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Isolation and Characterization of a New Type of Bufotoxin from the Skin of *Bufo americanus*[1]

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The occurrence of a new type of bufotoxin, in which L-glutamine replaces the arginine residue of hitherto known "bufotoxin," in the skin of the North American toad, *Bufo americanus*, is reported. Two novel bufotoxins, 3-suberoyl-L-glutamine esters of marinobufagin and telocinobufagin, were separated by column chromatography on silica gel, high-performance liquid chromatography and gel chromatography on Sephadex LH-20, and their structures were elucidated by degradative and synthetic studies.

Keywords——*Bufo americanus*; toad venom; marinobufagin 3-suberoyl-L-glutamine ester; telocinobufagin 3-suberoyl-L-glutamine ester; new type of bufotoxin; active ester method

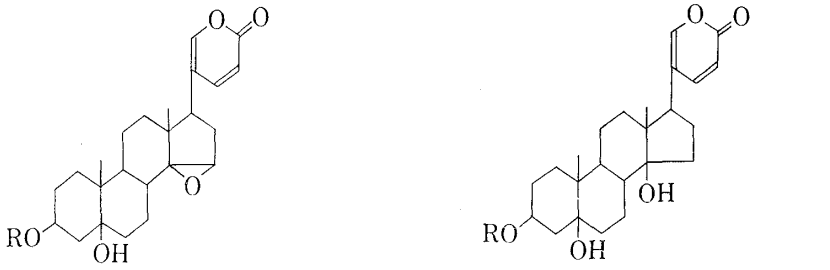
Recent studies in this laboratory disclosed the occurrence of three new types of bufotoxins in which succinoyl, adipoyl and pimeloyl groups replace the suberoyl residue of the so-called "bufotoxin", in the Japanese toad, *Bufo vulgaris formosus* BOULENGER[3] and the tropical toad, *Bufo marinus* (L.) SCHNEIDER.[4] In addition, the existence of bufogenin 3-sulfates[3,5] and analogous conjugates of cardenolide named cardenobufotoxin[3,6] was also demonstrated. The present paper deals with the isolation and characterization of a new type of bufotoxin having L-glutamine as an amino acid component from the skin of the North American toad, *Bufo americanus*.

Ten toads were sacrificed by freezing in dry ice-acetone, and the skins were immediately flayed off and extracted with ethanol. The ethanolic extract was concentrated *in vacuo* and the residue was subjected to dry column chromatography on silica gel, employing ethyl acetate–methanol as an eluent. Subsequent column chromatography on silica gel with chloroform–methanol–water (80:20:2.5) as a mobile phase resulted in satisfactory separation. When further purification was required, high-performance liquid chromatography on a reversed phase column, preparative thin-layer chromatography and gel chromatography on Sephadex LH-20 were effective.

A new bufotoxin (IV), mp 166—170° (dec.), $[\alpha]_D^{20}$ +15.1°, was separated as a colorless amorphous substance. This compound gave negative ninhydrin and Sakaguchi tests. Upon hydrolysis with 6N hydrochloric acid, glutamic acid was separated and identified using an amino acid analyzer. On enzymatic hydrolysis with a hog pancreas lipase preparation[3,7] followed by methylation with diazomethane, IV afforded marinobufagin 3-hemisuberate methyl ester (II), which was unequivocally characterized by direct comparison with an

1) Part CLVI of "Studies on Steroids" by T. Nambara; Part CLV: H. Hosoda, S. Miyairi, and T. Nambara, *Chem. Pharm. Bull.*, **28**, 1294 (1980). A part of this work has previously been presented as a preliminary communication: K. Shimada and T. Nambara, *Tetrahedron Lett.*, **1979**, 163.
2) Location: *Aobayama, Sendai 980, Japan.*
3) K. Shimada, Y. Fujii, E. Yamashita, Y. Niizaki, Y. Sato, and T. Nambara, *Chem. Pharm. Bull.*, **25**, 714 (1977).
4) K. Shimada and T. Nambara, *Chem. Pharm. Bull.*, **27**, 1881 (1979).
5) K. Shimada, Y. Fujii, and T. Nambara, *Tetrahedron Lett.*, **1974**, 2767; Y. Fujii, K. Shimada, and T. Nambara, *Chem. Ind.* (London), **1976**, 614.
6) Y. Fujii, K. Shimada, Y. Niizaki, and T. Nambara, *Tetrahedron Lett.*, **1975**, 3017.
7) The enzyme preparation also contained amylase and peptidase.

authentic sample.[8] In order to confirm the identify of the amino acid moiety, marinobufagin 3-suberoyl-D- and -L-glutamine, -glutamic acid and -L-isoglutamine esters were synthesized by the active ester method starting from marinobufagin 3-hemisuberate (I). The glutamic acid and isoglutamine conjugates were excluded as possible structures on the basis of thin-layer chromatographic behavior. Marinobufagin 3-suberoyl-L-glutamine ester was readily differentiated from the corresponding D-glutamine conjugate (V) as judged from the infrared spectra in chloroform. Direct comparison with the synthetic specimen led us to assign the structure marinobufagin 3-suberoyl-L-glutamine ester to the new bufotoxin (IV).



