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Bufadienolides. IX.[1] Isolation and Structure of Resibufagin[2,3]

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A new bufadienolide, resibufagin (I), has been isolated from both "thin-plate" Ch'an Su and "disk-like" one. Based on the chemical and spectral evidence, the structure of resibufagin (I) was assigned to be 3β-hydroxy-19-oxo-14β,15β-epoxy-5β-bufa-20,22-dienolide. Treatment of I with sodium borohydride afforded an alcohol, named resibufaginol (III). Oxidation of an acetate (II) obtained from I with chromium trioxide gave a corresponding 10-carboxylic acid (V), which was methylated to afford a 10-methyl carboxylate (VI). Treatment of I or II with hydroxylamine hydrochloride and sodium acetate gave the corresponding oxime, VII or VIII, respectively. Acetylation of VII or VIII with acetic anhydride and pyridine afforded the same acetyloxime (IX).

Furthermore, together I, there were obtained a bufogenin, which was identified to be marinobufagin (X), first isolation from Ch'an Su.

Resibufagin (I) isolated from Ch'an Su is the third bufadienolide having 10-formyl group. Also resibufaginol (III) obtained from I is the third bufadienolide having 10-alcohol group, and may be obtained from toad venoms on further examination.

Among some twenty bufadienolides hitherto known, thirteen of them were isolated[5] from the Chinese toad venom drug, Ch'an Su (Japanese name "Senso (蟾酥)"). We have also noted[6] recently the detection of unknown compounds from Ch'an Su using thin-layer chromatography (TLC) (Fig. 1). This report concerns the isolation and characterization of the new components.

The chloroform extract of "thin-plate" Ch'an Su[7] was chromatographed on silica gel.[8] Elution with *n*-hexane–acetone mixture afforded the known bufogenins and a mixture of unknown materials. This mixture agreed with X_4, which had previously been detected[6] and isolated[8] from Ch'an Su. Although X_4 on TLC by using of acetone–chloroform–*n*-hexane (3:3:4) solvent indicated one spot, it gave two spots by using of ether–ethyl acetate (6:4) solvent, as shown in Fig. 1. By rechromatography of the mixture, there was obtained two bufadienolides, one of which was found to be marinobufagin (X),[9] first isolated from Ch'an Su. The second compound, mp 210—212°, obtained as colorless needles from methanol, was named

1) Part VIII: Y. Kamano, H. Yamamoto, Y. Tanaka, and M. Komatsu, *Chem. Pharm. Bull.* (Tokyo), in press.
2) Part of this work was presented at the 12th symposium on the Chemistry of Natural Products of Japan, Sendai, October 8, 1968, "Abstracts of Paper," p. 166.
3) A preliminary communication: Y. Kamano, H. Yamamoto, K. Hatayama, Y. Tanaka, M. Shinohara, and M. Komatsu, *Tetrahedron Letters*, **1968**, 5669.
4) Location: *34-1, Takata 3-chome, Toshima-ku, Tokyo, 170-91, Japan.*
5) K. Meyer, *Pharm. Acta Helv.*, **24**, 222 (1949); K. Meyer, *Helv. Chim. Acta*, **35**, 2444 (1952); J. -P. Ruckstukl and K. Meyer, *ibid.*, **40**, 1270 (1957); P. Hofer and K. Meyer, *ibid.*, **43**, 1495 (1960); P. Hofer, H. Linde, and K. Meyer, *ibid.*, **43**, 1955 (1960); F. Bernoulli, H. Linde, and K. Meyer, *ibid.*, **45**, 240 (1962); H. Linde, P. Hofer, and K. Meyer, *ibid.*, **44**, 1243 (1966). See, Y. Kamano, "The chemistry of toad poisons" (*Kagaku No Ryoiki*, Vol. **24**, 339 (1970) (Nankodo, Tokyo)).
6) M. Komatsu, Y. Kamano, and M. Suzuki, *Bunseki Kagaku*, **14**, 1949 (1965).
7) At present, a "thin-plate" and "disk-like" Ch'an Su are on the market.
8) M. Komatsu and T. Okano, *Yakugaku Zasshi*, **87**, 712 (1967).
9) K. Meyer, *Helv. Chim. Acta*, **34**, 2147 (1951); S. Pataki and K. Meyer, *ibid.*, **38**, 1631 (1955); H. Schröter, R. Rees, and K. Meyer, *ibid.*, **42**, 1385 (1959).

resibufagin. Based on the following spectral and chemical evidence, structure I (3β-hydroxy-19-oxo-14β,15β-epoxy-5β-bufa-20, 22-dienolide) was assigned to the new bufadienolide.

