3-*endo*-Hydroxy-5-*endo*-carboxybicyclo[2.2.2]octane Lactone (6).—The published methods[6] were modified as follows. Bicyclo-[2.2.2]oct-2-ene-5-carboxylic acid[5,6] (100 g) in 720 ml of 30% (v/v) H_2SO_4 was stirred and heated at 110° for 1 hr. The mixture was cooled, poured onto ice, and extracted with $CHCl_3$. The extract was washed with 10% $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated under reduced pressure, yielding 74.3 g of **6**, mp 207–208°.

3-*endo*-Hydroxy-5-*endo*-carbamylbicyclo[2.2.2]octane (7).—A solution of 500 mg of **6** in 10 ml of MeOH and 10 ml of liquid NH_3 was heated at 110° for 12 hr. The reaction mixture was concentrated under reduced pressure, and the residue was recrystallized from hot $CHCl_3$–petroleum ether, yielding 500 mg of **7**, mp 188.5–189°. *Anal.* Calcd for $C_9H_{15}NO_2$: C, 63.88; H, 8.94; N, 8.28. Found: C, 64.09; H, 9.24; N, 8.50.

3-Keto-5-*endo*-carbamylbicyclo[2.2.2]octane (8).—A solution of 28.6 g of CrO_3 in 358 ml of 90% AcOH was added dropwise in the course of 2 hr to a stirred solution of 40 g of **7** in 385 ml of AcOH. After being stirred overnight, the mixture was concentrated under reduced pressure, diluted with 300 ml of H_2O, and extracted continuously overnight with $CHCl_3$. The extract was dried over $MgSO_4$, filtered, and concentrated under reduced pressure, yielding 26.3 g of crystallized product that was used in the next step. Recrystallization of a small sample of the product from MeOH–Et_2O gave **8**, mp 183–184°. *Anal.* Calcd for $C_9H_{13}NO_2$: C, 64.65; H, 7.84; N, 8.38. Found: C, 64.63; H, 7.93; N, 8.57.

3-Keto-5-*endo*-cyanobicyclo[2.2.2]octane (9).—A mixture of 1.15 g (7 mmol) of **8** and 1.45 g (7.7 mmol) of TsCl in 5 ml of pyridine was heated at 80° for 2 hr. The mixture was diluted with 5 ml of H_2O and concentrated; the residue as taken up in $CHCl_3$, and the solution was concentrated. After the residue was taken up in $CHCl_3$ and the solution was concentrated a second time, the same procedure was repeated with C_6H_6. The residue was then triturated with C_6H_6, and the mixture was filtered. The C_6H_6 extract was concentrated and the residue was recrystallized from $CHCl_3$–petroleum ether, yielding 0.55 g of **9**, mp 145–152° (subl 90–130°). *Anal.* Calcd for $C_9H_{11}NO$: C, 72.45; H, 7.43; N, 9.39. Found: C, 72.52; H, 7.36; N, 9.44.

3-Keto-5-*endo*-carbiminomethoxybicyclo[2.2.2]octane Hydrochloride (10).—A solution of 250 mg of **9** and 100 mg of MeOH in 5 ml of Et_2O was cooled and saturated with anhydrous HCl. The next day, the mixture was poured into 100 ml of Et_2O and filtered, yielding 220 mg of **10**, mp 170–175°.

3-Keto-5-*endo*-(2-imidazolyl)bicyclo[2.2.2]octane (11).—A solution of 107 g (0.49 mol) of **10** in 240 ml of MeOH was added dropwise in the course of 45 min to 144 g (1.08 mol) of β-aminoacetaldehye diethyl acetal at 55–60°. The mixture was stirred at room temperature for 1 hr and concentrated under reduced pressure, yielding a 239-g residue. The product was dissolved in 1 l. of 6 *N* HCl and heated under reflux for 1 hr. The solution was cooled, extracted with $CHCl_3$, made alkaline with concentrated NH_4OH, and extracted with $CHCl_3$. Concentration of the latter yielded a 60-g residue that was purified by adsorption on 550 g of silica gel packed in CH_2Cl_2, elution with 4% MeOH–CH_2Cl_2, and recrystallization from CH_2Cl_2–Et_2O, yielding 37.1 g of **11**, mp 144–147°. *Anal.* Calcd for $C_{11}H_{14}N_2O$: C, 69.44; H, 7.42; N, 14.73. Found: C, 69.63; H, 7.49; N, 14.70.

Registry No.—**2**, 30338-51-3; **4**, 30338-52-4; **5**, 30338-53-5; **6**, 20507-79-3; **7**, 30338-55-7; **8**, 30338-56-8; **9**, 30338-57-9; **10**, 30338-58-0; **11**, 30338-59-1.

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Tumor Inhibitors. LXV.[1] Bersenogenin, Berscillogenin, and 3-Epiberscillogenin, Three New Cytotoxic Bufadienolides from *Bersama abyssinica*[2]

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An ethanol extract of the fruits of *Bersama abyssinica* was found to show significant inhibitory activity against cells derived from human carcinoma of the nasopharynx carried in cell culture (KB). The isolation and structural elucidation of three new cytotoxic bufadienolides, berscillogenin (**1**), 3-epiberscillogenin (**2**), and bersenogenin (**3**), are reported. Mass spectrometry and elemental analysis indicated that all three compounds had a $C_{24}H_{30}O_6$ molecular formula. Chemical and spectral evidence support assignment of structure **1** (16β-hydroxyscilloglaucosidin) for berscillogenin. The same enone (**7**) was obtained from manganese dioxide oxidation of **1** and **2**, indicative that the compounds are C-3 epimers. Treatment of **3** with 80% acetic acid afforded both **1** and **2**. This reaction and the nmr spectrum of **3** indicated that it is a Δ^3-5β-hydroxy isomer of berscillogenin (**1**). The isolation and identification of hellebrigenin 3-acetate (**4**) and scilliglaucosidin (**5**) are also discussed.

