BIOORGANIC CHEMISTRY 1, 13–31 (1971

Tumor Inhibitors. LXIV.[1] Isolation and Structural Elucidation of Novel Bufadienolides, the Cytotoxic Principles of *Bersama abyssinica*

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Received January 29, 1971; accepted March 19, 1971

The isolation and structural elucidation of six new cytotoxic bufadienolides from *Bersama abyssinica* are reported. Bersaldegenin 1,3,5-orthoacetate (**1**), 16β-hydroxybersaldegenin 1,3,5-orthoacetate (**10**), bersamagenin 1,3,5-orthoacetate (**18**), and 16β-hydroxybersamagenin 1,3,5-orthoacetate (**19**) appear to be the first recognized naturally occurring orthoacetates. Interrelations of bersaldegenin 3-acetate (**5**) with **1** and of 16β-hydroxybersaldegenin 3-acetate (**15**) with **10** are described. The bersaldegenin derivatives **1** and **10** were interrelated with the corresponding bersamagenin derivatives **18** and **19** via the common reduction products **24** and **25**. A novel intramolecular facilitation of equilibration of bersaldegenin 1,3,5-orthoacetate (**1**) and bersaldegenin 1-acetate (**2**) is described, and a plausible rationalization which involves participation of the aldehyde group is presented. The parallelism between cytotoxic activity and inhibition of transport ATPase among the foregoing and other bufadienolides is noted, and a speculative rationalization is discussed.

In the course of a continuing search for tumor inhibitors of plant origin, alcoholic extracts of *Bersama abyssinica* Fresen. (Melianthaceae)[2] were found to show significant inhibitory activity against cells derived from human carcinoma of the nasopharynx carried in cell culture (KB).[3]

The isolation and characterization of two of the cytotoxic principles, hellebrigenin 3-acetate (**6**) and hellebrigenin 3,5-diacetate have been described earlier (*1*).

We report here in detail the isolation and structural elucidation of four novel naturally occurring bufadienolide orthoacetates and of two related acetates.[4] A preliminary report has been published of part of this work dealing with bersaldegenin 1,3,5-orthoacetate (**1**) (*2*).

Changes in the isolation procedures described earlier (*1*) have resulted in an easier separation of an enriched bufadienolide fraction containing the compounds discussed. All steps of the isolation were monitored by thin-layer chromatography, ultraviolet

[1] Part LXIII: S. M. Kupchan and E. Bauerschmidt, *Phytochemistry* **10**, 664 (1971).

[2] Stems, leaves, and fruits of *B. abyssinica* were collected in Ethiopia, January, 1968. The authors acknowledge with thanks receipt of the dried plant material from Dr. Robert E. Perdue, U.S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with U.S.D.A. by the Cancer Chemotherapy National Service Center (C.C.N.S.C.).

[3] Cytotoxicity was assayed under the auspices of the C.C.N.S.C. and the procedures were those described in *Cancer Chemotherapy Rep.* **25**, 1 (1962). We thank Professor D. Perlman for the testing in agar suspension.

[4] All of the new natural products have been named as derivatives of bersaldegenin (1β,3β,5β,14β-tetrahydroxy-19-oxobufa-20,22-dienolide) and bersamagenin (1β,3β,5β,14β-tetrahydroxybufa-20,22-dienolide).

spectroscopy (ca. 298 mμ, α-pyrone ring), and especially, by determination of the cytotoxicity of the fractions.

The preliminary fractionation of the alcoholic extract (A) of *B. abyssinica* is summarized in Fig. 1. Chromatography of fraction C on neutral alumina gave five fractions (I–N) possessing cytotoxic activity. Purification by crystallization and rechromatography afforded, in addition to the previously isolated hellebrigenin 3-acetate (**6**), six new bufadienolides, bersaldegenin 1,3,5-orthoacetate (**1**), bersaldegenin 3-acetate (**5**),

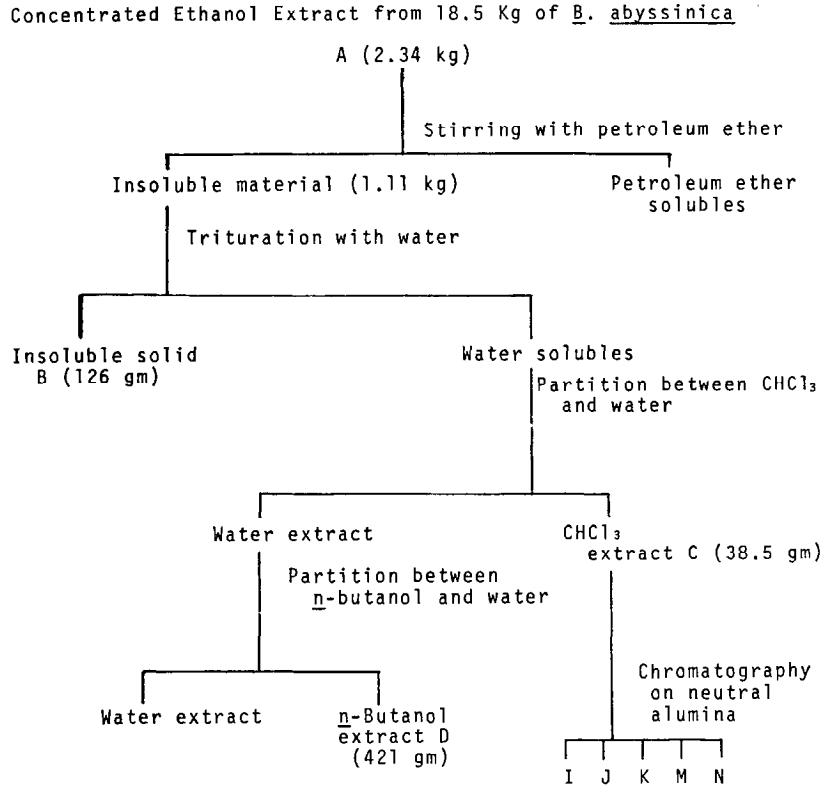


FIG. 1. Flow sheet for fractionation of cytotoxic extract of *B. abyssinica*.

16β-hydroxybersaldegenin 1,3,5-orthoacetate (**10**), 16β-hydroxybersaldegenin 3-acetate (**15**), bersamagenin 1,3,5-orthoacetate (**18**), and 16β-hydroxybersamagenin 1,3,5-orthoacetate[5] (**19**) (see Table 1).

The new compounds isolated showed all the ir and nmr spectral characteristics of bufadienolides. These spectral data and the analyses of compounds **1**, **10**, **18**, and **19** were the first indications that these compounds represented a new type of bufadienolide derivative. All four compounds showed loss of an acetic acid unit in their mass spectra yet possessed no other spectral properties of acetate esters. The most significant differences from the spectra of known bufadienolides and their derivatives were the presence of typical strong sharp bands in the ir spectra at 7.1 (orthoacetate methyl (*3*, *4*)), 7.69 and 8.85 μ (orthoacetate ether (*3*, *4*)), and of a singlet (τ 8.34–8.45,

[5] This compound was previously isolated from the fruits of *B. abyssinica* by Dr. R. J. Hemingway in this laboratory.

TABLE 1

CYTOTOXICITY OF NEW BUFADIENOLIDES

Compound	ED_{50}[a] (μg/ml)
1	7.1×10^{-2}
5	3.6×10^{-5}
10	2.2×10^{-1}
15	1.8×10^{-1}
18	1.7×10^{-2}
19	1.5×10^{-2}

[a] Dose which inhibits cell growth to 50% of control growth.

3H) in the nmr spectra corresponding to orthoacetate methyl (*5*) The mass spectra of **1** and **10** were very similar to that of hellebrigenin 3-acetate (**6**) and the elemental analysis of **1** supported a formula which possessed one more oxygen and two fewer hydrogen atoms than that of **6**. These observations strongly suggested the presence of an A-ring orthoacetate structure in these compounds.

Bersaldegenin 1,3,5-orthoacetate (**1**) was assigned the formula $C_{26}H_{32}O_7$ (M^+ 456) on the basis of elemental analysis and mass spectrum. The nmr spectrum contained signals for a deshielded tertiary methyl group (τ 8.41), two deshielded protons (τ 5.25, $W_H = 6$ Hz and τ 5.56, $W_H = 8.5$ Hz), a deuterium oxide exchangeable proton, and an aldehyde proton (τ −0.46). This compound could not be acetylated and all but three oxygens of the formula were accounted for by the pyrone ring, the aldehyde, and a tertiary hydroxyl group. Hydrolysis of **1** with 80% acetic acid gave a monoacetate (**2**) that showed an nmr spectrum in which the deshielded methyl group signal had been replaced by an acetate methyl signal (τ 8.04), one of the deshielded proton signals had moved upfield (τ 5.40, $W_H = 9$ Hz), and the other downfield (τ 3.82, $W_H = 6$ Hz). In addition, there were three deuterium oxide-exchangeable protons. This strongly suggested that **1** was an orthoacetate attached to two secondary and one tertiary oxygen functions. Acetylation of the monoacetate **2** gave a diacetate (**3**). The nmr spectrum of **3** contained two exchangeable proton signals and two acetate methyl group signals, confirming the presence of two tertiary hydroxyl groups. Mild alkaline hydrolysis of the monoacetate **2** and the diacetate **3** with sodium bicarbonate gave the same new C-10 formyl tetrahydroxybufadienolide, bersaldegenin (**4**).