From molecular weight determination (*m/e* 398) and elemental analysis the compound was found to have the formula $C_{24}H_{30}O_5$. The presence of an α-pyrone ring was indicated ultraviolet (UV) (λ_{max}^{MeOH}: 301 mμ (log ε=3.60)) and infrared (IR) spectra (ν_{max}^{KBr}: 1714, 1630, 1535 cm^{-1}) (Fig. 2). The structure was supported by the nuclear magnetic resonance (NMR) spectra,

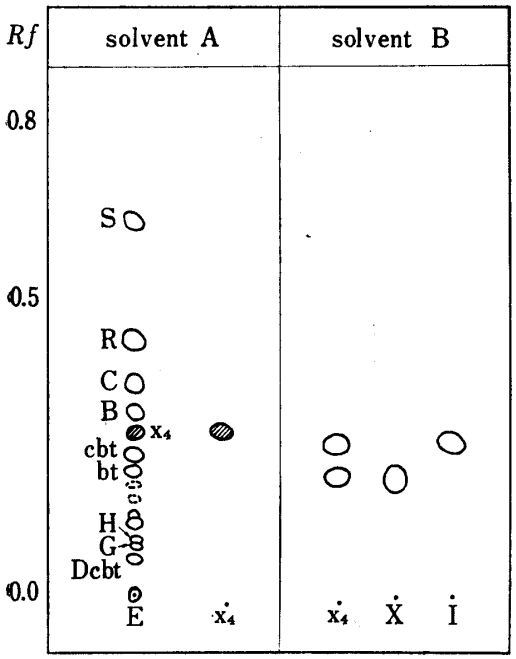


Fig. 1. Thin-Layer Chromatograms of Unknown Bufogenins in Ch'an Su

Plate: silica gel G
solvent: (A) Me_2CO–$CHCl_3$–*n*-hexane (3:3:4)
(C) Et_2O–AcOEt (6:4)
sample E: $CHCl_3$ extract of Ch'an Su(S=sterol, R=Resibufogenin, C=Cinobufagin B=Bufalin, cbt=Cinobufotalin, bt=Bufotalin, H=Hellebrigenin, G=Gamabufotalin, Dcbt=Desacetyl-cinobufotalin)
X_4: Unknown bufogenin mixture
I: resibufagin
X: marinobufagin

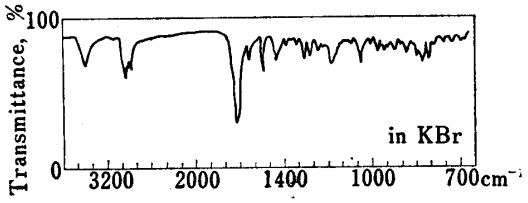


Fig. 2. Infrared Spectra of Resibufagin (I)

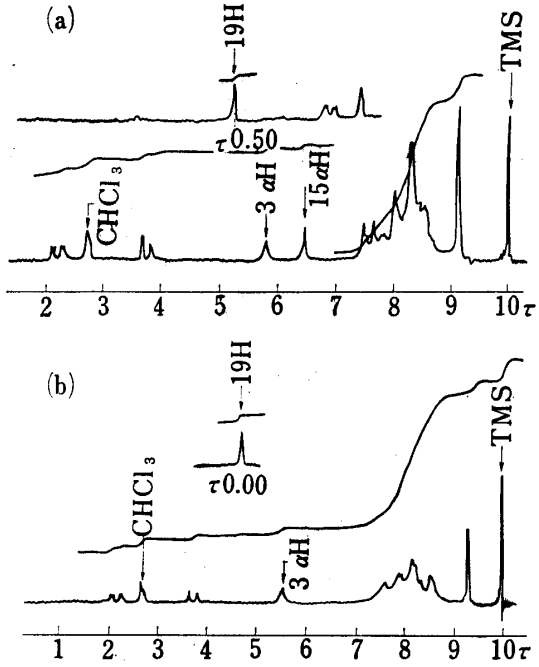


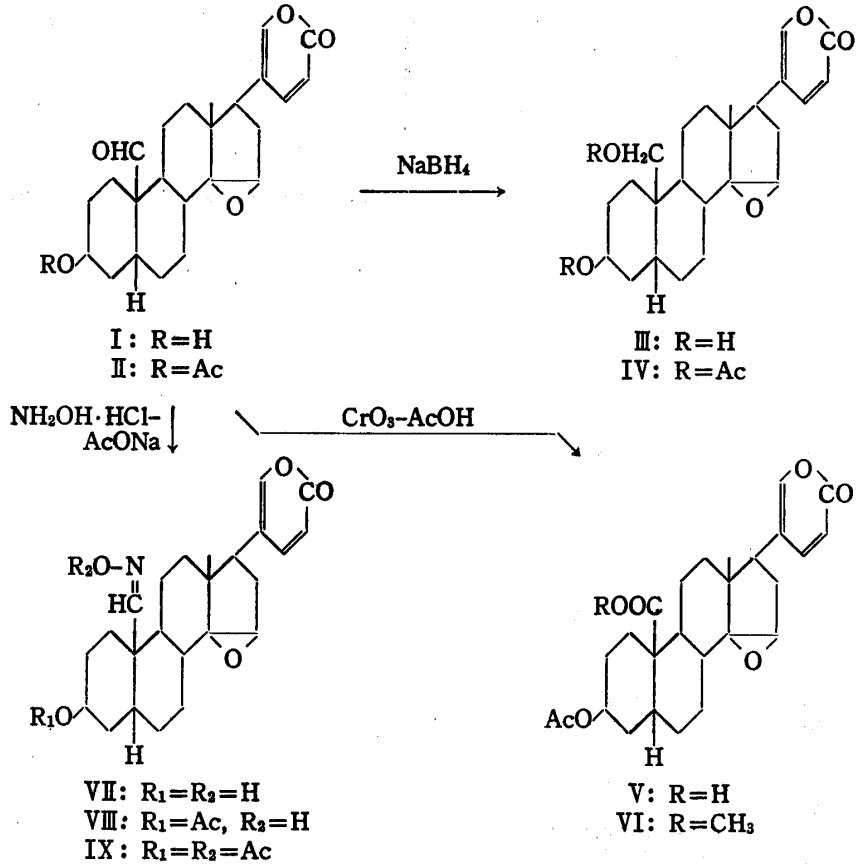
Fig. 3. Nuclear Magnetic Resonance Spectra of Resibufagin (I) (a) and Hellebrigenin (XIII) (b) in $CDCl_3$ at 60Mc.