In the course of a continuing search for tumor inhibitors of plant origin,[4] we found that extracts of the fruits of *Bersama abyssinica* Fresen. (*Melianthaceae*)[5] showed significant inhibitory activity against cells derived from human carcinoma of the nasopharynx carried in cell culture (KB).[6] Previously, we have reported systematic studies of the KB-inhibitory principles of the stem bark of *B. abyssinica* which led to the isolation and structural elucidation of the cytotoxic principles, hellebrigenin 3-acetate and hellebrigenin 3,5-diacetate,[7] as well as four novel naturally occurring bufadienolide orthoacetates and two related acetate esters.[1] The observation that hellebrigenin 3-acetate showed significant activity *in vivo* against the Walker intramuscular carcinosarcoma 256 in rats stimulated further

(1) Part LXIV: S. M. Kupchan, I. Ognyanov, and J. L. Moniot, *Bioorg. Chem.*, in press.

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(4) S. M. Kupchan, *Trans. N. Y. Acad. Sci.*, **32**, 85 (1970).

(5) Fruits of *B. abyssinica* were collected in Ethiopia, Jan 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 USDA by the Cancer Chemotherapy National Service Center (CCNSC).

(6) Cytotoxicity was assayed under the auspices of the CCNSC and the procedures were those described in *Cancer Chemother. Rep.*, **25**, 1 (1962). Cytotoxicity was also assayed by differential agar diffusion by Professor D. Perlman, University of Wisconsin; *cf.* D. Perlman and J. L. Schwartz, *J. Pharm. Sci.*, **58**, 633 (1969).

(7) S. M. Kupchan, R. J. Hemingway, and J. C. Hemingway, *J. Org. Chem.*, **34**, 3894 (1969).

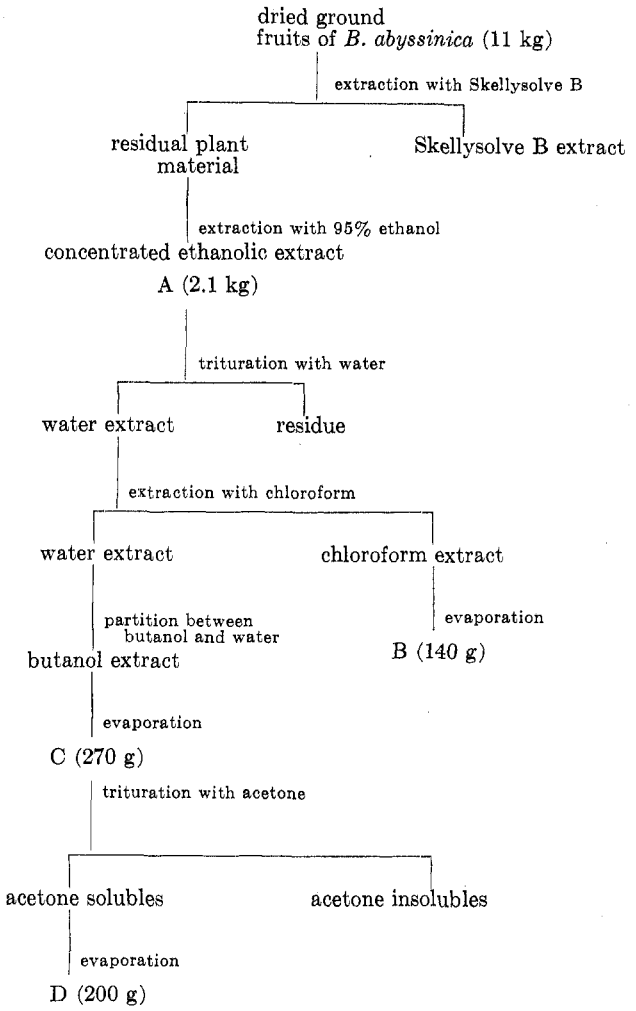
TABLE I

CYTOTOXICITY OF BUFADIENOLIDES FROM THE FRUITS OF *B. abyssinica* AGAINST CELL CULTURE (KB)

Compd	ED_{50}, μg/ml
1	2.8×10^{-2}
2	6.2×10^{-1}
3	4.6×10^{-3}
5	2.0×10^{-3}

CHART I

FRACTIONATION OF CYTOTOXIC EXTRACT FROM *B. abyssinica* FRESEN



studies into the cytotoxic constituents of *B. abyssinica*. We report here in detail the systematic fractionation of the active extract of the fruit of *B. abyssinica* and the isolation of three new cytotoxic A-ring unsaturated bufadienolides, berscillogenin (**1**), 3-epiberscillogenin (**2**), and bersenogenin (**3**), in addition to the previously known hellebrigenin 3-acetate (**4**)[7] and scilliglaucosidin (**5**)[8] (see Table I for cytotoxicity data).

