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Studies on Cardiotonic Steroids from the Skin of Japanese Toad[1]

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The isolation and characterization of the cardiotonic steroid constituents in the skin of the Japanese toad, *Bufo vulgaris formosus* Boulenger, has been carried out. Three new types of bufotoxins in which the succinoyl, adipoyl, and pimeloyl groups are displaced for the suberoyl residue of the so-called "bufotoxin," were separated and identified. In addition, the occurrence of bufogenin 3-sulfates and analogous conjugates of cardenolide named cardenobufotoxin was also demonstrated.

The ethanolic extract of the skin obtained from 1800 toads was separated by chromatography on Amberlite XAD-2 resin, silica gel, and Sephadex LH-20, followed by high-performance liquid chromatography to provide new cardiotonic steroid conjugates. The structures of these substances were elucidated to be resibufogenin 3-succinoylarginine ester (Id), gamabufotalitoxin homologs (IIg, IIk, IIn, IIp), bufalitoxin homologs (IIIc, IIIe, IIIg, IIIi), cinobufotoxin homologs (IVc, IVe, IVg, IVl), arenobufotoxin (Vd), cinobufotalitoxin (VIb), cardenobufotoxins (VIIId, VIIIf), bufogenin 3-sulfates (IIq, IIIj, Ve, VIId), and sarmentogenin 3-sulfate (VIIIg) by degradative and synthetic means.

Keywords——*Bufo vulgaris formosus* Boulenger; skin of toad; separation of conjugated bufogenins; high-performance liquid chromatography; cardiotonic steroid; hog pancreas lipase; bufotoxin homolog; cardenobufotoxin; bufadienolide 3-sulfate; cardenolide 3-sulfate

In 1922 Wieland, *et al.* separated from the toad venom the so-called "bufotoxin",[3] whose structure was subsequently assigned to bufotalin 14-suberoylarginine ester.[4] Kamano, *et al.*[5] reported the isolation of the bufogenin 3-hemisuberate from the Chinese drug *Ch'an Su* whereby they suggested that "bufotoxin" would be a conjugate linked to the C-3 position in a steroid molecule. In actuality, the complete structure was then unambiguously established to be the 3-suberoylarginine ester of bufogenin by degradative[6] and synthetic means.[7,8] In 1970 Meyer and his co-workers[9] disclosed the existence of the cardenolide and its

1) Part CXIX of "Studies on Steroids" by T. Nambara; Part CXVIII: M. Numazawa, N. Soeda, S. Moro, and T. Nambara, *Biochem. Pharmacol.*, "in press." Preliminary accounts of this work have been presented: K. Shimada, Y. Fujii, E. Mitsuishi, and T. Nambara, *Tetrahedron Letters*, **1974**, 467; *idem*, *Chem. Ind.* (London), **1974**, 342; *idem*, *Chem. Pharm. Bull.* (Tokyo), **22**, 1673 (1974); K. Shimada, Y. Fujii, and T. Nambara, *Tetrahedron Letters*, **1974**, 2767; *idem*, *Chem. Ind.* (London), **1974**, 963; K. Shimada, Y. Fujii, Y. Niizaki, and T. Nambara, *Tetrahedron Letters*, **1975**, 653; Y. Fujii, K. Shimada, Y. Niizaki, and T. Nambara, *ibid.*, **1975**, 3017; Y. Fujii, K. Shimada, and T. Nambara, *Chem. Ind.* (London), **1976**, 614; K. Shimada, Y. Sato, Y. Fujii, and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **24**, 1118 (1976).

2) Location: *Aobayama, Sendai.*

3) H. Wieland and R. Alles, *Ber.*, **55**, 1789 (1922); H. Wieland, G. Hesse, and R. Hüttel, *Ann*, **524**, 203 (1936); H. Wieland and H. Behringer, *ibid.*, **549**, 209 (1941).

4) *a*) K. Meyer, *Helv. Chim. Acta*, **32**, 1993 (1949); *b*) H.R. Urscheler, Ch. Tamm, and T. Reichstein, *ibid.*, **38**, 883 (1955).

5) Y. Kamano, H. Yamamoto, Y. Tanaka, and M. Komatsu, *Tetrahedron Letters*, **1968**, 5673.

6) H.O. Linde-Tempel, *Helv. Chim. Acta*, **53**, 2188 (1970).

7) *a*) G.R. Pettit and Y. Kamano, *Chem. Commun.*, **1972**, 45; *b*) *Idem*, *Experientia*, **28**, 768 (1972).

8) K. Shimada, Y. Fujii, and T. Nambara, *Chem. Ind.* (London), **1972**, 258; *idem*, *Chem. Pharm. Bull.* (Tokyo), **21**, 2183 (1973).

9) *a*) N. Höriger, H.H.A. Linde, and K. Meyer, *Helv. Chim. Acta*, **53**, 1503 (1970); *b*) N. Höriger, D. Živanov, H.H.A. Linde, and K. Meyer, *ibid.*, **53**, 1993 (1970); *c*) *Idem*, *ibid.*, **53**, 2051 (1970).

3-hemisuberate in *Ch'an Su*. This finding indicates the possible occurrence of analogous conjugates in the living animal. As a series of studies on the structure-activity relationship the syntheses of the cardiotonic steroid analogs have been carried out in this laboratory.[8,10] A particular interest in these respects prompted us to explore the cardiotonic steroid conjugates in the skin of Japanese toad, *Bufo vulgaris formosus* Boulenger. The present paper deals with the isolation and characterization of three new types of bufotoxins in which the succinoyl, adipoyl, and pimeloyl groups are displaced for the suberoyl residue of "bufotoxin". The occurrence of bufogenin 3-sulfates and analogous conjugates of cardenolide named cardenobufotoxin is also described.

Eighteen hundred toads collected in the northeastern district of Japan were sacrificed by freezing in dry ice–acetone, and the skins were immediately flayed and extracted with ethanol. The ethanolic extract was partitioned with the ether–water system and then with the ethyl acetate–water system as shown in Chart 1. The ether layer was concentrated *in vacuo* and the residue obtained was chromatographed on silica gel. Purification of the eluate by preparative thin-layer chromatography provided resibufogenin (Ia), bufalin (IIIa), cinobufagin (IVa), and digitoxigenin (IX).[11] In addition, gel filtration on Sephadex LH-20 using methanol as solvent afforded 14β, 15β-epoxy-"β"-anhydrodigitoxigenin (X).[11] In similar fashion gamabufotalin (IIa), arenobufagin (Va), and sarmentogenin (VIIIa)[11] were also separated from the ethyl acetate layer. These aglycones were unequivocally characterized by direct comparison with the authentic samples,[12] respectively.

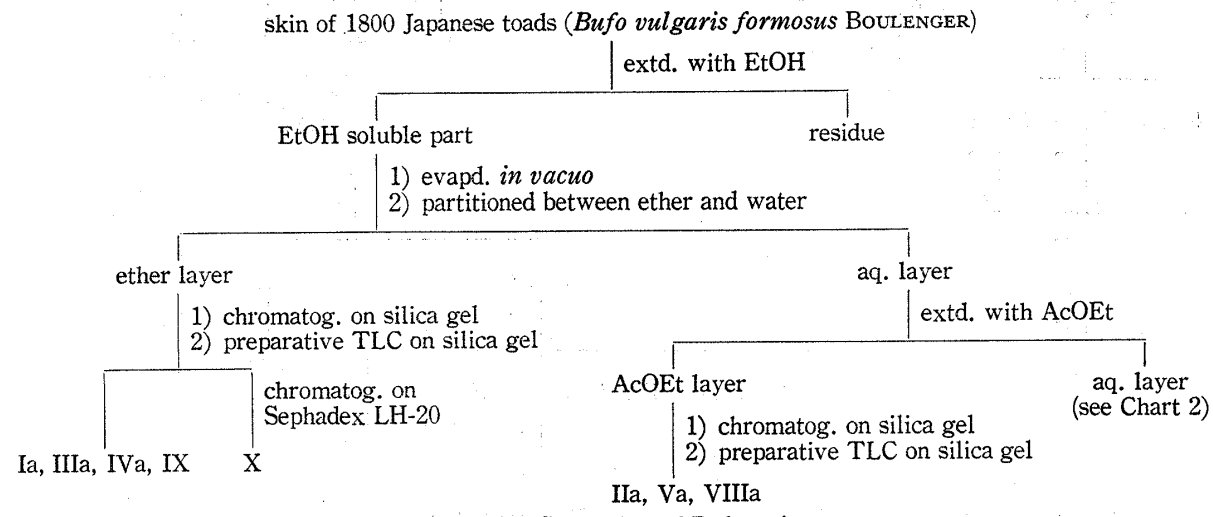


Chart 1. Separation of Bufogenins

Separation of the conjugated steroids was processed in the manner as shown in Chart 2. The aqueous layer was percolated through a column of Amberlite XAD-2 resin. After thorough washing with distilled water the conjugated steroid fraction was eluted with 70%, 80%, and 90% methanol, successively. Each eluate was submitted to dry column chromatography on silica gel employing ethyl acetate–methanol as eluent. Subsequent column chromatography on silica gel with use of chloroform–methanol–water (80:20:2.5) as mobile phase provided satisfactory separation. When further purification was required, gel filtration on Sephadex LH-20, high-performance liquid chromatography on the reversed phase column, and preparative thin-layer chromatography were effective.

10) K. Shimada and T. Nambara, *Chem. Pharm. Bull.* (Tokyo), **19**, 1073 (1971); T. Nambara, K. Shimada, and Y. Fujii, *ibid.*, **20**, 1424 (1972); *idem*, *ibid.*, **21**, 1031 (1973).
11) These cardenolides were isolated from *Ch'an Su* by Höriger, *et al.*[9] but their existence in the skin of toad has not yet been reported.
12) 14β,15β-Epoxy-"β"-anhydrodigitoxigenin was prepared from digitoxigenin as authentic sample.[13]
13) P. Hofer, H. Linde, and K. Meyer, *Helv. Chim. Acta*, **15**, 1041 (1962).

