Bufadienolides. 21. Synthesis of Cinobufagin from Bufotalin[1]

GEORGE R. PETTIT* AND YOSHIAKI KAMANO

Department of Chemistry, Arizona State University, Tempe, Arizona 85281

Received July 24, 1972

A synthetic method was developed for transformation of bufotalin (**1a**) to cinobufagin (**5b**). The procedure was based on dehydration of bufotalin acetate (**1b**) to olefin **2** which upon treatment with hypobromous or hypoiodous acid afforded halohydrin **4**. Treatment of halohydrin **4** with either activated alumina or pyridine yielded cinobufagin acetate (**5a**). Selective acid-catalyzed hydrolysis of diacetate **5a** was employed to obtain cinobufagin (**5b**). A number of other cinobufagin derivatives were prepared including deacetylcinobufagin (**5d**) and the new bufadienolide 3-epicinobufagin (**9a**).

Cinobufagin (**5b**) and deacetylcinobufagin (**5d**) are two prominent components of the Chinese medicinal preparation Ch'an Su.[2] The structure of bufotalin (**1a**), another important toad venom constituent, has recently been reconfirmed.[3] To provide further support for the structure of cinobufagin an unequivocal transformation of bufotalin (**1a**) to cinobufagin was undertaken. The interrelationship of bufotalin with cinobufagin by synthesis was also required as part of a projected total synthesis proceeding from 14-dehydrobufalin[4] *via* bufotalin to cinobufagin.

A partial synthesis of cinobufagin (**5b**) was realized by the following route. Bufotalin (**1a**) was isolated from the toad venom preparation Ch'an Su and acetylated. Dehydration of bufotalin acetate (**1b**) was easily performed in pyridine with thionyl chloride to yield olefin **2**. Direct epoxidation of olefin **2** using *m*-chloroperbenzoic acid gave as exclusive product 14α,15α-epoxide **3**, a new isomer of cinobufagin acetate. This result was completely analogous to our earlier experience with α-epoxidation of 14-dehydrobufalin.[4] Accordingly, the halohydrin route developed for synthesis of resibufogenin[4] was applied to the problem at hand. Hypobromous acid prepared *in situ* from *N*-bromoacetamide (NBA) or *N*-bromosuccinimide (NBS) was added to 14-dehydrobufotalin acetate (**2**), and the resulting bromohydrin (**4a**) was treated with basic alumina or pyridine to afford cinobufagin acetate (**5a**). Use of *N*-iodosuccinimide (NIS) and proceeding *via* iodohydrin **4b** led to comparable yields of cinobufagin acetate. Preparation of the hypohalous acid in aqueous acetone and epoxide formation in pyridine provided approximately 80% yields of cinobufagin acetate, while preparation of the halohydrin in dioxane containing a small amount of perchloric acid and ring closure with basic alumina proved less satisfactory and led to about 30% yields of epoxide **5a**.

Selective hydrolysis of cinobufagin acetate (**5a**) to the natural product (cinobufagin, **5b**) was not easily accomplished. Mild treatment of cinobufagin acetate with basic alumina, the basic ion-exchange resin CG-400, ammonium hydroxide, or aqueous potassium bicarbonate gave almost exclusively 3β-acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (**5c**) thereby illustrating sensitivity of the 16β-acetate to base. The same product (**5c**) was obtained using the enzyme Taka-diastase produced by *Aspergillus oryzae*. Fortunately, acid hydrolysis of cinobufagin acetate using Dowex-50 W-X8 gave both cinobufagin (**5b**) and deacetylcinobufagin (**5d**) accompanied by alcohol **5c**. Both cinobufagin and deacetylcinobufagin were found identical with authentic specimens isolated from Ch'an Su. The synthetic sample of cinobufagin was further characterized by preparation of 3,5-dinitrobenzoate (**5e**), cinnamate (**5f**), and succinate (**5g**) esters.

Preparation of deacetylcinobufagin was easily accomplished by hydrolysis of cinobufagin using CG-400 (OH^- form), ammonium hydroxide, Taka-diastase or Dowex-50 W-X8 (H^+ form).[5] The reverse reaction (**5d** → **5b**) was found possible using hot acetic acid, but as expected acetates **5c** and **5a** accompanied cinobufagin.

For the purpose of facilitating the possible isolation of 3-epicinobufagin (**9a**) from toad venom[6] oxidation of cinobufagin to cinobufagone (**6a**) was next viewed.[7] Oxidation of cinobufagin with chromium trioxide in acetic acid easily afforded cinobufagone (**6a**). Mild saponification of ester **6a** gave alcohol **6b**. The same product (**6b**) was prepared from deacetylcinobufagin (**5d**) by selective oxidation with chromium trioxide–pyridine or by *N*-bromoacetamide in methanol–pyridine–water. Chromic acid oxidation of either alcohol **6b** or deacetylcinobufagin provided diketone **7**. The ketone (**8**) isomeric to ketone **6a** was readily obtained by oxidizing alcohol **5c** with chromium trioxide in acetic acid.

Interpretation of the proton magnetic spectra of ketones **6**, **7**, and **8** combined with the partial synthesis of cinobufagin described herein provided further support for the structure of this substance and that of

(1) This investigation was supported by Public Health Service Research Grants CA-11451-02 and CA-10612-04 from the National Cancer Institute. We also wish to thank the National Science Foundation for Grant No. GB 4939 and GP 6979 which aided in purchase of the Varian MAT CH4B and SM1B mass spectrometers. For Bufadienolides (paper 20) and Steroids and Related Natural Products (paper 76), see Y. Kamano and G. R. Pettit, *Chem. Pharm. Bull.*, in press.

