K. Stöckel, and T. R. Watson [*Helv. Chim. Acta*, **62**, 412 (1979)] have discussed the origin of this fragment, i.



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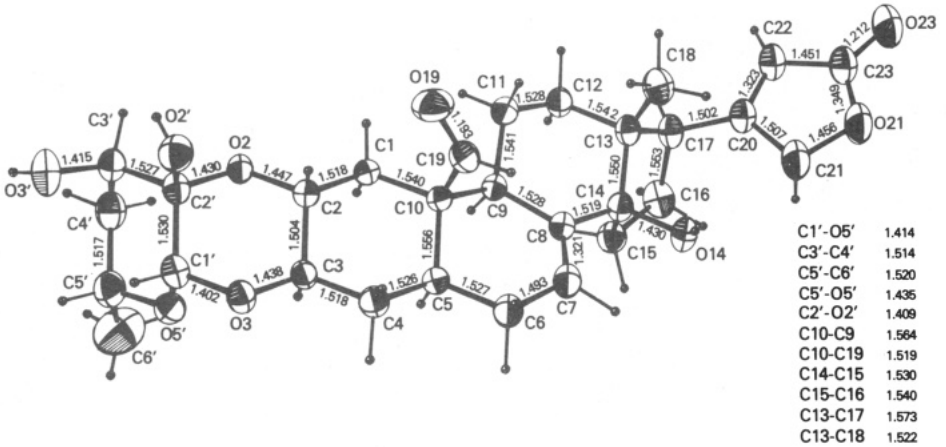

Figure 1. Perspective view of humistratin (**5**) with bond lengths (estimated standard deviations <0.004 Å).

been reported to be present in calactin (**1**),[10] calotropin (**3**),[10] proceroside,[13] syriobioside,[11] desglucosyrioside,[11] and afroside.[14] TLC comparison excluded the possibility that humistratin might be identified with any of these cardenolides.[15]

Besides the two prominent peaks at m/z 129 and 111 characteristic[14] of the 4,6-dideoxyhexosulose moiety, the chemical-ionization (methane as the reagent gas) mass spectrum of humistratin exhibited three large fragments at 403, 385, and 367, the first one indicative of the quasi-molecular ion of the genin. The molecular weight of humistratin thus obtained (129 + 403 − 2 = 530), combined with its elemental analytical data, leads to the molecular formula $C_{29}H_{38}O_9$. Humistratin therefore represents a new cardenolide whose hypothetical genin (named humistratagenin) possesses two less hydrogen atoms than does calotropagenin (**4**), the common genin of calactin (**1**) and calotropin (**3**).

In the electron-impact mass spectrum of humistratin, there were two small peaks visible at m/z 274 and 231, accompanied by their dehydration and decarbonylation products. These two fragments probably resulted from sequential loss, from a monodehydrated genin ($C_{23}H_{28}O_5$ = 384, which was not visible), of $C_6H_6O_2$ (m/z 110) comprising C(16), C(17), and the butenolide ring of the C_2H_3O (43) composed of C(14)–O and C(15), as seen more typically[16] in the electron-impact fragmentation of digitoxigenin. Examination of the electron-impact mass spectrum of calactin (**1**)[10] revealed that all the above-mentioned genin fragments of humistratin (including dehydration and decarbonylation products) had their corresponding counterparts[17] in the electron-impact mass spectrum of calactin, with each humistratagenin fragment smaller than its calotropagenin counterpart by 2 atomic mass units. These facts, together with the infrared absorption band of humistratin at 1729 cm^{-1} indicative of the presence of an aldehydic functionality,[18] suggest that humistratagenin may differ from calotropagenin (**4**) only by possessing an additional double bond in ring A, B, or C.