which exhibited signals at τ 2.24 (1H, dd, J=3 and 10 cps, 22-H), 2.77 (1H, dd, J=3 and 1 cps, 21-H) and 3.79 (1H, dd, J=10 and 1 cps, 23-H)[10,11] (Fig. 3 (a)). The appearance of a signal at a low field of τ 0.50 (1H, s) indicated the presence of a formyl group, the location of which was deduced to be C_{10} based on analogy with hellebrigenin (XIII), which showed the signal at τ 0.00[10,11] (Fig. 3 (b)). The 18-methyl proton showed a singlet at τ 9.14[10,11,12,13] (Fig. 3 (a),(b)). A signal at τ 6.49 (1H, s) (Fig. 3 (a)) was assignable to the tertiary proton at C_{15} in the 14β,15β-epoxy grouping, whose presence was clear by the IR spectral data (3040cm^{-1})[12,14] (Fig. 2).

10) S.M. Kupchan, R.H. Hemingway, and J.C. Hemingway, *Tetrahedron Letters*, **1968**, 149.
11) L. Gsell and Ch. Tamm, *Helv. Chim. Acta*, **52**, 551 (1969).
12) H. Linde, P. Hofer, and K. Meyer, *Helv. Chim. Acta*, **49**, 1243 (1966).
13) K. Tori and K. Aono, *Shionogi Kenkyusho Nempo*, **15**, 130 (1965).
14) H.B. Henbest, G.D. Meakings, B. Nicholls, and K.J. Taylor, *J. Chem. Soc.*, **1957**, 1459; J-P. Ruckstuhl and K. Meyer, *Helv. Chim. Acta*, **41**, 2121 (1958); H. Linde and K. Meyer, *ibid.*, **42**, 807 (1959).

The epoxy ring was also supported by the IR and NMR spectra of thec orresponding acetate (II), mp 195—199°.

Treatment of I with sodium borohydride afforded an alcohol, designated **resibufaginol** (III), mp 207—210°, which, on acetylation, yielded acetate (IV) as a colorless amorphous solid (Chart 1). Compound III exhibited the 19-methylene signal[13] as a part of AB type doublets at τ 6.07 and 6.48 (J=11 cps), whilst compound IV also exhibited the corresponding signals at τ 5.65 and 5.96 (J=12 cps).



I: R=H
II: R=Ac
III: R=H
IV: R=Ac
VII: R1=R2=H
VIII: R1=Ac, R2=H
IX: R1=R2=Ac
V: R=H
VI: R=CH3

Chart 1

Oxidation of II with chromium trioxide in acetic acid gave the corresponding 10-carboxylic acid (V) as a colorless amorphous solid, which was methylated with diazomethane to afford a 10-methyl carboxylate (VI), mp 209—211° (Chart 1).

Treatment of I with hydroxylamine hydrochloride and sodium acetate in aqueous alcohol gave the 10-oxime (VII), mp 262° (decomp.), as colorless needles from methanol. Treatment of II with hydroxylamine hydrochloride and sodium acetate in aqueous ethanol gave VIII, mp 260—261° (decomp.). Acetylation of VII or VIII with acetic anhydride–pyridine afforded an acetyl oxime (IX) as a coloress amorphous solid (Chart 1). The structural assignments for these new derivatives (V, VI, VII, VIII and IX) was based upon the UV, IR and NMR spectra.

Marinobufagin (X, 3β, 5-dihydroxy-14β, 15β-epoxy-5β-bufa-20, 22-dienolide), mp 222—224°, was obtained as colorless prisms from acetone. Analytical values and mass spectral determination (*m/e* 400) supported the formula $C_{24}H_{32}O_5$. The compound had the following spectral properties; λ_{max}^{MeOH} 300 mμ (log ε=3.61); ν_{max}^{KBr} 3400—3000 (OH), 3040 (15-H), 1760, 1640, 1540 cm^{-1} (α-pyrone ring); τ (10% solution in $CDCl_3$) 2.26 (1H, dd, J=3 and 10 cps, 22-H), 2.78 (1H, d, J=3 cps, 21-H), 3.77 (1H, d, J=10 cps, 23-H), 5.83 (1H, broad peak, 3-H), 6.48 (1H, s, 15-H), 9.02 (3H, s, 19-CH_3), 9.20 (3H, s, 18-CH_3). 3-Acetate (IX) showed

an infrared absorption at 3500 cm^{-1} (5-OH) and NMR signals at τ 7.90 (3H, s, -$OCOCH_3$) and 4.76 (1H, broad peak, 3-H). The results of paper chromatography (PPC) and color reactions for the compounds (X and XI) are consistant with those reported for marinobufagin (X)[9] and its acetate (XI).[9] On the basis of these data, X was identified as marinobufagin.

