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Isolation and Characterization of Cinobufagin 3-Glutaroyl-L-arginine Ester from *Bufo bufo gargarizans* CANTOR[1)]

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A new bufotoxin having glutaric acid as a dicarboxylic acid moiety was found in the Korean toad, *Bufo bufo gargarizans* CANTOR. This new bufotoxin was separated from the dried toad by usual chromatographic methods including reversed phase high-performance liquid chromatography, and its structure was elucidated to be cinobufagin 3-glutaroyl-L-arginine ester by degradative and synthetic means. In addition, seven known bufogenins and eight known bufotoxins were isolated and characterized.

Keywords——*Bufo bufo gargarizans*; toad venom; cinobufagin 3-glutaroyl-L-arginine ester; new bufotoxin; active ester method

The structure of the so-called "bufotoxin" has been considered to be bufogenin 3-suberoylarginine ester. Recent studies in this laboratory disclosed the existence of new bufotoxins having glutamine,[3)] histidine, 1-methylhistidine, and 3-methylhistidine[4)] instead of arginine as an amino acid component in the skin of the North American toad, *Bufo americanus*,[3)] and the Formosan toad, *Bufo melanostictus* SCHNEIDER.[4)] In addition, we isolated new bufotoxins, in which the succinoyl, adipoyl, and pimeloyl groups replace the suberoyl residue of "bufotoxin," from the skin of the Japanese toad, *Bufo vulgaris formosus* BOULENGER.[5)] No evidence, however, has been obtained for the presence of the glutaroyl homolog despite much effort. The present paper deals with the isolation and characterization of a new bufotoxin having glutaric acid as a dicarboxylic acid component from the dried body of the Korean toad, *Bufo bufo gargarizans* CANTOR.

Separation of bufogenins and bufotoxins from the Korean toad was carried out as shown in Chart 1. One hundred dried toads were pulverized and extracted with ethanol at room temperature for three months. The ethanolic extract was partitioned with the hexane–water system and then with the ethyl acetate–water system.[3-5)] The ethyl acetate layer was concentrated *in vacuo* and the residue obtained was chromatographed on silica gel. Purification of the dried eluate by high-performance liquid chromatography (HPLC) on a reversed phase column[6)] provided resibufogenin, cinobufagin, bufalin, bufotalin, cinobufotalin, marinobufagin, and gamabufotalin. These bufogenins were unequivocally characterized by direct comparison with authentic samples.[5)]

The aqueous layer was percolated through a column of Amberlite XAD-2 resin. After thorough washing with distilled water and 50% methanol, the conjugated steroid fraction was eluted with 90% methanol.[5)] The dried eluate was subjected to column chromatography on silica gel with ethyl acetate containing stepwise-increasing concentrations of methanol as the eluent system. Subsequent column chromatography on silica gel using chloroform–methanol–water (80 : 20 : 2.5) as a mobile phase followed by reversed phase HPLC[7)] provided satisfactory separation of bufotoxins. Cinobufagin 3-succinoyl-, 3-adipoyl-, 3-pimeloyl- and 3-