(2) A mixture corresponding to cinobufagin and cinobufotalin was isolated from toad venom by N. Kotake, *Justus Liebigs Ann. Chem.*, **465**, 11 (1928); H. Jensen and K. K. Chen, *J. Biol. Chem.*, **87**, 741 (1930); R. Tschesche and H. A. Offe, *Chem. Ber.*, **68**, 1998 (1935); and R. Tschesche and W. Haupt, *ibid.*, **70**, 43 (1937). Chromatographic resolution of the cinobufagin–cinobufotalin mixture was achieved by N. Kotake and K. Kuwada, *Nippon Kagaku Zasshi*, **58**, 838 (1937). For a summary of cinobufagin structural studies, refer to reviews by Y. Kamano, *Kagaku No Ryoiki*, **24**, 57 (1970), and R. Ode, Y. Kamano, and G. R. Pettit, "MTP International Review of Science, Organic Chemistry Series One," Vol. 8, W. D. Johns, Ed., Butterworths, London, 1972.

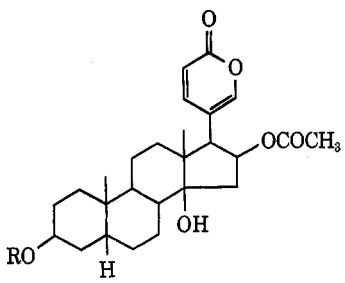
(3) G. R. Pettit, P. Brown, F. Bruschweiler, and L. E. Houghton, *Chem. Commun.*, 1566 (1970).

(4) G. R. Pettit, Y. Kamano, F. Bruschweiler, and P. Brown, *J. Org. Chem.*, **36**, 3736 (1971).

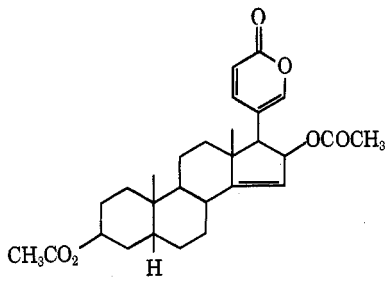
(5) Saponification of cinobufagin to desacetyl cinobufagin with aqueous potassium bicarbonate has been described by J.-T. Ruckstuhl and K. Meyer, *Helv. Chim. Acta*, **40**, 1270 (1957).

(6) Isolation of 3-epibufalin from the Japanese toad *Bufo formosus* Boulenger has been described by E. Iseli, M. Kotake, E. K. Weiss, and T. Reichstein, *ibid.*, **48**, 1093 (1965). A partial synthesis of 3-epibufalin has been summarized by C. Tamm, *ibid.*, **43**, 338 (1960).

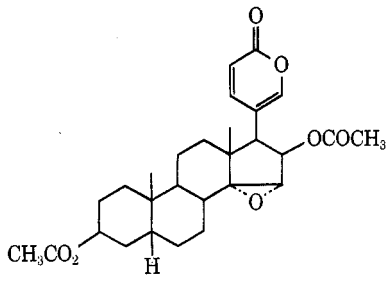
(7) Cinobufagone (**6a**) had been previously described: N. Kotake and K. Kuwada, *Sci. Pap. Inst. Phys. Chem. Res. Tokyo*, **32**, 1 (1937); J.-P. Ruckstuhl and K. Meyer, *Helv. Chim. Acta*, **41**, 2121 (1958).



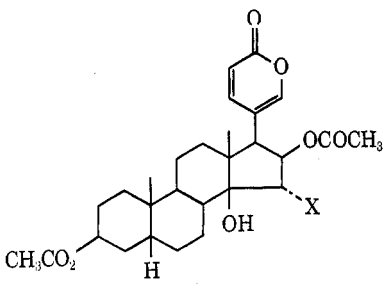
1a, R = H
b, R = $COCH_3$



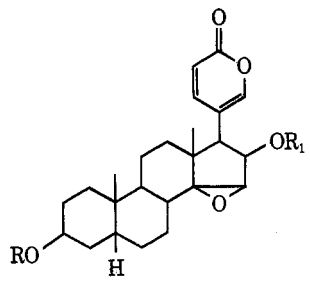
2



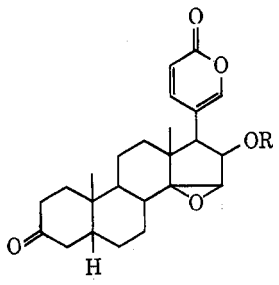
3



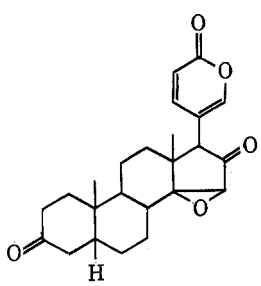
4a, X = Br
b, X = I



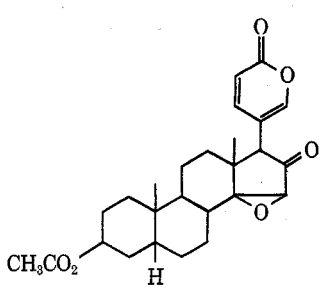
5a, R = R_1 = $COCH_3$
b, R = H, R_1 = $COCH_3$
c, R = $COCH_3$, R_1 = H
d, R = R_1 = H
e, R = 3,5-$(NO_2)_2C_6H_3CO$, R_1 = $COCH_3$
f, R = $C_6H_5CH{=}CHCO$, R_1 = $COCH_3$
g, R = $HO_2CCH_2CH_2CO$, R_1 = $COCH_3$



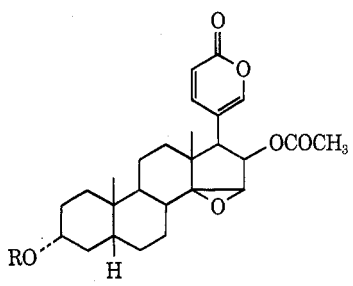
6a, R = $COCH_3$
b, R = H



7



8



9a, R = H
b, R = $COCH_3$

bufotalin (**1a**). For example, the 17α-proton signal of ketones **7** and **8** appeared as a sharp singlet as compared to the doublet (J = 6.5 Hz) of ketone **6**, thus verifying the D-ring substitution relationships.

