[Chem. Pharm. Bull. 27(8)1881—1886(1979)]

UDC 547.925.02.05 : 597.825.05.08

# Isolation and Characterization of Cardiotonic Steroid Conjugates from the Skin of *Bufo marinus* (L.) SCHNEIDER[1]

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(Received February 22, 1979)

The occurrence of new cardiotonic steroid conjugates in the skin of a tropical toad, *Bufo marinus* (L.) SCHNEIDER, is reported. These substances were separated by chromatography on Amberlite XAD-4 resin and silica gel, high-performance liquid chromatography, and gel chromatography on Sephadex LH-20. The sulfated bufogenins, marinobufagin 3-sulfate and resibufogenin 3-sulfate, were characterized by degradative and synthetic means. In addition, the structures of two new bufotoxins were elucidated as marinobufagin 3-pimeloylarginine ester and telocinobufagin 3-suberoylarginine ester.

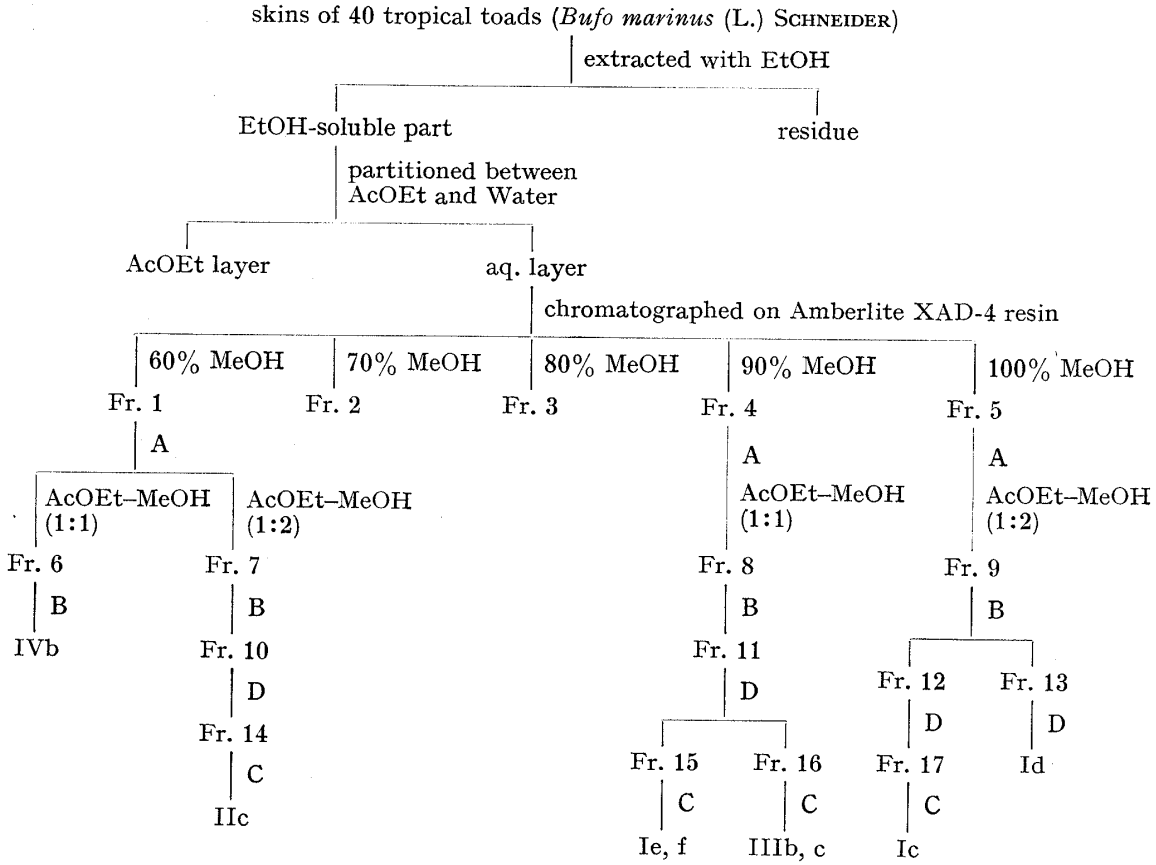
**Keywords**——*Bufo marinus* (L.) SCHNEIDER; skin of toad; separation of conjugated bufogenins; gel chromatography; high-performance liquid chromatography; telocinobufotoxin; marinobufotoxin; marinobufagin 3-pimeloylarginine ester; marinobufagin 3-sulfate; resibufogenin 3-sulfate