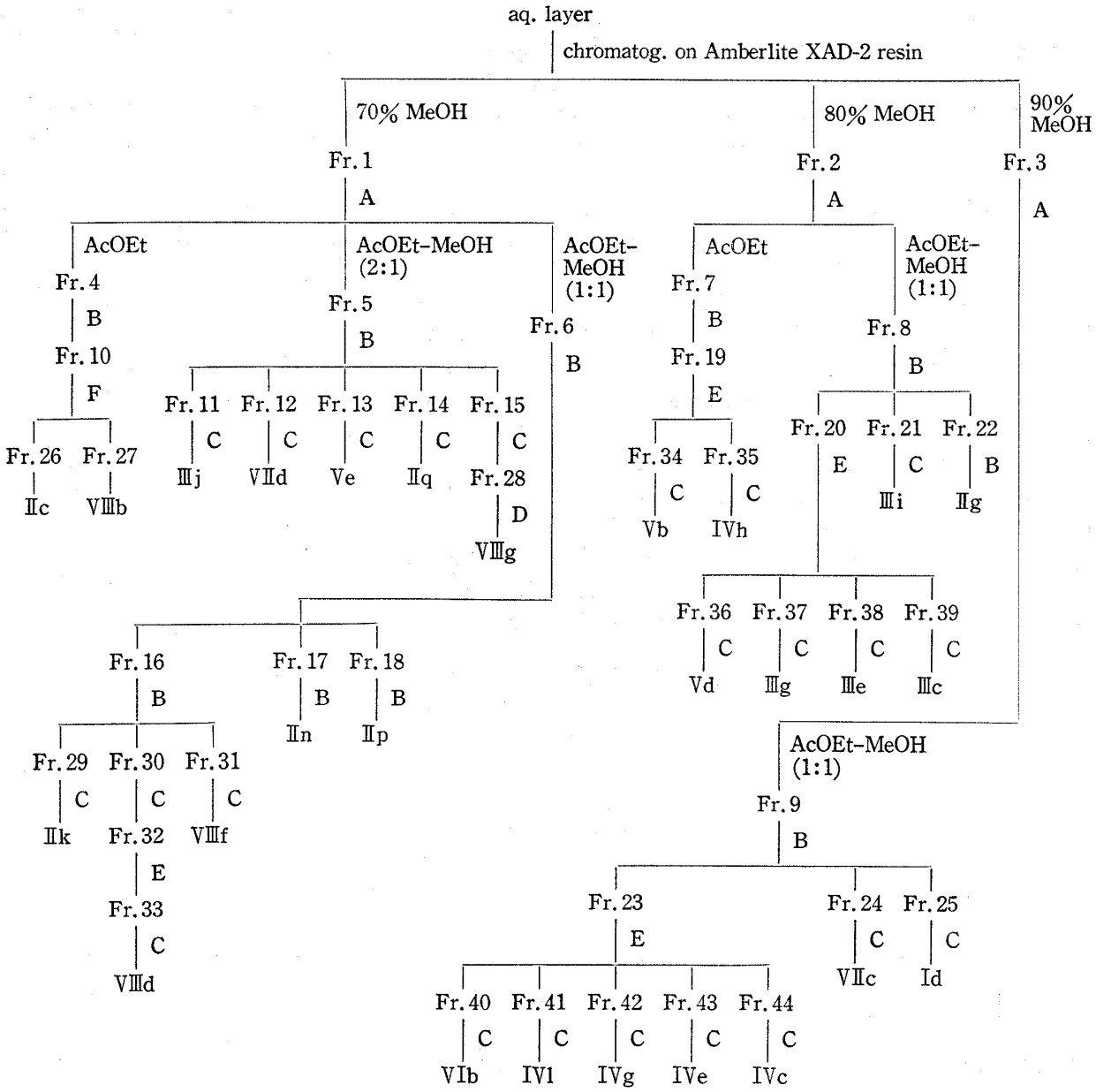


Chart 2. Separation of Bufogenin Conjugates

A new bufotoxin (Id), mp 210.5—212°, $[\alpha]_D^{19}+22.8°$, was first separated as colorless prisms. This substance showed a positive result with the Sakaguchi's reagent and a negative ninhydrin test. Inspection of the nuclear magnetic resonance spectrum revealed the existence of α-pyrone ring, epoxy and succinoyl groups. Upon hydrolysis with 6N hydrochloric acid arginine liberated was characterized by two-dimensional thin-layer chromatography. In order to confirm the presence of a peptide bond involving the α-amino group of arginine, Id was converted into the pyrimidine derivative (Ie) by treatment with acetylacetone–potassium bicarbonate and then with diazomethane.[6,8] Unfortunately Ie could not be obtained crystalline but its structure was unequivocally assignable on the basis of the nuclear magnetic resonance spectral data. Being subjected to enzymatic hydrolysis with the hog pancreas

lipase preparation (Sigma Chemical Co., St. Louis),[14] Id afforded resibufogenin 3-hemisuccinate (Ib), mp 163—165.5°, as colorless prisms. The hydrolyzate proved to be entirely identical with the synthetic sample which was readily prepared from resibufogenin (Ia) and succinic anhydride. Subsequent methylation with diazomethane in the usual manner yielded the methyl ester (Ic), which was also identified by direct comparison with the synthetic specimen. These evidences together led us to assign the new type bufotoxin to the structure 3-succinoylarginine ester of resibufogenin (Id).

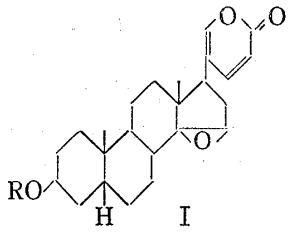
The second bufotoxin (IIg), mp 215—219°, $[\alpha]_D^{16}$ −2.2°, showed a positive result with the Sakaguchi's reagent and a negative test with ninhydrin. Hydrolytic cleavage with 6 N hydrochloric acid furnished arginine, which was characterized by thin-layer chromatography. Enzymatic hydrolysis with the hog pancreas lipase preparation, followed by methylation with diazomethane provided the methyl ester of gamabufotalin 3-hemisuberate (IIe), mp 190—193°, $[\alpha]_D^{14}$ −6.3°, as colorless needles. Subsequent acetylation with acetic anhydride and pyridine in the usual manner yielded the 11-acetate (IIf), mp 127—128°, $[\alpha]_D^{14}$ −6.7°, which was identified by comparison with the synthetic sample prepared from gamabufotalin (IIa) in three steps.[9b] The presence of a peptide bond involving the α-amino group of arginine in IIg was confirmed by leading to the pyrimidine derivative (IIh) in the manner as described above. These results lent a support to assign gamabufotalin 3-suberoylarginine ester named gamabufotalitoxin to IIg.

The third bufotoxin (IIk), mp 189—192° (decomp.), $[\alpha]_D^{18}$ −3.1°, was obtained as colorless amorphous substance. The peptide bond involving the α-amino group of arginine in IIk was ascertained by color tests and hydrolytic cleavage with hydrochloric acid. Enzymatic hydrolysis and subsequent methylation afforded the methyl ester of gamabufotalin 3-hemipimelate (IIi), mp 193—195°, $[\alpha]_D^{18}$ −10.0°, as colorless leaflets. The molecular ion and fragment ion peaks (*m/e* 558 (M^+), 175, 157)[15] in the mass spectrum justified to assign pimelic acid as dicarboxylic acid moiety. Acetylation of IIi in the usual way yielded the 11-acetate (IIj), which proved to be identical with the specimen prepared from gamabufotalin 11-acetate (IIb) and methyl hemipimelate by the N,N'-dicyclohexylcarbodiimide (DCC) method. It is evident from these results that the structure of IIk should be assignable to gamabufotalin 3-pimeloylarginine ester. It is noteworthy that this new bufotoxin contains a dicarboxylic acid of odd carbon number.

The fourth bufotoxin (IIn), mp 242—245°, $[\alpha]_D^{21}$ −16.4°, was isolated as colorless amorphous substance. Similar treatment with the enzyme preparation and then with diazomethane furnished the methyl ester of gamabufotalin 3-hemiadipate (III), mp 199—200°, $[\alpha]_D^{23}$ −15.2°, as colorless leaflets. Inspection of the mass spectrum (*m/e* 544 (M^+), 161, 143)[15] strongly implied the presence of adipic acid in IIn as dicarboxylate portion. Usual acetylation of III yielded the 11-acetate (IIm), mp 161—161.5°, which was characterized by comparison with the synthetic sample derived from gamabufotalin (IIa) in two steps. These evidences permitted us to assign the structure gamabufotalin 3-adipoylarginine ester to IIn.

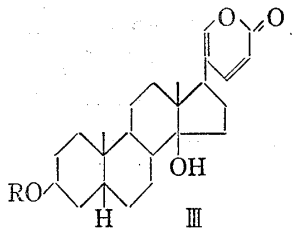
The fifth bufotoxin (IIp), mp 198—200°, $[\alpha]_D^{24}$ −6.6°, provided the methyl ester of gamabufotalin 3-hemisuccinate (IIo), mp 189—191.5°, $[\alpha]_D^{24}$ +7.1°, as colorless prisms, when hydrolyzed with the enzyme preparation and then methylated with diazomethane. The attached position of a succinoyloxy group to the steroid nucleus was deduced to be 3β rather than 11α as judged from the half-band width of 3α and 11β protons in the nuclear magnetic resonance spectrum (δ: 2.62 (4H, s, $-CO(CH_2)_2CO-$), 3.80 (1H, m, $W1/2=20$ Hz, 11β–H), 5.20 (1H, m, $W1/2=8$ Hz, 3α–H)). The mass fragmentation pattern (*m/e* 516 (M^+), 133, 115)[15] also rationalized to identify succinic acid as dicarboxylic acid component.

14) The enzyme preparation contains also amylase and peptidase.
15) P. Brown, Y. Kamano, and G.R. Pettit, *Org. Mass Spectrom.*, **6**, 613 (1972).



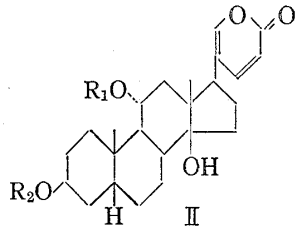
I

a : R = H
b : R = CO(CH$_2$)$_2$COOH
c : R = CO(CH$_2$)$_2$COOCH$_3$
d : R = CO(CH$_2$)$_2$COArg·OH
e : R = CO(CH$_2$)$_2$CONHCH(CH$_2$)$_3$NH–(2,4-dimethylpyrimidyl) (COOCH$_3$)



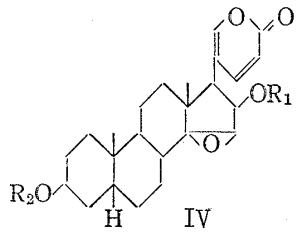
III

a : R = H
b : R = CO(CH$_2$)$_6$COOCH$_3$
c : R = CO(CH$_2$)$_6$COArg·OH
d : R = CO(CH$_2$)$_5$COOCH$_3$
e : R = CO(CH$_2$)$_5$COArg·OH
f : R = CO(CH$_2$)$_4$COOCH$_3$
g : R = CO(CH$_2$)$_4$COArg·OH
h : R = CO(CH$_2$)$_2$COOCH$_3$
i : R = CO(CH$_2$)$_2$COArg·OH
j : R = SO$_3$Na



II

a : R$_1$ = R$_2$ = H
b : R$_1$ = Ac, R$_2$ = H
c : R$_1$ = H, R$_2$ = CO(CH$_2$)$_6$COOH
d : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_6$COOH
e : R$_1$ = H, R$_2$ = CO(CH$_2$)$_6$COOCH$_3$
f : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_6$COOCH$_3$
g : R$_1$ = H, R$_2$ = CO(CH$_2$)$_6$COArg·OH
h : R$_1$ = H, R$_2$ = CO(CH$_2$)$_6$CONHCH(CH$_2$)$_3$NH–(2,4-dimethylpyrimidyl) (COOCH$_3$)
i : R$_1$ = H, R$_2$ = CO(CH$_2$)$_5$COOCH$_3$
j : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_5$COOCH$_3$
k : R$_1$ = H, R$_2$ = CO(CH$_2$)$_5$COArg·OH
l : R$_1$ = H, R$_2$ = CO(CH$_2$)$_4$COOCH$_3$
m : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_4$COOCH$_3$
n : R$_1$ = H, R$_2$ = CO(CH$_2$)$_4$COArg·OH
o : R$_1$ = H, R$_2$ = CO(CH$_2$)$_2$COOCH$_3$
p : R$_1$ = H, R$_2$ = CO(CH$_2$)$_2$COArg·OH
q : R$_1$ = H, R$_2$ = SO$_3$Na
r : R$_1$ = Ac, R$_2$ = SO$_3$Na



IV

a : R$_1$ = Ac, R$_2$ = H
b : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_6$COOCH$_3$
c : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_6$COArg·OH
d : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_5$COOCH$_3$
e : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_5$COArg·OH
f : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_4$COOCH$_3$
g : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_4$COArg·OH
h : R$_1$ = H, R$_2$ = CO(CH$_2$)$_2$COOH
i : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_2$COOH
j : R$_1$ = H, R$_2$ = CO(CH$_2$)$_2$COOCH$_3$
k : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_2$COOCH$_3$
l : R$_1$ = Ac, R$_2$ = CO(CH$_2$)$_2$COArg·OH

Chart 3

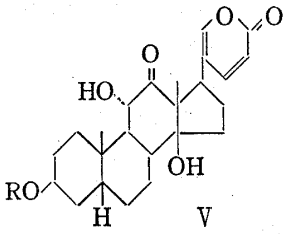
In addition, bufalitoxin (bufalin 3-suberoylarginine ester) (IIIc),[16] mp 200—205° (decomp.), $[\alpha]_D^{21}$ −5.5°, and its homologs (IIIe), mp 205—206.5° (decomp.), $[\alpha]_D^{21}$ −5.5°, (IIIg), mp 210—213° (decomp.), $[\alpha]_D^{22}$ +5.5°, and (IIIi), mp 240—241°, $[\alpha]_D^{23}$ −12.5°, were separated with success. By treatment with the enzyme preparation, followed by methylation, these were transfomed into bufogenin 3-hemidicarboxylate methyl esters (IIIb, IIId, IIIf, and IIIh), respectively. Among these four IIIf was further characterized by comparison with the synthetic specimen prepared from bufalin (IIIa). These results together with elemental analyses, nuclear magnetic resonance and mass spectral data clarified that the four new bufotoxins should be bufalitoxin (IIIc), bufalin 3-pimeloyl-(IIIe), 3-adipoyl-(IIIg), and 3-succinoyl-arginine (IIIi) esters.