Reduction of cinobufagone (**6a**) with sodium borohydride provided 3-epicinobufagin (**9a**) accompanied by cinobufagin (**5b**) in a 3:1 ratio. Acetylation of the 3α-alcohol (**9a**) gave acetate **9b**, and chromic acid oxidation led to the starting ketone **6a**. The series of reactions just described and the preceding synthetic interrelationship with bufotalin served to substantiate firmly structure **5b** for cinobufagin.

Experimental Section

Bufotalin, cinobufagin, and deacetylcinobufagin were isolated from Ch'an Su. The Chinese medicinal preparation, Ch'an Su, is generally prepared from venom of local toads such as *Bufo bufo gargarizans* Cantor and *Bufo melanostrictus* Schneider and is available in the Asian market in the "disk-like" (round cake, dark brown and hard) and "thin-plate" (thin, brownish black) forms. The Japanese variety is known as Senso.

Solvents were redistilled and ligroin refers to a fraction boiling at 60–80°. The Taka-diastase (Fisher Scientific Co.) was used as received. All solvent extracts of aqueous solutions were dried over sodium sulfate and concentrated or evaporated under reduced pressure using a rotatory evaporator. Basic alumina (Merck, Rahway, "Suitable for Chromatography") and silica gel (0.05–0.20 mm, E. Merck, Darmstadt) were employed for column chromatography. Silica gel thin-layer chromatography plates were supplied by E. Merck and acetone–chloroform–*n*-hexane (3:3:4) or ethyl acetate–*n*-hexane (9:1) or methanol–ethyl acetate–*n*-hexane (1:3:4) was employed as solvent, and the plate was developed with sulfuric acid or iodine.

All analytical samples were colorless and displayed a single spot on a thin layer chromatograph. The mutual identity of specimens prepared by different procedures or with natural products was established by mixture melting point determination and infrared spectral as well as thin-layer chromatographic comparisons. Melting points were determined with a micro hot-stage apparatus (Reichert, Austria) and are uncorrected. The ultraviolet (Perkin-Elmer, Model 400, methanol solution), infrared (potassium bromide pellets, Beckman IR-12 instrument), and pmr (deuteriochloroform solution with tetramethylsilane as internal standard, Varian A-60) were recorded by Miss K. Reimer The low resolution mass spectra were obtained by Messrs. Richard Scott and Eugene Kelley using an Atlas CH-4B instrument equipped with a molecular beam type inlet system. The results of elemental microanalyses were provided by the laboratories of Dr. A. Bernhardt, 5251 Elbach über Engelskirche, Muhheim (Ruhr) West Germany.

3β-Acetoxy-14-dehydrobufotalin (3β,16β-Diacetoxy-5β-bufa-14,20,22-trienolide) (2).—To a solution of bufotalin acetate (**1b**,

289 mg) in pyridine (19 ml) was added 1.9 ml of thionyl chloride. The mixture was allowed to stand for 25 min at room temperature, poured into ice–water, and extracted with chloroform. The chloroform extract was washed with water, 2% hydrochloric acid, and water and evaporated. The product (300 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (9:1) gave olefin **2** (122 mg), mp 178–179°, as colorless prisms from acetone–*n*-hexane. Recrystallization from the same solvent provided a pure sample melting at 204–207°: λ_{max} nm (log ϵ) 299 (3.71); ν_{max} cm^{-1} 1760, 1740 (ester CO and conjugated CO), 1660, 1550 (conjugated C=C), 1270, 1260, 1240 (ester C–O), 958, 750 (C=C); pmr δ 0.80 (3 H, s, 18-CH_3), 0.98 (3 H, s, 19-CH_3), 2.05 (3 H, s, 3-$OCOCH_3$), 2.65 (1 H, d, J = 9 Hz, 17-H), 3.37 (3 H, s, 16-$OCOCH_3$), 4.52 (1 H, d, J = 9 Hz, 16-H), 5.06 (1 H, broad s, 3-H), 5.45 (1 H, s, 15-H), 6.31 (1 H, d, J = 10 Hz, 23-H), 7.28 (1 H, d, J = 10 Hz, 22-H), 7.37 (1 H, s, 21-H); mass spectrum m/e 468 (M^+).

Anal. Calcd for $C_{28}H_{36}O_6$: C, 71.77; H, 7.74. Found: C, 71.91; H, 7.68.

3β,16β-Diacetoxy-14α,15α-epoxy-5β-bufa-20,22-dienolide (14α,15α-Epoxycinobufagin Acetate) (3).—To a solution of olefin **2** (80 mg) in 5 ml of chloroform, *m*-chloroperbenzoic acid (64 mg) was added, and the mixture was allowed to stand at room temperature for 30 min. After dilution with chloroform, the solution was poured into ice–water. The chloroform layer was washed with dilute sodium thiosulfate solution and water and concentrated to dryness. The product (80 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (9:1) provided 14α,15α-epoxide **3** (33 mg), as a colorless amorphous solid: λ_{max} nm (log ϵ) 302 (3.69); ν_{max} cm^{-1} 1750, 1740 (ester CO and conjugated CO), 1640, 1540 (conjugated C=C), 1260–1240, 1230 (ester C–O), 950, 750 (C=C); pmr δ 0.77 (3 H, s, 18-CH_3), 1.0 (3 H, s, 19-CH_3), 2.06 (3 H, s, 3-$OCOCH_3$), 2.54 (1 H, d, J = 9.5 Hz, 17-H), 3.46 (3 H, s, 16-$OCOCH_3$), 3.72 (1 H, s, 15-H), 3.91 (1 H, d, J = 9.5 Hz, 16-H), 5.08 (1 H, broad s, 3-H), 6.30 (1 H, d, J = 10 Hz, 23-H), 7.19 (1 H, d, J = 10 Hz, 22-H), 7.26 (1 H, s, 21-H); mass spectrum m/e 484 (M^+).

Anal. Calcd for $C_{28}H_{36}O_7$: C, 69.40; H, 7.48. Found: C, 69.53; H, 7.74.