The dried fruit of *B. abyssinica* was defatted by extracting continuously with Skellysolve B and the residue was extracted with 95% ethanol. The concentrated ethanolic extract (A) was triturated with water and the water was extracted extensively with chloroform (B) (see Chart I for summary). The aqueous layer was then extracted with butanol (C). Both fractions B and C were active (9KB) and possessed the ultraviolet chromophore characteristic for the dienolide ring (λ_{max} near 295 nm). Subsequently, it was found that by extracting the aqueous solution directly with a mixture of chloroform–butanol (7:3) a greater proportion of the bufadienolides was concentrated into one fraction and the isolation procedure was simplified. Crystalline material could be obtained directly by chromatography on neutral alumina of the chloroform–butanol solubles.

Filtration of the chloroform solubles (B) on neutral alumina and elution with chloroform–methanol provided the bufadienolide-rich fractions E and F. Rechromatography of fraction F on silicAR CC-7 afforded two new crystalline compounds, bersenogenin (**3**) and 3-epiberscillogenin (**2**). Similar rechromatography of fraction E provided hellebrigenin 3-acetate (**4**), identified by comparison with a sample previously isolated from stem bark,[7] and scilliglaucosidin (**5**), identified by comparison of its physical properties with those reported in the literature.[8]

Trituration of the butanol soluble fraction C with acetone, followed by successive chromatographies of the acetone solubles on neutral alumina and silicAR CC-7, afforded a fifth crystalline bufadienolide, berscillogenin (**1**).[9]

On the basis of elemental analyses and mass spectrometry,[10] berscillogenin (**1**), 3-epiberscillogenin (**2**), and bersenogenin (**3**) were assigned the same molecular formula, $C_{24}H_{30}O_6$. All three compounds had very similar ultraviolet and infrared spectra, which showed the characteristics of bufadienolides [uv max near 299 nm (ϵ 6000) and ir peaks near 2.90 (hydroxyl), 5.80 and 6.12 μ (complex carbonyl)]. The nmr spectra showed the characteristic dienolide ring proton signal pattern.[1,11,12]

A comparison of the nmr spectrum of berscillogenin (**1**) with the spectrum of scilliglaucosidin (**5**) was suggestive that the two compounds possessed very similar structures (see Table II). The two spectra were almost superimposable, with the major differences that berscillogenin possessed one more downfield methine proton at τ 5.18, an additional D_2O-exchangeable proton, and a doublet (J = 7 Hz) at τ 7.19 which was characteristic of a deshielded proton at C-17 when a hydroxyl group is located at C-16.[1] Both compounds showed a single unsplit signal in the olefinic region near τ 4.00. These observations and the cooccurrence of scilliglaucosidin (**5**) in *Bersama* fruits led to the hypothesis that **1** was a 16-hydroxyscilliglaucosidin.

Upon acetylation of berscillogenin (**1**), a diacetate (**6**) was obtained. Two downfield methine protons were deshielded, confirming the presence of two secondary hydroxyl groups. Oxidation of **1** with manganese dioxide afforded the enone **7** with an ultraviolet spectrum characteristic of a six-membered-ring α,β-unsaturated ketone. This reaction established that

(8) A. Stoll, A. v. Wartburg, and J. Renz, *Helv. Chim. Acta*, **36**, 1531 (1953).

(9) Berscillogenin (**1**), 3-epiberscillogenin (**2**), and bersenogenin (**3**) have also been isolated in these laboratories from the stem bark of *B. abyssinica* by similar procedures.

(10) We cordially thank Dr. D. Rosenthal, Research Triangle Institute, and Dr. W. E. Baitinger and Dr. W. L. Budde, Purdue University, for the mass spectra.

(11) (a) Ch. Tamm, *Forschr. Chem. Org. Naturst.*, **14**, 71 (1957); (b) L. F. Fieser and M. Fieser, "Steroids," Reinhold, New York, N. Y., 1959, p 728.

(12) v. L. Gsell and Ch. Tamm, *Helv. Chim. Acta*, **52**, 551 (1969).

TABLE II[a]

NUCLEAR MAGNETIC RESONANCE DATA

Compd	C-3	C-4	C-16	C-17	C-18	C-19	C-21	C-22	C-23
1	5.48 m	3.93 s	5.18 br t (8)	7.19 d (8)	9.00 s	0.08 s	2.49 dd (2, 1)	1.55 dd (2, 9.5)	3.68 dd (9.5, 1)
2	5.51 m	3.97 d (6)	5.18 br t (8)	7.25 d (8)	9.00 s	0.04 s	2.56 dd (2, 1)	1.52 dd (2, 9.5)	3.74 dd (9.5, 1)
3	4.10 br d (10)	4.32 d (10)	5.25 br t (8)	7.18 d (8)	8.97 s	−0.51 s	2.40 dd (2, 1)	1.30 dd (2, 9.5)	3.68 dd (9.5, 1)
5	5.49 m	3.99 s			9.11 s	0.05 s	2.45 d (2)	1.76 dd (2, 10)	3.67 d (10)
6[b]	4.60 m	4.08 s	4.38 m	7.05 d (8.5)	9.07 s	0.10 s	2.55 (2)	1.51 dd (2, 10)	3.77 d (10)
7		3.98 s	5.22 br t (7)	7.22 d (7)	9.00 s	−0.13 s	2.56 d (2)	1.61 dd (2, 10)	3.76 d (10)
8	4.14 br d	4.41 d			8.97 s		2.56 d (1.5)	2.22 dd (1.5, 10)	3.83 d (10)
9[c-e]	3.95–4.20 br d	4.15 d (10)		6.95 s	9.0 s	0.07 s		3.00 dd (2, 9)	3.69 d (9)
10[c,e]	4.0–4.22 m	4.5 d (10)	5.41 m	7.42 d (4)	9.11 s	−0.07 s		2.24 dd (2, 9)	3.87 d (9)