Examination of Dreiding models and biogenetic considerations suggested that the orthoacetate was attached to the axial functions at positions 1, 3, and 5 with the A-ring in a chair conformation. Comparison of the chemical shift and W_H values (Table 2) for the deshielded proton signals in the nmr spectra of **2**, **3**, and **4** with those in the spectra of the known compounds **6** and **7**, led to the assignment of the structures of bersaldegenin 1-acetate for **2**, bersaldegenin 1,3-diacetate for **3**, and bersaldegenin for **4**. (The narrower and lower field signal was assigned to the proton at the 1-position because of the lower number of protons on adjacent carbons and its proximity to the C-10 formyl carbonyl.) The orthoacetate structure of **1** was confirmed by its synthesis from the tetra-ol **4** via a procedure analogous to the formation of cyclohexane 1,3,5-orthoacetate (*6*). It was deemed likely that the orthoacetate ring was on the β-face of the molecule and this required a *cis* A/B-ring juncture. From the nmr data (W_H) it was known that the 3-OH was axial and, in order to demonstrate the *cis* A/B juncture

TABLE 2. NMR SPECTRAL DATA OF THE BUFADIENOLIDE DERIVATIVES[a]

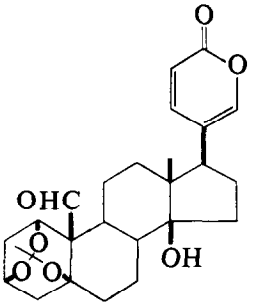
Compound	Acetate methyl				Methyl		Other protons				Dienolide protons			Aldehy
	Ortho-	C-1	C-3	C-16	C-18	C-19	C-1	C-3	C-16	C-17	C-21	C-22	C-23	C-19
1	8.41				9.14		5.25m W_H=6	5.56m W_H=8.5			2.50	1.79	3.61	-0.46
2		8.04			9.18		3.82m W_H=6	5.40m W_H=9			2.50	1.80	3.60	-0.50
3		7.96	7.96		9.18		3.91m W_H=6	4.55m W_H=10			2.55	1.81	3.67	-0.45
4					9.10		4.90m W_H=5	5.37m W_H=8.5			2.51	1.80	3.62	-0.70
5			8.01		9.14		5.00m W_H=5	4.55m W_H=9			2.40	1.75	3.57	-0.63
6			8.00		9.12			4.75m W_H=10			2.50	1.77	3.64	-0.40
7					9.08			5.54 W_H=9			2.54	1.80	3.66	-0.47
8a					9.03		6.10t J=8	5.84m W_H=10			2.58	1.77	3.60	4.60
9	8.57				9.05		5.36m W_H=6	5.61m W_H=8.5			3.44s	2.78d J=15	4.37d J=15	-0.29
10	8.45				9.03		5.16m[b]	5.61m W_H=8.5	5.16t J=7	7.18d J=7	2.50	1.51	3.71	-0.42
11	8.43			8.20	9.07		5.16m W_H=6	5.60m W_H=8.5	4.30t J=7	6.97d J=7	2.46	1.41	3.64	-0.44
12		8.05			9.01		3.84m W_H=6	5.46m W_H=8.5	5.15t J=7	7.17d J=7	2.47	1.47	3.68	-0.52
13		7.95	7.95	8.22	9.07		3.91m W_H=6	4.57m W_H=9	4.34t J=7	6.97d J=7	2.45	1.51	3.68	-0.43
14					9.01		5.00 W_H=7	5.42[b]	5.20	7.16d J=7	2.30	1.49	3.71	-0.68
15			8.02		9.00		5.02[b]	4.48 W_H=9	5.19	7.18d J=7	2.49	1.47	3.68	-0.63
16			8.01	8.20	9.05		5.05 W_H=7	4.98 W_H=9	4.21t J=7	6.95d J=7	2.39	1.34	3.64	-0.65
17	8.42				8.99		5.17	5.61	4.98t J=6	7.18d J=6	2.47	1.46	3.68	-0.50
18	8.35				9.09	8.64	5.98 W_H=6	5.58 W_H=8			2.52	1.70	3.61	
19	8.36				8.95	8.61	6.00 W_H=6	5.65 W_H=9	5.25t J=7	7.15d J=7	2.47	1.44	3.63	
20	8.34			8.18	8.98	8.60	5.96 W_H=7	5.55 W_H=9	4.21t J=7	6.90d J=7	2.37	1.37	3.61	
21 in $CDCl_3$	8.45				9.15		5.21[c]	5.71			2.74	2.15	3.70	
22	8.23				8.78		5.19[bc]	5.62 W_H=9	5.19		2.48	1.45	3.68	
23 in $CDCl_3$	8.48			8.12	9.10		5.23[c]	5.72 W_H=10	4.49		2.72	1.95	3.69	
24	8.35				8.83	8.61	6.00 W_H=7	5.47 W_H=10			5.00[d]		8.20[d]	
25	8.36				8.70	8.63	5.98[b]	5.50[e]	5.85		5.60[de]		8.00[d]	

[a] Determined in D_5-pyridine solution unless otherwise noted; W_H = width at half-height, s = singlet, d = dout t = triplet, m = multiplet.

[b] Overlap with C-16H signal. [c] Overlap with C-19H signal. [d] Two-proton multiplet.

[e] Overlap of C-3H and C-21H.

and the *cis* relationship of the 3-OH and C-10 formyl groups, formation of the 3,19-cycloacetal (*7*) was attempted. Treatment of the monoacetate **2** with methanolic hydrogen chloride, however, afforded the orthoacetate **1** in quantitative yield. In a further attempt to demonstrate these relationships, the tetra-ol **4** was treated under the same conditions and yielded a mixture of epimeric 3,19-cycloacetals in a ratio of 3:2 (**8a**, **8b**). Separation of the mixture gave one epimer (**8a**) in crystalline form and the other (**8b**) amorphous. The nmr spectrum of **8a** was similar to that of the starting material except that the C-19 proton signal now appeared at τ 4.60, the C-3 proton signal had moved upfield, and the C-1α proton now appeared as a higher field (τ 6.10,



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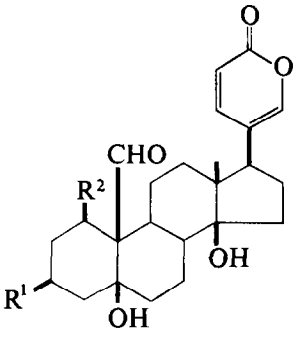
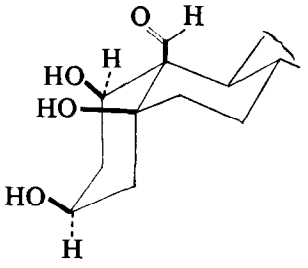
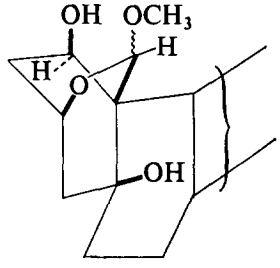


2: R^1 = OH; R^2 = CH_3COO
3: R^1 = R^2 = CH_3COO
4: R^1 = R^2 = OH
5: R^1 = CH_3COO; R^2 = OH
6: R^1 = CH_3COO; R^2 = H
7: R^1 = OH; R^2 = H



4a



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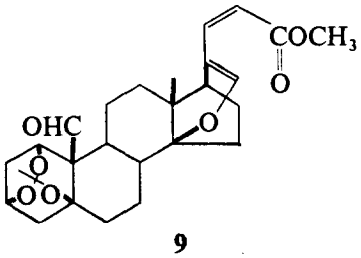


9

$J = 8$ Hz) triplet. In deuteriochloroform solution, the C-3H signal of **8a** appeared at τ 6.00 ($W_H = 10$ Hz) and the C-19H at τ 5.05, in good agreement with the signals for Δ^5-strophanthidin 3,19-cycloacetal (*8*). Formation of the cycloacetal in the 5β-steroidal series requires rings A and B to assume the boat configuration, causing the C-1α-equatorial proton of the starting material to become axial in the product (see **4a**). In order to prove that the remaining tertiary hydroxyl was located at C-14, and in a *cis* relationship with the dienolide ring, **1** was treated with base followed by acid, to obtain the 14,21-enol ether **9**. The nmr spectrum of **9** was fully consistent with the structure assigned and was analogous to that of hellebrigenin enol ether (*1*). Therefore, **1** was assigned the structure bersaldegenin 1,3,5-orthoacetate.[6]

Bersaldegenin 3-acetate (**5**) was assigned the formula $C_{26}H_{34}O_8$ (M^+ 474) on the basis of elemental analysis and mass spectrum. The ir spectrum was similar to that of monoacetates **2** and **6**. The nmr spectrum showed signals for a C-10 formyl group, an acetate methyl (τ 8.01), two deshielded protons (τ 5.00, $W_H = 5$ Hz and 4.55, $W_H = 9$ Hz) and three deuterium oxide-exchangeable protons. Acetylation of **5** with acetic anhydride–pyridine yielded diacetate **3**, identical with a sample obtained by acetylation of bersaldegenin 1-acetate. Hence **5** was a new C-10-formyl bufadienolide monoacetate, and comparisons of chemical shift and W_H values led to its characterization as bersaldegenin 3-acetate.