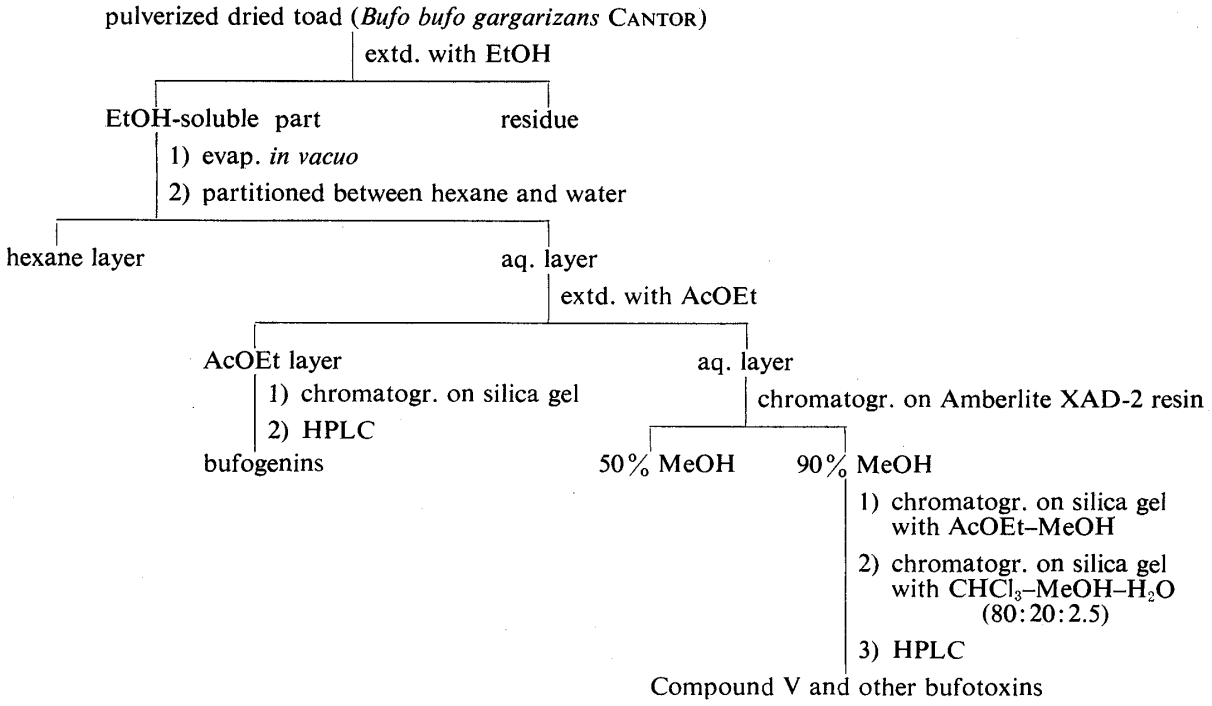
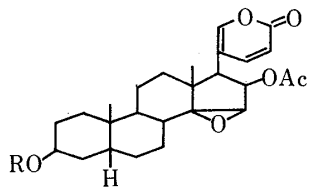


Chart 1. Separation of Bufadienolides



I: R=H

II: R=CO$(CH_2)_3$COOH

III: R=CO$(CH_2)_3$COOCH$_3$

IV: R=CO$(CH_2)_3$COO-C_6H_4-NO_2

V: R=CO$(CH_2)_3$CONHCH(COOH)$(CH_2)_3$NHC(=NH)NH$_2$

Chart 2

suberoyl-arginine esters, bufalin 3-succinoylarginine ester, gamabufotalin 3-succinoylarginine ester, gamabufotalitoxin, and vulgarobufotoxin were isolated and unambiguously identified by direct comparison with authentic samples.[5]

Besides these, a new bufotoxin (V) was isolated as a white amorphous substance. In the proton nuclear magnetic resonance (^{1}H-NMR) spectrum, V exhibited signals at δ: 0.83 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.89 (3H, s, $OCOCH_3$), 3.70 (1H, d, J=2 Hz, 15α-H), implying that the steroid moiety is cinobufagin. This compound gave negative ninhydrin and positive Sakaguchi tests. Hydrolysis with 6 N hydrochloric acid provided arginine, which was characterized by two-dimensional thin-layer chromatography (TLC). Upon enzymic hydrolysis with a hog pancreas lipase preparation followed by methylation with diazomethane, V afforded an oily product (III) whose mass spectrum (MS) showed characteristic peaks at m/z 570 (M^+), 147 ($(OH)_2^+C(CH_2)_3COOCH_3$), and 129 ($O^+\equiv C(CH_2)_3COOCH_3$). The absolute configuration of the amino acid was deduced to be L based on the specificity of the enzyme used.[4] On the basis of MS data, glutaric acid was concluded to be the dicarboxylic acid component of this bufotoxin.[8] These results prompted us to prepare an authentic sample for direct comparison. Cinobufagin 3-glutaroyl-L-arginine ester was synthesized by the active ester method[3,4] starting from cinobufagin (I). Treatment of cinobufagin 3-hemiglutarate (II) with the *p*-nitrophenol and *N*,*N*′-dicyclohexylcarbodiimide provided the *p*-nitrophenyl ester (IV) which, in turn, was condensed with L-arginine to give the desired cinobufagin 3-

glutaroyl-L-arginine ester. The ^{1}H-NMR spectrum and chromatographic behavior (HPLC and TLC) of the synthetic conjugate were identical with those of the natural product. The synthetic cinobufagin 3-hemiglutarate methyl ester (III) also showed the same MS and chromatographic behavior (HPLC and TLC) as III derived from the natural source. These results lent support to the structure cinobufagin 3-glutaroyl-L-arginine ester for V.