[a] Spectra were determined on a Varian HA-100 spectrometer in pyridine-d_5 unless otherwise indicated. Values are given in τ units relative to tetramethylsilane as internal standard. Multiplicity of signals is designated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; m, multiplet; br, broad. Numbers in parenthesis denote peak separations in hertz. [b] C-3 acetate, 8.01 s; C-16 acetate, 8.27 s. [c] Deuteriochloroform. [d] C-15, 4.12 s. [e] C-21 signal obscured by solvent.

the second secondary hydroxyl of **1** was allylic to a trisubstituted double bond in a six-membered ring. Furthermore, of all the possible positions for this partial structure on the steroid nucleus, only a Δ^4-3-hydroxy compound would show a broad multiplet for the methine proton on the allylic carbon. The C-3 hydroxyl of **1** was assigned the β configuration on the basis of the unsplit olefinic proton signal (C-4 H) which requires the same configuration as scilliglaucosidin (**5**). The remaining tertiary hydroxyl group was presumed to be β at C-14 by analogy with all other known bufadienolides.[11]

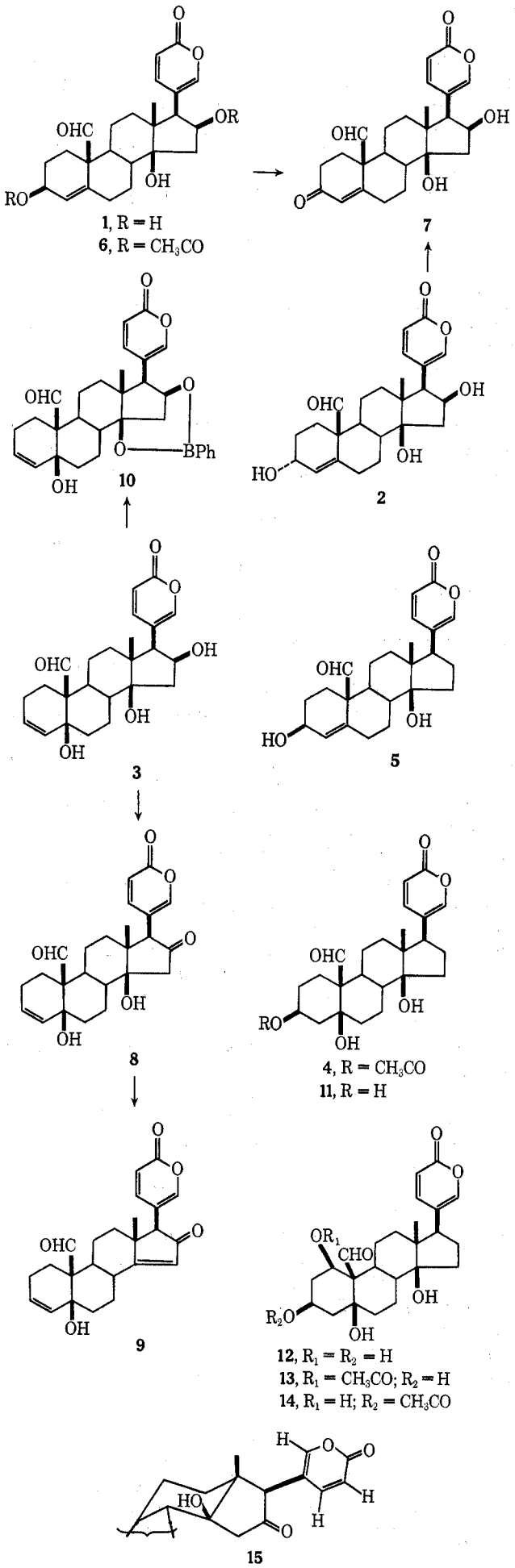
The nmr spectrum of **2** was very similar to the spectrum of berscillogenin (**1**), with the major difference that the olefinic proton signal near τ 4.00 for **2** appeared as a doublet (J = 6 Hz) instead of a singlet. This observation led to the hypothesis that **1** and **2** were epimeric at the allylic alcohol position. Oxidation of **2** with manganese dioxide afforded an enone identical to the one obtained from **1**. Thus **1** and **2** were shown conclusively to be C-3 epimers and, therefore, **2** was assigned the α configuration for the alcohol at C-3.

The nmr spectrum of bersenogenin (**3**) showed a signal for one downfield methine proton (τ 5.25, br t, J = 8 Hz) and a doublet at τ 7.18 (J = 8 Hz), indicative of a secondary hydroxyl function at C-16.[1] There were signals for three D_2O-exchangeable protons, one corresponding to the secondary hydroxyl at C-16 and two persumed to correspond to tertiary hydroxyls. One tertiary hydroxyl group was presumed to be at C-14, and the chemical shift of the C-19 aldehyde proton signal (τ −0.51) strongly suggested that the remaining tertiary hydroxyl was at C-5 (see nmr discussion). The C-5 position appeared most likely also from the standpoint that all other bufadienolides isolated from *Bersama* either possessed an oxygen function at C-5 or could have arisen from a dehydration reaction involving C-5. Signals for two olefinic protons were also notable in the nmr spectrum. The higher field proton signal appeared as a doublet (J = 10 Hz) at τ 4.32 and the other proton as a broad doublet (J = 10 Hz) at τ 4.1. This splitting pattern was consistent with a disubstituted cis double bond with one carbon attached to a methylene group and the other carbon to a quaternary center. It appeared reasonable that **3** was an isomeric A-ring allylic alcohol with a disubstituted double bond and a tertiary hydroxyl group, in contrast to the trisubstituted double bond and secondary hydroxyl group found in **1** and **2**. Further support for this structure was obtained by treatment of **3** with 80% acetic acid at 80° for 30 min, which afforded equal amounts of **1** and **2** along with an unidentified nonpolar product. This interconversion established that the tertiary hydroxyl was at C-5 and the double bond at C-3.