I: R=CO(CH$_2$)$_6$COOH
II: R=CO(CH$_2$)$_6$COOCH$_3$
III: R=CO(CH$_2$)$_6$COO-C$_6$H$_4$-NO$_2$
IV: R=CO(CH$_2$)$_6$CO-L-Gln
V: R=CO(CH$_2$)$_6$CO-D-Gln

VI: R=CO(CH$_2$)$_6$COOH
VII: R=CO(CH$_2$)$_6$COOCH$_3$
VIII: R=CO(CH$_2$)$_6$COO-C$_6$H$_4$-NO$_2$
IX: R=CO(CH$_2$)$_6$CO-L-Gln
X: R=CO(CH$_2$)$_6$CO-D-Gln

Chart 1

Another new bufotoxin (IX), mp 196—200°, $[\alpha]_D^{15}$ +6.9°, gave negative results with Sakaguchi's reagent and ninhydrin. Hydrolytic cleavage with 6 N hydrochloric acid furnished glutamic acid alone as an amino acid component. Enzymatic hydrolysis with the hog pancreas lipase preparation and subsequent methylation with diazomethane yielded telocinobufagin 3-hemisuberate methyl ester (VII) which was identified by direct comparison with an authentic sample.[8] In order to clarify the complete structure, telocinobufagin 3-suberoyl-D- and -L-glutamine esters were synthesized from telocinobufagin 3-hemisuberate (VI) by the active ester method. The synthetic L-glutamine conjugate proved to be identical with the natural product. These findings support the structure telocinobufagin 3-suberoyl-L-glutamine ester for the new bufotoxin (IX).

To the best of our knowledge this is the first recorded instance of a naturally occurring bufotoxin which contains an amino acid other than arginine. Pharmacological tests of these new bufotoxins are being conducted and the results will be the subject of a future communication.

Experimental[9]

Extraction of Steroidal Components——Ten toads (*Bufo americanus*) obtained from Fisher Scientific Co. (Itasca, Ill.) were sacrificed in dry ice-acetone. The skins were immediately flayed off and extracted

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

9) Melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 automatic polarimeter. Infrared spectra were obtained with a JASCO IRA-1 spectrometer. Mass spectral measurements were run on a Hitachi M-52G spectrometer. Amino acids were analyzed with a Hitachi 835 amino acid analyzer. Nuclear magnetic resonance (NMR) spectra were recorded on a JEOL PS-100 spectrometer at 100 MHz using tetramethylsilane as an internal standard. Abbreviations: s=singlet, d=doublet, q=quartet and m=multiplet. For preparative thin-layer chromatography (TLC), silica gel HF (E. Merck AG, Darmstadt) was used as an adsorbent. Silica gel (70—230 mesh) and silica gel H (E. Merck AG) were used for column chromatography with AcOEt–MeOH and $CHCl_3$–MeOH–H_2O (80: 20: 2.5), respectively. High-performance liquid chromatography (HPLC) was carried out on a Waters ALC/GPC 202 R401 chromatograph with a μBondapak C_{18} column (1 ft × 0.25 in i.d.) (Waters Assoc., Milford, Mass.).

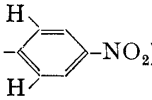
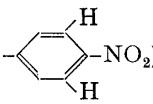
with EtOH (1 liter) for 6 months. After removal of insoluble materials by filtration through a layer of Celite, the filtrate was concentrated *in vacuo* below 50° to give a thick brown syrup (20 g).