Cinobufagin Acetate (5a). Method A. Using NBA.—In a typical experiment a solution of *N*-bromoacetamide (25 mg) in acetone (0.5 ml)–water (0.5 ml) was added to a solution of olefin **2** (25 mg) in acetone (4 ml). After stirring for 30 min, the mixture was allowed to stand for 20 hr at room temperature. A solution prepared from sodium sulfite (anhydrous, 25 mg) in water (1 ml) was added, and the mixture was poured into ice–water and extracted with chloroform. Following a wash with water the chloroform extract was evaporated to dryness. The crude bromohydrin (**4a**, 22 mg) was stirred in pyridine (1 ml) for 2 hr at room temperature. The solvent was evaporated, and the product was chromatographed on a column of silica gel. Elution with ligroin–acetone (19:1) provided 20 mg (80%) of cinobufagin acetate (**5a**) as needles melting at 202–205°.

In another experiment a solution of *N*-bromoacetamide (68 mg) in dioxane (1 ml) was added to a mixture prepared from 3β,16β-diacetoxy-5β-bufa-14,20,22-trienolide (70 mg) (**2**) in dioxane (3 ml) containing 70% perchloric acid (0.02 ml). Before adding a solution prepared from sodium sulfite (70 mg) and water (1.5 ml), the mixture was stirred 90 min at room temperature. The solution was concentrated under reduced pressure to approximately one-third of the original volume, poured into ice–water with stirring, and extracted with chloroform. The chloroform extract was washed with water and evaporated to dryness. The crude bromohydrin (**4a**, 75 mg), without further purification, was chromatographed on basic alumina (3 g). The fraction (27 mg) eluted by benzene–chloroform (19:1) was crystallized from acetone to afford 15.5 mg (22%) of cinobufagin acetate (**5a**) as needles melting at 203–205°.

Method B. Using NBS.—The preceding reaction (method A, pyridine procedure) was repeated using 50 mg of olefin **2** and 50 mg of *N*-bromosuccinimide. In this example the reaction time was 18 hr (after stirring for 10 min at room temperature), and the crude yield of bromohydrin (**4a**) was 52 mg. Treatment of bromohydrin **4a** with pyridine (2 ml), chromatography of the product on a column of silica gel, and elution with ligroin–acetone (19:1) provided 41 mg (82%) of cinobufagin acetate (**5a**), mp 202–204°.

Method C. Using NIS.—When *N*-iodosuccinimide (50 mg) was substituted for NBA as described in the second part of method A, olefin **2** (50 mg) led to 46 mg of crude iodohydrin (**4b**). Conversion of the iodohydrin (25 mg) into cinobufagin acetate by the basic alumina technique resulted in a 34% yield (17 mg) of product, mp 202–203°.

The pyridine (1 ml) route with iodohydrin **4b** (25 mg) provided an 83% yield (21 mg) of product (**5a**) melting at 201–204°.

The samples of cinobufagin acetate (**5a**) prepared by methods A–C were found identical with the acetate (**5a**) prepared from natural cinobufagin (**5b**).

3β-Acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (5c). Method A. Using Alumina.—The mixture prepared from cinobufagin acetate (**5a**, 20 mg) in benzene (3 ml)–ether (1.5 ml) and basic alumina (600 mg) was stirred at room temperature. After 24 hr, methanol (1 ml) was added, and stirring was continued for 18 hr. The solution obtained by filtration was concentrated, and the crude product (18 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (9:1 and 5:1) provided 13.5 mg of 16β-alcohol **5c** as needles melting at 207–209° (from methanol).

Method B. Using Basic Ion-Exchange Resin.—The mixture prepared from cinobufagin acetate (**5a**, 20 mg), methanol (5 ml), water (0.5 ml), and 100 mg of Amberlite CG-400 (OH^- form) ion-exchange resin was stirred for 2 hr at room temperature. After filtration the solution was concentrated to afford 19 mg of crude product which was chromatographed on a column of silica gel. Elution with ligroin–acetone (9:1 and 5:1) and recrystallization from methanol provided 11 mg of 16β-alcohol **5c** as needles melting at 207–208°.

Method C. Using Ammonium Hydroxide.—To a solution of 20 mg of cinobufagin acetate (**5a**) in ethanol (6 ml)–water (1 ml), 0.1 ml of 30% ammonium hydroxide solution was added. The mixture was allowed to stand for 40 hr at room temperature and then neutralized with dilute hydrochloric acid, poured into water, and extracted with chloroform. The chloroform extract was washed with water and concentrated to dryness. The crude product (19 mg) was chromatographed on a column of silica gel and 16β-alcohol **5c** was eluted with ligroin–acetone (9:1 and 5:1) the yield after recrystallization from methanol was 15 mg as needles.

Method D. Using Taka-diastase.—A solution composed of cinobufagin acetate (**5a**, 20 mg), Taka-diastase (1 g), and methanol (10 ml)–water (5 ml), was allowed to stand for 14 days at 30°. After dilution with water, the mixture was extracted with chloroform, and the extract was washed with water and concentrated to dryness. The crude product (21 mg) was purified as described above to yield 13 mg of 16β-alcohol **5c** as needles melting at 205–208°.

Method E. Using Potassium Bicarbonate.—To a solution of cinobufagin acetate **5a** (14 mg) in methanol (3 ml), 17 mg of potassium bicarbonate in water (0.5 ml) was added. The solution was allowed to stand for 3 days at 30° and then acidified (pH 3.0) with dilute sulfuric acid. After extraction with chloroform, the extract was washed with water and concentrated to dryness. Recrystallization of the product (10 mg) from methanol provided 8 mg of 16β-alcohol **5c** as needles melting at 206–207°. Each sample of 16β-alcohol **5c** prepared by methods A–E exhibited the following physical constants: λ_{max}^{ETOH} nm (log ϵ) 298 (3.74); ν_{max} cm^{-1} 3400 (OH), 1730, 1710, 1695 (ester CO and conjugated CO), 1630, 1538 (conjugated C=C), 1260, 1245, 1230–1220 (ester C–O), 960, 840, 790, 755 (C=C); pmr δ 0.80 (3 H, s, 18-CH_3), 0.99 (3 H, s, 19-CH_3, 2.05 (3 H, s, 3-$OCOCH_3$), 2.60 (1 H, d, J = 9 Hz, 17-H), 5.07 (1 H, broad s, 3-H), 6.18 (1 H, d, J = 10.5 Hz, 23-H), 7.25 (1 H, d, J = 3 Hz, 21-H), 7.95 (1 H, dd, J = 3 and 10.5 Hz, 22-H); mass spectrum m/e 442 (M^+).