Recent studies in this laboratory have identified three new types of bufotoxins, in which succinoyl, adipoyl and pimeloyl groups replace the suberoyl residue of the so-called "bufotoxin", in the Japanese toad, *Bufo vulgaris formosus* BOULENGER.[3] In addition, the existence of bufogenin 3-sulfates[3,4] and analogous conjugates of a cardenolide named cardenobufotoxin[3,5] in the toad was also demonstrated. The present paper deals with the isolation and characterization of new two bufotoxins and two bufogenin sulfates from the skin of a tropical toad, *Bufo marinus* (L.) SCHNEIDER.

Forty toads were sacrificed by freezing in dry ice-acetone, and the skins were immediately flayed and extracted with ethanol. The ethanolic extract was partitioned in an ethyl acetate-water system. The aqueous layer was percolated through a column of Amberlite XAD-4 resin. After thorough washing with distilled water the conjugated steroid fraction was eluted with stepwise increasing concentrations of methanol as shown in Chart 1. Each eluate was subjected to dry column chromatography on silica gel employing ethyl acetate–methanol as an eluent. Subsequent column chromatography on silica gel with chloroform–methanol–water (80 : 20 : 2.5) as a mobile phase provided satisfactory separation. When further purification was required, high-performance liquid chromatography on a reversed phase column and gel chromatography on Sephadex LH-20 were effective.

A new bufotoxin (Ic), mp 186—190°, $[\alpha]_D^{20}$ +22.0°, was separated as a colorless amorphous substance. This compound was positive with Sakaguchi's reagent and gave a negative ninhydrin test. Upon hydrolysis with 6 N hydrochloric acid Ic liberated arginine, which was characterized by two-dimensional thin-layer chromatography. On enzymatic hydrolysis with a hog pancreas lipase preparation[6] followed by methylation with diazomethane, Ic afforded

1) Part CXXXXVIII of "Studies on Steroids" by T. Nambara; Part CXXXXVII: S. Ikegawa, Y. Hino, M. Sunagawa, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **27**, 1864 (1979).
2) Location: *Aobayama, Sendai, 980, Japan.*
3) K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **25**, 714 (1977).
4) K. Shimada, Y. Fujii, and T. Nambara, *Tetrahedron Lett.*, **1974**, 2767; Y. Fujii, K. Shimada, and T. Nambara, *Chem. Ind.* (London), **1976**, 614.
5) Y. Fujii, K. Shimada, Y. Niizaki, and T. Nambara, *Tetrahedron Lett.*, **1975**, 3017.
6) The enzyme preparation also contains amylase and peptidase.




A : chromatography on silica gel; eluent, AcOEt–MeOH.
B : chromatography on silica gel; eluent, $CHCl_3$–MeOH–$H_2O$ (80 : 20 : 2.5).
C : chromatography on Sephadex LH-20; eluent, MeOH.
D : high-performance liquid chromatography.

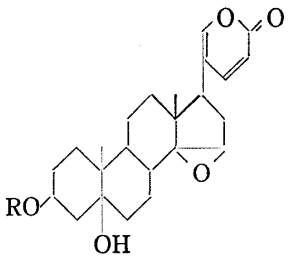
Chart 1. Separation of Bufogenin Conjugates

marinobufagin (Ia) and marinobufagin 3-hemipimelate methyl ester (Ib). The transformation products were identified by direct comparison with authentic samples. These findings led us to assign the structure marinobufagin 3-pimeloylarginine ester to the new bufotoxin (Ic).

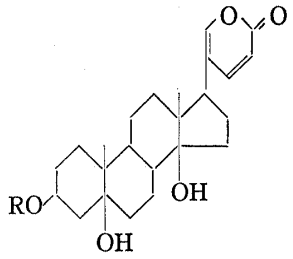
The second new bufotoxin (IIc), mp 194—198°, $[\alpha]_D^{20}$ +1.8°, was positive with Sakaguchi's reagent and negative with ninhydrin. Hydrolytic cleavage with 6 N hydrochloric acid furnished arginine as an amino acid component. Enzymatic hydrolysis with hog pancreas lipase preparation and subsequent methylation with diazomethane yielded telocinobufagin (IIa) and telocinobufagin 3-hemisuberate methyl ester (IIb).[7] These results led us to assign the structure telocinobufagin 3-suberoylarginine ester (telocinobufotoxin[8]) to IIc. In a similar fashion, marinobufagin 3-suberoylarginine ester (marinobufotoxin) (Id), mp 176—180°, was unequivocally characterized by degradative means.