Confirmation of the presence of the C-16 hydroxyl group was accomplished by a modification of the method of Van Wyk and Enslin used for the same purpose.[13] Oxidation of bersenogenin (**3**) with Jones reagent afforded the ketone **8** which was dehydrated with Florisil[14] to afford the five-membered-ring α,β-unsaturated ketone **9** [λ_{max} 232 nm (ϵ 13,800)]. These experiments confirmed the presence of both the C-14 and C-16 hydroxyl groups. The C-16 hydroxyl was demonstrated to be cis to the 14β hydroxyl by preparation of the 14,16-phenyl boronic ester.[15] Attempts to form a 14,16-cyclic sulfite of **1**, **2**, or **3** yielded intractable mixtures, presumably due to side reactions in the A ring. A further indication of the cis relationship between the C-14 and C-16 hydroxyl groups was the observation that for berscillogenin diacetate (**6**) the C-16 acetate methyl signal was shifted upfield to τ 8.27 which, according to Gsell and Tamm,[12] is indicative of a C-14β hydroxyl and C-16β acetate relationship.

The β configuration has been assigned to the C-17 dienolide ring on the basis of the similarity of the chemical shift and coupling constant of the C-17α proton to those of the cooccurring C-17β dienolides.[1,16] Formation of the phenyl boronic ester **10** caused a decreased in the $J_{16,17}$ coupling constant from 8 to 4 Hz. Examination of a Drieding model indicates that, if the C-16 and C-17 protons were trans, the dihedral angle between them would be close to 90°, and a coupling constant significantly lower than that observed would be expected. Furthermore, it had been previously noted that the C-22 proton signal for other 16β-hydroxybufadienolides in pyridine solution was

(13) A. J. Van Wyk and P. R. Enslin, *J. S. Afr. Chem. Inst.*, **21**, 33 (1968).
(14) R. H. Bible, Jr., and N. W. Atwater, *J. Org. Chem.*, **26**, 1336 (1961).
(15) M. Fieser and L. Fieser, "Reagents for Organic Synthesis," Vol. 2, Wiley-Interscience, New York, N. Y., 1969, p 317.
(16) Earlier studies have demonstrated a marked difference in the characteristics of C-17β and C-17α proton signals in the cardenolide series: D. Satoh, H. Ishii, K. Tori, T. Tozyo, and J. Morita, *Justus Liebigs Ann. Chem.*, **685**, 246 (1965).



shifted downfield by *ca.* 0.33 ± 0.4 ppm.[1] The C-21 and C-23 protons were only shifted slightly. This downfield shift appears to be diagnostic for 16β-hydroxyl groups and requires a cis relationship between the dienolide ring and the group at C-16. This same downfield shift was observed for bufadienolides **1**, **2**, and **3**.

Finally, it had been noted previously that the mass spectra for bufadienolides with a 16β-hydroxyl group showed an extra initial mode of fragmentation, resulting in a series of peaks 44 mass units (CO_2) lower than the fragmentation pattern for bufadienolides without 16β-hydroxyl group.[1] This fragmentation apparently involves both the hydroxyl group and the dienolide ring and requires a cis relationship. All three compounds (**1**, **2**, and **3**) showed this characteristic fragmentation pattern.

The above arguments have led to assignment of the structures 3β,14β,16β-trihydroxy-19-oxo-Δ^4-bufa-20,22-trienolide (**1**) for berscillogenin, 3α,14β,16β-trihydroxy-19-oxo-Δ^4-bufa-20,22-trienolide (**2**) for 3-epiberscillogenin, and 5β,14β,16β-trihydroxy-19-oxo-Δ^3-bufa-20,22-trienolide (**3**) for bersenogenin.

Nmr Spectral Correlations.—The nmr spectral data observed for this series of bufadienolides agrees well with the chemical shift–structural correlations reported earlier.[1,12] In addition, it has been observed that, for spectra obtained in pyridine-d_5 solution, it is possible to make reliable predictions of the numbers of hydroxyl groups cis–vicinal to the C-19 aldehyde group from its chemical shift (see Table III). In the absence

TABLE III
CHEMICAL SHIFT OF C-19 ALDEHYDE PROTON IN PYRIDINE-d_5 SOLUTION

Compd	Chemical shift (τ) of C-19 proton	Substituents
Berscillogenin (**1**)	0.08	3β-OH, Δ^4
3-Epiberscillogenin (**2**)	0.04	3α-OH, Δ^4
3-Dehydroberscillogenin (**7**)	−0.13	Δ^4
16-Dehydro-Δ^{14}-bersenogenin (**9**)	−0.40	5β-OH
Hellebrigenin (**11**)	−0.47	3β,5β-diOH
Bersaldegenin 1-acetate (**13**)	−0.48	1β-OAc, 3β,5β-diOH
Bersaldegenin 3-acetate (**14**)	−0.63	1β,5β-diOH, 3β-OAc
Bersaldegenin (**12**)	−0.70	1β,3β,5β-triOH

of cis–vicinal hydroxyl substituents, the aldehyde proton signal is observed near τ 0.00 with one group, near τ −0.45, and, with two groups, near τ −0.65.