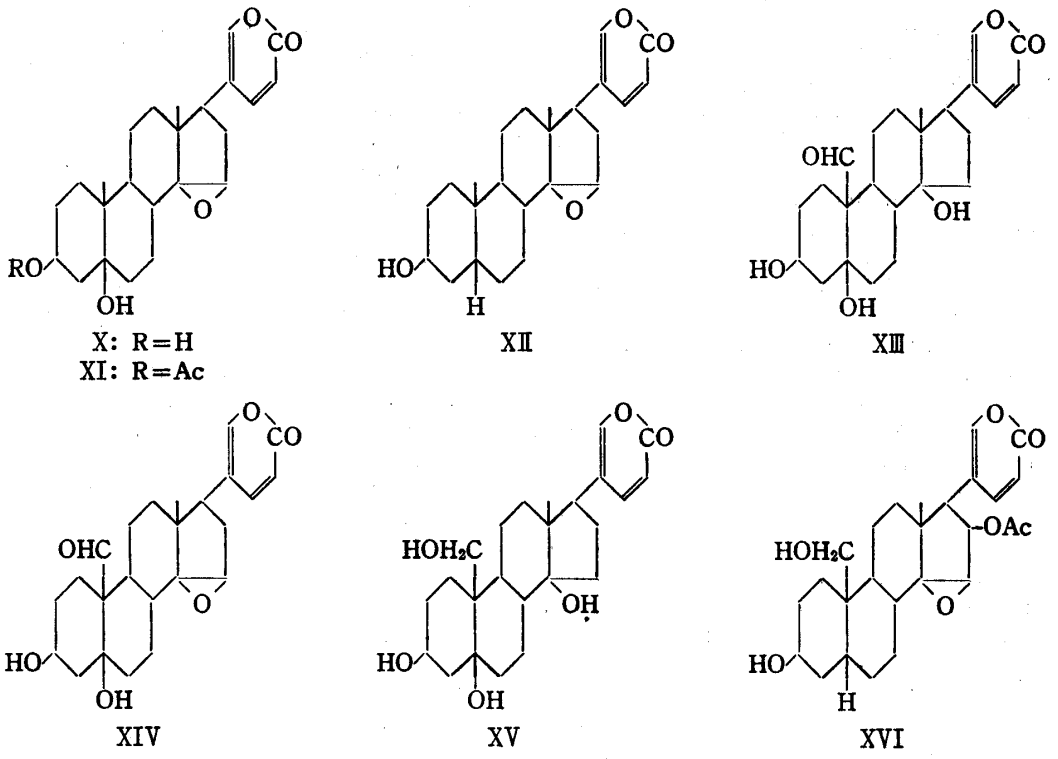


Chart 2

The chloroform extract of "disk-like" Ch'an Su was chromatographed in the same manner as described for the "thin-plate" Ch'an Su. By this means resibufagin (I) and marinobufagin (X) were again isolated.

Resibufagin (I) isolated from Ch'an Su is the third bufadienolide having a 10-formyl group (the others are hellebrigenin (XIII)[15] and bufotalinin (XIV)[15] (Chart 2)). Resibufaginol (III) obtained from I in the present studies corresponds to a 19-hydroxy derivative of resibufogenin (XII). Already hellebrigenol (XV) and cinobufaginol (XVI) (Chart 2) have been isolated from Ch'an Su by the Meyer group.[12] Therefore, resibufaginol (III) may also occur in toad venoms.

It is expected that resibufagin (I) and resibufaginol (III) will show pharmacological activities different from those of resibufogenin (XII).

Experimental

All melting points are uncorrected. IR spectra were determined in KBr pellets using a Nihon Bunko Model DS 301 spectrophotometer. NMR spectra were taken on a Hitachi-Perkin-Elmer R-20 High Resolution Spectrometer with tetramethyl silane as an internal standard. The chemical shifts were reported in τ values. The solvents used are $CDCl_3$ and C_5D_5N.

Mass spectra were recorded on Hitachi mass spectrometer Model RMU-6c at an ionizing potential of 70eV and at an evaporating temperature of 120—220°. Samples were injected directly into the ion source by using a vacuum lock system.

15) Hellebrigenin (VIII) was obtained from Ch'an Su (Ref. 4), and bufotalinin (XIV) was isolated from *Bufo bufo bufo* L. (H. Schröter, Ch. Tamm, T. Reichstein, and V. Deulofeu, *Helv. Chim. Acta*, **41**, 140 (1958)) and *Bnfo arenarum* Hensel (R. Rees, O. Schindler, V. Deulofeu, and T. Reichstein, *ibid.*, **42**, 2400 (1959)).