In addition, approximately 500 mg of sulfated bufogenins was separated from 40 toads by the chromatographic methods reported in the previous paper.[3] A portion of the sulfate was further purified by high-performance liquid chromatography using methanol-0.5% ammonium carbonate as the mobile phase. On gel chromatography on Sephadex LH-20 using methanol,[9] a new bufogenin sulfate was resolved into two fractions to provide Ie, mp 156—160°, $[\alpha]_D^{20}$ +7.0°, as colorless prisms and If, mp 160—163°, $[\alpha]_D^{20}$ +12.1°, as an amorphous

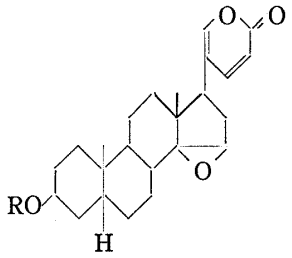
7) H.O. Linde-Tempel, *Helv. Chim. Acta*, **53**, 2188 (1970).
8) The existence of telocinobufotoxin in toad venom was suggested by Linde-Tempel,[7] but it could not be isolated.
9) K. Shimada and T. Nambara, *J. Chromatogr.*, **174**, 440 (1979).



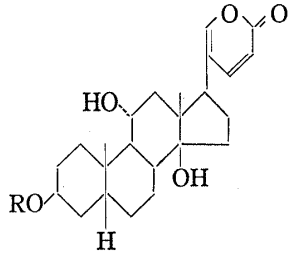
Ia: R=H
Ib: R=$CO(CH_2)_5COOCH_3$
Ic: R=$CO(CH_2)_5COArg\cdot OH$
Id: R=$CO(CH_2)_6COArg\cdot OH$
Ie: R=$SO_3NH_4$
If: R=$SO_3Na$



IIa: R=H
IIb: R=$CO(CH_2)_6COOCH_3$
IIc: R=$CO(CH_2)_6COArg\cdot OH$



IIIa: R=H
IIIb: R=$SO_3NH_4$
IIIc: R=$SO_3Na$



IVa: R=H
IVb: R=$SO_3Na$

Chart 2

substance. These compounds were positive with the barium-rhodizonate reagent (sulfate ions) and showed infrared absorption bands at 1220 and 1055 $cm^{-1}$ due to the sulfate group. Comparison of the carbon-13 NMR signal ($\delta$ 77.6, C-3) of the conjugates with that ($\delta$ 69.0, C-3) of bufogenin indicated that the sulfate linkage is at C-3 rather than C-5. Upon solvolysis in the usual manner[10] both sulfates yielded marinobufagin, which was identified by thin-layer chromatography and mass spectrometry. Compound Ie showed infrared absorption bands at 3200 and 1400 $cm^{-1}$ due to the ammonium group, and If was found to be a sodium salt by flame analysis. These data support assignment of the structure marinobufagin 3-sulfate ammonium salt to Ie and marinobufagin 3-sulfate sodium salt to If. Indeed, the two sulfate salts could be readily converted into each other by treatment with cation-exchange resin. Authentic samples were prepared from marinobufagin by sulfation with chlorosulfonic acid and pyridine followed by percolating the aqueous solution through ion-exchange resin of the desired cationic form. The naturally occurring sulfates proved to be identical with the corresponding synthetic specimens.

Another new bufogenin sulfate, resibufogenin 3-sulfate ammonium salt (IIIb), mp 153—156°, $[\alpha]_D^{20}$ +1.5°, was separated and its structure was elucidated by degradative and synthetic means similar to those described above. Gamabufotalin 3-sulfate sodium salt (IVb), mp 146—147°, was also isolated by chromatography on silica gel and identified by direct comparison with an authentic sample.