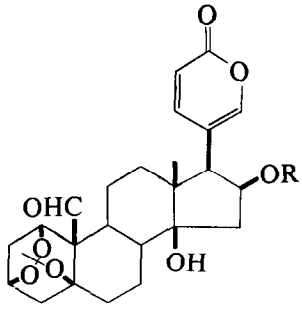
16β-Hydroxybersaldegenin 1,3,5-orthoacetate (**10**) was shown by elemental analysis and mass spectrum to have the formula $C_{26}H_{32}O_8$ (M^+ 472). The ir and nmr spectra were analogous to those of orthoacetate **1** except for the presence of a signal in the nmr spectrum of **10** for an additional deshielded proton (broadened triplet, τ 5.16, $J = 7$ Hz). Double resonance experiments showed this signal was coupled to a one proton doublet at τ 7.18 ($J = 7$ Hz). This indicated that the extra oxygen in **10** was a secondary hydroxyl group at position 16 (*7*).

Acetylation of orthoacetate **10** led to monoacetate **11**. The nmr spectrum showed a signal for the C-16 proton as a broadened triplet at lower field (τ 4.30, $J = 7$ Hz). By procedures similar to those described earlier, hydrolysis of the orthoacetate **10** gave a new C-10-formyl bufadienolide monoacetate, which was characterized as 16β-hydroxybersaldegenin 1-acetate[7] (**12**) on the basis of similar chemical behavior to **1** and **2** and spectral comparisons. Methanolic hydrogen chloride treatment of the 1-acetate **12** gave a quantitative yield of orthoacetate **10**, as expected. Acetylation of **12** gave the 1,3,16-triacetate **13**. Mild alkaline hydrolysis converted the 1-acetate **12** to a new C-10-formyl pentahydroxy bufadienolide, 16β-hydroxybersaldegenin (**14**), which was, in turn, converted back to the naturally occurring orthoacetate **10** via treatment with ethyl orthoacetate and hydrogen chloride. The 14-OH was assigned the β-configuration by analogy with all known naturally occurring bufadienolides and cardenolides (*10*). The 16-OH was demonstrated to be *cis* to the 14-OH by formation of the 14,16-cyclic sulfite **17**. The coupling constant ($J_{16,17} = 7$ Hz) of the C-16 proton signals in the nmr spectra of the 16-hydroxybersaldegenin derivatives and the solvent shifts discussed below indicated that the 16α and 17-protons were *cis*. Consequently, the dienolide ring of these compounds was β-oriented, as in the bersaldegenin series. On the basis of these observations **10** was assigned the structure 16β-hydroxybersaldegenin 1,3,5-orthoacetate.

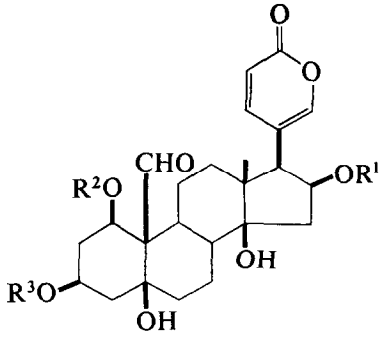
16β-Hydroxybersaldegenin 3-acetate (**15**) was assigned the formula $C_{26}H_{34}O_9$ on the basis of elemental analysis. The spectral data of **15** were very similar to those of

[6] The independent isolation from *Melianthus comosus* and structural elucidation of **1** has recently been reported (*9*).

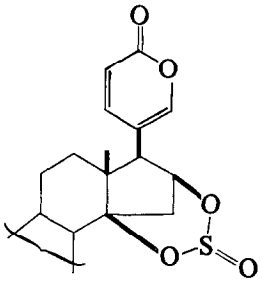
[7] This compound was also isolated as a natural product from fraction D.



10: R = H
11: R = CH_3CO



12: $R^1 = R^3 = H$; $R^2 = CH_3CO$
13: $R^1 = R^2 = R^3 = CH_3CO$
14: $R^1 = R^2 = R^3 = H$
15: $R^1 = R^2 = H$; $R^3 = CH_3CO$
16: $R^1 = R^3 = CH_3CO$; $R^2 = H$



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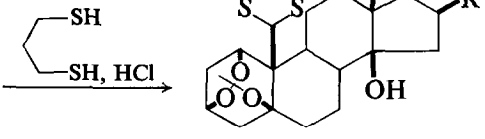
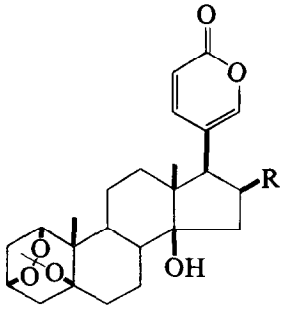
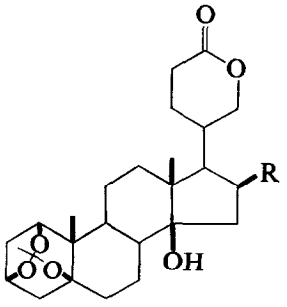


1 and **10**

21: R = H
22: R = OH
23: R = CH_3COO



18: R = H
19: R = OH
20: R = CH_3COO



24: R = H
25: R = OH

bersaldegenin 3-acetate (**5**) and indicated that the compound was the 16β-OH derivative of **5**. Acetylation of **15** yielded diacetate **16** and the triacetate **13**, also obtained from the 1-acetate, **12**. Thus, the oxygenation pattern was the same as in **10**, and by comparison of the chemical shifts and W_H values for the signals of the protons on carbon-bearing acetate with those of **10**, **12**, **13**, and the corresponding bersaldegenin derivatives, **15** was assigned the 16β-hydroxybersaldegenin 3-acetate structure (**15**).

Bersamagenin 1,3,5-orthoacetate (**18**) and 16β-hydroxybersamagenin 1,3,5-orthoacetate (**19**) were assigned the formulae $C_{26}H_{34}O_6$ and $C_{26}H_{34}O_7$, respectively. The spectral data of these compounds indicated that **18** and **19** were the C-10 methyl analogs of orthoacetates **1** and **10**. Acetylation of **19** gave the expected 16β-acetate **20**. Attempted acid hydrolyses under the conditions used for the C-10 formyl orthoacetates were unsuccessful, and only starting materials were recovered. In order to prove the stereochemical similarity of **18** and **19** to the bersaldegenin orthoacetates **1** and **10**, the latter were converted to the respective 19-propane dithioacetals **21** and **22**, which were then subjected to Raney nickel hydrogenolysis (*11*) to afford the crystalline products **24** and **25**, respectively. This treatment had converted the C-10 formyl groups of **1** and **10** to methyl groups. However, the spectral properties of the products indicated the loss of the α-pyrone chromophore in the process. These data indicated that simultaneous hydrogenation of the dienolides to the 20,21,22,23-tetrahydro derivatives had taken place. Similar Raney nickel treatment of the natural products **18** and **19** afforded tetrahydro derivatives **24** and **25**, respectively, identical to those obtained via the thioacetals.