Komatsu *et al.*[9] isolated resibufogenin from the venom of this Korean toad, but no further investigation has been done. It is of interest that cinobufotoxin homologs having dicarboxylic acid residues with consecutive carbon numbers (C_4–C_8) have been isolated from the toad. To the best of our knowledge, this is the first reported instance of a naturally occurring bufotoxin which contains glutaric acid as a dicarboxylic acid component. Pharmacological tests of this new bufotoxin are being conducted, and the results will be the subject of a future communication.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. Low- and high-resolution MS spectral measurements were run on Hitachi M-52G and JEOL JMS-O1SG-2 spectrometers, respectively. ^{1}H-NMR spectra were recorded on a JEOL FX-100 spectrometer at 100 MHz with tetramethylsilane as an internal standard. Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, t=triplet, and m=multiplet. For TLC, Silica gel HF_{254} (E. Merck AG, Darmstadt) was used as an adsorbent unless otherwise stated. Silica gel 60 (70—230 mesh) and Silica gel H (E. Merck AG) were used for column chromatography. Amberlite XAD-2 resin was purchased from Rohm and Haas Co. (Philadelphia, PA). A hog pancreas lipase preparation and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and Nakarai Chemicals Ltd. (Kyoto), respectively. HPLC was carried out on a Waters ALC/GPC 202 chromatograph equipped with a UV detector (254 nm) and a TSKgel ODS-120T column (25 cm × 0.4 cm i.d.) (Toyo Soda Co., Tokyo) at a flow rate of 1 ml/min unless otherwise stated.

Extraction of Steroidal Components——One hundred dried bodies (1.7 kg) of the Korean toad, *Bufo bufo gargarizans* CANTOR, obtained in South Korea, were crushed and pulverized. The resulting powder was extracted with EtOH (50 l) for 3 months at room temperature. After removal of insoluble materials by filtration through a layer of Celite, the filtrate was concentrated *in vacuo* below 50 °C and partitioned with the hexane–H_2O system and then with the AcOEt–H_2O system, three times each. Sterols and fatty acids were extracted into the hexane layer. Bufogenins and their conjugates were extracted into the AcOEt and aqueous layers, respectively.

Column Chromatography on Amberlite XAD-2 Resin——The aqueous layer was concentrated *in vacuo* below 50 °C to remove the organic solvent and diluted with H_2O (10 l). The resulting solution was percolated through a column packed with Amberlite XAD-2 resin (40 cm × 2.5 cm i.d.) to adsorb bufogenin conjugates. The column was washed successively with H_2O (15 l) and 50% MeOH (1 l), and then eluted with 90% MeOH.

Isolation of Bufogenins——The AcOEt layer was concentrated *in vacuo* to afford a brown residue (1 g) which, in turn, was chromatographed on silica gel 60 (40 cm × 2.5 cm i.d.) with benzene containing stepwise-increasing concentrations of AcOEt. Further purification of the dried eluate obtained with benzene–AcOEt (2 : 1) by reversed phase HPLC (CH_3CN–H_2O (4 : 5—2 : 5))[6] gave the following bufogenins: resibufogenin (< 1 mg), cinobufagin (26 mg), bufalin (4 mg), bufotalin (4 mg), cinobufotalin (< 1 mg), marinobufagin (< 1 mg), and gamabufotalin (5 mg). These bufogenins were identified by direct comparison with authentic samples.