16) Bufalitoxin has been synthesized by Pettit, *et al.*[7a]

Cinobufotoxin (cinobufagin 3-suberoylarginine ester) (IVc),[17] mp 170—175° (decomp.), $[\alpha]_D^{16}$ +5.6°, and its homologs (IVe), mp 175—178° (decomp.), $[\alpha]_D^{16}$ +6.3°, (IVg), mp 183—186° (decomp.), $[\alpha]_D^{17}$ +4.7°, and (IVl), mp 200—202.5°, $[\alpha]_D^{15}$ +27.8°, were also isolated. Upon enzymatic hydrolysis and subsequent methylation these bufotoxins were led to the methyl esters of cinobufagin 3-hemisuberate (IVb), 3-hemipimelate (IVd), 3-hemiadipate (IVf), and 3-hemisuccinate (IVk), respectively. The usual criteria, *i.e.* elemental analyses and spectral properties justified the structural assignment of cinobufotoxin (IVc), cinobufagin 3-pimeloyl-(IVe), 3-adipoyl-(IVg), and 3-succinoyl-arginine (IVl) esters to these four bufotoxins.

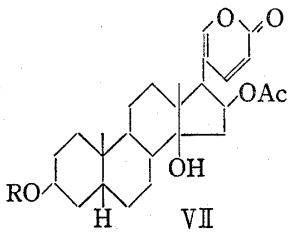
In similar fashion, arenobufotoxin (arenobufagin 3-suberoylarginine ester) (Vd),[19] mp 182—184° (decomp.), $[\alpha]_D^{23}$ −33.3°, cinobufotalitoxin (cinobufotalin 3-suberoylarginine ester) (VIb), mp 176—178° (decomp.), $[\alpha]_D^{16}$ −5.4°, and vulgarobufotoxin (bufotalin 3-suberoyl-arginine ester) (VIIc),[21] mp 205—206.5°, $[\alpha]_D^{20}$ +2.4°, were unequivocally characterized by degradative means.

Additional new bufotoxin analogs (VIIId), mp 171—173°, $[\alpha]_D^{16}$ +14.2°, and (VIIIf), mp 184—187°, $[\alpha]_D^{17}$ +9.6°, which exhibited a characteristic ultraviolet absorption (λ_{max}^{MeOH} 217 nm) and a positive Kedde's test for the butenolide ring, were isolated with success. Both conjugated cardenolides showed a positive result with the Sakaguchi's reagent and a negative test with ninhydrin. Treatment of VIIId with the enzyme preparation and then with diazomethane provided the methyl ester of sarmentogenin 3-hemisuberate (VIIIc), mp 115—116°,



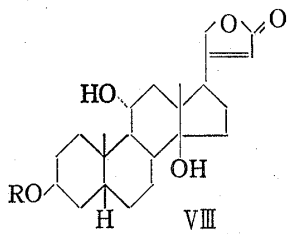
V

a : R=H
b : R=CO(CH$_2$)$_6$COOH
c : R=CO(CH$_2$)$_6$COOCH$_3$
d : R=CO(CH$_2$)$_6$COArg·OH
e : R=SO$_3$Na



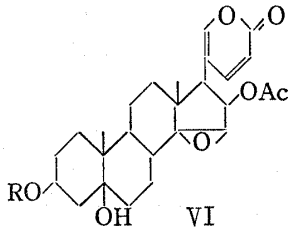
VII

a : R=H
b : R=CO(CH$_2$)$_6$COOCH$_3$
c : R=CO(CH$_2$)$_6$COArg·OH
d : R=SO$_3$Na



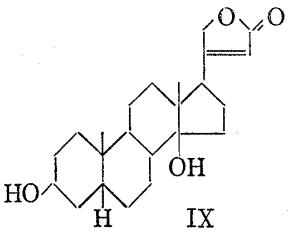
VIII

a : R=H
b : R=CO(CH$_2$)$_6$COOH
c : R=CO(CH$_2$)$_6$COOCH$_3$
d : R=CO(CH$_2$)$_6$COArg·OH
e : R=CO(CH$_2$)$_5$COOCH$_3$
f : R=CO(CH$_2$)$_5$COArg·OH
g : R=SO$_3$Na

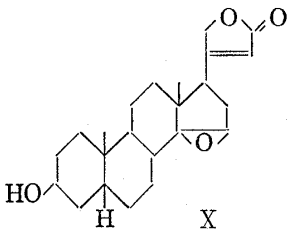


VI

a : R=CO(CH$_2$)$_6$COOCH$_3$
b : R=CO(CH$_2$)$_6$COArg·OH



IX



X

Chart 4

17) Cinobufotoxin was isolated from the Japanese toad by Ohno, *et al.* but its complete structure has not yet been elucidated.[18]
18) S. Ohno and M. Komatsu, *Yakugaku Zasshi*, **73**, 651, 796 (1953).
19) Arenobufotoxin was first isolated from the toad venom by Chen, *et al.*[20] but its purity and structure still remained uncertain.
20) K.K. Chen, H. Jensen, and A.L. Chen, *J. Pharmacol. Exptl. Therap.*, **43**, 13 (1931); K.K. Chen and A.L. Chen, *ibid.*, **49**, 514, 529 (1933).
21) Vulgarobufotoxin was first isolated from the toad venom by Wieland, *et al.*[3] and was synthesized by Pettit, *et al.*[7a]

$[\alpha]_D^{16}$ +25.0°, as colorless leaflets, whose structure was verified by the nuclear magnetic resonance (δ: 3.75 (1H, m, $W1/2$=20 Hz, 11β-H), 5.04 (1H, m, $W1/2$=10 Hz, 3α-H)) and mass (*m/e* 560 (M^+), 189, 171)[15] spectral data. Hydrolytic cleavage with 5% hydrochloric acid in aqueous methanol under the mild conditions yielded an aglycone, which was identified as sarmentogenin (VIIIa). Based upon these results the structure sarmentogenin 3-suberoylarginine ester was assigned to VIIId.

Another new bufotoxin analog (VIIIf) was similarly transformed into the methyl ester of sarmentogenin 3-hemipimelate (VIIIe). The dicarboxylic acid moiety in VIIIe was deduced to be pimelic acid from the mass spectrum exhibiting the parent ion and fragment ion peaks at *m/e* 546 (M^+), 175, and 157.[15] From these findings we arrived at the conclusion that VIIIf should be sarmentogenin 3-pimeloylarginine ester. This is the first recorded instance of the conjugated cardenolide separated from the animal source. The novel type of cardenolide derived from the toad venom is named cardenobufotoxin.

The presence of 3-hemidicarboxylates of bufadienolide and cardenolide in the living animal was also disclosed. Gamabufotalin 3-hemisuberate (IIc), arenobufagin 3-hemisuberate (Vb),[22] and sarmentogenin 3-hemisuberate (VIIIb) isolated from the skin were transformed into the methyl esters (IIe, Vc, and VIIIc), whose structures were established by comparison with the authentic samples. Desacetylcinobufagin 3-hemisuccinate (IVh) was led to the methyl ester (IVj) and further to the 16-acetate (IVk), which was unequivocally identified by direct comparison with the authentic specimen.

Different type conjugates of bufadienolide and cardenolide were then separated. Conjugate (IIIj), mp 165.5—166.5°, $[\alpha]_D^{23}$ −33.1°, resisted to usual acetylation, exhibited a positive test with barium-rhodizonate reagent responsible for sulfate ions[23] and infrared absorption bands at 1220, 1055 cm^{-1} due to the sulfate group. Solvolysis in the usual manner[24] furnished bufalin (IIIa), which was characterized by thin-layer chromatography and mass spectrum. The nuclear magnetic resonance spectral properties (δ: 4.71 (1H, m, 3α-H)) rationalized to put the sulfate linkage at C-3 in the bufogenin. These data lent a support to assign the structure bufalin 3-sulfate and prompted us to prepare the authentic sample for direct comparison. Condensation of bufalin (IIIa) with sulfuric acid was readily attained by treatment with DCC.[25] The crude product obtained was purified by chromatography to provide the desired sodium bufalin 3-sulfate (IIIj), mp 165—166.5°, $[\alpha]_D^{23}$ −35.7°, which proved to be entirely identical with the natural product in every respect.

Conjugate (VIId), mp 156—158°, $[\alpha]_D^{21}$ −32.2°, exhibited a positive test with barium-rhodizonate reagent and an infrared absorption at 1060 cm^{-1}, and yielded bufotalin (VIIa) by usual solvolysis. Consequently, the structure bufotalin 3-sulfate was assigned to VIId.

Conjugate (Ve), mp 196—201°, $[\alpha]_D^{21}$ +72.9°, showed an infrared absorption at 1060 cm^{-1} and afforded arenobufagin (Va) as aglycone by solvolysis. The position of a sulfate linkage to the bufogenin was elucidated to be 3β from the nuclear magnetic resonance spectrum (δ: 4.32 (1H, d, J=10 Hz, 11β-H), 4.72 (1H, m, $W1/2$=10 Hz, 3α-H)). On the basis of these data Ve was identified as arenobufagin 3-sulfate.

Conjugate (IIq), mp 146—147°, $[\alpha]_D^{26}$ −8.3°, exhibited an infrared absorption band at 1060 cm^{-1} and on usual solvolysis provided gamabufotalin (IIa) as aglycone. The position of a sulfate group attached to the bufogenin was deduced to be 3β from the nuclear magnetic resonance spectrum (δ: 4.78 (1H, m, $W1/2$=10 Hz, 3α-H), 4.94 (1H, m, $W1/2$=20 Hz, 11β-H))

22) Gamabufotalin 3-hemisuberate and arenobufagin 3-hemisuberate were isolated from *Ch'an Su* by Höriger, *et al.*[9b]
23) D.P. Burma, *Anal. Chim. Acta*, **9**, 513 (1953); J.J. Schneider and M.L. Lewbart, *J. Biol. Chem.*, **222**, 787 (1956).
24) S. Burstein and S. Lieberman, *J. Biol. Chem.*, **233**, 331 (1958).
25) R.O. Mumma, C.P. Hoiberg, and W.W. Weber II, *Steroids*, **14**, 67 (1969).

of the 11-acetate (IIr) formed by usual acetylation. In consequence, the structure gamabufotalin 3-sulfate was assignable to IIq.

Conjugate (VIIIg), mp 149—151°, $[\alpha]_D^{20}$ +56.1°, showed an ultraviolet absorption (λ_{max}^{MeOH} 218 nm) and a positive Kedde's test for the butenolide ring. This substance exhibited a positive result with barium-rhodizonate reagent and an infrared absorption at 1050 cm^{-1}. Solvolysis in the usual manner afforded sarmentogenin (VIIIa) as aglycone. As judged from the nuclear magnetic resonance spectral data (δ: 3.70 (1H, m, $W1/2=19$ Hz, 11β-H), 4.75 (1H, m, $W1/2=11$ Hz, 3α-H)) the position of a sulfate group was deduced to be 3β. These evidences lent a support to assign the structure sarmentogenin 3-sulfate to VIIIg.

The occurrence of several new types of conjugated bufogenins in the skin of Japanese toad has been demonstrated. First, it is to be noted that the homologous succinoyl-, adipoyl-, and pimeloyl-arginine esters are common conjugate forms of bufogenin as the hitherto known suberoylarginine ester. Among these the pimeloyl derivative is of particular interest in view of its biosynthesis in the toad. No evidence, however, could be obtained for the presence of the glutaroyl homolog despite of much efforts. Secondly, the existence of the cardenolide and its conjugate in the living animal has been disclosed. It should be emphasized that the cardenolide as well as the bufadienolide is distributed in both animal and plant kingdoms. Thirdly, this is the first reported isolation of the sulfates of bufadienolide and cardenolide from an animal source. It is also noteworthy that the cardiotonic steroid as well as the hormonal steroid occurs in the form of a sulfate in the animal kingdom. In view of the accumulating evidences for active participation of the steroid conjugates, the physiological significance of cardiotonic steroid sulfates in the living animal is an attractive problem to be solved.

The pharmacological tests of these new cardiotonic steroids are being conducted and the results will be the subject of a future communication.

Experimental[26]

Extraction of Steroidal Components——Eighteen hundred Japanese toads (*Bufo vulgaris formosus* Boulenger) collected in the northeastern district of Japan from the end of March to the beginning of April were sacrificed by freezing in dry ice–acetone. The skins were immediately teared off and extracted with EtOH (100 liter) for a month at 5°. After filtration through a layer of Celite the extract was concentrated to *ca.* 20 liter *in vacuo* below 50°, and partitioned with the ether–H_2O and then AcOEt–H_2O systems three times, respectively (Chart 1).