Intramolecular Facilitation

Aqueous acetic acid treatment at 90°C of either a bersaldegenin (C-10 formyl) orthoacetate or a bersaldegenin 1-acetate led to a 1:1 equilibrium mixture of the

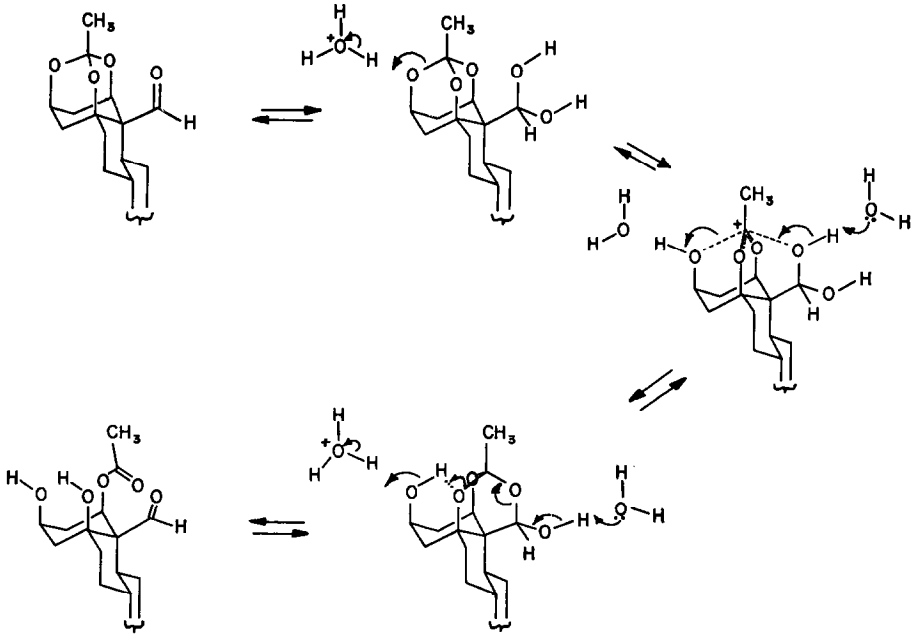


FIG. 2. Equilibration of bersaldegenin 1,3,5-orthoacetate (**1**) and bersaldegenin 1-acetate (**2**).

orthoacetate and the 1-acetate. The C-3 acetates (**5**, **15**) were not detectable in these equilibrium mixtures. (Indeed, **5** and **15** were recovered unchanged after having been subjected to the same conditions.) This behavior of the bersaldegenin derivatives was in marked contrast to the behavior of the bersamagenin (C-10 methyl) orthoacetates which were unaltered by treatment under the same conditions. Treatment of the bersaldegenin 1-acetates (**2**, **12**) with methanolic hydrogen chloride resulted in quantitative conversion to the corresponding orthoacetates; however, the bersaldegenin 3-acetates were unchanged under these conditions also. The enhanced reactivity of the bersaldegenin compounds may be attributable to participation of the aldehyde group (presumably via the intramolecularly solvated cation depicted in Fig. 2) which allows

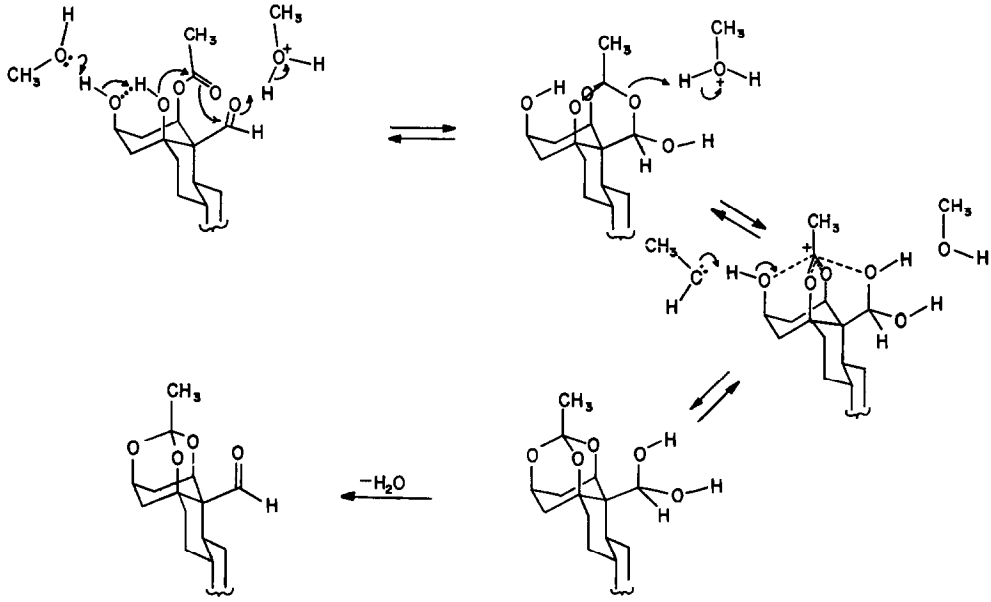
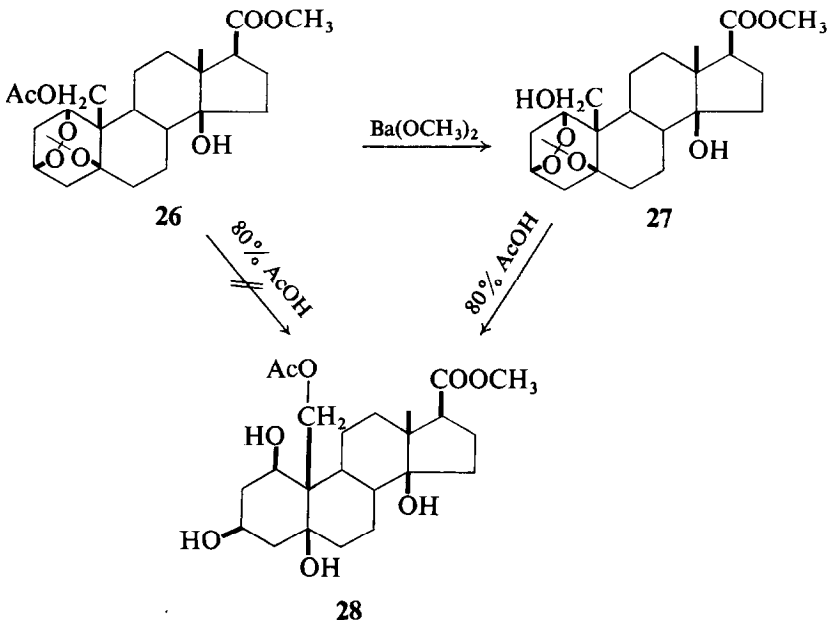


FIG. 3. Orthoacetate formation in methanolic hydrogen chloride.

facile interconversion of the two isomeric orthoacetates. In the orthoacetate formation under anhydrous conditions, participation of the aldehyde again leads to facile interconversion of the isomeric orthoacetates (Fig. 3). However, the irreversible loss of water under these anhydrous conditions drives the orthoacetate formation to completion.

Support for the view that C-19 oxygenated functions can participate in 1,3,5-orthoacetate solvolysis came from the observations of Anderson and Koekemoer (*9*). These authors observed that treatment of **26** with 80% acetic acid effected no change, whereas similar treatment of the 19-ol **27** resulted in a shift of the equilibrium entirely towards the open form, giving **28**. The mechanism of the reaction was visualized as proceeding through the opening of the symmetric 1,3,5-orthoacetate followed by the formation of a new orthoacetate incorporating the C-19 OH group.

The facile interconversion of the bersaldegenin 1-acetates and orthoacetates by mild acid treatment led to consideration of the possibility that the orthoacetates could have been formed during the isolation procedure. However, absorption of the 1-acetates **2** and **12** and the 3-acetate **5** on columns of neutral alumina and quantitative elution



after 48 hr gave only the starting materials **2**, **12**, and **5**, respectively. This observation and the results of thin-layer chromatographic examination of the crude extracts support the view that the orthoacetates are, indeed, naturally occurring compounds.

NMR Spectral Correlations

The nmr data recorded in Table 2 agree well with those presented by Gsell and Tamm (*12*) in a recent exhaustive survey of the nmr spectra of bufadienolides. However, some additional correlations are noteworthy.

The bersaldegenins and bersamagenins possess the same substitution and stereochemistry in ring A and in the rest of the molecule but differ in the nature of the C-10 substituent, the former having a formyl group and the latter, a methyl group. The C-10 formyl group influences the chemical shifts of the neighboring protons in a manner independent of the solvent used (Table 3). The C-1α-H signal was shifted downfield ca. 0.75 ppm, while the signal of the more distant C-3α-H was shifted downfield to a smaller extent (ca. 0.02 ppm). The C-18 methyl and orthoacetate methyl signals were both shifted upfield to a small extent (ca. 0.07 ppm). The direction and magnitude of these shifts may reflect hindered rotation of the formyl group. The diamagnetic shift of both methyl signals suggested that their protons lay in the conical shielding regions (13) on both sides of the plane of the trigonal carbonyl carbon. Two conformations of the aldehyde meet these requirements; however, the one with the carbonyl oxygen atom closer to C-1 is favored because of the magnitude of the paramagnetic shift of the C-1α proton.