Isolation of Bufotoxins——The 90% MeOH fraction in Amberlite XAD-2 resin chromatography was concentrated *in vacuo* to give a brown residue (1 g). This was loaded onto silica gel 60 (27 cm × 1.8 cm i.d.) and then eluted successively with AcOEt (300 ml), AcOEt–MeOH (4 : 1) (250 ml), AcOEt–MeOH (2 : 1) (300 ml), AcOEt–MeOH (1 : 1) (300 ml), AcOEt–MeOH (1 : 2) (400 ml), and MeOH (100 ml). The eluate with AcOEt–MeOH (1 : 2) was concentrated *in vacuo* below 50 °C and the residue obtained was subjected to column chromatography on Silica gel H (25 cm × 0.8 cm i.d.) with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) as an eluent. The dried eluate was further purified by reversed phase HPLC with MeOH–H_2O (10 : 7). Three successive separations using this HPLC system gave a new compound exhibiting a single peak (t_R 18 min) on the chromatogram. Treatment of the dried eluate with MeOH–ether gave cinobufagin 3-glutaroyl-L-arginine ester (V) (2 mg) as a white amorphous substance. The compound showed the same ^{1}H-NMR spectrum and chromatographic behavior as a synthetic sample. HPLC: MeOH–H_2O (10 : 7) t_R 18 min, CH_3CN–H_2O (1 : 2) t_R 15.6 min, tetrahydrofuran–H_2O (2 : 5) t_R 15 min. TLC: $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) *Rf* 0.17.

Eight known bufotoxins were isolated from the AcOEt–MeOH (1 : 1 and 1 : 2) fractions in the same manner as

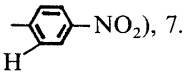
fotalin 3-succinoylarginine ester (2 mg), gamabufotalitoxin (2 mg), and vulgarobufotoxin (1 mg). These bufotoxins were identical with the corresponding authentic samples.

Hydrolysis of V with 6 N HCl——Compound V (< 1 mg) obtained from the natural source was heated with 6 N HCl (0.5 ml) in a sealed tube at 100 °C for 10 h. A portion of the resulting solution was subjected to two-dimensional TLC on Silica gel G (E. Merck AG) with $CHCl_3$–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 acids on the TLC plate.

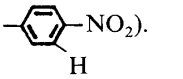
Enzymic Hydrolysis of V——Compound V (1 mg) obtained from the natural source was dissolved in MeOH–1% NaCl (1 : 9) (2.5 ml) and incubated with a hog pancreas lipase preparation (1 mg) at 37 °C for 2 h. The incubation mixture was concentrated *in vacuo* and extracted with AcOEt. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and then evaporated down *in vacuo*. The residue was dissolved in MeOH (0.1 ml) and treated with an ethereal solution of CH_2N_2. After usual work-up, the crude product obtained was purified by preparative TLC with benzene–AcOEt (4 : 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.28 was eluted with AcOEt to give III (< 1 mg) as a colorless oily substance. The product was identical with the synthetic sample in terms of the MS and chromatographic behavior. HPLC: MeOH–H_2O (3 : 1) t_R 15 min, CH_3CN–H_2O (4 : 3) t_R 22 min; μPorasil column (Waters Assoc., Milford, MA), hexane–tetrahydrofuran (7 : 3) t_R 7.5 min. TLC: benzene–AcOEt (4 : 1) *Rf* 0.28.

Synthesis of Cinobufagin 3-Hemiglutarate (II), Its Methyl Ester (III) and Its *p*-Nitrophenyl Ester (IV)——A solution of I[5] (20 mg) and glutaric anhydride (40 mg) in pyridine (2 ml) was refluxed for 24 h. The reaction mixture was poured into ice-water and extracted with AcOEt. The organic layer was washed with 5% HCl and H_2O, dried over anhydrous Na_2SO_4, and evaporated down *in vacuo*. The residue obtained was subjected to preparative TLC with benzene–AcOEt (1 : 4) as a developing solvent. Elution of the adsorbent corresponding to the spot of *Rf* 0.30 with AcOEt gave II (10 mg) as a colorless oily substance. ^{1}H-NMR ($CDCl_3$/CD_3OD) δ: 0.81 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.89 (3H, s, $OCOCH_3$), 2.10—2.50 (4H, m, $COCH_2$–×2), 2.80 (1H, d, J=10 Hz, 17α-H), 3.70 (1H, s, 15α-H), 5.05 (1H, br s, 3α-H), 5.45 (1H, d, J=10 Hz, 16α-H), 6.20 (1H, d, J=12 Hz, 23-H), 7.20 (1H, d, J=3 Hz, 21-H), 8.00 (1H, dd, J=12, 3 Hz, 22-H). Compound II (2 mg) was dissolved in MeOH (0.1 ml) and treated with an ethereal solution of CH_2N_2 in the usual manner. III (2 mg) was obtained as a colorless oily substance. ^{1}H-NMR ($CDCl_3$) δ: 0.81 (3H, s, 18-CH_3), 0.99 (3H, s, 19-CH_3), 1.88 (3H, s, $OCOCH_3$), 2.35 (4H, t, J=10 Hz, $COCH_2$–×2), 2.78 (1H, d, J=10 Hz, 17α-H), 3.64 (1H, d, J=2 Hz, 15α-H), 3.66 (3H, s, $COOCH_3$), 5.08 (1H, br s, 3α-H), 5.44 (1H, dd, J=10, 2 Hz, 16α-H), 6.18 (1H, d, J=12 Hz, 23-H), 7.12 (1H, d, J=3 Hz, 21-H), 7.86 (1H, dd, J=12, 3 Hz, 22-H). MS *m/z*: 570 (M^+), 147, 129. High-resolution MS *m/z*: 570.2792 [M^+] (Calcd for $C_{32}H_{42}O_9$ 570.2827).