Anal. Calcd for $C_{26}H_{34}O_6$: C, 70.18; H, 7.68. Found: C, 70.56; H, 7.74.

Cinobufagin (5b).—A mixture prepared from cinobufagin acetate (**5a**, 200 mg) in ethanol (50 ml)–water (10 ml) and 2 g of the acidic ion-exchange resin Dowex 50W-X8 (H^+ form) was stirred for 40 hr at room temperature. The solution obtained by filtration was evaporated to dryness. The crude product (230 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (19:1, 9:1, 5:1, and 3:1) provided 27 mg of **cinobufagin** (**5b**, as prisms from acetone melting at 213–215°), 20 mg of **3β-acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide** (**5c**, as needles from methanol melting at 206–207°), and 11 mg of **deacetylcinobufagin** (**5d**, as a colorless amorphous solid melting at 153–160°), in addition to unreacted starting material (**5a**, 120 mg).

The samples of cinobufagin (**5b**) and deacetylcinobufagin (**5d**) were found identical with natural specimens isolated from Ch'an Su. The sample of 16β-alcohol **5c** was found identical with material obtained by methods A–E, as described above.

Cinobufagin 3,5-dinitrobenzoate (**5e**) was prepared using cinobufagin (60 mg) in pyridine (1 ml), and 3,5-dinitrobenzoyl chloride (60 mg). After 18 hr at room temperature, the mixture was poured into ice–water and extracted with chloroform. The solvent extract was washed with dilute hydrochloric acid and water and concentrated to give 68 mg of crude product which was chromatographed on a column of silica gel. Recrystallization of the ligroin–acetone (9:1) fraction from acetone–methanol afforded an analytical sample (51 mg) as needles melting at 159–162°: $\lambda_{max}^{CHCl_3}$ nm (log ε) 294 (3.58); ν_{max} cm^{-1} 3100 (CH), 3040 (CH), 1760–1740, 1720 (ester CO and conjugated CO), 1630, 1600, 1500 (conjugated C=C), 1550 (conjugated C=C and C–NO_2), 1340 (C–NO_2), 1270–1260, 1240 (ester C–O and epoxy C–O), 980 (C=C), 870 (C–N), 835 (epoxy C–O), 757 (C=C); pmr δ 0.84 (3 H, s, 18-CH_3), 1.09 (3 H, s, 19-CH_3), 1.91 (3 H, s, 16-$OCOCH_3$), 2.84 (1 H, d, J = 9 Hz, 17-H), 3.69 (1 H, s, 15-H), 5.42 (1 H, broad s, 3-H), 5.54 (1 H, d, J = 9 Hz, 16-H), 6.22 (1 H, d, J = 11 Hz, 23-H), 7.19 (1 H, d, J = 3 Hz, 21-H), 7.94 (1 H, dd, J = 11 and 3 Hz, 22-H), 9.18 (3 H, m, aromatic protons); mass spectrum m/e 636 (M^+).

Anal. Calcd for $C_{33}H_{36}O_{11}N_2$: C, 62.24; H, 5.69; N, 4.56. Found: C, 62.47; H, 5.95; N, 4.38.

Cinobufagin cinnamate (**5f**) was obtained when cinobufagin (40 mg) in pyridine (4 ml) was treated with cinnamoyl chloride (40 mg) for 20 hr at room temperature. The product was isolated as described for 3,5-dinitrobenzoate ester **5e**, and the analytical sample was recyrstallized from methanol to afford 35 mg of ester **5f** decomposing at 250°: $\lambda_{max}^{CHCl_3}$ nm (log ε) 283 (5.88) and 302 (3.68); ν_{max} cm^{-1} 3100, 3040 (CH), 1760–1740, 1720 (ester O=C and conjugated CO), 1640, 1540 (conjugated C=C of α-pyrone ring), 1580, 1500 (conjugated C=C of phenyl group), 1270, 1240, 1220 (ester C–O and epoxy C–O), 955 (C=C), 910 (C=C), 850 (epoxy C–O), 770, 750 (C=C); pmr δ 0.82 (3 H, s, 18-CH_3), 1.02 (3 H, s, 19-CH_3), 1.89 (3 H, s, 16-$OCOCH_3$), 2.79 (1 H, d, J = 9.5 Hz, 17-H), 3.66 (1 H, s, 15-H), 5.22 (1 H, broad s, 3-H), 5.47 (1 H, d, J = 9.5 Hz, 16-H), 6.21 (1 H, d, J = 11 Hz, 23-H), 7.70–7.10 (about 8 H, m, 21-H and annamoyl group), 7.90 (1 H, dd, J = 11 and 3 Hz, 22-H); mass spectrum m/e 572 (M^+).

Anal. Calcd for $C_{35}H_{40}O_7$: C, 73.40; H, 7.04. Found: C, 73.11; H, 6.90.