Column Chromatography on Amberlite XAD-2 Resin——The aqueous layer was evaporated *in vacuo* below 50° for removal of the organic solvent and diluted with H_2O (450 liter). The resulting solution was percolated through a column packed with Amberlite XAD-2 resin (Rohm and Haas Co., Philadelphia) (150×15 cm i.d.), washed with H_2O (100 liter), and then eluted successively with 20% MeOH (1.5 liter), 40% MeOH (1.5 liter), 70% MeOH (5 liter), 80% MeOH (5 liter), 90% MeOH (5 liter), and anhydrous MeOH (5 liter) (Chart 2).

Isolation of Resibufogenin 3-Succinoylarginine Ester (Id)——Fr. 3 was concentrated under the reduced pressure to afford a brown residue (30 g), which was chromatographed on silica gel (500 g) and eluted succes-

26) All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO Model DIP–SL automatic polarimeter in $CHCl_3$ unless otherwise specified. Ultraviolet (UV) spectra were obtained by a Hitachi Model 124 spectrometer and infrared (IR) spectra by Hitachi Model 215, JASCO Model IRA-1 and IR–S spectrometers. Mass spectral measurements were run on Hitachi Model RMU-7 and RMU-6E spectrometers. Nuclear magnetic resonance (NMR) spectra were recorded using tetramethylsilane as an internal standard on Hitachi Model R-20A and JEOL Model PS-100 spectrometers at 60 MHz and 100 MHz, respectively. Abbreviation used s= singlet, d=doublet, q=quartet, and m=multiplet. For preparative thin-layer chromatography (TLC) silica gel HF_{254} (E. Merck AG, Darmstadt) was used as an adsorbent. Silica gel (70—230 mesh) (E. Merck AG) was used for column chromatography with AcOEt–MeOH and silica gel H (E. Merck AG) for column chromatography with $CHCl_3$–MeOH–H_2O (80: 20: 2.5). High-performance liquid chromatography (HPLC) was carried out on a Waters Model ALC/GPC 202/401 using μ-Bondapak C_{18} column (Waters Associates Inc., Milford).

sively with AcOEt (2.5 liter), AcOEt–MeOH (4: 1) (3 liter), AcOEt–MeOH (2: 1) (3 liter), AcOEt–MeOH (1: 1) (6 liter), AcOEt–MeOH (1: 2) (3 liter), and MeOH (3 liter). Fr. 9 (25 g) was chromatographed on silica gel (150×6 cm i.d.) using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as solvent to give Fr. 23, Fr. 24, and Fr. 25. Fr. 25 (300 mg) was further purified by gel filtration on Sephadex LH-20 (100×3 cm i.d.) using MeOH as eluent. The eluate was recrystallized from MeOH to give Id (200 mg) as colorless prisms. mp 210.5—212°. $[\alpha]_D^{19}$ +22.8° (c=0.15, $CHCl_3$–MeOH (1: 1)). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{34}H_{48}O_8N_4 \cdot 2H_2O$: C, 60.33; H, 7.74; N, 8.28. Found: C, 60.82; H, 7.29; N, 7.92. NMR (5% solution in CD_3OD) δ: 0.78 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 2.58 (4H, s, $-CO(CH_2)_2CO-$), 3.56 (1H, s, 15α-H), 5.03 (1H, m, 3α-H), 6.20 (1H, d, J=9 Hz, 23-H), 7.40 (1H, d, J=2.5 Hz, 21-H), 7.83 (1H, q, J=9, 2.5 Hz, 22-H).

Synthesis of Resibufogenin 3-Hemisuccinate (Ib)——To a solution of resibufogenin (Ia) (50 mg) in pyridine (2 ml) was added succinic anhydride (100 mg) and refluxed for 16 hr. The resulting solution was diluted with ice-water and then extracted with ether. The organic layer was washed with 5% HCl and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was submitted to preparative TLC using $CHCl_3$–MeOH (20: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (Rf 0.50) with AcOEt–MeOH (10: 1) and recrystallization of the eluate from MeOH–ether gave Ib (15 mg) as colorless prisms. mp 162—166.5°. $[\alpha]_D^{17}$ −11.4° (c=0.09, MeOH). *Anal.* Calcd. for $C_{28}H_{36}O_7$: C, 69.40; H, 7.48. Found: C, 69.11; H, 7.45.

Synthesis of Resibufogenin 3-Hemisuccinate Methyl Ester (Ic)——To a solution of Ib (50 mg) in ether was added an ethereal solution of CH_2N_2 and allowed to stand at room temperature for 1 hr. On usual work-up the residue obtained was submitted to preparative TLC using benzene–AcOEt (3: 1) as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.40) was eluted with AcOEt. Unfortunately the eluate (Ic) (15 mg) could not be crystallized but was substantially homogeneous according to TLC.

Enzymatic Hydrolysis of Id——Id (70 mg) was dissolved in 80% MeOH (10 ml)–1% NaCl (50 ml) and incubated with hog pancreas lipase preparation (Sigma Chemical Co., St. Louis) (70 mg) at 37° for 8 hr. The incubation mixture was concentrated and extracted with CH_2Cl_2–ether (1: 1). The extract was submitted to preparative TLC using $CHCl_3$–MeOH (70: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.10) with AcOEt and recrystallization of the eluate from MeOH–ether gave Ib (15 mg) as colorless prisms. mp 163—165.5°. NMR (4% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.64 (4H, s, $-CO(CH_2)_2CO-$), 3.50 (1H, s, 15α-H), 5.10 (1H, m, 3α-H), 6.20 (1H, d, J=10 Hz, 23-H), 7.18 (1H, d, J=2 Hz, 21-H), 7.73 (1H, q, J=10, 2 Hz, 22-H). Mixed melting point on admixture with the synthetic sample showed no depression, and IR, NMR spectra and chromatographic behaviors of two samples were entirely identical.

A solution of Ib (15 mg) in MeOH (10 ml) was treated with an ethereal solution of CH_2N_2 in the manner as described above. After usual work-up the crude product obtained was purified by preparative TLC to give Ic (10 mg). NMR (0.5% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 2.64 (4H, s, $-CO(CH_2)_2CO-$), 3.53 (1H, s, 15α-H), 3.69 (3H, s, $-COOCH_3$), 5.15 (1H, m, 3α-H), 6.26 (1H, d, J=11 Hz, 23-H), 7.26 (1H, d, J=2 Hz, 21-H), 7.80 (1H, q, J=11, 2 Hz, 22-H). The IR, NMR spectra and chromatographic behaviors were entirely identical with those of the synthetic sample.

Transformation of Id into Pyrimidine Derivative (Ie)——A solution of Id (70 mg) in acetylacetone–$KHCO_3$[6,8] (4 ml) was allowed to stand at 50° for 48 hr. The resulting solution was extracted with $CHCl_3$–MeOH (10:1), and the organic layer was separated and concentrated *in vacuo*. The residue obtained was dissolved in MeOH (1 ml) and treated with an ethereal solution of CH_2N_2 in the usual manner. The crude product was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (4: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^2Rf 0.30) was eluted with AcOEt. Unfortunately the eluate (Ie) (20 mg) could not be crystallized but was substantially homogeneous according to TLC. UV λ_{max}^{EtOH} nm: 237, 301. NMR (4% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 2.26 (6H, s, pyrimidine-CH_3), 2.59 (4H, s, $-CO(CH_2)_2CO-$), 3.50 (1H, s, 15α-H), 3.69 (3H, s, $-COOCH_3$), 5.05 (1H, m, 3α-H), 6.15 (1H, d, J=9 Hz, 23-H), 6.20 (1H, s, pyrimidine-H), 7.25 (1H, d, J=2.5 Hz, 21-H), 7.75 (1H, q, J=9, 2.5 Hz, 22-H).

Hydrolysis of Bufotoxin with 6N HCl——Bufotoxin (1 mg) was heated with 6N HCl (0.5 ml) in a sealed tube at 110° for 10 hr. A portion of the resulting solution was submitted 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 detection of arginine on the TLC plate.

Isolation of Gamabufotalin 3-Suberoylarginine Ester (IIg)——Fr. 2 (18 g) was chromatographed on silica gel (300 g) and eluted successively with AcOEt (1.5 liter), AcOEt–MeOH (4: 1) (1 liter), AcOEt–MeOH (2: 1) (3 liter), AcOEt–MeOH (1: 1) (4.5 liter), AcOEt–MeOH (1: 2) (3 liter), and MeOH (3 liter). Fr. 8 (10 g) was chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as solvent to give Fr. 20, Fr. 21, and Fr. 22. Fr. 22 (5 g) was repeatedly chromatographed on silica gel with $CHCl_3$–MeOH–H_2O (80: 20: 2.5) and the eluate was reprecipitated from MeOH–ether to give IIg (233 mg) as colorless amorphous substance. mp 215—219°. $[\alpha]_D^{16}$ −2.2° (c=0.68, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{38}H_{58}O_9N_4$: C, 63.84; H, 8.18; N, 7.84. Found: C, 63.46; H, 8.62; N, 7.69. NMR (0.5% solution in CD_3OD) δ: 0.76 (3H, s, 18-CH_3), 1.11 (3H, s, 19-CH_3), 3.70 (1H, m, 11β-H), 5.12 (1H, m, 3α-H), 6.31 (1H, d, J=10 Hz, 23-H), 7.49 (1H, d, J=2 Hz, 21-H), 8.01 (1H, q, J=10, 2 Hz, 22-H).

Synthesis of Gamabufotalin 3-Hemisuberate Methyl Ester 11-Acetate (IIf)——To a solution of gamabufotalin (IIa) (100 mg) in pyridine–benzene (1: 1) (4 ml) was added Ac_2O (0.19 ml) and stirred for 24 hr at room temperature. The resulting solution was neutralized with 5% HCl and extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (1: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.30) with AcOEt and recrystallization of the eluate from MeOH gave IIb (30 mg) as colorless needles. mp 139—146° (lit. mp 143—148°).[27] NMR (2% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.96 (3H, s, 11α-$OCOCH_3$), 4.12 (1H, m, 3α-H), 4.85 (1H, m, 11β-H), 6.19 (1H, d, J=10 Hz, 23-H), 7.18 (1H, d, J=2 Hz, 21-H), 7.70 (1H, q, J=10, 2 Hz, 22-H).

To a solution of IIb (25 mg) in pyridine (1 ml) was added suberic anhydride (50 mg) and allowed to stand at 70° for 30 hr. The resulting solution was diluted with ice-water and then extracted with ether. The organic layer was washed with 5% HCl and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was submitted to preparative TLC using AcOEt as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.08) was eluted with AcOEt to give IId (20 mg). The crude product was submitted to further elaboration without purification.

IId (20 mg) was treated with CH_2N_2 in the usual manner. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (3: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.44) with AcOEt and recrystallization of the eluate from hexane–AcOEt gave IIf (7 mg) as colorless needles. mp 128—129.2° (lit. mp 127—130°).[9b]

Transformation of IIg into IIf——IIg (100 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (1: 2) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.50) with AcOEt and recrystallization of the eluate from AcOEt gave IIe (32 mg) as colorless needles. mp 190—193° (lit. mp 189—193°).[9b] $[\alpha]_D^{14}$ −6.3° (c=0.16). NMR (4% solution in $CDCl_3$) δ: 0.74 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 3.62 (3H, s, -$COOCH_3$), 3.70 (1H, m, 11β-H), 5.08 (1H, m, 3α-H), 6.20 (1H, d, J=10 Hz, 23-H), 7.20 (1H, d, J=2.5 Hz, 21-H), 7.75 (1H, q, J=10, 2.5 Hz, 22-H).

IIe (30 mg) was acetylated with Ac_2O (1 ml) and pyridine (2 ml) in the usual manner. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (3: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.50) with AcOEt and recrystallization of the eluate from hexane–AcOEt gave IIf (15 mg) as colorless needles. mp 127—128°. $[\alpha]_D^{14}$ −6.7° (c=0.15). NMR (2.5% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.95 (3H, s, 11α-$OCOCH_3$), 3.63 (3H, s, -$COOCH_3$), 4.90 (1H, m, 11β-H), 5.06 (1H, m, 3α-H), 6.21 (1H, d, J=10 Hz, 23-H), 7.19 (1H, d, J=2 Hz, 21-H), 7.72 (1H, q, J=10, 2 Hz, 22-H). Mixed melting point on admixture with the synthetic sample showed no depression, and IR, NMR spectra and chromatographic behaviors of two samples were entirely identical.