The chemical shifts of the low field signals corresponding to the protons at positions 21, 22, and 23 on the dienolide ring were solvent dependent. In deuteriochloroform the signals appeared at τ 2.76 $\pm$ 0.02 ppm (overlapped dd, $J = 2$ and 1 Hz) for the C-21 proton, at τ 2.19 $\pm$ 0.04 ppm (dd, $J = 10$ and 2 Hz) for the C-22 proton, and at τ 3.74 (dd, $J = 10$ and 1 Hz) for the C-23 proton. In pyridine solution these signals were shifted downfield as expected (*12*); however, in the presence of a 16β-hydroxyl

TABLE 3

INFLUENCE OF THE C-10 SUBSTITUENT ON THE CHEMICAL SHIFT OF NEIGHBORING PROTONS

C-10 CH_3 to C-10 CHO		$\Delta(CH_3 - CHO)$ in ppm			
Compound	Solvent	C-1H	C-3H	C-18Me	Orthoac. Me
18 – 1	DPy	−0.73	−0.02	+0.05	+0.06
	$CDCl_3$	−0.69	−0.05	+0.07	+0.07
19 – 10	DPy	−0.84	−0.04	+0.08	+0.09
	$CDCl_3$	−0.70	−0.01	+0.11	+0.05
20 – 11	DPy	−0.81	+0.03	+0.09	+0.09
	$CDCl_3$	−0.73	−0.04	+0.06	−0.02

or acetoxyl group the signal for the C-22 proton was shifted further downfield by ca. 0.33 ± 0.04 ppm (Table 2), while the signals of the C-21 and C-23 protons were shifted to a smaller extent (ca. ±0.07 ppm). This observed specific increase in solvent shift is diagnostic for the 16β-substituted bufadienolides of this series and may indicate a favored conformation of the dienolide ring.

Finally, the aldehyde proton signal of compounds which possess free hydroxyls at positions 1β and 5β (**4, 5, 14, 15, 16**) appeared at τ −0.60 to −0.70, while for all other C-10 formyl compounds it appeared at τ −0.40 to −0.50 when spectra were measured in D_5-pyridine solutions. Additional data illustrating the influence of *cis*-vicinal oxygen functions upon the chemical shift of the aldehyde proton are presented in another report (*25*).

Mass Spectra

Both the high and low resolution mass spectra of the new bufadienolide derivatives have been measured. As expected, all showed a series of fragmentation peaks corresponding to combinations of successive losses of the substituents (OH, OAc, CH_3) on the bufadienolide skeleton. Fragmentation of the α-pyrone ring and the C-10 formyl group resulted in peaks 28 mass units (CO) lower. The 1,3,5-orthoacetates had essentially the same spectra as the corresponding monoacetoxy-bufadienolides after the initial loss of H_2O (−18) from the latter.

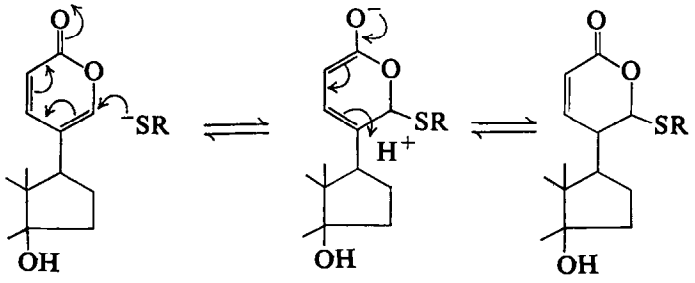
The 16-hydroxy- but not 16-acetoxy-bufadienolides showed an extra initial mode of fragmentation resulting in a series of peaks 44 mass units (CO_2) lower than the simple fragmentation pattern described above. Further study of the mechanism of this interesting fragmentation, which apparently involves interaction of the C-16 hydroxyl group with the α-pyrone ring, is in progress.

Speculative Mechanism of Cytotoxic Activity

The reaction of α,β-unsaturated lactones with thiols has been suggested to play a key role in several biological growth-regulatory phenomena. The selective growth-inhibitory action of δ-hexenolactone on certain animal tissues was shown to be antagonized by cysteine (*14*). Spectrophotometric and colorimetric studies showed that direct and reversible reaction took place between the lactone and the thiol grouping, and it was proposed that δ-hexenolactone exerts its effect on cellular proliferation mainly through its reactivity with sulfhydryl groups essential to enzyme function. Similar studies of a variety of unsaturated lactone antibiotics led to similar proposals concerning their mode of action (*15–17*). The inhibition of plant growth by proto-

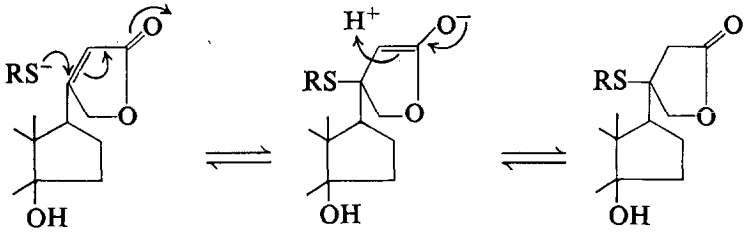
anemonin (*18*), heliangine (*19*), and vernolepin (*20*) is prevented by BAL and other sulfhydryl compounds, and has been attributed to reaction of the inhibitors with sulfhydryl enzymes. Recently, a study of the tumor-inhibitory α-methylene lactones with model biological nucleophiles revealed that thiols were the most reactive of the nucleophiles investigated, and that successive thiol addition to bis unsaturated lactones resulted in marked diminution in the biological activity of the adducts (*21*, *22*). The tumor-inhibitory α-methylene lactones were shown to inhibit the sulfhydryl enzyme, phosphofructokinase and evidence was presented to indicate that the inhibition resulted from their reaction with the sulfhydryl groups of the enzyme (*23*). When substituted endocyclic α,β-unsaturated γ-lactones were exposed to model polyfunctional biological nucleophiles, the principal products were identified as the thio ethers resulting from 1,4-addition of thiols across the substituted conjugated carbonyl systems (*24*).

In an earlier study, direct comparison of the cytotoxic activity (KB) with the brain ATPase inhibitory activity of various cardenolides indicated a close parallel in the relative effectiveness of the compounds in each test (*8*). The cytotoxic bufadienolides of *B. abyssinica*, which have been characterized in this and related reports (*1*, *25*), have also been found to inhibit brain ATPase as well as heart ATPase preparations (*26*). Comparison of the cytotoxic activity and transport ATPase inhibitory activity in both enzyme systems indicates a close parallel in the relative effectiveness of the bufadienolides in each test. It has been shown that the active transport of many amino acids is inhibited by cardiotonic steroids, possibly because of the interrelationship between amino acid transport and sodium transport (*27*, *28*). Inasmuch as tumor cells are very active in accumulating amino acids (*29*), presumably because of their high amino acid requirements for growth, it seems plausible that the inhibition in growth of nasopharyngeal carcinoma cells in culture by cardenolides and bufadienolides may be attributable to their effectiveness in inhibiting ATPase-mediated amino acid accumulation. Sulfhydryl groups have been shown to be involved in the mechanism of action of a nerve cell transport ATPase (*30*), and it is likely that the same groups play equally important roles in the actions of other ATPases. In view of the aforementioned demonstrated selective reactivity of α,β-unsaturated lactones toward thiols, it is tempting to speculate that bufadienolides may exert much of their ATPase inhibitory activity (and cytotoxicity) via an addition reaction with a specific receptor sulfhydryl group, such as a cysteine thiol residue, as illustrated in Scheme 1. The hypothesis is



SCHEME 1

supported by the demonstrated importance of the intact bufadienolide ring system for maximal effects on movements of sodium and potassium across the human red cell membrane; thus, scillaren A was found to have 100 times the activity of hexahydroscillaren A in the inhibition of potassium influx into the cells (*31*). Cardenolides presumably may act via a similar Michael-type reaction with the same receptor sulf-



SCHEME 2

hydryl group, as illustrated in Scheme 2. This hypothesis is supported by the demonstrated substantial diminution in ATPase inhibitory activity (*32*) and cytotoxicity (*8*) which results from hydrogenation of the lactone double bond. Furthermore, in a model experiment, β-methyl-$\Delta^{\alpha,\beta}$-butenolide has been shown to undergo addition of cysteine in the manner exemplified by Scheme 2 (*24*). Similar experiments designed to effect addition of model simple thiols to cardenolides have thus far been unsuccessful (*31, 33*). We postulate, however, that increased reactivity of the sulfhydryl group in a polyfunctional protein molecule, as well as catalytic enzymatic interactions, could favor the addition reaction *in vivo*.

While the unsaturated lactone is a *necessary* function for maximal ATPase inhibition, this function alone is clearly not sufficient to impart inhibitory activity. Thus, the cytotoxic unsaturated lactones elephantopin, euparotin acetate, vernolepin, and withaferin A (*22*) showed no inhibitory activity whatsoever toward ATPase preparations (*34*). It is likely that different α,β-unsaturated lactones exert their growth-inhibitory activities by interaction with enzymatic sulfhydryl groups in varying environments, and, indeed, in different enzymes. A key factor in the effectiveness of a given bufadienolide or cardenolide as an ATPase inhibitor is apt to be the degree to which the steroid residue fits the specificity requirements of the receptor site which incorporates the target sulfhydryl group.

Studies of the 1,4-addition of thiols to cardiotonic steroids and other cytotoxic unsaturated lactones are in progress, and will be reported in due course.