Isolation of Marinobufagin 3-Suberoyl-L-glutamine Ester (IV)——The residue described above was chromatographed on silica gel (39 cm × 2 cm i.d.) and eluted successively with hexane (200 ml), AcOEt (200 ml), AcOEt–MeOH (1: 1) (200 ml), and MeOH (200 ml). The eluate from AcOEt–MeOH (1: 1) was rechromatographed on silica gel (25 cm × 1 cm i.d.) with $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.20 was eluted with $CHCl_3$–MeOH (5: 1). The eluate was further subjected to gel chromatography on Sephadex LH-20 (35 cm × 1.5 cm i.d.) using MeOH as a solvent. Treatment of the eluate with MeOH–ether gave IV (5 mg) as a colorless amorphous substance. mp 166—170° (dec.). $[\alpha]_D^{20}$ +15.1° (*c*=0.11 in $CHCl_3$/MeOH (1: 1)). NMR (1.25% solution in $CD_3OD/CDCl_3$ (1: 1)) δ: 0.80 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 3.55 (1H, s, 15α-H), 5.25 (1H, m, 3α-H), 6.30 (1H, d, *J*=10 Hz, 23-H), 7.30 (1H, d, *J*=2 Hz, 21-H), 7.85 (1H, q, *J*=10, 2 Hz, 22-H).

Isolation of Telocinobufagin 3-Suberoyl-L-glutamine Ester (IX)——The ethanolic extract was repeatedly chromatographed on silica gel in the manner described above. The fraction was further purified by HPLC using CH_3CN–0.5% $NH_4H_2PO_4$ as a mobile phase. The eluate was concentrated *in vacuo* below 50° and then applied to a Sephadex LH-20 column (35 cm × 1.5 cm i.d.) using MeOH as a developing solvent. Treatment of the eluate with MeOH–ether gave IX (3 mg) as a colorless amorphous substance. mp 196—200°. $[\alpha]_D^{15}$ +6.9° (*c*=0.29 in MeOH). NMR (1.5% solution in CD_3OD) δ: 0.70 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 5.25 (1H, m, 3α-H), 6.25 (1H, d, *J*=10 Hz, 23-H), 7.30 (1H, d, *J*=2 Hz, 21-H), 7.90 (1H, q, *J*=10, 2 Hz, 22-H).

Hydrolysis of Bufotoxin with 6 N HCl——Bufotoxin (1 mg) was heated with 6 N HCl (0.5 ml) in a sealed tube at 100° for 8 hr. A portion of the resulting solution was applied to the amino acid analyzer.

Enzymatic Hydrolysis of IV——Compound IV (1 mg) was dissolved in 80% MeOH (1 ml)–1% NaCl (5 ml) and incubated with the hog pancreas lipase preparation (Sigma Chemical Co., St. Louis, Mo.) (5 mg) at 37° for 12 hr. The incubation mixture was concentrated *in vacuo*, and the residue was dissolved in MeOH (1 ml) and then treated with an ethereal solution of CH_2N_2. After usual work-up the crude product obtained was purified by preparative TLC using benzene–AcOEt (1: 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.50 was eluted with AcOEt and the eluate was recrystallized from ether to give II as colorless leaflets. The mass spectrum (*m/e*: 570 (M^+), 189, 171) and chromatographic behavior of II were identical with those of the corresponding authentic sample (mp 105°).[8]

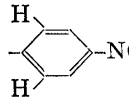
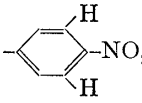
Synthesis of Marinobufagin 3-Hemisuberate *p*-Nitrophenyl Ester (III)——N,N′-Dicyclohexylcarbodiimide (30 mg) was added to a solution of marinobufagin 3-hemisuberate (I) (50 mg) and *p*-nitrophenol (30 mg) in AcOEt (3 ml) and the solution was allowed to stand at room temperature for 12 hr. The resulting solution was evaporated down *in vacuo* and the residue was purified by preparative TLC using benzene–AcOEt (5: 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.30 was eluted with AcOEt to give III (40 mg) as a pale yellow oily substance. NMR (5% solution in $CDCl_3$) δ: 0.80 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 3.50 (1H, s, 15α-H), 5.20 (1H, m, 3α-H), 6.20 (1H, d, *J*=10 Hz, 23-H), 7.10 (1H, d, *J*=2 Hz, 21-H), 7.20 (2H, d, *J*=10 Hz, ), 7.70 (1H, q, *J*=10, 2 Hz, 22-H), 8.20 (2H, d, *J*=10 Hz, ).

Synthesis of Marinobufagin 3-Suberoyl-L-glutamine Ester (IV)——L-Glutamine (50 mg) in H_2O (5 ml) was added to a solution of III (40 mg) in pyridine (5 ml) and the solution was allowed to stand at room temperature for 1 hr. The resulting solution was evaporated down *in vacuo* and the residue was purified by preparative TLC using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.20 was eluted with $CHCl_3$–MeOH (5: 1). The eluate was further subjected to gel chromatography on Sephadex LH-20 (35 cm × 1.5 cm i.d.) using MeOH as a solvent. Treatment of the eluate with MeOH–ether gave IV (20 mg) as a colorless amorphous substance. mp 166—170° (dec.). $[\alpha]_D^{20}$ +15.0° (*c*=0.10 in $CHCl_3$/MeOH (1: 1)). *Anal.* Calcd for $C_{37}H_{52}N_2O_{10}\cdot 1\ 1/2H_2O$: C, 62.43; H, 7.79; N, 3.94. Found: C, 62.31; H, 7.80; N, 3.55.