The occurrence of new bufotoxins and bufogenin 3-sulfates in the skin of the tropical toad has thus been demonstrated. To the best of our knowledge this is the first reported isolation of marinobufagin 3-pimeloylarginine ester, which has a dicarboxylic acid moiety other than suberic acid. No evidence was obtained for the presence of the succinoyl and adipoyl homologs in the skin of the tropical toad, although their existence in the Japanese

10) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).

toad has already been demonstrated.[3] It should be noted that significant amounts of bufogenin 3-sulfates were separated from the tropical toad. Considering the previous findings on the Japanese toad,[3,4] the sulfate appears to be a commonly occurring conjugate form of cardiotonic steroids in the toad. It is also noteworthy that steroid sulfate salts which differ only in the cationic moiety were distinguishable by gel chromatography on Sephadex LH-20 with methanol.

Of the new cardiotonic steroid conjugates thus obtained, marinobufotoxin and marinobufagin 3-pimeloylarginine ester were tested for their cardiotonic activities using isolated frog heart (Straub's preparation).[11] No significant difference was observed in cardiotonic activities between the two homologs, and contraction of the heart was brought about at a concentration of $10^{-6}$ g/ml. Further studies on the isolation of cardiotonic steroids from the toad venom are being conducted in this laboratory; the details will be reported in the near future.

## Experimental[12]

**Extraction of Steroidal Components**——Forty tropical toads (*Bufo marinus* (L.) SCHNEIDER) obtained from Mogul-ED Co. (Oshkosh, Wis.) were sacrificed by freezing in dry ice-acetone. The skins were immediately flayed off and extracted with EtOH (2 l) for 6 months. After removal of insoluble materials by filtration through a layer of Celite the filtrate was concentrated *in vacuo* below 50°. The residue was partitioned in an AcOEt–$H_2O$ system three times (Chart 1).

**Column Chromatography on Amberlite XAD-4 Resin**——The aqueous layer was concentrated *in vacuo* below 50° to remove the organic solvent and diluted with $H_2O$ to five times the original volume. The resulting solution was percolated through a column packed with Amberlite XAD-4 resin (Rohm and Haas Co., Philadelphia, Pa.) (150×15 cm i.d.), washed with $H_2O$ (5 l), and then eluted successively with 1 l each of 60%, 70%, 80%, 90% and 100% MeOH.

**Isolation of Marinobufagin 3-Pimeloylarginine Ester (Ic)**——Fr. 5 was concentrated *in vacuo* to afford a brown residue (10 g) which in turn was chromatographed on silica gel (200 g) and eluted successively with AcOEt (500 ml), AcOEt–MeOH (1: 1) (500 ml), AcOEt–MeOH (1: 2) (500 ml) and MeOH (500 ml). Fr. 9 (1.5 g) was again chromatographed on silica gel (42×1.5 cm i.d.) using $CHCl_3$–MeOH–$H_2O$ (80: 20: 2.5) to give Fr. 12 (30 mg). Fr. 12 was further purified by HPLC followed by gel chromatography on Sephadex LH-20 (35×1.5 cm i.d.). The eluate was reprecipitated from MeOH–$H_2O$ to give Ic (5 mg) as a colorless amorphous substance. mp 186—190°. $[\alpha]_D^{20}$ +22.0° ($c$=0.23). *Anal.* Calcd. for $C_{37}H_{54}N_4O_9 \cdot H_2O$: C, 61.99; H, 7.87; N, 7.82. Found: C, 61.64; H, 7.46; N, 7.92. NMR (2.5% solution in $CD_3OD$) $\delta$: 0.82 (3H, s, 18-$CH_3$), 1.00 (3H, s, 19-$CH_3$), 3.65 (1H, s, 15α-H), 5.20 (1H, m, 3α-H), 6.30 (1H, d, $J$=10 Hz, 23-H), 7.42 (1H, d, $J$=2 Hz, 21-H), 7.90 (1H, q, $J$=10, 2 Hz, 22-H).

**Synthesis of Marinobufagin 3-Hemipimelate Methyl Ester (Ib)**——To a solution of marinobufagin (Ia) (10 mg) and methyl hemipimelate (10 mg) in pyridine (2 ml) was added N,N′-dicyclohexylcarbodiimide (20 mg) and the solution was allowed to stand at room temperature for 3 days. The resulting solution was extracted with AcOEt. The organic layer was washed successively with 5% HCl, 5% $NaHCO_3$ and $H_2O$, dried over anhydrous $Na_2SO_4$, and evaporated. The crude product obtained was subjected to preparative TLC using benzene–AcOEt (1: 1) as a developing solvent. The adsorbent corresponding to the spot ($Rf$ 0.45) was eluted with AcOEt. The eluate (Ib) (2 mg) could not be crystallized but did behave homogeneously on TLC and HPLC. MS $m/e$: 556 ($M^+$), 175, 157.