Transformation of IIg into Pyrimidine Derivative (IIh)——A solution of IIg (100 mg) in acetylacetone–$KHCO_3$ (4 ml) was allowed to stand at 50° for 48 hr. The resulting solution was evaporated *in vacuo*, treated with CH_2N_2 in the manner as described in Ie, and then extracted with AcOEt. The crude product obtained was submitted to preparative TLC using AcOEt as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.35) was eluted with AcOEt and the eluate was reprecipitated from ether to give IIh (14 mg) as colorless amorphous substance. mp 126—128.5°. $[\alpha]_D^{14}$ −15.3° (c=0.10). *Anal.* Calcd. for $C_{44}H_{64}O_9N_4$: C, 66.64; H, 8.14; N, 7.07. Found: C, 66.11; H, 8.16; N, 6.65. NMR (4% solution in $CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 2.30 (6H, s, pyrimidine-CH_3), 3.70 (3H, s, -$COOCH_3$), 3.70 (1H, m, 11β-H), 5.10 (1H, m, 3α-H), 6.27 (1H, d, J=10 Hz, 23-H), 6.32 (1H, s, pyrimidine-H), 7.22 (1H, d, J=2 Hz, 21-H), 7.76 (1H, q, J=10, 2 Hz, 22-H).

Isolation of Gamabufotalin 3-Pimeloylarginine Ester (IIk)——Fr. 1 (10 g) was chromatographed on silica gel (150 g) and eluted successively with AcOEt (1.5 liter), AcOEt–MeOH (4: 1) (1 liter), AcOEt–MeOH (2: 1) (3 liter), AcOEt–MeOH (1: 1) (4 liter), AcOEt–MeOH (1: 2) (2 liter), and MeOH (2 liter). Fr. 6 (5 g) was chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as solvent to give Fr. 16, Fr. 17, and Fr. 18. Fr. 16 (900 mg) was chromatographed again on silica gel with $CHCl_3$–MeOH–H_2O (80: 20: 2.5) to give Fr. 29, Fr. 30, and Fr. 31. Fr. 29 (80 mg) was further purified by gel filtration on Sephadex LH-20 using MeOH as eluent. The eluate was reprecipitated from MeOH–acetone to give IIk (30 mg) as colorless amorphous substance. mp 189—192° (decomp.). $[\alpha]_D^{18}$ −3.1° (c=0.16, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{37}H_{56}O_9N_4 \cdot 1/2H_2O$: C, 62.60; H, 8.09; N, 7.89. Found: C, 62.69; H, 7.77; N, 7.76. NMR (5% solution in CD_3OD) δ: 0.75 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 3.70 (1H, m, 11β-H), 5.05 (1H, m, 3α-H), 6.25 (1H, d, J=10 Hz, 23-H), 7.45 (1H, d, J=2 Hz, 21-H), 7.95 (1H, q, J=10, 2 Hz, 22-H).

Synthesis of Gamabufotalin 3-Hemipimelate Methyl Ester 11-Acetate (IIj)——To a solution of gamabufotalin 11-acetate (IIb) (15 mg) and methyl hemipimelate (15 mg) in pyridine (1 ml) was added DCC (10 mg) and allowed to stand at room temperature for 5 days. The resulting solution was extracted with AcOEt, washed with 5% HCl, 5% $NaHCO_3$, and H_2O, dried over anhydrous Na_2SO_4, and evaporated.

27) G.R. Pettit and Y. Kamano, *J. Chem. Soc.* (*C*), 1973, 725.

The crude product obtained was submitted to preparative TLC using benzene–AcOEt (6: 1) as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.70) was eluted with AcOEt and the eluate was further purified by gel filtration on Sephadex LH-20 using MeOH as eluent to give IIj (5 mg) s,11 α-$OCOCH_3$), as colorless oil. NMR (0.5% solution in $CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 1.97 (3H, 3.65 (3H, s, -$COOCH_3$), 4.90 (1H, m, 11β-H), 5.08 (1H, m, 3α-H), 6.22 (1H, d, J=9 Hz, 23-H), 7.18 (1H, d, J=2 Hz, 21-H), 7.70 (1H, q, J=9, 2 Hz, 22-H).

Transformation of IIk into IIj——IIk (20 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^5Rf 0.30) with AcOEt and recrystallization of the eluate from ether gave IIi (5 mg) as colorless leaflets. mp 193—195°. $[\alpha]_D^{18}$ −10.0° (c=0.05). *Anal.* Calcd. for $C_{32}H_{46}O_8$: C, 68.79; H, 8.39. Found: C, 68.77; H, 8.21. Mass Spectrum *m/e*: 558 (M^+), 175, 157. NMR (1% solution 'n $CDCl_3$) δ: 0.75 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 3.64 (3H, s, -$COOCH_3$), 3.84 (1H, m, 11β-H), 5.10 (1H, m, 3α-H), 6.24 (1H, d, J=10 Hz, 23-H), 7.76 (1H, q, J=10, 2 Hz, 22-H).

IIi (3 mg) was acetylated with Ac_2O (0.5 ml) and pyridine (1 ml) in the usual manner. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (6: 1) as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.70) was eluted with AcOEt to give IIj as colorless oil. The IR, NMR spectra and chromatographic behaviors were entirely identical with those of the synthetic sample.

Isolation of Gamabufotalin 3-Adipoylarginine Ester (IIn)——Fr. 17 (500 mg) was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) and the eluate was reprecipitated from MeOH to give IIn (20 mg) as colorless amorphous substance. mp 242—245°. $[\alpha]_D^{21}$ −16.4° (c=0.09, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{36}H_{54}O_9N_4$: C, 62.95; H, 7.93; N, 8.16. Found: C, 62.58; H, 7.83; N, 8.16. NMR (1.3% solution in CD_3OD) δ: 0.72 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 3.64 (1H, m, 11β-H), 5.02 (1H, m, 3α-H), 6.19 (1H, d, J=10 Hz, 23-H), 7.33 (1H, d, J=2 Hz, 21-H), 7.85 (1H, q, J=10, 2 Hz, 22-H).

Synthesis of Gamabufotalin 3-Hemiadipate Methyl Ester 11-Acetate (IIm)——To a solution of IIb (30 mg) and methyl hemiadipate (30 mg) in pyridine (2 ml) was added DCC (15 mg) and allowed to stand at room temperature for 10 days in the manner as described in IIj. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (6: 1) as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.68) was eluted with AcOEt and the eluate was further purified by gel filtration on Sephadex LH-20 using MeOH as eluent to give IIm (10 mg) as colorless prisms. mp 159—161°. $[\alpha]_D^{23}$ −8.0° (c= 0.08). *Anal.* Calcd. for $C_{33}H_{46}O_9$: C, 67.55; H, 7.90. Found: C, 67.37; H, 7.44.

Transformation of IIn into IIm——IIn (15 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.35) with AcOEt and recrystallization of the eluate from ether gave IIl (6 mg) as colorless leaflets. mp 199—200°. $[\alpha]_D^{23}$ −15.2° (c=0.05). *Anal.* Calcd. for $C_{31}H_{44}O_8$: C, 68.36; H, 8.14. Found: C, 68.05; H, 7.97. Mass Spectrum *m/e*: 544 (M^+), 161, 143. NMR (1% solution in $CDCl_3$) δ: 0.72 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), 3.61 (3H, s, -$COOCH_3$), 3.75 (1H, m, $W1/2$=20 Hz, 11β-H), 5.20 (1H, m, $W1/2$=9 Hz, 3α-H), 6.23 (1H, d, J=10 Hz, 23-H), 7.75 (1H, q, J=10, 2 Hz, 22-H).

IIl (3 mg) was acetylated with Ac_2O (0.5 ml) and pyridine (0.5 ml) in the usual manner. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (6: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.70) with AcOEt and recrystallization of the eluate from ether gave IIm (2 mg) as colorless prisms. mp 161—161.5°. NMR (0.5% solution in $CDCl_3$) δ: 0.77 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.91 (3H, s, 11α-$OCOCH_3$), 3.60 (3H, s, -$COOCH_3$), 4.96 (1H, m, $W1/2$= 20 Hz, 11β-H), 5.04 (1H, m, $W1/2$=10 Hz, 3α-H), 6.16 (1H, d, J=9 Hz, 23-H), 7.10 (1H, d, J=2 Hz, 21-H), 7.64 (1H, q, J=9, 2 Hz, 22-H). Mixed melting point on admixture with the synthetic sample showed no depression, and IR, NMR spectra and chromatographic behaviors of two samples were entirely identical.

Isolation of Gamabufotalin 3-Succinoylarginine Ester (IIp)——Fr. 18 (250 mg) was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as solvent an d the eluate was reprecipitated from MeOH–acetone to give IIp (25 mg) as colorless amorphous substance. mp 198—200°. $[\alpha]_D^{24}$ −6.6° (c=0.08, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{34}H_{50}O_9N_4 \cdot 2H_2O$: C, 58.77; H, 7.83; N, 8.06. Found: C, 59.08; H, 7.59; N, 7.34. NMR (1% solution in CD_3OD) δ: 0.75 (3H, s, 18-CH_3), 1.10 (3H, s, 19-CH_3), 2.60 (4H, s, -$CO(CH_2)_2CO$-), 3.68 (1H, m, 11β-H), 5.04 (1H, m, 3α-H), 6.23 (1H, d, J=9 Hz, 23-H), 7.37 (1H, d, J=2 Hz, 21-H), 7.89 (1H, q, J=9, 2 Hz, 22-H).

Transformation of IIp into Gamabufotalin 3-Hemisuccinate Methyl Ester (IIo)——IIp (20 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.10) with AcOEt and recrystallization of the eluate from ether gave IIo (5 mg) as colorless prisms. mp 189—191.5°. $[\alpha]_D^{24}$ +7.1° (c=0.07). *Anal.* Calcd. for $C_{29}H_{40}O_8$: C, 67.42; H, 7.80. Found: C, 67.49; H, 7.51. Mass Spectrum *m/e*: 516 (M^+), 133, 115. NMR (0.5% solution in $CDCl_3$) δ: 0.74 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 2.62 (4H, s, -$CO(CH_2)_2CO$-), 3.67

(3H, s, $-COOCH_3$), 3.80 (1H, m, $W1/2=20$ Hz, 11β-H), 5.20 (1H, m, $W1/2=8$ Hz, 3α-H), 6.20 (1H, d, $J=10$ Hz, 23-H), 7.70 (1H, q, $J=10$, 2 Hz, 22-H).

Isolation of Bufalin 3-Suberoylarginine Ester (IIIc)——Fr. 20 (100 mg) which exhibited a single spot on TLC was separated by HPLC (flow rate: 1 ml/min) using MeOH–H_2O (2: 1) as solvent to give Fr. 36, Fr. 37, Fr. 38, and Fr. 39. Fr. 39 (15 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–AcOEt to give IIIc (5 mg) as colorless amorphous substance. mp 200—205° (decomp.) (lit. mp 204—211°).[7a] $[\alpha]_D^{21}$ −5.5° ($c=0.09$, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{38}H_{58}O_8N_4\cdot 2H_2O$: C, 62.10; H, 8.50; N, 7.62. Found: C, 62.08; H, 8.15; N, 7.35. NMR (1.5% solution in CD_3OD) δ: 0.72 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 6.20 (1H, d, $J=9$ Hz, 23-H), 7.30 (1H, d, $J=2.5$ Hz, 21-H), 7.85 (1H, q, $J=9$, 2.5 Hz, 22-H).

Transformation of IIIc into Bufalin 3-Hemisuberate Methyl Ester (IIIb)——IIIc (1 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. The adsorbent corresponding to the spot (Rf 0.39) was eluted with AcOEt to give IIIb as colorless oil. Mass Spectrum m/e: 556 (M^+), 189, 171. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic samples.[9b]

Isolation of Bufalin 3-Pimeloylarginine Ester (IIIe)——Fr. 38 (20 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–H_2O to give IIIe (5 mg) as colorless amorphous substance. mp 205—206.5° (decomp.). $[\alpha]_D^{21}$ −5.5° ($c=0.18$, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{37}H_{56}O_8N_4\cdot 3/2H_2O$: C, 62.42; H, 8.35; N, 7.87. Found: C, 62.27; H, 7.89; N, 7.71. NMR (1.5% solution in CD_3OD) δ: 0.72 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 4.26 (1H, m, Arg–CH), 5.06 (1H, m, 3α-H), 6.27 (1H, d, $J=10$ Hz, 23-H), 7.41 (1H, d, $J=2.5$ Hz, 21-H), 7.97 (1H, q, $J=10$, 2.5 Hz, 22-H).

Degradation of IIIe——IIIe (1 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^2Rf 0.62) was eluted with AcOEt to give IIId as colorless oil. Mass Spectrum m/e: 542 (M^+), 175, 157.