It has been suggested that one conformer of the dienolide ring of 16β-hydroxybufadienolides is favored, due to steric interactions.[1] This was based on the observed specific increase in the solvent shift of the C-22 dienolide ring proton signal of 16β-hydroxybufadienolides when the nmr spectrum was measured in pyridine. Further support for the preference for one conformation was obtained from the nmr spectrum of the 16-oxobufadienolide **8**. In this case the C-21 and C-23 proton signals are shifted only slightly, while the C-22 proton signal is shifted to a higher field by about τ 0.9. This strongly suggests that the C-22 proton and not the C-21 proton lies in the conical shielding region above the C-16 carbonyl group as shown in **15**.

Experimental Section

Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are corrected. Infrared spectra were determined on a Perkin-Elmer 257 and 337 recording spectrophotometers. Ultraviolet asorption spectra were determined on a Coleman Model EPS-3T recording spectrophotometer. Specific rotations were determined on a Rudolph Model 30 polarimeter. Skellysolve B refers to the hydrocarbon fraction with bp 60–68°. Evaporations were carried out under reduced pressure at temperatures of less than 40°. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich.

Extraction and Preliminary Fraction of *Bersama abyssinica*.—Ground dry fruits of *Bersama abyssinica* (11 kg) were extracted continuously with Skellysolve B. The residue was extracted with 95% ethanol for 16 hr and the ethanol extract evaporated under reduced pressure to yield a dark green gum (A, 2.1 kg). The extract was triturated with water (14 l.) and the gummy insolubles were removed by filtration. The water extract (in 2-l. portions) was extracted with fifteen to twenty 500-ml portions of chloroform (which was distilled and reused) until the chloroform soluble materials were removed from the water. The chloroform solubles were combined and evaporated to afford fraction B (140 g). A portion of the remaining water soluble fraction (8 l.) was partitioned between water and 1-butanol to afford a 1-butanol extract which was evaporated to yield C (270 g). The 1-butanol extract (C) was triturated with acetone and the filtrate was evaporated to afford D (200 g).

Isolation of Bufadienolides.—The chloroform solubles B were filtered rapidly through neutral alumina (activity 1) by absorbing from chloroform onto 300 g of absorbent, adding to a column of 2.2 kg of alumina, and eluting with chloroform (0.75 l.), 1% methanol in chloroform (3.7 l.), 3% methanol in chloroform (4.5 l.), and 5% methanol in chloroform (5.1 l.). Fractions were combined on the basis of thin layer chromatography on silica gel (E. Merck) plates, developed with chloroform–acetone–methanol (4:1:0.3), and visualized by spraying with a 3% ceric sulfate in 3 *N* sulfuric acid solution followed by heating. Fraction E (12 g) was obtained by evaporation of the fraction eluted with 1% methanol in chloroform and fraction F (13.6 g) by evaporation of the 3% methanol in chloroform eluate. Fraction E was rechromatographed on SilicAR CC-7 (1.5 kg). The column was eluted with chloroform (4 l.) and the solvent was evaporated to give G (0.81 g). The solvent was changed to 1% methanol in chloroform (4 l.) and the elutate evaporated to afford H (2.1 g). Rechromatography of G on neutral alumina afforded a crystalline fraction. Recrystallization from methanol afforded colorless prisms of hellebrigenin 3-acetate (**4**), identified by comparison of its infrared and nmr spectra with a sample previously isolated from stem bark.[7] Fraction H was crystallized from acetone–hexane to afford 1.2 g of colorless prisms, mp 235–245°. Recrystallization from methanol afforded colorless prisms, mp 240–243°, $[\alpha]^{25}D$ +128° (*c* 0.93, $CHCl_3$). The melting point and infrared, ultraviolet, and nmr spectral data were identical with those reported for scilliglaucosidin (**5**).[8] Fraction F was rechromatographed on SilicAR CC-7 (1.2 kg) by elution with 4% methanol in chloroform (4 l.). Evaporation of the solvent gave I (1.5 g). By elution with 6% methanol in chloroform (6 l.) and evaporation of the solvent, fraction J (0.2 g) was obtained. Fraction I was crystallized from acetone to yield colorless prisms (1.1 g) of **3**, mp 189–190° dec. Recrystallization from methanol afforded rhombs, mp 202–204° dec, and from chloroform, needles: mp 226–230° dec; $[\alpha]^{26}D$ +108° (*c* 1.42, $CHCl_3$); uv max (MeOH) 298 nm (ε 6500); ir (KBr) 3.31, 3.39, 3.46, 3.65, 5.85, 6.14, 6.47, 6.72, 6.89, 7.14, 7.59, and 7.96 μ; mass spectrum *m/e* 414 (M^+), 396, 378, 370, 368, 360, 350, 330, 324, 307.

Anal. Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.59; H, 7.39.

Crystallization of fraction J from methanol afforded 0.080 g of **2** as colorless rhombs: mp 213–215° dec; $[\alpha]^{26}D$ +84° (*c* 0.94, CH_3OH); uv max (95% C_2H_5OH) 299 mμ (ε 6000); ir (KBr) 3.00, 3.39, 5.82, 6.15, 6.50, 6.91, 7.05, 7.82, 7.99, 8.71, 9.05, 9.15, and 9.90 μ; mass spectrum *m/e* 414 (M^+), 396, 378, 370, 368, 360, 350, 330, 324, 307.

Anal. Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.39; H, 7.43.