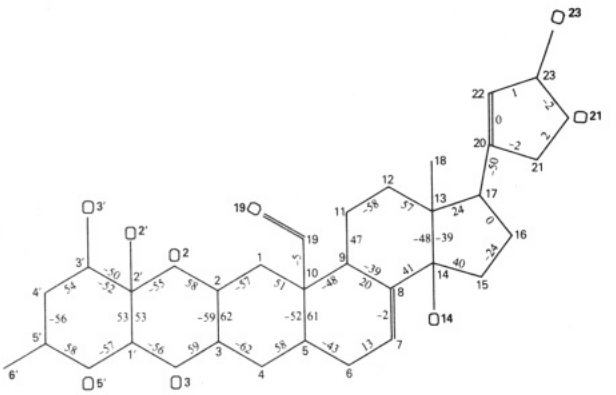

Figure 2. Torsion angles of humistratin (**5**).

Comparison of the ^1H NMR and ^{13}C NMR spectra of diacetylhumistratin with those of diacetylcalactin (**2**)[11] confirmed the above structural model of humistratin. In addition, the small coupling constant (J = 3 Hz) for a triplet at δ 5.76 in the ^1H NMR spectrum of diacetylhumistratin suggests that the proton on C(3′) has an equatorial orientation, the hydroxyl group on the same carbon of humistratin thus being axial, as found in calactin (**1**), but in contrast to calotropin (**3**), whose hydroxyl group at 3′ is equatorial.[19] All the other NMR signals from the sugar moiety of diacetylhumistratin had virtually the same chemical shifts as the corresponding signals of diacetylcalactin (**2**), which can be taken as evidence that the 4,6-dideoxyhexosulose moiety of humistratin has the 1′*S*,2′*S*,3′*R*,5′*R* configuration, as recently determined[20] for gomphoside and afroside.

Two olefinic carbon peaks at δ 121.0 and 139.7 (doublet and singlet, respectively, in off-resonance ^1H decoupled) in the ^{13}C NMR spectrum of diacetylhumistratin indicate that the double bond in ring A, B, or C is trisubstituted. Of the six possible positions where the trisubstituted double bond can be located, Δ^7 appears to be the most compatible[21] with the chemical shift (δ 5.73) of the olefinic

(12) R. G. Coombe and T. R. Watson, *Aust. J. Chem.*, **17**, 92 (1964).
(13) F. Brüschweiler, W. Stöcklin, K. Stöckel, and T. Reichstein, *Helv. Chim. Acta*, **52**, 2086 (1969).
(14) H. T. A. Cheung, R. G. Coombe, W. T. L. Sidwell, and T. R. Watson, *J. Chem. Soc., Perkin Trans. 1*, 64 (1981).
(15) Except syriobioside, the authentic sample of which was not available.
(16) E. Flaskamp and H. Budzikiewicz, *Biomed. Mass Spectrom.*, **4**, 354 (1977).
(17) The same series of ions are more prominently represented in the electron-impact spectrum of uscharidin, which also contains calotropagenin (**4**; see ref 10).
(18) The other carbonyl absorption band at 1737 cm^{-1} is due to a butenolide ring [see O. P. Mittal, C. Tamm, and T. Reichstein, *Helv. Chim. Acta*, **45**, 907 (1962)].
(19) The axial proton on C(3′) of diacetylcalotropin gives rise to a quartet with larger coupling constants (J_{aa} = 10 Hz, J_{ae} = 6 Hz; see ref 11).
(20) The same configuration has been extended to the 4,6-dideoxyhexosulose moiety in calactin (**1**), syriobioside, desglucosyrioside, and syrioside (see H. T. A. Cheung and T. R. Watson, *J. Chem. Soc., Perkin Trans. 1*, 2162 (1980).
(21) The substituent constants used to calculate the chemical shift for the olefinic proton at six possible positions were from C. Pascual, J. Meier, and W. Simon, *Helv. Chim. Acta*, **49**, 164 (1966).