EXPERIMENTAL

Melting points were determined on a Thomas–Hoover capillary melting-point apparatus and are corrected. Infrared spectra were determined on Beckman Model IR-5A and IR-9 recording spectrophotometers. Ultraviolet spectra were determined on a Beckman Model DK-2A recording spectrophotometer. Nuclear magnetic resonance spectra were determined on a Varian A-60A spectrometer using tetramethylsilane as internal standard. Specific rotations were determined on a Zeiss–Winkel polarimeter and are approximated to the nearest degree. Petroleum ether refers to that fraction with bp 60–68°C. Evaporations were carried out at temperatures less than 40°C under reduced pressure. Thin-layer chromatography was carried out on silica gel and aluminum oxide plates (E. Merck) and chromatograms were visualized by spraying with a 3% $Ce(SO_4)_2$–3 *N* H_2SO_4 solution followed by heating. Solvent systems used were: A, $CHCl_3$:MeOH, 40:1; B, $CHCl_3$:MeOH, 40:3 and C, $CHCl_3$:MeOH, 40:5. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

Extraction and preliminary fractionation. Coarsely ground stems (wood and bark) of *B. abyssinica* (18.5 kg) were extracted continuously with two charges of 95% EtOH.

The extract was concentrated to a dark semisolid (A, 2.34 kg), and the noncytotoxic waxes and paraffins were removed by stirring with petroleum ether. The petroleum ether insoluble part (1.11 kg) was triturated with water (5 × 2 liters) and the insoluble material B (126 g) removed by filtration. The aqueous filtrate was extracted with $CHCl_3$ (10 × 5 liters) followed by *n*-butanol (10 × 2 liters). Evaporation of the $CHCl_3$ extract yielded fraction C (38.5 g), and evaporation of the *n*-butanol extract, fraction D (421 g). Preliminary assay showed that the cytotoxic activity was concentrated practically entirely in fractions C and D.

Isolation. The $CHCl_3$ extract C was chromatographed on neutral alumina (Woelm, Act. III, 1.8 kg) to yield 14 fractions (E–R), six of which (I–N) showed marked cytotoxic activity. Trituration of fraction I with MeOH followed by slow evaporation of the MeOH solution yielded a mixture of oily material and long fine crystals. The needles were filtered, washed, and recrystallized from MeOH, to give long fine needles of bersamagenin 1,3,5-orthoacetate (**18**, 71 mg): mp 241–244°C (dec); $[\alpha]_D^{28}$ −90° ($c = 1.07$, $CHCl_3$); λ_{max}^{KBr} 5.78, 6.10, 6.47, 7.14, 7.69, and 8.87 μ; mass spectrum *m/e* 442 (M^+), 424, 382, 364, 346, 339, and 321. (Found: C, 70.45; H, 7.89; $C_{26}H_{34}O_6$ requires: C, 70.56; H, 7.74%.) Trituration of fraction *J* (1.5 g) with cold MeOH, removal of the insoluble material, and evaporation of the MeOH solution gave bersaldegenin 1,3,5-orthoacetate (**1**, 190 mg), which was recrystallized several times from MeOH to give rhombic crystals: mp 282–287°C (dec); $[\alpha]_D^{22}$ −24° ($c = 0.85$, $CHCl_3$); λ_{max}^{KBr} 5.80, 5.88, 6.11, 6.49, 7.10, 7.69, and 8.81 μ; mass spectrum, *m/e* 456 (M^+), 378, 368, 350, 335, 332, and 307. (Found: C, 68.05; H, 7.04; $C_{26}H_{32}O_7$ requires: C, 68.40; H, 7.07%.) Fraction K was crystallized from MeOH to yield colorless crystals of **6** (109 mg): mp 236–240°C (dec), identical to an authentic sample of hellebrigenin 3-acetate (**6**) upon examination by mp, tlc, ir, and nmr spectroscopy. Fraction M was triturated with EtOAc, to yield insoluble and soluble portions. On recrystallization twice from MeOH, the insoluble part yielded colorless crystals of bersaldegenin 3-acetate (**5**, 547 mg): mp 283–287°C (dec); $[\alpha]_D^{22}$ −6° ($c = 1.19$, $CHCl_3$–MeOH, 5:1); λ_{max}^{KBr} 5.81, 6.11, 6.48, 7.83, and 8.85 μ; mass spectrum *m/e* 474 (M^+), 456, 396, 378, 368, 350, 335, and 307. (Found: C, 65.71; H, 7.29; $C_{26}H_{34}O_8$ requires: C, 65.80; H, 7.22%.) The EtOAc soluble part of fraction M was evaporated and recrystallization of the residue three times from MeOH yielded colorless fine plates of 16β-hydroxybersaldegenin 1,3,5-orthoacetate (**10**, 136 mg): mp 282–287°C (dec); $[\alpha]_D^{28}$ +15° ($c = 0.95$, $CHCl_3$); λ_{max}^{KBr} 5.73, 5.78, 6.09, 6.45, 7.14, 7.70, and 8.81 μ; mass spectrum *m/e* 472 (M^+), 454, 428, 394, 366, 348, 333, and 305. (Found: C, 65.91; H, 6.92; $C_{26}H_{32}O_8$ requires: C, 66.08; H, 6.83%.) Rechromatography of the mother liquors from fraction M on silica gel afforded a crude material which, after two recrystallizations from MeOH, yielded large colorless rhombic crystals of 16β-hydroxybersamagenin 1,3,5-orthoacetate (**19**, 55.2 mg): mp 293–295°C (dec); $[\alpha]_D^{28}$ +33° ($c = 1.38$, $CHCl_3$); λ_{max}^{KBr} 5.80, 6.12, 6.48, 7.15, 7.69, and 8.93 μ; mass spectrum *m/e* 458 (M^+), 440, 380, 362, 337, and 319. (Found: C, 67.84; H, 7.43; $C_{26}H_{34}O_7$ requires: C, 68.10; H, 7.47%.) The semicrystalline fraction N was dissolved in MeOH and slowly evaporated, to give 16β-hydroxybersaldegenin 3-acetate (**15**, 32.6 mg) which after two recrystallizations yielded colorless rhombic crystals: mp 293–298°C (dec); $[\alpha]_D^{28}$ +25° ($c = 1.20$, $CHCl_3$); λ_{max}^{KBr} 5.75, 6.09, 6.49, 7.81, and 9.01 μ; mass spectrum *m/e* 490 (M^+), 472, 454, 446, 394, 376, 348, 333, and 330. (Found: C, 63.61; H, 6.73; $C_{26}H_{34}O_9$ requires: C, 63.66; H, 6.99%.)

Acetylation of Bufadienolides

Bersaldegenin 1,3-diacetate (**3**). A solution of bersaldegenin 3-acetate (**5**, 100 mg) in Ac_2O (0.7 ml) and pyridine (2.0 ml) was kept at 25°C for 48 hr. Methanol (5 ml)

was added and the solution was evaporated by warming under a stream of N_2. Separation of product by tlc on silica gel yielded the diacetate **3** (72.8 mg). Two recrystallizations from MeOH afforded colorless plates: mp 255–258°C (dec); $[\alpha]_D^{22}$ −14° ($c = 1.29$, $CHCl_3$); λ_{max}^{KBr} 5.73, 5.80, 6.11, 6.47, 7.94, and 8.00; mass spectrum *m/e* 516 (M^+), 455, 396, 378, 350, 335, and 307. (Found: C, 65.10; H, 6.99; $C_{28}H_{36}O_9$ requires: C, 65.10; H, 7.02%.) Bersaldegenin 1-acetate (8.0 mg) was acetylated under the same conditions, and after recrystallization from MeOH gave crystals of **3** (6.5 mg): mp 253–258°C (dec). The product was identical (R_f, ir, mixture mp) with the diacetate **3** obtained from bersaldegenin 3-acetate.

16β-Acetoxybersaldegenin 1,3-diacetate (**13**). 16β-Hydroxybersaldegenin 1-acetate (**12**, 7.0 mg) was acetylated by the above procedure. The crude product, which crystallized spontaneously from MeOH, was recrystallized from MeOH to yield long needles of **13** (6.1 mg): mp 278–283°C (dec); λ_{max}^{KBr} 5.75, 6.09, 6.49, and 8.00 μ; mass spectrum *m/e* 575 (M^+), 514, 454, 394, 376, 348, 333, and 305. (Found: C, 62.75; H, 6.90; $C_{30}H_{38}O_{11}$ requires: C, 62.71; H, 6.67%.)

16β-Acetoxybersaldegenin 3-acetate (**16**). 16β-Hydroxybersaldegenin 3-acetate (**15**, 13 mg) was acetylated by the above procedure. Separation of the crude amorphous product by tlc on silica gel afforded the triacetate, **13** (1.7 mg), identical to that obtained from **12**, and a second product, the diacetate **16** (5.0 mg). After recrystallization from MeOH, **16** was obtained as colorless crystals: mp 248–253°C (dec); λ_{max}^{KBr} 5.74, 5.81, 6.15, 6.49, and 6.89 μ; mass spectrum *m/e* 532 (M^+), 472, 454, 394, 376, 348, 333, and 305. (Found: C, 63.21; H, 6.76; $C_{28}H_{36}O_{10}$ requires: C, 63.14; H, 6.81%.)