The acetone solubles D were chromatographed on neutral alumina (2 kg, activity I) by elution with chloroform (6 l.) followed by 20% methanol in chloroform (6 l.). Evaporation of the latter fraction gave K (6.9 g). Rechromatography of a portion of K (3.0 g) on SilicAR CC-7 (300 g) by elution with 4% methanol in chloroform afforded 0.135 g of 1. Crystallization from methanol gave colorless rhombs: mp 214–216° dec; $[\alpha]^{26}D$ +42° (*c* 1.0, CH_3OH); uv max (95% C_2H_5OH) 299 nm (ε 6000); ir (KBr) 2.84, 2.93, 3.05, 3.38, 3.48, 5.83, 6.01, 6.20, 6.50, 6.90, and 8.80 μ; mass spectrum *m/e* 414 (M^+) 396, 378, 370, 368, 360, 350, 330, 324, 307.

Anal. Calcd for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.45; H, 7.25.

Interconversion of Bufadienolides.—A solution of bersenogenin (**3**, 100 mg) in 80% acetic acid was stirred and heated at 80° for 30 min. The solvent was removed by evaporation and the residue dissolved in 20 ml of chloroform, which was washed with 5% sodium bicarbonate solution and saturated salt solution and dried (Mg SO_4). The residue (99 mg) was chromatographed on SilicAR CC-7 (15 g) and eluted with 1% methanol–chloroform. The solvent was evaporated to afford an oil (63 mg) which was not characterized. The solvent was changed to 2% methanol–chloroform and a second fraction was collected (7 mg). Crystallization from methanol afforded berscillogenin (**1**). Continued elution with 4% methanol–chloroform afforded a third fraction (7 mg) which was crystallized from methanol to afford 3-epiberscillogenin (**2**). Both compounds were identified by melting point, mixture melting point, and infrared and mass spectral comparisons.

3-Dehydroberscillogenin (**7**). **A.**—A solution of berscillogenin (**1**, 36 mg) in chloroform (5 ml) was stirred at room temperature with manganese dioxide (0.464 g) for 48 hr, centrifuged, and decanted. The manganese dioxide[17] was washed with hot acetone (20 ml) and the combined supernatant solutions were evaporated and chromatographed on silica gel tlc plates to afford recovered berscillogenin (16 mg) and the enone **7** (12 mg). Crystallization from methanol gave colorless rhombs: mp 258–264° dec; uv max (CH_3OH) 240 nm (ε 14,000) and 298 (6000); mass spectrum *m/e* 412 (M^+), 394, 384, 376, 368, 350, 348, 321, 303.

Anal. Calcd for $C_{24}H_{28}O_6$: 412.1885. Found: 412.1895.

B.—The allylic oxidation of 3-epiberscillogenin (18 mg) using the same procedure as for berscillogenin also gave the enone **7** (4 mg), shown to be identical with the product obtained from berscillogenin by melting point, tlc, infrared, ultraviolet, and mass spectral comparisons.

Berscillogenin 3,16-Diacetate (**6**).—Acetic anhydride (0.5 ml) was added dropwise to a solution of **1** (19.5 mg) in dry pyridine (1.5 ml) and the mixture was stirred for 18 hr at room temperature. The solution was evaporated under a stream of nitrogen, methanol added (2 ml), and the solution evaporated again under nitrogen to afford a solid, which was crystallized from methanol as colorless rhombs (17 mg): mp 242–244° dec; uv max (CH_3OH) 299 nm (ε 6200); ir (KBr) 6.12 and 7.98 μ; mass spectrum *m/e* 498 (M^+), 438, 420, 410, 378, 360, 349, 335, 331, 317.

Anal. Calcd for $C_{28}H_{34}O_8$: C, 67.47; H, 6.87. Found: C, 67.32; H, 6.87.

16-Dehydrobersenogenin (**8**).—To 20 ml of acetone flushed with nitrogen was added bersenogenin (**3**, 50 mg). The solution was cooled to 10° and 0.04 ml of Jones reagent[18] (0.11 mmol of chromium trioxide) was added in one portion. The ice bath was removed and the stirred reaction mixture was allowed to warm to room temperature over a period of 15 min. The green solution was poured into 400 ml of water which was extracted with six 20-ml portions of chloroform. The combined chloroform extracts were washed with saturated salt solution, dried (Mg SO_4), and evaporated to afford a clear oil (51 mg). Preparative thin layer chromatography on silica gel, followed by crystallization from methanol afforded ketone **8** (15 mg) as colorless rosettes: mp 220–222° dec; uv max (95% C_2H_5OH) 299 nm (ε 4000); ir (KBr) 2.83, 2.94, 3.41, 3.48, 5.75 (sh), 5.82, 6.50, and 6.90 μ; mass spectrum *m/e* 412 (M^+), 394, 376, 367, 366, 365, 348, 257.

Anal. Calcd for $C_{24}H_{28}O_6$: C, 69.89; H, 6.84. Found: C, 69.56; H, 6.84.

16-Dehydro-Δ^{14}-bersenogenin (**9**).—To a solution of **8** (30 mg) in 20 ml of benzene–chloroform (1:1) was added Florisil[15] (0.2 g). The slurry was refluxed with stirring for 10 hr and filtered, and the residue chromatographed on preparative tlc silica gel plates to afford enone **9** as a colorless solid (12 mg). Crystallization from methanol gave colorless rhombs: mp 239–241°; uv max

(17) O. Mancera, G. Rosenkranz, and F. Sondheimer, *J. Chem. Soc.*, 2189 (1953).