A solution of II (20 mg) in AcOEt (2 ml) was treated with *p*-nitrophenol (40 mg) and *N*,*N*′-dicyclohexylcarbodiimide (20 mg), and the reaction mixture was allowed to stand at room temperature for 24 h. After removal of the precipitate by filtration, the filtrate was evaporated down *in vacuo* and the residue was purified by preparative TLC with benzene–AcOEt (4 : 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.28 was eluted with AcOEt to give cinobufagin 3-hemiglutarate *p*-nitrophenyl ester (IV) (10 mg) as a yellow oily substance. ^{1}H-NMR ($CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.83 (3H, s, $OCOCH_3$), 2.10—2.50 (4H, m, $COCH_2$–×2), 2.70 (1H, d, J=10 Hz, 17α-H), 3.56 (1H, d, J=2 Hz, 15α-H), 5.06 (1H, br s, 3α-H), 5.40 (1H, dd, J=10, 2 Hz, 16α-H), 6.10 (1H, d, J=12 Hz, 23-H), 7.06 (1H, d, J=3 Hz, 21-H), 7.13 (2H, d, J=10 Hz,



), 7.80 (1H, dd, J=12, 3 Hz, 22-H), 8.11 (2H, d, J=10 Hz,



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Synthesis of Cinobufagin 3-Glutaroyl-L-arginine Ester (V)——L-Arginine (30 mg) in H_2O (3 ml) was added to a solution of IV (30 mg) in pyridine (3 ml), and the reaction mixture was allowed to stand at room temperature for 24 h. The resulting solution was diluted with H_2O (200 ml) and percolated through a column packed with Amberlite XAD-2 resin (23 cm × 1.7 cm i.d.). The column was washed with water (500 ml), the desired compound was eluted with MeOH, and the solvent was evaporated off *in vacuo*. The residue obtained was subjected to column chromatography (25 cm × 0.8 cm i.d.) on silica gel 60 with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5). The dried eluate was recrystallized from MeOH–ether to give V (20 mg) as a white amorphous substance. mp 195—200 °C, $[\alpha]_D^{23}$ +8.0° (c=0.13, MeOH), *Anal.* Calcd for $C_{37}H_{52}N_4O_{10}\cdot 2H_2O$: C, 59.34; H, 7.54; N, 7.48. Found: C, 59.10; H, 7.19; N, 7.32. ^{1}H-NMR ($CDCl_3$/CD_3OD) δ: 0.83 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 1.89 (3H, s, $OCOCH_3$), 2.10—2.50 (4H, m, $COCH_2$–×2), 2.86 (1H, d, J=10 Hz, 17α-H), 3.70 (1H, d, J=2 Hz, 15α-H), 5.08 (1H, br s, 3α-H), 5.45 (1H, dd, J=10, 2 Hz, 16α-H), 6.21 (1H, d, J=12 Hz, 23-H), 7.24 (1H, d, J=3 Hz, 21-H), 7.96 (1H, dd, J=12, 3 Hz, 22-H).

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