A solution of IIIe (1.5 mg) in 10% HCl–MeOH (1: 1) (1 ml) was allowed to stand at room temperature for 48 hr and the resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC to give IIIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Bufalin 3-Adipoylarginine Ester (IIIg)——Fr. 37 (30 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–H_2O to give IIIg (9 mg) as colorless amorphous substance. mp 210—213° (decomp.). $[\alpha]_D^{22}$ +5.5° ($c=0.09$, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{36}H_{54}O_8N_4\cdot 2H_2O$: C, 61.17; H, 8.27; N, 7.93. Found: C, 61.22; H, 7.84; N, 8.01. NMR (1.5% solution in CD_3OD) δ: 0.72 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 6.25 (1H, d, $J=10$ Hz, 23-H), 7.38 (1H, d, $J=2$ Hz, 21-H), 7.95 (1H, q, $J=10$, 2 Hz, 22-H).

Synthesis of Bufalin 3-Hemiadipate Methyl Ester (IIIf)——To a solution of bufalin (IIIa) (25 mg) and methyl hemiadipate (25 mg) in pyridine (1 ml) was added DCC (15 mg) and allowed to stand at room temperature for 6 days in the manner as described in IIj. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (Rf 0.22) with AcOEt and recrystallization of the eluate from ether gave IIIf (10 mg) as colorless needles. mp 168—169°. *Anal.* Calcd. for $C_{31}H_{44}O_7$: C, 70.43; H, 8.39. Found: C, 70.42; H, 8.27.

Transformation of IIIg into IIIf——IIIg (5 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.50) with AcOEt and recrystallization of the eluate from ether gave IIIf (3 mg) as colorless needles. mp 166—168°. $[\alpha]_D^{16}$ −3.1° ($c=0.16$). Mass Spectrum m/e: 528 (M^+), 161, 143. NMR (0.5% solution in $CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 3.58 (3H, s, $-COOCH_3$), 5.00 (1H, m, 3α-H), 6.15 (1H, d, $J=10$ Hz, 23-H), 7.18 (1H, d, $J=2$ Hz, 21-H), 7.75 (1H, q, $J=10$, 2 Hz, 22-H). Mixed melting point on admixture with the synthetic sample showed no depression, and IR, NMR spectra and chromatographic behaviors of two samples were entirely identical.

Isolation of Bufalin 3-Succinoylarginine Ester (IIIi)——Fr. 21 (100 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was recrystallized from aq. MeOH to give IIIi (47 mg) as colorless needles. mp 240—241°. $[\alpha]_D^{23}$ −12.5° ($c=0.08$, 80% MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{34}H_{50}O_8N_4\cdot 3/2H_2O$: C, 60.96; H, 7.98; N, 8.37. Found: C, 61.05; H, 7.64; N, 7.98. NMR (0.5% solution in $CDCl_3$–CD_3OD (1: 1)) δ: 0.72 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 2.64 (4H, s, $-CO(CH_2)_2CO-$), 5.15 (1H, m, 3α-H), 6.30 (1H, d, $J=10$ Hz, 23-H), 7.28 (1H, d, $J=3$ Hz, 21-H), 7.92 (1H, q, $J=10$, 3 Hz, 22-H).

Transformation of IIIi into Bufalin 3-Hemisuccinate Methyl Ester (IIIh)——IIIi (30 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted

to preparative TLC using benzene–AcOEt (4: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.50) with AcOEt and recrystallization of the eluate from ether gave IIIh (6.8 mg) as colorless needles. mp 165—166°. $[\alpha]_D^{14.5}$ −14.9° (c=0.13). *Anal.* Calcd. for $C_{29}H_{40}O_7$: C, 69.57; H, 8.05. Found: C, 69.08; H, 7.91. Mass Spectrum *m/e*: 500 (M^+), 133, 115. NMR (0.7% solution in $CDCl_3$) δ: 0.73 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.68 (4H, s, $-CO(CH_2)_2CO-$), 3.74 (3H, s, $-COOCH_3$), 5.19 (1H, m, 3α-H), 6.32 (1H, d, J=10 Hz, 23-H), 7.28 (1H, d, J=3 Hz, 21-H), 7.88 (1H, q, J=10, 3 Hz, 22-H).

Isolation of Cinobufagin 3-Suberoylarginine Ester (IVc)——Fr. 23 (250 mg) which exhibited a single spot on TLC was separated by HPLC (flow rate: 1 ml/min) using MeOH–H_2O (2: 1) as solvent to give Fr. 40, Fr. 41, Fr. 42, Fr. 43, and Fr. 44. Fr. 44 (22 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–ether to give IVc (14 mg) as colorless amorphous substance. mp 170—175° (decomp.). $[\alpha]_D^{16}$ +5.6° (c=0.09, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{40}H_{58}O_{10}N_4 \cdot H_2O$: C, 62.15; H, 7.82; N, 7.25. Found: C, 62.23; H, 7.80; N, 7.11. NMR (2.5% solution in CD_3OD) δ: 0.82 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.87 (3H, s, 16β-$OCOCH_3$), 2.93 (1H, d, J=9 Hz, 17α-H), 3.74 (1H, s, 15α-H), 4.26 (1H, m, Arg–CH), 5.06 (1H, m, 3α-H), 5.49 (1H, d, J=9 Hz, 16α-H), 6.23 (1H, d, J=10 Hz, 23-H), 7.35 (1H, d, J=2 Hz, 21-H), 8.01 (1H, q, J=10, 2 Hz, 22-H).

Transformation of IVc into Cinobufagin 3-Hemisuberate Methyl Ester (IVb)——IVc (5 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (20: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.28) with AcOEt and recrystallization of the eluate from ether gave IVb (2 mg) as colorless leaflets. mp 105—107°. Mass Spectrum *m/e*: 612 (M^+), 189, 171. NMR (0.5% solution in $CDCl_3$) δ: 0.83 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.91 (3H, s, 16β-$OCOCH_3$), 2.81 (1H, d, J=9 Hz, 17α-H), 3.66 (4H, s, 15α-H and $-COOCH_3$), 5.06 (1H, m, 3α-H), 5.42 (1H, d, J=9 Hz, 16α-H), 6.14 (1H, d, J=10 Hz, 23-H), 7.06 (1H, d, J=2.5 Hz, 21-H), 7.77 (1H, q, J=10, 2.5 Hz, 22-H). Mixed melting point on admixture with the authentic sample[5] showed no depression, and mass spectra and chromatographic behaviors of two samples were entirely identical.

Isolation of Cinobufagin 3-Pimeloylarginine Ester (IVe)——Fr. 43 (30 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–ether to give IVe (10 mg) as colorless amorphous substance. mp 175—178° (decomp.). $[\alpha]_D^{16}$ +6.3° (c=0.08, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{39}H_{56}O_{10}N_4 \cdot H_2O$ C, 61.72; H, 7.70; N, 7.38. Found: C, 61.58; H, 7.52; N, 7.67. NMR (2.5% solution in CD_3OD) δ: 0.84 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.92 (3H, s, 16β-$OCOCH_3$), 2.91 (1H, d, J=9 Hz, 17α-H), 3.72 (1H, s, 15α-H), 5.10 (1H, m, 3α-H), 5.51 (1H, d, J=9 Hz, 16α-H), 6.25 (1H, d, J=10 Hz, 23-H), 7.30 (1H, d, J=2 Hz, 21-H), 8.02 (1H, q, J=10, 2 Hz, 22-H).

Transformation of IVe into Cinobufagin 3-Hemipimelate Methyl Ester (IVd)——IVe (5 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (20: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.13) with AcOEt and recrystallization of the eluate from ether gave IVd (2 mg) as colorless needles. mp 111—112.5°. $[\alpha]_D^{18}$ +8.9° (c=0.11). *Anal.* Calcd. for $C_{34}H_{46}O_9$: C, 68.20; H, 7.74. Found: C, 67.97; H, 8.05. Mass Spectrum *m/e*: 598 (M^+), 175, 157. NMR (0.5% solution in $CDCl_3$) δ: 0.84 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.92 (3H, s, 16β-$OCOCH_3$), 2.84 (1H, d, J=9 Hz, 17α-H), 3.70 (4H, s, 15α-H and $-COOCH_3$), 5.14 (1H, m, 3α-H), 5.51 (1H, d, J=9 Hz, 16α-H), 6.25 (1H, d, J=10 Hz, 23-H), 7.20 (1H, d, J=2.5 Hz, 21-H), 7.94 (1H, q, J=10, 2.5 Hz, 22-H).

Isolation of Cinobufagin 3-Adipoylarginine Ester (IVg)——Fr. 42 (28 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from ether to give IVg (13 mg) as colorless amorphous substance. mp 183—186° (decomp.). $[\alpha]_D^{17}$ +4.7° (c=0.11, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{38}H_{54}O_{10}N_4 \cdot 3/2H_2O$: C, 60.54; H, 7.62; N, 7.43. Found: C, 60.67; H, 7.25; N, 7.17. NMR (2.5% solution in CD_3OD) δ: 0.83 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.88 (3H, s, 16β-$OCOCH_3$), 2.93 (1H, d, J=9 Hz, 17α-H), 3.72 (1H, s, 15α-H), 4.25 (1H, m, Arg–CH), 5.05 (1H, m, 3α–H), 5.46 (1H, d, J=9 Hz, 16α-H), 6.19 (1H, d, J=10 Hz, 23-H), 7.29 (1H, d, J=2 Hz, 21-H), 7.97 (1H, q, J=10, 2 Hz, 22-H).

Synthesis of Cinobufagin 3-Hemiadipate Methyl Ester (IVf)——To a solution of cinobufagin (IVa) (35 mg) and methyl hemiadipate (50 mg) in pyridine (1 ml) was added DCC (40 mg) and allowed to stand at room temperature for 7 days in the manner as described in IIj. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (2: 1) as developing solvent. The adsorbent corresponding to the spot (Rf 0.68) was eluted with AcOEt and the eluate was further purified by gel filtration on Sephadex LH-20. Recrystallization of the eluate from ether gave IVf (10 mg) as colorless prisms. mp 187—190°. $[\alpha]_D^{14}$ +13.6° (c=0.11). *Anal.* Calcd. for $C_{33}H_{44}O_9$: C, 67.79; H, 7.59. Found: C, 67.59; H, 7.47.

Transformation of IVg into IVf——IVg (10 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^2Rf 0.46) with AcOEt and recrystallization of the eluate from ether gave IVf (5 mg) as colorless prisms. mp 186—187°. Mass Spectrum *m/e*: 584 (M^+), 161, 143. NMR (1.3% solution in $CDCl_3$) δ: 0.80 (3H, s,

18-CH_3), 0.98 (3H, s, 19-CH_3), 1.84 (3H, s, 16β-$OCOCH_3$), 2.75 (1H, d, J=9 Hz, 17α-H), 3.60 (4H, s, 15α-H and -$COOCH_3$), 5.04 (1H, m, 3α-H), 5.40 (1H, d, J=9 Hz, 16α-H), 6.12 (1H, d, J=9 Hz, 23-H), 7.15 (1H, d, J=2 Hz, 21-H), 7.82 (1H, q, J=9, 2 Hz, 22-H). Mixed melting point on admixture with the synthetic sample showed no depression, and IR, NMR spectra and chromatographic behaviors of two samples were entirely identical.

Isolation of Cinobufagin 3-Succinoylarginine Ester (IVl)——Fr. 41 (40 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was recrystallized from MeOH to give IVI (32.2 mg) as colorless prisms. mp 200—202.5°. $[\alpha]_D^{15}$ +27.8° (c=0.11). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{36}H_{50}O_{10}N_4 \cdot 3/2H_2O$: C, 59.57; H, 7.36; N, 7.83. Found: C, 59.78; H, 7.18; N, 7.57. NMR (2.5% solution in CD_3OD) δ: 0.86 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), 1.92 (3H, s, 16β-$OCOCH_3$), 2.63 (4H, s, -$CO(CH_2)_2CO$-), 2.95 (1H, d, J=9 Hz, 17α-H), 3.76 (1H, s, 15α-H), 5.07 (1H, m, 3α-H), 5.49 (1H, d, J=9 Hz, 16α-H), 6.23 (1H, d, J=10 Hz, 23-H), 7.32 (1H, d, J=2 Hz, 21-H), 7.98 (1H, q, J=10, 2 Hz, 22-H).