Cinobufagin succinate (**5g**) was prepared from cinobufagin (50 mg) and succinic anhydride (100 mg) in pyridine (15 ml). The mixture was heated at reflux 2 hr, poured into ice–water, and extracted with chloroform, and the extract was washed with dilute sulfuric acid and water. Following removal of solvent, the residue (65 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (3:1) and recrystallization from acetone provided 39 mg of needles (**5g**): mp 258–260°; λ_{max}^{ETOH} nm (log ε) 297 (3.75); ν_{max} cm^{-1} 3400–3100 (OH of COOH), 3040 (CH), 1740, 1728, 1720 (ester CO and conjugated CO), 1675 (COOH), 1620, 1540 (conjugated C=C), 1270, 1245, 1230 (ester C–O and epoxy C–O), 954 (C=C), 832 (epoxy C–O), 800, 750 (C=C); pmr (10% solution in $CDCl_3$) δ 0.82 (3 H, s, 18-CH_3), 0.97 (3 H, s, 19-CH_3), 1.89 (3 H, s, 16-$OCOCH_3$), 2.68 (4 H, s, CH_2CH_2), 2.81 (1 H, d, J = 9 Hz, 17-H), 3.68 (1 H, s, 15-H), 5.14 (1 H, broad s, 3-H), 5.46 (1 H, d, J = 9 Hz, 16-H), 6.20 (1 H, d, J = 11 Hz, 23-H), 7.15 (1 H, d, J = 3 Hz, 21-H), 7.90 (1 H, dd, J = 11 and 3 Hz, 22-H), 11.15 (1 H, broad peak, COOH); mass spectrum m/e 542 (M^+).

Anal. Calcd for $C_{30}H_{38}O_9$: C, 66.40; H, 7.06. Found: C, 66.24; H, 7.36.

Deacetylcinobufagin (**5d**). **Method A.**—The mixture prepared from cinobufagin (**5b**, 20 mg), benzene (3 ml), chloroform (1 ml), methanol (0.5 ml), and basic alumina was allowed to react and the product isolated as described above (method A) for preparation of 16β-alcohol **5c**. Chromatography of the crude product (17 mg) on a column of silica gel and elution with ligroin–acetone (3:1) provided 11 mg of alcohol **5d** as an amorphous solid.

In the following series of experiments alcohol **5d** was obtained from cinobufagin (**5b**) using methods B–D summarized for hydrolysis of cinobufagin acetate (**5a**) to 16β-alcohol **5c**, and elution of the silica gel column with ligroin–acetone (3:1). Cinobufagin (18 mg), in methanol (4.5 ml)–water (0.8 ml), was treated with 100 mg of CG-400 (OH^- form) to yield 9 mg of amorphous solid. The same yield was realized from cinobufagin (15 mg), ethanol (5 ml), water (0.5 ml), and 0.09 ml of 30% ammonium hydroxide solution. When a mixture of cinobufagin (30 mg), methanol (15 ml), water (7 ml), and Taka-diastase (1.5 g) was used 14 mg of amorphous deacetylcinobufagin was obtained.

Method B.—A mixture of 3β-acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (**5c**, 18 mg), ethanol (5 ml), water (1 ml), and 0.2 g of Dowex 50W-X8 (H^+ form) was stirred for 40 hr at room temperature. The crude product (15 mg) was chromatographed on a column of silica gel. Elution with ligroin–acetone (5:1 and 3:1) yielded 10 mg of amorphous **5d** and 4 mg of starting material.

The specimens of deacetylcinobufagin obtained by methods A and B were found identical with the natural material isolated from Ch'an Su.

Acetylation of Deacetylcinobufagin (**5d**).—A solution of deacetylcinobufagin (**5d**, 100 mg) in glacial acetic acid (2 ml) was heated at reflux for 60 min. After evaporation of solvent the the residue was chromatographed on a column of silica gel. Elution with ligroin–acetone (19:1, 9:1, 5:1, and 3:1) provided 15 mg of **cinobufagin acetate** (**5a**, as needles melting a 202–204°), 21 mg of **cinobufagin** (**5b**, as prisms melting at 213–215°), and 27 mg of **3β-acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide** (**5c**, as needles melting at 206–208°), in addition to 33 mg of starting material (**5d**).

The sample of cinobufagin (**5b**) obtained in this experiment was found identical with the specimen isolated from Ch'an Su. Also, the samples of cinobufagin acetate (**5a**) and 3β-acetoxy-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (**5c**) were identical with the same materials prepared from natural cinobufagin.

Cinobufagone (**3-Oxocinobufagin**, **6a**).—To a solution prepared from cinobufagin (**5b**, 500 mg) and glacial acetic acid (5 ml) was added with stirring chromic acid (110 mg)–acetic acid (6 ml). The oxidation was conducted at 15–18° for 4 hr. After decomposition of excess reagent wih methanol (0.5 ml with stirring), the mixture was poured into ice–water and extracted with chloroform. The combined extract was washed with water and concentrated to dryness. Recrystallization of the residue (410 mg) from methanol led to 370 mg of 3-oxocinobufagin (**6a**) as needles: mp 235–236°; λ_{max}^{ETOH} nm (log ε) 297.5 (3.75); ν_{max} cm^{-1} 3040 (CH), 1740, 1725, 1700–1685 (ester, conjugated CO and ketone CO), 1635, 1540 (conjugated C=C), 1250, 1240, 1220 (ester C–O and epoxy C–O), 963 (C=C), 840 (epoxy C–O), 785, 750 (C=C); pmr δ 0.95 (3 H, s, 18-CH_3), 1.06 (3 H, s, 19-CH_3), 1.89 (3 H, s, 16-$OCOCH_3$), 2.82 (1 H, d, J = 9 Hz, 17-H), 3.68 (1 H, s, 15-H), 5.47 (1 H, d, J = 9 Hz, 16-H), 6.19 (1 H, d, J = 10.5 Hz, 23-H), 7.18 (1 H, d, J = 3 Hz, 21-H), 7.90 (1 H, dd, J = 10.5 and 3 Hz, 22-H); mass spectrum m/e 440 (M^+).

Anal. Calcd for $C_{26}H_{32}O_6$: C, 70.88; H, 7.32. Found: C, 70.57; H, 7.19.

3-Oxo-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (**6b**). **Method A. From Acetate 6a.**—The solution prepared from 3-oxocinobufagin (**6a**, 70 mg), methanol (56 ml), water (26 ml), and potassium bicarbonate (78 mg) was allowed to stand for 4 days at room temperature and then poured into ice–water. After acidification with dilute sulfuric acid and extraction with chloroform, the extract was washed with water and concentrated. The crude product (68 mg) was chromatographed on a column of silica gel and the fraction eluted with ligroin–acetone (3:1) was recrystallized from acetone to afford 44 mg of 3-oxo-16β-hydroxy-14β,15β-epoxy-5β-bufa-20,22-dienolide (**6b**) as needles, dec pt 233°.