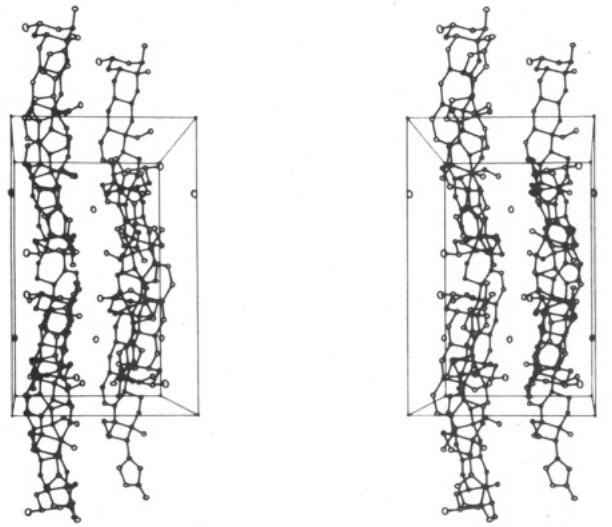

Figure 3. Stereoscopic unit-cell packing diagram. H atoms are omitted for clarity. There is one molecule of water of solvation for each two molecules of humistratin (**5**).

proton assigned to the trisubstituted double bond.

Unequivocal proof of the chemical structure of humistratin was obtained by a direct single-crystal X-ray crystallography. The crystal conformation, atomic nomenclature, and bond lengths are shown in Figure 1 (ORTEP[22] drawing). The torsion angles are given in Figure 2. The bond lengths furnish unambiguous evidence that the trisubstituted double bond is between C(7) and C(8). The torsion angles show that humistratin and calactin (**1**) share the same ring junctures except that the B–C fusion is quasi-trans in humistratin. The torsion angle also shows that humistratin has the 3′-hydroxyl cis to the 2′-hydroxyl, as found in calactin (**1**). Humistratin (**5**) is thus equivalent to 7-dehydrocalactin. Its hypothetical sugar, 4,6-dideoxy-β-D-*glycero*-D-*glycero*-2-hexosulopyranose, is doubly linked at 1 and 2, through acetal (glycosidic) and hemiketal bonds, to positions 3β and 2α, respectively, of humistratagenin, 2α,3β,14-trihydroxy-19-oxo-5α,14β-carda-7,20-(22)-dienolide, to form a dioxane ring with the new chiral center at C(2′) *S*. The six-membered rings, apart from ring B, all adopt fairly standard chair conformations and ring B has the usual cyclohexene monoplanar[23] conformation. The D ring of the steroid moiety has a 14-envelope conformation (the parameters given by Altona et al.[24] are P_m = 41.4, Δ = 35.6) and the lactone ring is almost flat.

There appears to be no intramolecular hydrogen bonding, but four types of hydrogen bonds link the molecules of humistratin (**5**) into a three-dimensional network to form a crystal. The water molecule, which is present in humistratin crystals as a molecule of crystallization, lies on a twofold axis parallel to *c* and is linked to O(2) in each of two cardenolide molecules. The linkage O(2′)–O(19) reinforces the interaction. The molecules are also linked along the *b* screw axis by the O(3′)–O(23) hydrogen bond and effectively along a primitive axis by the O(2′)–O(14) bond between molecules related by the screw axis, parallel to *b* through (1/4, 0, 1/4). A stereo diagram showing the packing is given as Figure 3.

There are a number of Δ^7 steroids known from natural sources,[25] but humistratin (**5**) appears to be the first cardenolide to possess unsaturation at C(7)–C(8).[26] Although its close structural resemblance to calactin (**1**), which is considered[27] to be one of the most effective cardenolides for the induction of emesis, suggests that humistratin (**5**) may have a comparably high emetic potential, it remains to be determined to what extent the presence of Δ^7 unsaturation affects the emeticity of this new cardenolide. Isolation of 7,8-epoxycardenolides has recently been reported from other *Asclepias* species.[11,28] It would be of interest, from a biogenetic standpoint, to know if *A. humistrata* also produces a 7,8-epoxycardenolide for which humistratin (**5**) may serve as a precursor. This study represents the first X-ray structural determination of an asclepiadaceous cardenolide with a doubly linked hexosulose moiety.[29]