Synthesis of Cinobufagin 3-Hemisuccinate Methyl Ester (IVk)——To a solution of cinobufagin (IVa) (30 mg) in pyridine (1 ml) was added succinic anhydride (50 mg) and allowed to stand at 70° for 72 hr in the manner as described in Ib. The crude product obtained was treated with CH_2N_2 in the usual manner and then submitted to preparative TLC using benzene–AcOEt (3: 1) as developing solvent. The adsorbent corresponding to the spot (Rf 0.25) was eluted with AcOEt to give IVk (12 mg) as colorless oil.

Transformation of IVl into Cinobufagin 3-Hemisuccinate Methyl Ester (IVk)——IVl (20 mg) was treated with enzyme in the manner as described in Ib. The crude product obtained was submitted to preparative TLC using $CHCl_3$–MeOH (15: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (Rf 0.42) with AcOEt–$CHCl_3$ (1: 1) and recrystallization of the eluate from ether gave cinobufagin 3-hemisuccinate (IVi) (9 mg) as colorless leaflets. mp 248—255°. NMR (1.5% solution in $CDCl_3$) δ: 0.86 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 1.94 (3H, s, 16β-$OCOCH_3$), 2.71 (4H, s, -$CO(CH_2)_2CO$-), 2.85 (1H, d, J=9 Hz, 17α-H), 3.69 (1H, s, 15α-H), 5.16 (1H, m, 3α-H), 5.50 (1H, d, J=9 Hz, 16α-H), 6.25 (1H, d, J=9 Hz, 23-H), 7.18 (1H, d, J=2 Hz, 21-H), 7.92 (1H, q, J=9, 2 Hz, 22-H). Mixed melting point on admixture with the authentic sample showed no depression and chromatographic behaviors of two samples were entirely identical.

IVi (8 mg) was treated with CH_2N_2 in the manner as described in Ic. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^2Rf 0.50) was eluted with AcOEt–$CHCl_3$ (1: 1) to give IVk (5.5 mg) as colorless oil. $[\alpha]_D^{15}$ +27.8° (c=0.11). Mass Spectrum m/e: 556 (M^+), 133, 115. NMR (0.8% solution in $CDCl_3$) δ: 0.90 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.87 (3H, s, 16β-$OCOCH_3$), 2.61 (4H, s, -$CO(CH_2)_2CO$-), 2.77 (1H, d, J=9 Hz, 17α-H), 3.66 (4H, s, 15α-H and -$COOCH_3$), 5.08 (1H, m, 3α-H), 5.38 (1H, d, J=9 Hz, 16α-H), 6.15 (1H, d, J=10 Hz, 23-H), 7.11 (1H, d, J=2.5 Hz, 21-H), 7.84 (1H, q, J=10, 2.5 Hz, 22-H). The mass spectra and chromatographic behaviors were entirely identical with those of the synthetic sample.

Isolation of Arenobufagin 3-Suberoylarginine Ester (Vd)——Fr. 36 (35 mg) was further purified by HPLC and gel filtration on Sephadex LH-20 and the eluate was reprecipitated from ether to give Vd (25 mg) as colorless amorphous substance. mp 182—184° (decomp.). $[\alpha]_D^{23}$ −33.3° (c=0.09, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal. Calcd.* for $C_{38}H_{56}O_{10}N_4 \cdot 2H_2O$: C, 59.67; H, 7.91; N, 7.33. Found: C, 59.69; H, 7.65; N, 7.06. NMR (1.7% solution in CD_3OD) δ: 0.93 (3H, s, 18-CH_3), 1.21 (3H, s, 19-CH_3), 4.26 (3H, m, 11β-H, 17α-H, and Arg–CH), 5.06 (1H, m, 3α-H), 6.29 (1H, d, J=10 Hz, 23-H), 7.50 (1H, d, J=2 Hz, 21-H), 7.88 (1H, q, J=10, 2 Hz, 22-H).

Transformation of Vd into Arenobufagin 3-Hemisuberate Methyl Ester (Vc)——Vd (22.9 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^2Rf 0.47) was eluted with CH_2Cl_2 to give Vc (10 mg) as colorless prisms. mp 119—119.5° (lit. mp 117—120°).[9b] Mass Spectrum m/e: 586 (M^+), 189, 171. NMR (1.3% solution in $CDCl_3$) δ: 0.91 (3H, s, 18-CH_3), 1.18 (3H, s, 19-CH_3), 3.63 (3H, s, -$COOCH_3$), 4.04 (1H, m, 17α-H), 4.28 (1H, d, J=11 Hz, 11β-H), 5.03 (1H, m, 3α-H), 6.23 (1H, d, J=10 Hz, 23-H), 7.37 (1H, d, J=2 Hz, 21-H), 7.70 (1H, q, J=10, 2 Hz, 22-H).

Isolation of Cinobufotalin 3-Suberoylarginine Ester (VIb)——Fr. 40 (50 mg) was further purified by HPLC, followed by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–ether to give VIb (20 mg) as colorless amorphous substance. mp 176—178° (decomp.). $[\alpha]_D^{15}$ −5.4° (c=0.09, MeOH). Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{40}H_{58}O_{11}N_4 \cdot 3/2H_2O$: C, 60.20; H, 7.71; N, 7.02. Found: C, 60.01; H, 7.48; N, 6.65. NMR (2.5% solution in CD_3OD) δ: 0.84 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.88 (3H, s, 16β-$OCOCH_3$), 2.95 (1H, d, J=9 Hz, 17α-H), 3.76 (1H, s, 15α-H), 4.28 (1H, m, Arg–CH), 5.14 (1H, m, 3α-H), 5.49 (1H, d, J=9 Hz, 16α-H), 6.25 (1H, d, J=10 Hz, 23-H), 7.37 (1H, d, J=2 Hz, 21-H), 8.03 (1H, q, J=10, 2 Hz, 22-H).

Transformation of VIb into Cinobufotalin 3-Hemisuberate Methyl Ester (VIa)——VIb (6 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (20: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^5Rf 0.19) was eluted with AcOEt to give VIa as colorless needles. mp 132.5—134.5° (lit. mp 132—136°).[9b] $[\alpha]_D^{17}$ +17.8° (c=0.03). Mass Spectrum m/e: 628 (M^+), 189, 171.

NMR (1.3% solution in $CDCl_3$) δ: 0.82 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.86 (3H, s, 16β-$OCOCH_3$), 2.76 (1H, d, J=9 Hz, 17α-H), 3.60 (4H, s, 15α-H and -$COOCH_3$), 5.20 (1H, m, 3α-H), 5.39 (1H, d, J=9 Hz, 16α-H), 6.13 (1H, d, J=10 Hz, 23-H), 7.07 (1H, d, J=2 Hz, 21-H), 7.81 (1H, q, J=10, 2 Hz, 22-H).

Isolation of Bufotalin 3-Suberoylarginine Ester (VIIc)——Fr. 24 (160 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was reprecipitated from MeOH to give VIIc (40 mg) as colorless amorphous substance. mp 205.5—206.5° (lit. mp 198—202°).[4b] $[\alpha]_D^{20}$ +2.4° (c=0.21, MeOH) (lit. $[\alpha]_D^{26}$ +2.0° (c=0.9939, MeOH).[4b] Sakaguchi test: positive, ninhydrin test: negative. *Anal.* Calcd. for $C_{40}H_{60}O_{10}N_4 \cdot H_2O$: C, 61.99; H, 8.06; N, 7.23. Found: C, 62.28; H, 7.37; N, 7.15. NMR (2.5% solution in CD_3OD) δ: 0.76 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.81 (3H, s, 16β-$OCOCH_3$), 4.22 (1H, m, Arg-CH), 5.00 (1H, m, 3α-H), 5.43 (1H, m, 16α-H), 6.10 (1H, d, J=9 Hz, 23-H), 7.30 (1H, d, J=2 Hz, 21-H), 8.13 (1H, q, J=9, 2 Hz, 22-H).

Transformation of VIIc into Bufotalin 3-Hemisuberate Methyl Ester (VIIb)——VIIc (10 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using benzene–AcOEt (10: 1) as developing solvent. Elution of the adsorbent corresponding to the spot (^5Rf 0.33) with AcOEt and recrystallization of the eluate from ether–hexane gave VIIb (5 mg) as colorless needles. mp 171—173° (lit. mp 170—173°).[9b] Mass Spectrum m/e: 614 (M^+), 189, 171. NMR (2.6% solution in $CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.88 (3H, s, 16β-$OCOCH_3$), 3.78 (3H, s, -$COOCH_3$), 5.14 (1H, m, 3α-H), 5.56 (1H, m, 16α-H), 6.19 (1H, d, J=10 Hz, 23-H), 8.03 (1H, q, J=10, 2.5 Hz, 22-H).

Isolation of Sarmentogenin 3-Suberoylarginine Ester (VIIId)——Purification of Fr. 30 (30 mg) by gel filtration on Sephadex LH-20 afforded Fr. 32, which in turn was separated by HPLC (flow rate: 1 ml/min) using MeOH–H_2O (2: 1) as solvent to give Fr. 33. Further purification by gel filtration on Sephadex LH-20 and reprecipitation of the eluate from MeOH–ether gave VIIId (15 mg) as colorless amorphous substance. mp 171—173°. $[\alpha]_D^{16}$ +14.2° (c=0.11, MeOH). Sakaguchi test: positive, ninhydrin test: negative, Kedde test: positive. *Anal.* Calcd. for $C_{37}H_{58}O_9N_4 \cdot H_2O$: C, 61.64; H, 8.39; N, 7.77. Found: C, 61.25; H, 8.11; N, 7.34. UV λ_{max}^{MeOH} nm: 217. NMR (2.5% solution in CD_3OD) δ: 0.89 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 2.09 (1H, m, 17α-H), 3.70 (1H, m, 11β-H), 4.26 (1H, m, Arg-CH), 5.06 (1H, m, 3α-H), 5.90 (1H, m, 22-H).

Degradation of VIIId——VIIId (10 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (^3Rf 0.35) with AcOEt and recrystallization of the eluate from ether gave VIIIc (5 mg) as colorless leaflets. mp 115—116°. $[\alpha]_D^{16}$ +25.0° (c=0.08). *Anal.* Calcd. for $C_{32}H_{48}O_8$: C, 68.54; H, 8.63. Found: C, 68.25; H, 8.86. UV λ_{max}^{MeOH} nm: 216. Mass Spectrum m/e: 560 (M^+), 189, 171. NMR (2.5% solution in $CDCl_3$) δ: 0.88 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.84 (1H, m, 17α-H), 3.62 (3H, s, -$COOCH_3$), 3.75 (1H, m, $W1/2$= 20 Hz, 11β-H), 4.82, 4.90 (each 1H, d, J=18 Hz, 21-CH_2), 5.04 (1H, m, $W1/2$=10 Hz, 3α-H), 5.85 (1H, m, 22-H).

To a solution of VIIIc (2 mg) in MeOH (0.5 ml) was added 10% HCl (0.5 ml) and allowed to stand at room temperature for 72 hr. The resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC to give VIIIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Sarmentogenin 3-Pimeloylarginine Ester (VIIIf)——Fr. 31 (15 mg) was repeatedly purified by gel filtration on Sephadex LH-20. The eluate was reprecipitated from MeOH–ether to give VIIIf (8 mg) as colorless amorphous substance. mp 184—187°. $[\alpha]_D^{17}$ +9.6° (c=0.15, MeOH). Sakaguchi test: positive, ninhydrin test: negative, Kedde test: positive. *Anal. Calcd.* for $C_{36}H_{56}O_9N_4 \cdot 2H_2O$: C, 59.65; H, 8.34; N, 7.73. Found: C, 59.98; H, 8.13; N, 7.27. UV λ_{max}^{MeOH} nm: 217. NMR (1.5% solution in CD_3OD) δ: 0.90 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 2.88 (1H, m, 17α-H), 3.72 (1H, m, 11β-H), 4.26 (1H, m, Arg-CH), 5.92 (1H, m, 22-H).

Degradation of VIIIf——VIIIf (3 mg) was treated with enzyme and then with CH_2N_2 in the manner as described in Id. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^3Rf 0.30) was eluted with AcOEt to give VIIIe as colorless oil. Mass Spectrum m/e: 546 (M^+), 175, 157. NMR (0.4% solution in $CDCl_3$) δ: 0.91 (3H, s, 18-CH_3), 1.09 (3H, s, 19-CH_3), 2.88 (1H, m, 17α-H), 3.67 (3H, s, -$COOCH_3$), 3.76 (1H, m, $W1/2$=20 Hz, 11β-H), 4.86 (2H, m, 21-CH_2), 5.10 (1H, m, $W1/2$=10 Hz, 3α-H), 5.86 (1H, m, 22-H).