11) T. Shigei, H. Tsuru, and N. Ishikawa, *Experientia*, 33, 258 (1977).

12) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured in MeOHwith a JASCO Model DIP-4 automatic polarimeter. Infrared (IR) spectra were obtained with a JASCO Model IRA-1 spectrometer. Mass spectral measurements were run on a Hitachi Model M-52G spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded using tetramethylsilane as an internal standard on JEOL Model PS-100 and FX-100 spectrometers at 100 MHz ($^1H$) and 25.047 MHz ($^{13}C$), respectively. Abbreviations: s=singlet, d=doublet, q=quartet and m=multiplet. Flame analysis was performed with a Hitachi Model 208 atomic absorption spectrophotometer. For preparative thin-layer chromatography (TLC), silica gel HF (E. Merck AG, Darmstadt) was used as an adsorbent. Silica gel (70—230 mesh) and silica gel H (E. Merck AG) were used for column chromatography with AcOEt–MeOH and $CHCl_3$–MeOH–$H_2O$ (80: 20: 2.5), respectively. High-performance liquid chromatography (HPLC) was carried out on a Waters Model ALC/GPC 202 R401 chromatograph with a μ-Bondapak $C_{18}$ column (1 ft.×0.25 in. i.d.) (Waters Associates, Milford, Mass.).

**Enzymatic Hydrolysis of Ic**——Ic (1.5 mg) was dissolved in 80% MeOH (1 ml)–1% NaCl (5 ml) and incubated with hog pancreas lipase preparation (Sigma Chemical Co., St. Louis, Mo.) (5 mg) at 37° for 12 hr. The incubation mixture was concentrated *in vacuo*, and the residue was dissolved in MeOH (1 ml), then treated with an ethereal solution of $CH_2N_2$. After usual work-up the crude product obtained was purified by preparative TLC using benzene–AcOEt (1: 1) as a developing solvent. The adsorbents corresponding to the spots (*Rf* 0.15 and 0.50) were eluted with AcOEt to give Ia and Ib, respectively. The mass spectra and chromatographic behavior of these compounds were identical with those of the corresponding authentic samples.

**Hydrolysis of Bufotoxin with 6N HCl**——Bufotoxin (1 mg) was heated with 6 N HCl (0.5 ml) in a sealed tube at 100° for 8 hr. A portion of the resulting solution was subjected to two-dimensional TLC (20×20 cm) on silica gel G using $CHCl_3$–MeOH–17% $NH_4OH$ (2: 2: 1) and phenol–$H_2O$ (3: 1) as developing solvents. Ninhydrin reagent was used for the detection of amino acid on the TLC plate.

**Isolation of Telocinobufagin 3-Suberoylarginine Ester (Telocinobufotoxin) (IIc)**——Fr. 1 was concentrated *in vacuo* to afford a brown residue (5 g), which in turn was chromatographed on silica gel (200 g) and eluted successively with AcOEt (500 ml), AcOEt–MeOH (1: 1) (500 ml), AcOEt–MeOH (1: 2) (500 ml) and MeOH (500 ml). Fr. 7 (3 g) was again chromatographed on silica gel (41×1.5 cm i.d.) using $CHCl_3$–MeOH–$H_2O$ (80: 20: 2.5) to give Fr. 10 (30 mg). Fr. 10 was further purified by HPLC followed by gel chromatography on Sephadex LH-20 (35×1.5 cm i.d.). The eluate was reprecipitated from MeOH–ether to give IIc (5 mg) as a colorless amorphous substance. mp 194—198°. $[\alpha]_D^{20}$ +1.8° ($c$=0.58). *Anal.* Calcd. for $C_{38}H_{58}N_4O_9 \cdot 2\ 1/2\ H_2O$: C, 60.06; H, 8.36; N, 7.37. Found: C, 59.98; H, 7.88; N, 7.49. NMR (2% solution in $CD_3OD$–$CDCl_3$ (1: 1)) $\delta$: 0.70 (3H, s, 18-$CH_3$), 0.95 (3H, s, 19-$CH_3$), 5.30 (1H, m, 3α-H), 6.30 (1H, d, $J$=10 Hz, 23-H), 7.35 (1H, d, $J$=2 Hz, 21-H), 8.00 (1H, q, $J$=10, 2 Hz, 22-H).