The progress in column chromatography and the course of reaction were followed by thin-layer chromatography (TLC), which was performed with silica gel G plates using the following solvent system; (A) acetone–$CHCl_3$–*n*-hexane (3:3:4), (B) acetone–$CHCl_3$ (3:7) and (C) ether–AcOEt (6:4). Visualisation of spots was effected by spraying conc. H_2SO_4, followed by heating.

Paper chromatography (PPC) was performed using TOYO paper No. 51A by adopting the descending development method according to a manner described by Ruckstuhl and Meyer.[16]

The solvent system used was C_6H_6–$CHCl_3$ (6:4) (The mobile phase)/formamide (The stationary phase) and the reagent was $SbCl_3$–$CHCl_3$ solution. The all column chromatography was followed by adopting the dry method[8] using silica gel (Wakogel C-200).

Extraction of Bufogenins and Isolation of Unknown Bufogenins——a) Form "Thin-Plate" Ch'an Su: The powder of "thin-plate" Ch'an Su (600 g) was extracted three times under stirring with $CHCl_3$ (*ca.* 5l.) at 50—60° for 7—8 hr. The extract was concentrated *in vacuo* to be reduced to jelly, mixed with silica gel (Wakogel C-200, *ca.* 100 g) and the mixture after removal of the solvent *in vacuo* was charged on the top of a column ($\phi 11 \times 60$ cm) of silica gel (1.5 kg). The column was successively eluted with *n*-hexane–acetone (3:1), acetone and MeOH to give the following fractions; *i.e.* sterol (15 g), the mixture of resibufogenin, cinobufagin and bufalin (49 g), the mixture of cinobufagin and bufalin (23 g), the mixture of bufalin, bufotalin, cinobufotalin, and unknown bufogenins (15 g), the mixture of the other polar bufogenins containing the other unknown materials (*ca.* 25 g).

Then the mixture (15 g) of bufalin, bufotalin, cinobufotalin and unknown bufogenins was rechromatographed on silica gel (450 g) using the same solvents as above to afford bufalin (2.3 g, mp 233—236° from MeOH), bufotalin (5.8 g, mp 213—214° from AcOEt), cinobufotalin (4.0 g, mp 250—255° from acetone) and unknown bufogenin (1.2 g, crystals from MeOH), respectively. Although the crystals on TLC using solvent A showed one spot at *Rf* 0.26, this revealed two spots at *Rf* 0.25 (corresponding the compound I) and 0.20 (corresponding the compound X) on TLC using solvent C. Besides, this mixture of unknown bufogenins was agreed with X_4, which was detected on TLC[6] and isolated[8] from Ch'an Su by the authors. Thus, this mixture (1.2 g) was again separated by column chromatography on silica gel (36 g) using *n*-hexane–acetone (3:1) to give the following two compounds, I (450 mg) and X (380 mg), respectively.

b) From "Dsik-Like" Ch'an Su: The powder of "disk-like" Ch'an Su (160 g) was extracted with $CHCl_3$ by the same manner as described a). The extract afforded a fraction containing the unknown bufogenins (4.5 g) by column chromatography on silica gel (500 g) which was eluted by adopting the similar method as a) using *n*-hexane–acetone mixture, acetone and MeOH. By rechromatography (silica gel, 135 g, solvent: *n*-hexane–acetone (3:1)) of the mixture, it was obtained 0.3 g of unknown materials (crystals). Then the unknown materials was again chromatographed on silica gel (12 g) using *n*-hexane–acetone (3:1) to give compound I (125 mg) and X (98 mg), respectively.

Based on the following evidence, compound X was identified to be marinobufagin (X), whilst compound I was assigned to a new bufogenin, 3β-hydroxy-19-oxo-14β, 15β-epoxy-5β-bufa-20, 22-dienolide and named resibufagin (I).

Marinobufagin (X)——A colorless prisms (from acetone), mp 222—224°. Mass Spectrum, *m/e*: 400 (M^+). *Anal.* Calcd. for $C_{24}H_{32}O_5$: C, 71.99; H, 8.05. Found: C, 71.68; H, 8.03. UV λ_{max}^{MeOH} mμ (log ε): 300 (3.61). IR ν_{max}^{KBr} cm^{-1}: 3400—3300 (OH), 3040 (C_{15}-H), 1760, 1740, 1720 (conjugated CO of α-pyrone ring), 1640, 1540 (conjugated C=C of α-pyrone ring), 952, 796 (C=C). TLC: *Rf* 0.15 (solvent A), 0.29 (solvent B), 0.20 (solvent C); Color, pink–pinkish purple–brownish purple. PPC: *Rf* 0.70; color, greyish brown. Color reaction with 84% H_2SO_4: brown–greyish brown–olivish brown–dark green–grey.