To a solution of VIIIe in MeOH (0.5 ml) was added 10% HCl (0.5 ml) and allowed to stand at room temperature for 72 hr. The resulting solution was extracted with ether. The organic layer was washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC to give VIIIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Gamabufotalin 3-Hemisuberate (IIc)——Purification of Fr. 4 (200 mg) by column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (80: 20: 2.5) afforded Fr. 10 (40 mg), which in turn was separated by preparative TLC using $CHCl_3$–MeOH–AcOH (95: 5: 0.2) as developing solvent to give Fr. 26 and Fr. 27. Fr. 26 was reprecipitated from ether to give IIc (5 mg) as colorless amorphous substance. mp 188—190°

lit. mp 187—191°).[9b] NMR (0.5% solution in $CDCl_3$) δ: 0.74 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 3.76 (1H, m, 11β-H), 5.08 (1H, m, 3α-H), 6.25 (1H, d, J=9 Hz, 23-H), 7.75 (1H, q, J=9, 2.5 Hz, 22-H).

Methylation of IIc——IIc (2 mg) was treated with CH_2N_2 in the usual manner and the crude product was purified by preparative TLC to give IIe (1 mg) as colorless oil. Mass Spectrum *m/e*: 572 (M^+), 189, 171. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Arenobufagin 3-Hemisuberate (Vb)——Purification of Fr. 7 (30 mg) by column chromatography on silica gel with $CHCl_3$–MeOH–H_2O (80: 20: 2.5) afforded Fr. 19 (20 mg), which in turn was separated by HPLC (flow rate: 2 ml/min) using MeOH–0.02M $NH_4H_2PO_4$ (2: 1) as solvent to afford Fr. 34 and Fr. 35. Fr. 34 (5 mg) was further purified by gel filtration on Sephadex LH-20 to give Vb (2 mg) as colorless oil. NMR (1% solution in $CDCl_3$) δ: 0.92 (3H, s, 18-CH_3), 1.20 (3H, s, 19-CH_3), 4.08 (1H, m, 17α-H), 4.29 (1H, d J=10 Hz, 11β-H), 5.04 (1H, m, 3α-H), 6.22 (1H, d, J=10 Hz, 23-H), 7.30 (1H, d, J=2 Hz, 21-H), 7.59 (1H, q, J=10, 2 Hz, 22-H).

Methylation of Vb——Vb (1 mg) was treated with CH_2N_2 in the usual manner and the crude product was purified by preparative TLC to give Vc (0.5 mg) as colorless prisms. mp 115—116°. Mixed melting point on admixture with the authentic sample showed no depression and mass spectra of two samples were entirely identical.

Isolation of Desacetylcinobufagin 3-Hemisuccinate (IVh)——Fr. 35 (13 mg) was further purified by gel filtration on Sephadex LH-20 to give IVh (6 mg) as colorless oil. NMR (0.5% solution in $CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.66 (4H, s, –$CO(CH_2)_2CO$–), 3.59 (1H, s, 15α-H), 4.76 (1H, d, J= 9 Hz, 16α-H), 5.15 (1H, m, 3α-H), 6.23 (1H, d, J=9 Hz, 23-H), 7.97 (1H, q, J=9, 2 Hz, 22-H).

Transformation of IVh into IVk——IVh (6 mg) was treated with CH_2N_2 in the usual manner and the crude product was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (10: 3: 3) as developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.33) with AcOEt and recrystallization of the eluate from ether gave IVj (3 mg) as colorless needles. mp 208—210°. $[\alpha]_D^{17}$ +21.1° (c=0.19). *Anal.* Calcd. for $C_{29}H_{38}O_8$: C, 67.68; H, 7.44. Found: C, 67.45; H, 7.63. Mass Spectrum *m/e*: 514 (M^+), 133, 115. NMR (2.5% solution in $CDCl_3$) δ: 0.79 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.63 (4H, s, –$CO(CH_2)_2CO$–), 3.57 (1H, s, 15α-H), 3.67 (3H, s, –$COOCH_3$), 4.70 (1H, d, J=9 Hz, 16α-H), 5.10 (1H, m, 3α-H), 6.18 (1H, d, J=10 Hz, 23-H), 7.17 (1H, d, J=2.5 Hz, 21-H), 7.95 (1H, q, J=10, 2.5 Hz, 22-H).

IVj (1 mg) was acetylated with Ac_2O (1 ml) and pyridine (2 ml) in the usual manner. The crude product obtained was submitted to preparative TLC using cyclohexane–acetone–$CHCl_3$ (20: 3: 3) as developing solvent. The adsorbent corresponding to the spot (^2Rf 0.12) was eluted with AcOEt to give IVk (0.5 mg) as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Sarmentogenin 3-Hemisuberate (VIIIb)——Fr. 27 was evaporated *in vacuo* to give VIIIb (20 mg) as colorless oil. NMR (0.5% solution in $CDCl_3$) δ: 0.90 (3H, s, 18-CH_3), 1.08 (3H, s, 19-CH_3), 3.80 (1H, m, 11β-H), 4.82 (1H, m, 21-CH_2), 5.08 (1H, m, 3α-H), 5.84 (1H, m, 22-H).

Methylation of VIIIb——VIIIb (10 mg) was treated with CH_2N_2 in the usual manner and the crude product was purified by preparative TLC to give VIIIc (5 mg) as colorless leaflets. mp 115—116°. Mixed melting point on admixture with the authentic sample showed no depression and the chromatographic behaviors of two samples were entirely identical.

Isolation of Bufalin 3-Sulfate (IIIj)——Fr. 5 (1 g) was repeatedly chromatographed on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) to give Fr. 11, Fr. 12, Fr. 13, Fr. 14, and Fr. 15. Fr. 11 (30 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was reprecipitated from MeOH–acetone to give IIIj (5 mg) as colorless amorphous substance. mp 165.5—166.5°. $[\alpha]_D^{23}$ −33.1° (c=0.05, MeOH). Ba^{2+}-rhodizonate test: positive. IR ν_{max}^{KBr} cm^{-1}: 1220, 1055 (SO_2). NMR (1.3% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.72 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.45 (1H, m, 17α-H), 4.71 (1H, m, 3α-H), 6.23 (1H, d, J=10 Hz, 23-H), 7.23 (1H, d, J=2 Hz, 21-H), 7.87 (1H, q, J=10, 2 Hz, 22-H). The IR, NMR spectra and chromatographic behaviors were entirely identical with those of the synthetic sample. An aq. solution of IIIj (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, washed with 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated. The crude product obtained was purified by preparative TLC using benzene–AcOEt (1: 1) as developing solvent to give IIIa as colorless oil. The chromatographic behaviors and mass spectra were entirely identical with those of the authentic sample.

Synthesis of IIIj——To a solution of IIIa (20 mg) and DCC (80 mg) in N,N-dimethylformamide (DMF) (2 ml) was added conc. H_2SO_4 (11.4 mg)–DMF (1 ml) under ice-cooling and allowed to stand for a few min. The resulting solution was diluted with ether and extracted with 5% NaOH. The aq. layer was diluted with H_2O, percolated through a column packed with Amberlite XAD-2 resin, washed with H_2O, and then eluted with MeOH. The eluate was purified by chromatography on silica gel using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as solvent, followed by gel filtration on Sephadex LH-20. Reprecipitation of the eluate from MeOH–acetone gave IIIj (7 mg) as colorless amorphous substance. mp 165—166.5°. $[\alpha]_D^{23}$ −35.7° (c= 0.06, MeOH). *Anal.* Calcd. for $C_{24}H_{33}O_7SNa \cdot 3/2H_2O$: C, 55.91; H, 7.04. Found: C, 55.75; H, 6.95.

Isolation of Bufotalin 3-Sulfate (VIId)——Fr. 12 (25 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was reprecipitated from MeOH–ether to give VIId (3 mg) as colorless amorphous

substance. mp 156—158°. $[\alpha]_D^{21}$ −32.2° (c=0.05, MeOH). Ba^{2+}-rhodizonate test: positive. IR ν_{max}^{KBr} cm^{-1}: 1060 (SO_2). NMR (0.5% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.77 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.90 (3H, s, 16β-$OCOCH_3$), 4.67 (1H, m, 3α-H), 5.45 (1H, m, 16α-H), 6.16 (1H, d, J=10 Hz, 23-H), 7.26 (1H, d, J=2 Hz, 21-H), 8.13 (1H, q, J=10, 2 Hz, 22-H). Solvolysis of VIId (1 mg) in the manner as described in IIIj gave VIIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Arenobufagin 3-Sulfate (Ve)——Fr. 13 (25 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was recrystallized from MeOH–acetone to give Ve (3 mg) as colorless needles. mp 196—201° (decomp.). $[\alpha]_D^{21}$ +72.9° (c=0.05, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1060 (SO_2). NMR (0.5% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.90 (3H, s, 18-CH_3), 1.15 (3H, s, 19-CH_3), 4.32 (1H, d, J=10 Hz, 11β-H), 4.72 (1H, m, $W1/2$=10 Hz, 3α-H), 6.30 (1H, d, J=10 Hz, 23-H), 7.42 (1H, d, J=2 Hz, 21-H), 7.84 (1H, q, J=10, 2 Hz, 22-H).

Solvolysis of Ve (1 mg) in the manner as described in IIIj gave Va as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Isolation of Gamabufotalin 3-Sulfate (IIq)——Fr. 14 (30 mg) was further purified by gel filtration on Sephadex LH-20 and the eluate was reprecipitated from MeOH–acetone to give IIq (10 mg) as colorless amorphous substance. mp 146—147°. $[\alpha]_D^{26}$ −8.3° (c=0.09, MeOH). IR ν_{max}^{KBr} cm^{-1}: 1060 (SO_2). NMR (1.3% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.75 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 4.78 (1H, m, 3α-H), 6.28 (1H, d, J=9 Hz, 23-H), 7.30 (1H, d, J=2 Hz, 21-H), 7.88 (1H, q, J=9, 2 Hz, 22-H).

Solvolysis of IIq (1 mg) in the manner as described in IIIj gave IIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

Acetylation of IIq——IIq (3 mg) was acetylated with Ac_2O (1 ml) and pyridine (1 ml) in the usual manner and the crude product obtained was submitted to preparative TLC using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as developing solvent. Elution of the adsorbent corresponding to the spot (Rf 0.40) was eluted with $CHCl_3$–MeOH (4: 1) and reprecipitation of the eluate from MeOH–ether gave IIr (2 mg) as colorless amorphous substance. mp 176—179°. $[\alpha]_D^{18}$ −12.5° (c=0.06, MeOH). NMR (0.5% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.78 (3H, s, 18-CH_3), 1.02 (3H, s, 19-CH_3), 1.97 (3H, s, 11α-$OCOCH_3$), 4.78 (1H, m, $W1/2$=10 Hz, 3α-H), 4.94 (1H, m, $W1/2$=20 Hz, 11β-H), 6.30 (1H, d, J=10 Hz, 23-H), 7.29 (1H, d, J=2.5 Hz, 21-H), 7.89 (1H, q, J=10, 2.5 Hz, 22-H).

Isolation of Sarmentogenin 3-Sulfate (VIIIg)——Fr. 15 (18 mg) was purified by gel filtration on Sephadex LH-20 with MeOH to give Fr. 28 (12 mg), which in turn was submitted to gel filtration on Sephadex LH-20 using H_2O as eluent. Reprecipitation of the eluate from MeOH–ether gave VIIIg (2 mg) as colorless amorphous substance. mp 149—151°. $[\alpha]_D^{20}$ +56.1° (c=0.08, MeOH). Kedde test: positive, Ba^{2+}-rhodizonate test: positive. UV λ_{max}^{MeOH} nm: 218. IR ν_{max}^{KBr} cm^{-1}: 1050 (SO_2). NMR (0.5% solution in CD_3OD–$CDCl_3$ (1: 4)) δ: 0.90 (3H, s, 18-CH_3), 1.07 (3H, s, 19-CH_3), 2.92 (1H, m, 17α-H), 3.70 (1H, m, $W1/2$=19 Hz, 11β-H), 4.75 (1H, m, $W1/2$=11 Hz, 3α-H), 4.97 (2H, m, 21-CH_2), 5.90 (1H, m, 22-H).

Solvolysis of VIIIg (1 mg) in the manner as described in IIIj gave VIIIa as colorless oil. The mass spectra and chromatographic behaviors were entirely identical with those of the authentic sample.

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