In an analogous experiment, 3-oxocinobufagin (20 mg), ethanol (7 ml), water (1 ml), and 0.2 ml of 30% ammonium hydroxide was allowed to remain at room temperature 40 hr. Isolation of crude product (22 mg) and subsequent purification as just described gave 13 mg of recrystallized alcohol **6b**, dec pt 231–233°. The same product was obtained in 12-mg (dec pt 232–233°) yield employing 3-oxocinobufagin (20 mg), methanol (7 ml), water (0.5 ml), and 0.10 g of CG-400 (OH^- form). The reaction period in this example was 2 hr at room temperature.

Method B. From Deacetylcinobufagin (**5d**) **Using Chromic Acid–Pyridine.**—To a solution of chromic acid (15 mg) in pyridine (0.2 ml) was added (dropwise with stirring) a solution of deacetylcinobufagin (**4a**, 50 mg) in pyridine (1 ml). Stirring was continued for 8 hr and the mixture was poured into ice–water, acidified with dilute hydrochloric acid, and extracted with chloroform. The extract was washed with water and evaporated to provide 53 mg of crude product which was chromatographed on

a column of silica gel. Elution with ligroin–acetone (3:1) gave a fraction which recrystallized from acetone to yield (32 mg) of ketone **6b** as needles, dec pt 231–233°.

Method C. From Deacetylcinobufagin (5d) Using *N*-Bromoacetamide.—To a solution (at 10°) of deacetylcinobufagin (50 mg) in methanol (6 ml), pyridine (2 ml), and water (0.2 ml) was added 45 mg of *N*-bromoacetamide. The mixture was allowed to stand for 18 hr at 10–15° in the dark, poured into ice–water, and extracted with chloroform. Following washing with water, the solvent extract was concentrated to dryness, and the crude product (48 mg) was purified as described in method B to afford 41 mg of ketone **6b**, dec pt 230–232°. Each sample of ketone **6b** prepared in this series of experiments corresponded to the following data: λ_{max}^{ETOH} nm (log ϵ) 297 (3.72); ν_{max} cm^{-1} 3440 (OH), 3040 (CH), 1720, 1710, 1700 (conjugated CO and ketone CO), 1640, 1535 (conjugated C=C), 1230 (epoxy C–O), 955 (C=C), 835 (epoxy C–O), 790, 755 (C=C); pmr δ 0.84 (3 H, s, 18-CH$_3$), 1.06 (3 H, s, 19-CH$_3$), 2.63 (1 H, d, J = 9 Hz, 17-H), 3.61 (1 H, s, 15-H), 4.77 (1 H, d, J = 9 Hz, 16-H), 6.19 (1 H, d, J = 10 Hz, 23-H), 7.22 (1 H, d, J = 3 Hz, 21-H), 7.98 (1 H, dd, J = 10 and 3 Hz, 22-H); mass spectrum m/e 398 (M$^+$).

Anal. Calcd for $C_{24}H_{30}O_5$: C, 72.33; H, 7.58. Found: C, 72.59; H, 7.42.

3,16-Dioxo-14β,15β-epoxy-5β-bufa-20,22-dienolide (7). Method A. From Alcohol 6b.—To a solution of 3-oxo-16β-hydroxybufadienolide **6b** (117 mg) in acetic acid (5 ml) was added (gradually with stirring during 4 hr at room temperature) a solution of chromium trioxide (0.05 g) in acetic acid (2 ml)–water (0.05 ml). After stirring an additional 2 hr excess chromic acid was decomposed with methanol (2 ml). The mixture was poured into ice–water and extracted with chloroform, and the extract was washed with water and concentrated to dryness. The residue (110 mg) was chromatographed on a column of silica gel and the fraction eluted by ligroin–acetone (9:1) was recrystallized from methanol–acetone to give diketone **7** (66 mg, needles, dec pt 218.5°): λ_{max} nm (log ϵ) 298 (3.73); ν_{max} cm^{-1} 3040 (CH), 1740, 1720–1700 (conjugated CO and ketone CO), 1650, 1550 (conjugated C=C), 1520 (epoxy C–O), 955 (C=C), 840 (epoxy C–O), 790, 760 (C=C); pmr δ 1.08 (3 H, s, 18-CH$_3$), 1.28 (3 H, s, 19-CH$_3$), 3.40 (1 H, s, 17-H), 3.49 (1 H, s, 15-H), 6.29 (1 H, d, J = 10 Hz, 23-H), 7.11 (1 H, dd, J = 10 and 3 Hz, 22-H), 7.26 (1 H, d, J = 3 Hz, 21-H); mass spectrum m/e 396 (M$^+$).

Anal. Calcd for $C_{24}H_{28}O_5$: C, 72.70; H, 7.12. Found: C, 72.46; H, 7.02.

Method B. From Deacetylcinobufagin (5d).—Oxidation of deacetylcinobufagin (**5d**, 40 mg) in acetic acid (3 ml) with chromium trioxide (25 mg) in acetic acid (1 ml) water (0.03 ml) was perfomed as illustrated by method A (above). Silica gel column chromatography of the crude product (42 mg) and recrystallization provided 24 mg of 3,16-diketone **7** as needles decomposing at 216–218° and identical with the sample prepared by method A.