**Enzymatic Hydrolysis of IIc**——IIc (2 mg) was hydrolyzed enzymatically and then methylated with $CH_2N_2$ as described for Ic. After usual work-up the crude product obtained was purified by preparative TLC using benzene–AcOEt (1: 2) as a developing solvent. The adsorbents corresponding to the spots (*Rf* 0.10 and 0.40) were eluted with AcOEt to give telocinobufagin (IIa) and telocinobufagin 3-hemisuberate methyl ester (IIb), respectively. The physical data and chromatographic behavior of these compounds were identical with those of the corresponding authentic samples.[7]

**Isolation of Marinobufagin 3-Suberoylarginine Ester (Marinobufotoxin) (Id)**——Fr. 9 (1.5 g) was chromatographed on silica gel as described in Ic. The eluate of Fr. 13 (30 mg) was recrystallized from aq. MeOH to give Id (10 mg) as a colorless amorphous substance. mp 176—180° (Lit. mp 174—179°).[7] The structure was elucidated to be marinobufotoxin by degradative means. Marinobufotoxin (*ca.* 5 g) was also obtained from Fr. 1—4.

**Isolation of Marinobufagin 3-Sulfate Ammonium Salt (Ie) and Marinobufagin 3-Sulfate Sodium Salt (If)**——Fr. 4 was concentrated *in vacuo* to afford a brown residue (7 g), which in turn was chromatographed on silica gel (200 g) and eluted successively with AcOEt (500 ml), AcOEt–MeOH (1: 1) (500 ml), AcOEt–MeOH (1: 2) (500 ml) and MeOH (500 ml). Fr. 8 (100 mg) was chromatographed on silica gel (35×1 cm i.d.) using $CHCl_3$–MeOH–$H_2O$ (80: 20: 2.5) to give Fr. 11 (20 mg). The fraction was further purified by HPLC using MeOH–0.5% ammonium carbonate as the mobile phase. The eluate was concentrated *in vacuo* at 60° and then applied to a Sephadex LH-20 column (35×1.5 cm i.d.) using MeOH as a developing solvent. The effluent corresponding to 35—45 ml elution volume was concentrated *in vacuo* and the eluate was recrystallized from MeOH–ether to give Ie (3 mg) as colorless prisms. mp 156—160° (dec.). $[\alpha]_D^{20}$ +7.0° ($c$=0.37). $Ba^{2+}$-rhodizonate test: positive. *Anal.* Calcd. for $C_{24}H_{35}NO_8S \cdot H_2O$: C, 55.90; H, 7.23; N, 2.72; S, 6.22. Found: C, 55.70; H, 7.39; N, 2.26; S, 6.69. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3200, 1400 ($NH_4^+$), 1220, 1055 ($SO_2$). NMR (2.5% solution in $CD_3OD$–$CDCl_3$ (1: 1)) $\delta$: 0.80 (3H, s, 18-$CH_3$), 1.00 (3H, s, 19-$CH_3$), 3.65 (1H, s, 15α-H), 4.90 (1H, m, 3α-H), 6.30 (1H, d, $J$=10 Hz, 23-H), 7.30 (1H, d, $J$=2 Hz, 21-H), 7.90 (1H, q, $J$=10, 2 Hz, 22-H). $^{13}C$-NMR (5% solution in $CD_3OD$) $\delta$: 75.0 (5-C), 77.6 (3-C).

The effluent corresponding to 50—60 ml elution volume was concentrated *in vacuo* and the eluate was reprecipitated from MeOH–ether to give If (5 mg) as a colorless amorphous substance. mp 160—163° (dec.). $[\alpha]_D^{20}$ +12.1° ($c$=0.33). *Anal.* Calcd. for $C_{24}H_{31}NaO_8S \cdot H_2O$: C, 55.37; H, 6.39; S, 6.16. Found: C, 55.54; H, 6.52; S, 6.29. The physical data and chromatographic behavior of Ie and If were identical with those of the corresponding synthetic samples.