Experimental Section

The melting point (uncorrected) was determined on a Hoover capillary apparatus. The IR spectrum was obtained on a Shimadzu 420 spectrophotometer with a reflection beam condenser. The mass spectra (MS) were taken at Mass Spectrometer Laboratory, Department of Chemistry, University of Georgia, using a Finnigan 4000 Quadrupole GC–mass spectrometer by direct insertion at 70 eV. The ionization chamber was operated at a pressure of 5.5×10^{-7} torr on the electron-impact (EI) mode and of ca. 3.8×10^{-5} torr on the chemical-ionization (CI) mode. The source temperature was 290 °C, and the probe temperature was programmed to increase ca. 0.7 °C/starting with 35 °C. The ^1H NMR spectrum was recorded on a Nicolet NT360 spectrometer. The ^{13}C NMR spectrum was recorded on a JEOL FX60 spectrometer. Both ^1H and ^{13}C shifts are given in parts per million relative to Me_4Si (δ 0), and coupling constants are in hertz. The combusion analysis was performed at Institute for Chemical Research, Kyoto University, Uji, Japan. TLC was carried out on precoated silica gel G, 0.25 mm in thickness (Brinkmann Instruments, Inc.), with CH_2Cl_2–MeOH–H_2O (90:9:1) or EtOAc–MeOH (97:3). Visualization of cardenolide spots was effected by spraying first with a saturated solution of 2,2′,4,4′-tetranitrobiphenyl in C_6H_6 and then with a 10% solution of KOH in 50% aqueous MeOH.

Isolation of Humistratin (5). The leaves and stems of *Asclepias humistrata* Walt., collected in March 1979 ca. 6 km south of Highlands Hammock State Park, Hardee Co., FL, were dried in the oven at 80 °C for 24 h and separately pulverized. A batch of ca. 200 g was extracted for 34 h in a Soxhlet extractor with 3 L of 80% aqueous MeOH (increasing percentage of MeOH, beginning with 50%). The filtrate was shaken with petroleum ether (3 × 1.5 L) and the petroleum ether layer was back-extracted with 80% aqueous MeOH (3 × 450 mL). The combined aqueous MeOH extracts were concentrated in vacuo to 200 mL of aqueous suspension, which was shaken successively with Et_2O (3 × 400 mL), $CHCl_3$ (4 × 400 mL), and $CHCl_3$–EtOH (3:2, 6 × 250 mL). Each organic layer was washed successively with H_2O, 2 N Na_2CO_3, and H_2O (160 mL each) and evaporated in vacuo to dryness. After TLC monitoring, the $CHCl_3$ extracts from two batches of leaves and two batches of stems were combined (ca. 2.3 g in total), adsorbed onto 10 g of silica gel ("Baker Analyzed" reagent, 60–200

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(25) Notably, the ecdysones show the structural similarities of 2- and 14-oxygenation; cf. K. Nakanishi, T. Goto, S. Ito, S. Natori, and S. Nozoe, "Natural Product Chemistry", Academic Press, New York, 1974, Vol. 1, Chapter 6.
(26) The cardenolides known prior to 1967 are listed in review by J. H. Hoch, "A Survey of Cardiac Glycosides and Genins", University of South Carolina Press, Columbia, SC, 1961; B. Singh and R. P. Rastogi, *Phytochemistry*, **9**, 315 (1970).
(27) S. S. Duffey, *Proc. Int. Congr. Entomol., 15th*, **1976**, 323 (1977).
(28) H. T. A. Cheung, T. R. Watson, J. N. Seiber, and C. Nelson, *J. Chem. Soc., Perkin Trans. 1*, 2169 (1980).
(29) There are two previous X-ray structure determinations of nonasclepiadaceous cardenolides containing similar doubly linked sugar moieties: elaedendroside A (S. M. Kupchan, I. Uchida, K. Shimada, B. Y. Fei, D. M. Stevens, A. T. Sneden, R. W. Miller, and R. F. Bryan, *J. Chem. Soc., Chem. Commun.*, 255, 1977) and affinoside B [T. Yamauchi, K. Miyahara, F. Abe, and T. Kawasaki, *Chem. Pharm. Bull.*, *27*, 2463 (1979)].