3β-Acetoxy-16-oxo-14β,15β-epoxy-5β-bufa-20,22-dienolide (8).—A solution of 16β-alcohol **5c** (85 mg) in acetic acid (1 ml) was treated with a solution of chromium trioxide (40 mg) in acetic acid (1 ml). Stirring was continued for 1 hr at room temperature. After standing for 16 hr, methanol (0.5 ml) was added, and the mixture was poured into ice–water and extracted with chloroform. The combined extract was washed with water, and solvent was removed. Silica gel column chromatography of the crude product (88 mg), elution with ligroin–acetone (9:1), and recrystallization from acetone–methanol led to 62 mg of 3β-acetoxy-16-ketone **8** as needles: mp 226–228°; λ_{max} nm (log ϵ) 297.5 (3.72); ν_{max} cm^{-1} 3060 (CH), 1760–1720, 1700 (ester CO, conjugated CO, and ketone CO), 1643, 1542 (conjugated C=C), 1265, 1252, 1235 (ester C–O and epoxy C–O), 955 (C=C), 863 (epoxy C–O), 756 (C=C); pmr (10% solution in $CDCl_3$) δ 0.97 (3 H, s, 18-CH$_3$), 1.04 (3 H, s, 19-CH$_3$), 2.08 (3 H, s, 16-OCOCH$_3$), 2.61 (1 H, s, 17-H), 3.52 (1 H, s, 15-H), 5.08 (1, H, broad s, 3-H), 6.24 (1 H, d, J = 10 Hz, 23-H), 7.29 (1 H, d, J = 3 Hz, 21-H), 7.45 (1 H, dd, J = 10 and 3 Hz, 22-H); mass spectrum m/e 440 (M$^+$).

Anal. Calcd for $C_{26}H_{32}O_6$: C, 70.89; H, 7.23. Found: C, 71.01; H, 7.29.

3-Epicinobufagin (3α-Hydroxy-16β-acetoxy-14β,15β-epoxy-bufa-20,22-dienolide) (9a).—To a solution of 3-oxocinobufagin (**6a**, 210 mg) in dioxane (21 ml)–water (7 ml) was added a solution of sodium borohydride (200 mg) in dioxane (12 ml)–water (4 ml). The mixture was allowed to stand for 3 hr at room temperature, poured into ice–water, acidified with dilute sulfuric acid, and extracted with chloroform. The combined extract was washed with water and concentrated to dryness. Chromatography of the crude product (225 mg) on a column of silica gel and elution with ligroin–acetone (5:1) provided 60 mg of 3-epicinobufagin (**9a**), mp 137–139° (as needles from methanol), and 20 mg of cinobufagin (**5b**), mp 214–218°, which were found identical with authentic samples. In addition, 100 mg of unreacted starting material (**6a**) was recovered. The 3-epicinobufagin exhibited λ_{max} nm (log ϵ) 298 (3.74); ν_{max} cm^{-1} 3540 (OH), 3040 (CH), 1740, 1720–1700 (ester CO and conjugated CO), 1630, 1540 (conjugated C=C), 1250, 1220 (ester C–O and epoxy C–O), 957 (C=C), 833 (epoxy C–O), 785, 750 (C=C); pmr δ 0.82 (3 H, s, 18-CH$_3$), 0.97 (3 H, s, 19-CH$_3$), 2.19 (3 H, s, 16-OCOCH$_3$), 2.82 (1 H, d, J = 9 Hz, 17-H), 3.66 (2 H, broad s, 15-H and 3-H), 5.42 (1 H, d, J = 9 Hz, 16-H), 6.19 (1 H, d, J = 9.5 Hz, 23-H), 7.17 (1 H, d, J = 2.5 Hz, 21-H), 7.89 (1 H, dd, J = 9.5 and 2.5 Hz, 22-H); mass spectrum m/e 422 (M$^+$).

Anal. Calcd for $C_{26}H_{34}O_6$: C, 70.56; H, 7.74. Found: C, 70.56; H, 7.77.

A specimen of **3-epicinobufagin acetate (9b)** was prepared from 50 mg of 3-epicinobufagin (**9a**) and acetic anhydride (1 ml)–pyridine (0.7 ml). Silica gel column chromatography of the crude product (53 mg) and elution with ligroin–acetone (9:1) led to 44 mg of diacetate **9b** as an amorphous solid: λ_{max} nm (log ϵ) 298 (3.71); ν_{max} cm^{-1} 3040 (CH), 1760, 1740, 1720 (ester CO and conjugated CO), 1645, 1545 (conjugated C=C), 1250–1220 (strong peak; ester C–O and epoxy C–O), 950 (C=C), 830 (epoxy C–O), 785, 755 (C=C); pmr δ 0.82 (3 H, s, 18-CH$_3$), 0.97 (3 H, s, 19-CH$_3$), 1.91 (3 H, s, 16-OCOCH$_3$), 2.05 (3 H, s, 3-OCOCH$_3$), 2.81 (1 H, d, J = 9 Hz, 17-H), 3.67 (1 H, s, 15-H), 4.71 (1 H, broad peak, 3-H), 5.45 (1 H, d, J = 9 Hz, 16-H), 6.19 (1 H d, J = 9.5 Hz, 23-H), 7.14 (1 H, d, J = 2.5 Hz, 21-H), 7.89 (1 H, dd, J = 9.5 and 2.5 Hz, 22-H); mass spectrum m/e 484 (M$^+$).

Anal. Calcd for $C_{28}H_{36}O_7$: C, 69.40; H, 7.48. Found: C, 69.52; H, 7.47.

Oxidation of 3-Epicinobufagin (9a).—The chromic acid oxidation of 3α-alcohol **9a** (20 mg) was carried out using chromium trioxide (10 mg in acetic acid–water) as described above for preparation of ketone **6a**. Chromatographic purification afforded 13 mg of 3-oxocinobufagin (**6a**, mp 234–236°), which was found identical with the material prepared from cinobufagin.

Registry No.—**1a**, 471-95-4; **2**, 36615-06-2; **3**, 36615-07-3; **5a**, 4026-97-5; **5b**, 470-37-1; **5c**, 4026-96-4; **5d**, 4026-95-3; **5e**, 36635-92-4; **5f**, 36615-11-9; **5g**, 36615-12-0; **6a**, 6869-66-5; **6b**, 36615-14-2; **7**, 36615-15-3; **8**, 36615-16-4; **9a**, 36121-84-3; **9b**, 36615-18-6.