Resibufagin (14β,15β-Epoxy-3β-hydroxy-19-oxo-5β-bufa-20,22-dienolide) (I)——A colorless needles (from MeOH), mp 210—212°. Mass Spectrum, *m/e*: 398 (M^+). *Anal.* Calcd. for $C_{24}H_{30}O_5$: C, 72.34; H, 7.59. Found: C, 72.33; H, 7.88. UV λ_{max}^{MeOH} mμ (log ε): 301 (3.60). IR ν_{max}^{KBr} cm^{-1}: 3500 (OH), 3040 (C_{15}-H), 1745, 1714, 1700 (conjugated CO of α-pyrone ring), 1630, 1535 (conjugated C=C of α-pyrone ring), 954, 805 (C=C). NMR (10% solution in $CDCl_3$) τ: 0.50 (1H, s, 19-CHO), 2.24 (1H, dd, J=10 and 3 cps, 22-H), 2.77 (1H, dd, J=3 and 1 cps, 21-H), 3.79 (1H, dd, J=10 and 1 cps, 23-H), 5.81 (1H, broad peak, 3-H), 6.49 (1H, s, 15-H), 9.14 (3H, s, 18-CH_3). TLC: *Rf* 0.16 (solvent A), 0.31 (solvent B), 0.25 (solvent C); color, yellow–yellowish brown–brown. PPC: *Rf* 0.78; color, greyish brown. Color reaction with 84%-H_2SO_4: yellow–orange–brown–greyish brown–blackish brown. Since the compound was unstable in acetone, recrystallization should be followed using MeOH as solvent.

Acetylation of X——X (80 mg) was acetylated with Ac_2O (1.4 ml)–pyridine (2 ml) by the usual means to give acetylmarinobufagin (XI) (72 mg), mp 198—200°, as colorless prisms from acetone–*n*-hexane. UV λ_{max}^{MeOH} mμ (log ε): 300 (3.37). IR ν_{max}^{KBr} cm^{-1}: 3550 (C_5-OH), 3040 (C_{15}-H), 1750, 1725, 1695 (ester CO and conjugated CO), 1635, 1540 (conjugated CO), 1270 1260, 1225 (ester CO), 955, 798 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.25 (1H, dd, J=10.8 and 3.3 cps. 22-H), 2.77 (1H, d, J=3 cps, 21-H), 3.77 (1H, d, J=10.8 cps, 23-H), 4.76 (1H, broad peak, 3-H), 6.46 (1H, s, 15-H), 7.91 (3H, s, 3-$OCOCH_3$), 8.99 (3H, s, 19-CH_3), 9.20 (3H, s, 18-CH_3). TLC: *Rf* 0.30 (solvent A), 0.37 (solvent C); color, pink–pinkish purple–brownish purple.

16) J.-P. Ruckstuhl and K. Meyer, *Helv. Chim. Acta*, **40**, 1270 (1957).

Acetylation of I——I (90 mg) was acetylated with Ac_2O (1.5 ml)–pyridine (2.2 ml) in the usual manner. Recrystallization from MeOH gave acetylresibufagin (3β-acetoxy-14β, 15β-epoxy-19-oxo-5β-bufa-20, 22-dienolide) (II) (85 mg), as colorless prisms, mp 195—199°. *Anal.* Calcd. for $C_{26}H_{32}O_6$: C, 70.89; H, 7.32. Found: C, 70.66; H, 7.25. UV λ_{max}^{MeOH} mμ (log ε): 301 (3.29). IR ν_{max}^{KBr} cm^{-1}: 3040 (C_{15}-H), 1740, 1720, 1710 (ester CO and conjugated CO), 1630, 1532 (conjugated C=C), 1250, 1230, 1220 (ester C-O), 948, 785 (C=C). NMR (10% solution in $CDCl_3$) τ: 0.48 (1H, s, 19-CHO), 2.25 (1H, dd, J=9 and 3 cps, 22-H), 2.77 (1H, d, J=3 cps, 21-H), 3.78 (1H, d, J=9 cps, 23-H), 4.88 (1H, broad peak, 3-H), 6.46 (1H, s, 15-H), 7.96 (3H, s, 3-$OCOCH_3$), 9.14 (3H, s, 18-CH_3). TLC: *Rf* 0.37 (solvent A), 0.47 (solvent C); color, yellow–yellowish brown–brown.