An aq. solution of Ie or If (1 mg) was adjusted to pH 1 with 50% $H_2SO_4$ and extracted with AcOEt (5 ml). The organic layer was allowed to stand at 37° for 12 hr. The resulting solution was washed with 5% $NaHCO_3$ and $H_2O$, dried over anhydrous $Na_2SO_4$, and evaporated. The crude product was purified by preparative TLC using benzene–AcOEt (1: 1) as a developing solvent to give Ia as a colorless oil. The chromatographic behavior and mass spectra were identical with those of an authentic sample. Ie and If were converted into each other by passing the aq. solution through a Dowex 50W-X8 ($Na^+$ or $NH_4^+$ form, 100—200 mesh) column. Marinobufagin 3-sulfate (*ca.* 500 mg) was also obtained from Frs. 1—3 and 5.

**Synthesis of Ie and If**——To a solution of Ia (30 mg) in pyridine (1 ml) was added chlorosulfonic acid (0.2 ml) in pyridine (2 ml) under ice-cooling and the solution was stirred for 30 min. The reaction mixture was poured into ice-water (20 ml). The resulting solution was adsorbed on Amberlite XAD-2 resin (30×1.5 cm i.d.), washed with $H_2O$ and then eluted with MeOH. The eluate was concentrated *in vacuo* and passed

through a Dowex 50W-X8 ($NH_4^+$ form) column as described above to give Ie (10 mg) as colorless prisms. mp 155—159° (dec.). If was prepared from Ie by passing the aq. solution through a Dowex 50W-X8 ($Na^+$ form) column.

**Isolation of Resibufogenin 3-Sulfate Ammonium Salt (IIIb) and Resibufogenin 3-Sulfate Sodium Salt (IIIc)**——Fr. 16 (10 mg) was purified by gel chromatography on a Sephadex LH-20 column (35×1.5 cm i.d.) using MeOH as a solvent. The effluent corresponding to 35—45 ml elution volume was concentrated *in vacuo* and the eluate was recrystallized from MeOH–ether to give IIIb (2 mg) as colorless prisms. mp 153—156°. $[\alpha]_D^{20}$ +1.5° ($c$=0.27). $Ba^{2+}$-rhodizonate test: positive. *Anal.* Calcd. for $C_{24}H_{35}NO_7S \cdot 1\ 2/3\ H_2O$: C, 56.33; H, 7.55; N, 2.74. Found: C, 56.16; H, 7.67; N, 2.61. IR $\nu_{max}^{KBr}$ $cm^{-1}$: 3200, 1400 ($NH_4^+$), 1220, 1055 ($SO_2$). NMR (2.5% solution in $CDCl_3$–$CD_3OD$ (1: 1)) $\delta$: 0.80 (3H, s, 18-$CH_3$), 1.00 (3H, s, 19-$CH_3$), 3.60 (1H, s, 15$\alpha$-H), 4.75 (1H, m, 3$\alpha$-H), 6.30 (1H, d, $J$=10 Hz, 23-H), 7.30 (1H, d, $J$=2 Hz, 21-H), 7.90 (1H, q, $J$=10, 2 Hz, 22-H). The effluent corresponding to 50—60 ml elution volume was concentrated *in vacuo* to give IIIc (3 mg) as a colorless oil. IIIc was converted to IIIb by passing the aq. solution through a Dowex 50W-X8 ($NH_4^+$ form) column. IIIc was characterized as an Na salt by flame analysis. Solvolysis of IIIb or IIIc (1 mg) in the manner described for Ie gave resibufogenin (IIIa) as a colorless oil. The mass spectra and chromatographic behavior were identical with those of an authentic sample.

**Isolation of Gamabufotalin 3-Sulfate Sodium Salt (IVb)**——Fr. 6 (200 mg) was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH–$H_2O$ (80: 20: 2.5) to give IVb (10 mg) as a colorless amorphous substance. mp 146—147° (Lit. mp 146—147°).[3] IVb was shown to be gamabufotalin 3-sulfate Na salt by direct comparison with an authentic sample. Solvolysis of IVb (1 mg) in the manner described for Ie gave gamabufotalin (IVa) as a colorless oil. The mass spectra and chromatographic behavior were identical with those of an authentic sample.

**Acknowledgement** The authors are indebted to the staff of the central analytical laboratory of this Institute for elemental analyses and spectral measurements. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, which is gratefully acknowledged.