16β-Acetoxybersamagenin 1,3,5-orthoacetate (**20**). 16β-Hydroxybersamagenin 1,3,5-orthoacetate (**19**, 10 mg) was acetylated as above and the product was crystallized from MeOH to afford colorless needles of **20** (5.5 mg): mp 293–295°C (dec); λ_{max}^{KBr} 5.78, 6.10, 6.47, 7.15, 7.69, 8.00, 8.90, and 9.07 μ; mass spectrum *m/e* 500 (M^+), 458, 440, 427, 397, 380, 362, 337, and 319. (Found: C, 67.21; H, 7.19; $C_{28}H_{36}O_8$ requires: C, 67.18; H, 7.25%.)

16β-Acetoxybersaldegenin 1,3,5-orthoacetate (**11**). Acetylation of **10** (9.5 mg) by the above procedure, and recrystallization from MeOH gave rhombic crystals of **11** (5.9 mg): mp 310–312°C (dec); λ_{max}^{KBr} 5.81, 6.11, 6.49, 7.15, 7.69, 8.02, and 9.11 μ; mass spectrum *m/e* 514 (M^+), 486, 472, 454, 436, 426, 366, 348, and 305. (Found: C, 65.66; H, 6.64; $C_{28}H_{34}O_9$ requires: C, 65.41; H, 6.66%.)

Acid Cleavage of Orthoacetates

Bersaldegenin 1-acetate (**2**). A solution of bersaldegenin 1,3,5-orthoacetate (**1**, 190 mg) in 80% HOAc was heated for 3 hr at 90°C in a sealed tube; the reaction mixture was diluted with water (50 ml) and extracted with $CHCl_3$ (10 × 20 ml). The $CHCl_3$ extract was washed successively with water, 2% aq $NaHCO_3$, and water, and evaporated to yield a residue which showed three spots (R_f 0.20, 0.73, and 0.95) on tlc in solvent B. The mixture was separated by tlc on silica gel and the zone of R_f 0.20 gave crude **2** (56.3 mg), which was recrystallized from methanol to afford fine colorless plates (43.2 mg): mp 241–244°C (dec); $[\alpha]_D^{28}$ +0° ($c = 1.14$, $CHCl_3$–MeOH, 5:1); λ_{max}^{KBr} 5.78, 6.10, 6.45, 6.99, and 8.00 μ; mass spectrum *m/e* 474 (M^+), 456, 396, 378, 360, 350, 332, and 307. (Found: C, 65.79; H, 7.14; $C_{26}H_{34}O_8$ requires: C, 65.80; H, 7.22%.)

16β-Hydroxybersaldegenin 1-acetate (**12**). The orthoacetate **10** (28 mg) was hydrolysed by the above procedure (the reaction mixture was extracted with *n*-butanol instead of $CHCl_3$) to yield a residue which after separation by tlc on silica gel and recrystallization from methanol afforded fine white crystals of **12** (10.2 mg): mp 254–258°C (dec); λ_{max}^{KBr} 5.78, 6.11, 6.49, 7.86, and 8.93 μ; mass spectrum *m/e* 490 (M^+), 454,

446, 430, 412, 394, 386, 376, 366, 348, 333, 330, and 305. (Found: C, 63.72; H, 7.09; $C_{26}H_{34}O_9$ requires: C, 63.66; H, 6.99%.)

Attempted acid cleavage of bersamagenin orthoacetates (**18** *and* **19**). The C-10 methyl orthoacetates **18** and **19** (4 mg each) were heated separately for 3 hr at 90°C in 80% HOAc (0.5 ml) in sealed tubes. The solvents were evaporated and the residues showed spots on tlc corresponding only to the unchanged starting materials **18** and **19**, respectively.

Alkaline Hydrolysis of Acetates

Bersaldegenin (**4**). A solution of $NaHCO_3$ (100 mg) in water (15 ml) was added to a solution of bersaldegenin 3-acetate (**5**, 100 mg) in MeOH (30 ml) and the mixture was kept under N_2 at 25°C for 48 hr. The solution was concentrated to 10 ml, the white precipitate filtered, washed with water (5 ml), and the filtrate was evaporated and extracted with methanol (3 × 5 ml). The precipitate and methanol extract were combined and evaporated to give a residue (91 mg) which, after two recrystallizations from methanol, afforded long colorless needles of **4** (48 mg): mp 250–252°C (dec); $[\alpha]_D^{22}$ −3° ($c = 0.73$, $CHCl_3$–MeOH, 5:1); λ_{max}^{KBr} 5.83, 6.10, 6.47, 8.84, and 9.30 μ; mass spectrum *m/e* 432 (M^+), 414, 396, 368, 352, 335, and 307. (Found: C, 66.70; H, 7.43; $C_{24}H_{32}O_7$ requires: C, 66.65; H, 7.46%.)

Small scale experiments with bersaldegenin 1-acetate (**2**, 2 mg) or bersaldegenin 1,3-diacetate (**3**, 2 mg), yielded products identical (R_f, ir) in both cases with bersaldegenin, **4**.

16β-Hydroxybersaldegenin (**14**). 16β-Hydroxybersaldegenin 3-acetate (**15**, 20 mg) was hydrolysed by the above procedure, and the residue purified by tlc on silica gel. Recrystallization from MeOH–$CHCl_3$ gave crystals of **14** (8.1 mg): mp 247–253°C (dec); λ_{max}^{KBr} 5.80, 6.12, 6.49, 8.28, and 8.81 μ. (Found: C, 64.07; H, 7.26; $C_{24}H_{32}O_8$ requires: C, 64.27; H, 7.19%.)

Small scale experiments with 16β-hydroxybersaldegenin 1-acetate yielded a product identical with **14** by R_f, ir, mixture mp.

Bersaldegenin 1,3,5-orthoacetate 14,21-enol ether (**9**). A solution of the orthoacetate **1** (50 mg) in MeOH (3 ml), $CHCl_3$ (2 ml), and 2% NaOH in MeOH (3 ml) was kept for 70 min at 25°C, when tlc showed full conversion of **1** to a product with R_f 0.73 (silica gel, solvent A). The mixture was cooled to 0°C, acidified with excess 5% H_2SO_4 and, after 15 min, diluted with water and extracted with chloroform (3 × 20 ml). The chloroform extract was washed with water, evaporated and the residue was crystallized from methanol. Recrystallization from MeOH–$CHCl_3$ gave long needles of **9** (38.1 mg): mp 279–282°C (dec); λ_{max}^{MeOH} 299 mμ; λ_{max}^{KBr} 5.81, 5.89, 6.21, 6.97, 7.15, 7.69, and 8.62 μ; mass spectrum *m/e* 470 (M^+), 442, 382, 364, 349, 336, and 332. (Found: C, 69.01; H, 7.35; $C_{27}H_{34}O_7$ requires: C, 68.92; H, 7.28%.)

Synthesis of Orthoacetates

Bersaldegenin 1,3,5-orthoacetate (**1**). Bersaldegenin (**5**, 2.5 mg) was suspended in ethyl orthoacetate (0.5 ml) and $CHCl_3$ (0.2 ml) and six drops of a benzene solution saturated with HCl were added. After three days at 25°C the solution was evaporated and the residue separated by tlc on silica gel to give a product (1.5 mg) which on crystallization from MeOH gave **1**, identical (mp, mixture mp, ir, R_f) with authentic bersaldegenin 1,3,5-orthoacetate.

Bersaldegenin 1,3,5-orthoacetate (**1**) *from bersaldegenin 1-acetate* (**3**). (a) A solution of 1-acetate **2** (17.4 mg) in MeOH (1 ml) was treated with 2 drops of 10% methanolic HCl and the mixture kept for 2 hr, at 25°C. The solvent was evaporated and the

residue (15.7 mg) was recrystallized from MeOH to yield **1** (8.3 mg), identical (mp, mixture mp, ir, R_f) with authentic bersaldegenin 1,3,5-orthoacetate.

(b) The 1-acetate (**2**, 4.0 mg) was heated to 90°C in a sealed ampule with 0.5 ml of 80% HOAc for 3 hr. After removal of solvent, the residue showed bands on tlc (solvent B) corresponding to starting material (R_f 0.20) and the orthoacetate **1** (R_f 0.73). The less-polar material was isolated preparatively by tlc and after crystallization from methanol was identical (mp, mixture mp, R_f, ir) with an authentic sample of bersaldegenin 1,3,5-orthoacetate (**1**).

(c) Bersaldegenin 3-acetate (**5**), upon treatment as above with methanolic HCl, was converted very slowly to dehydration products. After 5 days, the mixture still contained unchanged starting material.