mesh), put on top of a column (150 g of silica gel, 3.6 cm in diameter, wet packing in C_6H_6), and eluted in 800-mL fractions with C_6H_6, C_6H_6–Et_2O (1:2), and increasing percentage of $CHCl_3$–EtOAc–MeOH (1:1:1) in Et_2O: 1%, 2.7%, 7.4% (2×), 20%, 30% (2×), 40%, 50%, 75%, 100%. The eluents were allowed to stand in test tubes at room temperature until precipitation occurred as a result of gradual evaporation of solvents. The 7.4% fractions thus gave colorless needle crystals (140 mg) of **5**, mp 246–251 °C dec. Thin-layer chromatography of the crystalline material showed identical behavior with the major constituent of the crude extract and no other cardenolides. **5**: IR (KBr) λ_{max} 3500 (OH), 2945 (CH), 1778, 1737 (C=O), 1729 (C=O), 1659, 1620 (C=C), 1448, 1423, 1379, 1345, 1309, 1273, 1221, 1170, 1137, 1113, 1091, 1029, 1017, 987, 956, 923, 899, 868, 817 cm^{-1}; MS (EI), m/z (relative intensity) 366 [[G(= M − 128) − $2H_2O$]$^+$, 0.24], 356 [(G − H_2O − CO)$^+$, 0.24], 338 [[G− $2H_2O$ − CO)$^+$, 0.41], 320 [(G − $3H_2O$ − CO)$^+$, 0.20], 274 [(G − 110 − H_2O)$^+$, 0.038], 256 [(274 − H_2O)$^+$, 0.11], 246 [(274 − CO)$^+$, 0.038], 238 [(274 − $2H_2O$)$^+$, 0.071], 231 [(274 − 43)$^+$, 0.45], 228 [(274 − H_2O − CO)$^+$, 0.24], 213 [(231 − H_2O)$^+$, 0.31], 203 [(231 − CO)$^+$, 0.52], 185 [(231 − H_2O − CO)$^+$, 0.82], 128 [($C_6H_8O_3$)$^+$, 77], 113 [(128 − CH_3)$^+$, 64]; MS (CI, methane), m/z (relative intensity) 403 [(G + H)$^+$, 20], 385 [(G + H − H_2O)$^+$, 53], 367 [(G + H − 2 H_2O)$^+$, 52], 129 [(M + H − G)$^+$, 100], 111 [(M + H − G − H_2O)$^+$, 16].

Anal. Calcd for $C_{29}H_{38}O_9 \cdot {}^1/_2H_2O$: C, 64.55; H, 7.29. Found: C, 64.90; H, 7.52.

Osazone Reaction[30] of 5 for Methylreductinic Acid. **5** (1 mg) was dissolved in 0.1 N HCl saturated with (2,4-dinitrophenyl)hydrazine (1 mL) by adding ethanol (ca. 0.5 mL) and warming to 76 °C. The orange-red precipitate, formed after 2 days of occasional warming and shaking, was filtered off and washed with 0.1 N HCl and H_2O. It developed a deep violet-blue color in EtOH upon addition of KOH.