(18) C. Djerassi, R. R. Engle, and A. Bowers, *J. Org. Chem.*, **21**, 1547 (1956).

(95% C_2H_5OH) 232 nm (ε 13,800) and 300 (5100); ir (KBr) 2.95, 3.35, 3.40, 3.50, 5.72 (sh), 5.81, 5.90, and 6.22 μ; mass spectrum *m/e* 394 (M^+), 376, 358, 348, 347, 257.

Anal. Calcd for $C_{24}H_{26}O_5$: 394.1780. Found: 394.1782.

Bersenogenin 14,16-Phenylboronate (10).—To a solution of bersenogenin (**3**, 19.5 mg) in dry acetone was added phenylboronic acid (5.74 mg) and the solution was allowed to stand at room temperature for 7 min. On addition of hexane (1 ml) crystals formed. The product was collected by filtration and recrystallized from chloroform–hexane to give the cyclic boronic ester (**10**, 13 mg) as needles: mp 221–224°; uv max (CH_3OH) end absorption, 298 nm (ε 6100); mass spectrum *m/e* 423 ($M - C_6H_5$), 405, 377.

Anal. Calcd for $C_{30}H_{33}O_6B$: C, 72.00; H, 6.60; B, 2.20. Found: C, 71.84; H, 6.73; B, 2.12.

Registry No.—**1**, 30344-95-7; **2**, 30344-96-8; **3**, 30344-97-9; **5**, 510-62-3; **6**, 30344-99-1; **7**, 30345-00-7, **8**, 30345-01-8; **9**, 30345-02-9; **10**, 30345-03-0; **11**, 465-90-7; **12**, 23044-69-1; **13**, 23044-67-9; **14**, 23044-72-6.

Identification and Synthesis of the Four Compounds Comprising the Boll Weevil Sex Attractant[1a]

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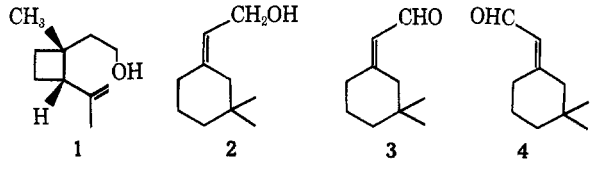
Four terpenoid compounds, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol, (**1**), *Z*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneethanol (**2**), *Z*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (**3**), and *E*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (**4**), were identified as the components of the male sex pheromone of the boll weevil. The synthesis and structural assignments of the four compounds are also reported.

Insect sex attractants (pheromones) are currently of considerable interest since they may provide a generally nontoxic method of surveying and controlling insect populations.[2] The growing concern over the environmental pollution and ecological imbalance caused by insecticides has further stimulated interest in this area.

A pheromone complex emitted by live male boll weevils (*Anthonomus grandis* Boheman) elicits a response by female weevils in laboratory assays.[3] The volatile components of this complex and other compounds were obtained by steam distillation of the crude extracts of 4.5 million weevils and 54.7 kg of weevil feces. The concentrated dichloromethane extract of the steam distillate mimicked the attractiveness of live males in laboratory tests.[4]

We have now isolated, identified, and synthesized four terpenoid compounds (**1**, **2**, **3**, **4**) which account for all of the attractancy of live male weevils. The response by females to mixtures of the synthetic compounds is identical with their response to corresponding mixtures of the natural compounds.



1 2 3 4

The extract of the steam distillate from weevils and their feces was fractionated by column chromatography on Carbowax 20M coated[5] silica gel. None of the individual fractions from this column were attractive to females, but the combination of two of the fractions was as active as the original distillate. Each of these two fractions was then separately fractionated on a column containing Adsorbosil-CABN (25% $AgNO_3$ on silica gel).

Various recombinations of all the fractions from both $AgNO_3$–silica gel columns yielded two fractions, one from each column, that were attractive together but totally unattractive separately. Each of these latter two active fractions was then fractionated by glpc on Carbowax 4000 and SE-30. Three components were collected which were attractive when all three were combined but which were unattractive individually or in pairs. Rechromatography on Carbowax 4000, SE-30, and a 50-ft support coated open tubular (SCOT) column showed two of these components to be pure (**1**, **2**) and the third to consist of two compounds (**3**, **4**). Concentrations of compounds **1**, **2**, **3**, and **4** in fecal material, determined by glpc, were 0.76, 0.57, 0.06, and 0.06, respectively. Concentrations in weevils were about tenfold less. Compound **1** was identified as (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol on the basis of mass, ir, and nmr spectra.[6] The cis configuration was assigned by comparison with the nmr spectrum of the synthetic cis isomer (*vide infra*). The optical rotation was measured on 11 mg of the pure natural compound. The specific rotation was estimated to be about +50° (±10°).

Scheme I outlines the synthesis of *cis*-2-isopropenyl-1-methycyclobutaneethanol. The photocycloaddition of isoprene and 3-buten-2-one produced several products, many of them in greater yield than the desired isomer of 2-methyl-2-vinylcyclobutyl methyl ketone **5**. Compounds **7**, **8**, **9**, **10**, **11**, and **12** were tentatively iden-

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(5) Mention of a proprietary product in this paper does not constitute an endorsement of this product by the U. S. Department of Agriculture.

(6) A preliminary report of this work discussing the isolation and identification was published by J. H. Tumlinson, D. D. Hardee, R. C. Gueldner, A. C. Thompson, P. A. Hedin, and J. P. Minyard, *Science*, **166**, 1010 (1969).