16β-Hydroxybersaldegenin 1,3,5-orthoacetate (**10**). (a) The 1-acetate **12** (1.0 mg) was heated to 90°C with 80% HOAc in a sealed ampule for 3 hr, and the solvent was then evaporated. Separation of the products by tlc (Solvent C) on silica gel gave two bands of equal intensity, R_f 0.26 (unchanged **12**) and R_f 0.63, identical to **10** in three solvent systems.

(b) A solution of **12** (4.0 mg) in MeOH (1 ml) was treated with 10% methanolic HCl (3 drops) and the mixture kept for 3 hr. The product (1.9 mg) on isolation and recrystallization from MeOH was identical by R_f, mixture mp, and ir spectrum with authentic **10**.

(c) A suspension of **14** (2.0 mg) in ethyl orthoacetate (1 ml) and $CHCl_3$ (0.5 ml) was treated with 3 drops of benzene solution saturated with HCl. After seven days at room temperature, tlc (solvent C) on silica gel showed a spot R_f (0.63) identical with that of the orthoacetate **10**.

Bersaldegenin 1,3,5-orthoacetate 14β,16β-cyclic sulfite (**17**). A solution of 16β-hydroxybersaldegenin 1,3,5-orthoacetate (**10**, 40 mg) in pyridine (1.5 ml) was cooled to −15°C and treated with thionyl chloride (5 drops). The mixture was kept at −5°C overnight, then evaporated and mixed with ice water (5 ml). The precipitate was filtered and washed with water to yield a crude product (47 mg). After column chromatography on silica gel (6.0 g) with 7% MeOH in $CHCl_3$ as eluent, recrystallization from MeOH–$CHCl_3$ gave colorless crystals of **17** (32 mg): mp 181–182°C (dec); λ_{max}^{KBr} 5.76, 6.11, 6.49, 7.15, and 7.69 μ; mass spectrum *m/e* 518 (M^+), 458, 438, 430, 378, 360, 345, and 317. (Found: C, 60.01; H, 5.80; S, 6.16; $C_{26}H_{30}O_9S$ requires: C, 60.22; H, 5.83; S, 6.17%.)

Bersaldegenin 3,19-cycloacetal (**8a** *and* **8b**). To a solution of bersaldegenin (**4**, 40 mg) in MeOH (5 ml) was added 1 drop of 10% methanolic HCl solution at 25°C and the mixture kept for 1 hr, and evaporated. Preparative tlc on alumina (solvent A) yielded one solid (R_f 0.25, 18.8 mg) and one oily product (R_f 0.38, 11.2 mg).

The solid product, after crystallization from acetone and then from MeOH, yielded colorless rhombohedral crystals of **8a** (10.5 mg): mp 246–248°C (dec); $[\alpha]_D^{22}$ −23° ($c = 0.56$, $CHCl_3$); λ_{max}^{KBr} 5.81, 6.11, 6.49, 7.15, 7.69, and 8.80 μ; mass spectrum *m/e* 446 (M^+), 414, 396, 378, 360, 352, 332, and 307. (Found: C, 67.25; H, 7.62; $C_{24}H_{34}O_7$ requires: C, 67.24; H, 7.68%.)

Attempts to crystallize the amorphous product (**8b**) were unsuccessful; however, the homogeneous product had uv, ir, and mass spectra which were very similar to those of **8a**.

C-19 Thioacetal Formation

(a) *Bersaldegenin 1,3,5-orthoacetate C-19-propylenethioacetal* (**21**). The orthoacetate **1** (40 mg) in a mixture of MeOH (2.0 ml), $CHCl_3$ (0.5 ml), and propane dithiol

(0.5 ml) was treated with 10% methanolic HCl (10 drops), and the mixture was kept for 16 hr. The reaction was monitored by following the development of the tlc spot (R_f 0.19, solvent A) corresponding to **21**. The solution was evaporated under N_2 and the residue separated by tlc on silica gel. The zone of R_f 0.19 yielded crude amorphous thioacetal **21** (23 mg). Attempts to crystallize **21** from different solvents were unsuccessful. The ir spectrum (2.86, 3.38, 5.80, 6.10, 6.47, 7.15, 7.61, and 8.81 μ) and the nmr spectrum (Table 2) of this homogeneous material supported the proposed structure.

(b) *16β-Hydroxybersaldegenin 1,3,5-orthoacetate C-19-thioacetal* (**22**) *and 16β-acetoxybersaldegenin 1,3,5-orthoacetate C-19-thioacetal* (**23**). 16β-Acetoxybersaldegenin 1,3,5-orthoacetate (**11**, 35 mg) was treated as above and yielded two tlc (solvent B) bands. The first (R_f 0.80), upon recrystallization from MeOH, yielded crystals of **23** (11.0 mg): mp 230–240°C (dec); λ_{max}^{KBr} 2.89, 3.38, 5.81, 6.10, 6.49, 7.17, 7.72, and 8.00 μ; mass spectrum *m/e* 604 (M^+), 586, 544, 530, 526, 508, 469, 452, 427, 408, 348, 330 and 305. (Found: C, 61.43; H, 6.62; S, 7.39; $C_{31}H_{40}O_8S_2$ requires: C, 61.58; H, 6.67; S, 7.25%.) The second band (R_f 0.35), upon recrystallization from MeOH gave crystals of **22** (11.8 mg): mp 285–290°C (dec); λ_{max}^{KBr} 2.92, 3.39, 5.78, 6.10, 6.47, 7.17, 7.72, 8.83, and 8.97 μ; mass spectrum *m/e* 562 (M^+), 544, 526, 518, 500, 484, 466, 452, 440, 366, 348, 330, and 305. (Found: C, 62.15; H, 6.93; S, 11.15; $C_{29}H_{38}O_7S_2$ requires: C, 61.91; H, 6.81; S, 11.18%.)

Hydrogenation and Hydrogenolysis

1. *21,22,23,24-Tetrahydrobersamagenin 1,3,5-orthoacetate* (**24**). (a) *From the thioacetal.* A solution of **21** (21.4 mg) in acetone (10 ml) was shaken vigorously at room temperature with freshly prepared Raney nickel W-2 catalyst (*35*) (50 mg) for 5 hr. Fresh catalyst (200 mg) was added and the mixture was shaken for an additional 7 hr. TLC showed two nonfluorescent spots, but none of the starting material or of the expected product, **18**. The catalyst was centrifuged and washed with MeOH (5 × 30 ml), and the filtrate and washings were combined and evaporated. The residue (12.5 mg) was preparatively chromatographed on silica gel plates (solvent B) and the zone with R_f 0.77 yielded a crystalline product (1.3 mg) identical by mixture mp, R_f, and ir spectrum with the material obtained by similar treatment of **18**.

(b) *From the natural product.* A solution of orthoacetate **18** (42 mg) in MeOH (5 ml) and catalyst (400 mg) were shaken for 2.5 hr at 60°C and worked up as above. The residue (35.2 mg) was chromatographed as above and after two recrystallizations from MeOH gave crystals of **24** (12.3 mg): mp 241–245°C (dec); λ_{max}^{MeOH} end absorption only; λ_{max}^{KBr} 2.83, 3.38, 5.76, 7.15, 7.69 and 8.81 μ; mass spectrum *m/e* 446 (M^+), 428, 387, 368, 350, and 313. (Found: C, 70.00; H, 8.64; $C_{26}H_{38}O_6$ requires: C, 69.93; H, 8.58%.)

2. *21,22,23,24-Tetrahydro 16-β-hydroxybersamagenin 1,3,5-orthoacetate* (**25**). (a) *From the thioketal.* A solution of **22** (16.4 mg) in methanol (2 ml) and Raney nickel catalyst (150 mg) were shaken in a sealed tube at 70°C for 2 hr. Workup and preparative chromatography as above led to isolation of the tlc band of R_f 0.57 (solvent B), which, after successive crystallizations from acetone and MeOH, yielded crystals of **25** [0.9 mg, identical by mixture mp, R_f, and ir spectrum with **25** obtained from 16β-hydroxybersamagenin 1,3,5-orthoacetate (**19**)].

(b) *From the natural product.* 16β-Hydroxybersamagenin 1,3,5-orthoacetate (**19**, 27.8 mg) was treated as above, and after recrystallization of the product from MeOH afforded crystals of **25** (9.8 mg): mp 252–255°C (dec); λ_{max}^{MeOH} end absorption only; λ_{max}^{KBr} 2.89, 3.38, 5.73, 7.15, 7.69, and 8.87 μ; mass spectrum *m/e* 462 (M^+), 444, 426,

402, 400, 384, and 366. (Found: C, 67.42; H, 8.35; $C_{26}H_{38}O_7$ requires: C, 67.51; H, 8.28%.)

ACKNOWLEDGMENT

This investigation was supported by grants from the National Institutes of Health (CA-11718 and HE-12957) and the American Cancer Society (T-275), and a contract with Chemotherapy, National Cancer Institute (PH-43-64-551). I.O. was research associate, 1967–1969, on leave from the Institute of Organic Chemistry, Bulgarian Academy of Sciences.

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