Diacetylhumistratin (6). **5** (15 mg) was dissolved in pyridine (0.3 mL) and Ac_2O (0.2 mL) and kept under N_2 at room temperature for 7 days. The reaction solution was free of pyridine by evaporating it several times with C_6H_6. The resultant dried residue was adsorbed onto 300 mg of Al_2O_3 ("Baker Analyzed" reagent), applied to a column [6 g of Al_2O_3, 1 cm in diameter, dry packing in C_6H_6–EtOAc (99:1)], and eluted in 20-mL fractions with C_6H_6–EtOAc (99:1), C_6H_6–Et_2O–EtOAc (45:44:1), and Et_2O–EtOAc (99:1, 4×). Evaporation of the third fraction gave ca. 15 mg of **6** as white powder: ^1H NMR (360 MHz, $CDCl_3$) δ 0.78 (3 H, s, H-18), 1.22 (3 H, d, J = 6, H-6′), 2.06 (3 H, s, OAc), 2.13 (3 H, s, OAc), 2.43 (1 H, dd, $J_{1\beta,1\alpha}$ = 13, $J_{1\beta,2\beta}$ = 5, H-1β), 2.81 (1 H, t, J = 5, H-17), 3.9–4.2 (3 H, m, H-2, H-3, H-5′), 4.80 (1 H, dd, $J_{21,21}$ = 18, $J_{21,22}$ = 1.5, H-21), 4.81 (1 H, s, H-1′), 4.94 (1 H, dd, $J_{21,22}$ = 18, $J_{21,22}$ = 1.5, H-21), 5.73 (1 H, br s, $W_{1/2}$ = 14, H-7), 5.76 (1 H, t, J = 3, H-3′), 5.93 (1 H, s, H-22), 9.71 (1 H, d, J = 1, H-19); ^{13}C NMR (15 MHz, $CDCl_3$) δ 38.8 (C-1), 70.6* (C-2), 70.8* (C-3), 34.7 (C-4), 39.5 (C-5), 29.4 (C-6), 121.0 (C-7), 139.7 (C-8), 44.5 (C-9), 52.2 (C-10), 23.2 (C-11), 39.2 (C-12), 50.6 (C-13), 85.0 (C-14), 32.9 (C-15), 27.8 (C-16), 50.1 (C-17), 15.9 (C-18), 205.6 (C-19), 174.2** (C-20), 73.4 (C-21), 118.0 (C-22), 173.8** (C-23), 93.2 (C-1′), 95.4 (C-2′), 70.4* (C-3′), 35.0 (C-4′), 66.6 (C-5′), 20.9 (C-6′), 20.8, 21.7 (Me of OAc), 168.6, 168.8 (C=O of OAc). The presence of pairs of peaks of equal size attributable to acetyl groups assures that this product is a diacetate. Values with one and two asterisks may be interchanged. Assignment of the ^{13}C signals of **6** was based on the off-resonance decoupling experiment and the substituent effects[31] of Δ^7 on the reported assignments[11] of diacetylcalactin (**2**).

X-ray Structure Determination of 5. **5** was crystallized as prismatic needles from moist Et_2O–$CHCl_3$–EtOAc–MeOH. The axial labeling adopted was chosen to order the axial lengths as $a < b < c$. The space group is $B22_12$ (no. 20), with a = 12.216 (1) Å, b = 19.216 (1) Å, c = 22.970 (2) Å, V = 5392.04 Å^3, D_{calcd} = 1.329 g cm^{-3}, and D_{obsd} = 1.31 (1) g cm^{-3} for Z = 8 (asymmetric unit, $C_{29}H_{38}O_9 \cdot {}^1/_2H_2O$; asymmetric unit weight, 539.26). Intensity measurements were made on a Nonius CAD-4 diffractometer using graphite-monochromated Cu K_α radiation (λ = 1.5418 Å). A crystal measuring ca. 0.4 × 0.2 × 0.1 mm was used for data collection. Of 3010 independent reflections measured for θ < 73.82°, 2427 were considered to be observed [$I > 1\sigma(I)$].

The phase problem was solved by using MULTAN.[32] Practically the entire molecule was visible in the E map calculated from the phase set with the best weighted figure of merit. (The three MULTAN tests, ABSFOM, PSIZERO, and RKARLE, were weighted 0.5:1.5:1.0.) The only atoms missing were two on the lactone ring and the methyl carbon atom at C(6′). There was a fairly strong extra peak at a special position near the main molecule. This peak was omitted from the initial model. After isotropic least-squares refinement, the missing atoms were found in a difference map, which also showed the previously mentioned extra peak. The most likely explanation appeared to be that there was a molecule of water of crystallization since water possesses the necessary twofold symmetry. Further refinement justified the insertion of the peak and a difference map showed the peaks appropriate to hydrogen atoms. All other expected hydrogen atoms were also found. Final refinement utilized anisotropic thermal parameters for C and O and isotropic parameters for H. The final R factor was 3.4%. Programs used in the refinement were from the XRAY72 system.[33]

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Registry No. **5**, 81340-34-3; **5** hemihydrate, 81371-04-2; **6**, 81340-35-4.

Supplementary Material Available: Atomic positional and thermal parameters for the heavier atoms (Table I), atomic parameters for the hydrogen atoms (Table II), bond angles for the heavier atoms (Table III), and bond lengths and angles and symmetry operations for the hydrogen bonds (Table IV) (4 pages). Ordering information is given on any current masthead page. Tables of observed and calculated structure factors (18 pages) were submitted to the referees and may be obtained from J.V.S.

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