Reduction of I with $NaBH_4$——To a solution of I (125 mg) dissolved in 20 ml of 80% EtOH, a solution of $NaBH_4$ (75 mg) dissolved in 10 ml of 80% EtOH, was gradually added at −5° and the mixture was allowed to stand for 5—6 min. After acidification (pH 3) with dil. H_2SO_4 aq. at −5—0°, the reaction mixture was poured into H_2O and was concentrated *in vacuo* to a half of the original volume and extracted with $CHCl_3$. The extract was successively washed with dil. $NaHCO_3$ aq. and H_2O, dried over anhydrous Na_2SO_4. Recrystallization of the crude product from MeOH gave 14β, 15β-epoxy-3β, 19-dihydroxy-5β-bufa-20,22-dienolide (III) (121 mg), which was named resibufaginol, mp 207—210°, as colorless prisms. Mass Spectrum *m/e*: 400 (M^+). *Anal.* Calcd. for $C_{24}H_{32}O_5$: C, 71.97; H, 8.05. Found: C, 72.05; H, 8.01. UV λ_{max}^{MeOH} mμ (log ε): 301 (3.78). IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 3040 (CH), 1745, 1725, 1710 (conjugated CO of α-pyrone ring), 1634, 1540 (conjugated C=C of α-pyrone ring), 957, 810 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.20 (1H, dd, J=11 and 3 cps, 22-H), 2.76 (1H, d, J=3 cps, 21-H), 3.74 (1H, d, J=11 cps, 23-H), 5.86 (1H, broad peak, 3-H), 6.07 and 6.48 (2H, AB quartet, J=11 cps, 19-CH_2OH), 6.44 (1H, s, 15-H), 9.23 (3H, s, 18-CH_3). TLC: *Rf* 0.05 (solvent A), 0.07 (solvent: acetone–$CHCl_3$-cyclohexane–HCOOH (3: 3: 4: 0.1)); color, yellow–yellowish brown.

Acetylation of III——III (60 mg) was acetylated with Ac_2O (1.0 ml)–pyridine (1.8 ml) in the usual manner. The crude product was purified by column chromatography with silica gel (2 g) using *n*-hexane–acetone (15:1) to give 3β, 19-diacetoxy-14β, 15β-epoxy–5β-bufa-20, 22-dienolide (IV) (45 mg), as a colorless amorphous solid, which revealed one spot at *Rf* 0.62 (color: yellow–yellowish brown) on TLC using solvent A. *Anal.* Calcd. for $C_{26}H_{34}O_6$: C, 70.56; H, 7.74. Found: C, 70.84; H, 7.90. UV λ_{max}^{MeOH} mμ (log ε): 302 (3.22). IR ν_{max}^{KBr} cm^{-1}: 3060, 3020 (CH), 1745—1680 (conjugated CO and ester CO; broad), 1635, 1540 (conjugated C=C), 1260—1240 (ester C-O), 958, 810 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.15 (1H, dd, J=10 and 2.5 cps, 22-H), 2.69 (1H, d, J=2.5 cps, 21-H), 3.70 (1H, d, J=10 cps, 23-H), 4.78 (1H, broad peak, 3-H), 5.65 and 5.96 (2H, AB quartet, J=12 cps, 19-CH_2OAc), 6.43 (1H, s, 15-H), 7.89 (3H, s, 3- or 19-$OCOCH_3$), 7.92 (3H, s, 3- or 19-$OCOCH_3$), 9.21 (3H, s, 18-CH_3).

Oxidation of II with CrO_3–AcOH——To a solution of II (100 mg) dissolved in AcOH (10 ml), a solution of CrO_3 (75 mg) in AcOH (5 ml) was added and the mixture was allowed to stand for 15 hr at room temperature. Excess of CrO_3 was reduced with MeOH, the mixture was diluted with H_2O and extracted with $CHCl_3$. The extract was washed with H_2O, and dried over anhydrous Na_2SO_4. The residue (105 mg) obtained by $CHCl_3$ extraction was chromatographed on silica gel (6 g) with *n*-hexane–acetone (7:1) to give 3β-acetoxy-10-carboxy-14β, 15β-epoxy–5β-bufa-20, 22-dienolide (V) (65 mg) as a colorless amorphous solid. UV λ_{max}^{MeOH} mμ (log ε): 300 (3.54). IR ν_{max}^{KBr} cm^{-1}: 3400—3100 (carboxyl OH), 1760, 1750—1710, 1690 (ester, carboxyl and conjugated CO), 1630, 1539 (conjugated C=C), 1258, 1236 (ester C-O), 956, 794 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.32 (1H, dd, J=9.5 and 2 cps, 22-H), 2.85 (1H, d, J=2 cps, 21-H), 3.87 (1H, d, J=9.5 cps, 23-H), 4.98 (1H, broad peak, 3-H), 6.57 (1H, s, 15-H), 8.00 (3H, s, 3-$OCOCH_3$), 9.20 (3H, s, 18-CH_3). TLC: *Rf* 0.28 (solvent A); color, yellow–orange–yellowish brown.

Methylation of V——V (55 mg) was methylated with CH_2N_2 in ether in the usual manner. The crude ester (52 mg) obtained thus was purified by column chromatography over silica gel (3 g) in *n*-hexane–acetone (7: 1) to give 45 mg of 3β-acetoxy-10-methoxycarbonyl-14β, 15β-epoxy-5β-bufa-20, 22-dienolide, VI, mp 209—211°, as colorless prisms. UV λ_{max}^{MeOH} mμ (log ε): 300 (3.40). IR ν_{max}^{KBr} cm^{-1}: 3038 (C_{15}-H), 1740, 1728, 1715, 1690 (ester, methylcarbonyl and conjugated CO), 1633, 1540 (conjugated C=C), 1260, 1240, 1220 (ester C-O), 955, 795 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.20 (1H, dd, J=10.5 and 3 cps, 22-H), 2.74 (1H, d, J=3 cps, 21-H), 3.75 (1H, d, J=10.5 cps, 23-H), 4.90 (1H, broad peak, 3-H), 6.31 (3H, s, 10-$COOCH_3$), 6.50 (1H, s, 15-H), 9.17 (3H, s, 18-CH_3). *Anal.* Calcd. for $C_{24}H_{30}O_6$: C, 69.54; H, 7.30. Found: C, 69.71; H, 7.33. TLC: *Rf* 0.56 (solvent A); color, blue–bluish green–yellowish green.

3β-Hydroxy-19-hydroxyimino-14β,15β-epoxy-5β-bufa-20,22-dienolide (VII)——To a solution of I (80 mg) dissolved in 10 ml of 80% EtOH, $NH_2OH \cdot HCl$ (35 mg) and $AcONa \cdot 3H_2O$ (40 mg) were added and the mixture was refluxed for 2 hr. After dilution with H_2O, the solution was allowed to stand overnight. The white precipitate was filtrated, washed with H_2O, and dried *in vacuo*. Recrystallization from MeOH gave 69 mg of VII, mp 262° (decomp.), as colorless needles. UV λ_{max}^{MeOH} mμ (log ε): 302 (3.48). IR ν_{max}^{KBr} cm^{-1}: 3400, 3300—3200 (OH), 3020 (C_{15}-H), 1740, 1730, 1720—1700, 1690—1680 (conjugated CO and C=N of oxime), 1630, 1535 (conjugated C=C), 960 (N-O), 955, 802 (C=C). NMR (10% solution in C_5D_5N)

τ: 1.99 (1H, s, 19-C(=N-OH)-H), 2.20 (1H, dd, J=10 and 3 cps, 22-H), 2.64 (1H, d, J=3 cps, 21-H), 3.73 (1H, d,

J=3 cps, 23-H), 5.72 (1H, broad peak, 3-H), 6.48 (1H, s, 15-H), 9.24 (3H, s, 18-CH_3). *Anal.* Calcd. for $C_{24}H_{31}O_5N$: C, 69.71, H, 7.56; N, 3.39. Found: C, 69.94, H, 7.48; N, 3.51. TLC: *Rf* 0.04 (solvent A); color, yellow–yellowish green–green.

3β-Acetoxy-19-hydroxyimino-14β,15β-epoxy-5β-bufa-20, 22-dienolide (VIII)——To a solution of II (60 mg) dissolved in 7.5 ml of 80% EtOH, $NH_2OH \cdot HCl$ (25 mg) and $AcONa \cdot 3H_2O$ (30 mg) were added and the mixture was refluxed for 2 hr. The reaction solution was treated in the manner analogous to the formation of VII to give 47 mg of VIII, mp 260—261° (decomp.) from EtOH–H_2O, as colorless needles. UV λ_{max}^{MeOH} mμ (log ε): 301 (3.35). IR ν_{max}^{KBr} cm^{-1}: 3320 (OH of oxime), 3040 (C_{15}-H), 1733, 1710, 1700—1680 (ester and conjugated CO and C=N of oxime), 1630, 1538 (conjugated C=C), 1260, 1240, 1230 (ester C-O), 970 (N=C), 955, 810 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.17 (1H, dd, J=10 and 3 cps, 22-H), 2.27 (1H, s, 19-C$\overset{N-OH}{H}$), 2.72 (1H, d, J=3 cps, 21-H), 3.74 (1H, d, J=10 cps, 23-H), 4.89 (1H, broad peak, 3-H), 6.50 (1H, s, 15-H), 7.97 (3H, s, 3-$OCOCH_3$), 9.24 (3H, s, 18-CH_3). *Anal.* Calcd. for $C_{26}H_{33}O_6N$: C, 68.55; H, 7.30; N, 3.08. Found: C, 68.67; H, 7.32; N, 2.99. TLC: *Rf* 0.24 (solvent A); color, yellow–yellowish green–green.

3β-Acetoxy-19-acetoxyimino-14β,15β-epoxy-5β-bufa-20,22-dienolide (IX)——On acetylation with Ac_2O and pyridine in a usual manner, both VII and VIII afforded, after purification by chromatography, a corresponding oxime acetate (IX) as a colorless amorphous solid. UV λ_{max}^{MeOH} mμ (log ε): 300 (3.21). IR ν_{max}^{KBr} cm^{-1}: 3350 (OH of oxime), 3010 (C_{15}-H), 1740, 1725, 1710, 1700—1690 (ester and conjugated CO and C=N of oxime), 1630, 1535 (conjugated C=C), 1260, 1230 (ester C-O), 966 (N=C), 955, 795 (C=C). NMR (10% solution in $CDCl_3$) τ: 2.35 (1H, dd, J=10 and 3 cps, 22-H), 2.84 (1H, d, J=3 cps, 21-H), 3.85 (1H, d, J=10 cps, 23-H), 5.00 (1H, broad peak, 3-H), 6.58 (1H, s, 15-H), 7.91 (3H, s, 19-CH=N-$OCOCH_3$), 8.00 (3H, s, 3-$OCOCH_3$), 9.18 (3H, s, 18-CH_3).

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