Synthesis of Marinobufagin 3-Suberoyl-D-glutamine Ester (V)——D-Glutamine (10 mg) in H_2O (1 ml) was added to a solution of III (30 mg) in pyridine (2 ml) and the solution was treated in the manner described for IV. Treatment of the eluate with MeOH–ether gave V (10 mg) as a colorless amorphous substance. mp 125—143° (dec.). $[\alpha]_D^{20}$ +6.3° (*c*=0.82 in $CHCl_3$/MeOH (1: 1)). *Anal.* Calcd for $C_{37}H_{52}N_2O_{10}\cdot 11/2H_2O$: C, 62.43; H, 7.79; N, 3.94. Found: C, 62.10; H, 7.69; N, 3.74.

TLC Comparison of IV with Marinobufagin 3-Suberoyl-D-glutamic Acid, -L-glutamic Acid and -L-isoglutamine Esters——D-Glutamic acid, L-glutamic acid or L-isoglutamine (2 mg) in H_2O (0.5—1 ml) was added to a solution of marinobufagin 3-hemisuberate *p*-nitrophenyl ester (III) (3 mg) in pyridine (2 ml) and the resulting solution was allowed to stand at room temperature for 12 hr. The reaction mixture was evaporated down under an N_2 gas stream and the residue was subjected to TLC using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as a developing solvent. Each conjugate showed a spot at *Rf* 0.10, clearly distinguishable from that of IV (*Rf* 0.20).

Enzymatic Hydrolysis of IX——Compound IX (1 mg) was dissolved in 80% MeOH (1 ml)–1% NaCl (5 ml) and incubated with the hog pancreas lipase preparation (5 mg) at 37° for 12 hr. The incubation mixture was concentrated *in vacuo*, and the residue was dissolved in MeOH (1 ml) and then treated with an ethereal solution of CH_2N_2. After usual work-up, the crude product obtained was purified by preparative TLC using benzene-AcOEt (1: 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.35 was eluted with AcOEt to give VII. The mass spectrum (*m/e* 572 (M^+), 189, 171) and chromatographic behavior were identical with those of the corresponding authentic sample (mp 128—129°).[8]

Synthesis of Telocinobufagin 3-Hemisuberate *p*-Nitrophenyl Ester (VIII)——N,N′-Dicyclohexylcarbodiimide (10 mg) was added to a solution of telocinobufagin 3-hemisuberate (VI) (20 mg) and *p*-nitrophenol (10 mg) in AcOEt (3 ml), and the solution was allowed to stand at room temperature for 12 hr. The resulting solution was concentrated *in vacuo* and the residue obtained was purified by preparative TLC using benzene–AcOEt (1: 1) as a developing solvent. Elution of the adsorbent corresponding to the spot (*Rf* 0.10) with AcOEt and recrystallization of the eluate from ether gave VIII (15 mg) as pale yellow prisms. mp 148—152°. $[\alpha]_D^{28}$ +27.7° (c=0.09 in CH_2Cl_2). *Anal.* Calcd for $C_{38}H_{49}NO_{10}\cdot 1/2H_2O$: C, 66.26; H, 7.32; N, 2.03. Found: C, 66.15; H, 7.16; N, 2.14. NMR (2% solution in $CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 5.35 (1H, m, 3α-H), 6.30 (1H, d, J=10 Hz, 23-H), 7.20 (1H, d, J=2 Hz, 21-H), 7.30 (2H, d, J=10 Hz, ), 7.85 (1H, q, J=10, 2 Hz, 22-H), 8.30 (2H, d, J=10 Hz, ).

Synthesis of Telocinobufagin 3-Suberoyl-L-glutamine Ester (IX)——L-Glutamine (10 mg) in H_2O (2 ml) was added to a solution of VIII (14 mg) in pyridine (5 ml) and the solution was allowed to stand at room temperature for 5 hr. The resulting solution was evaporated down *in vacuo* and the residue was purified by preparative TLC using $CHCl_3$–MeOH–H_2O (80: 20: 2.5) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.33 was eluted with $CHCl_3$–MeOH (5: 1). The eluate was further subjected to gel chromatography on Sephadex LH-20 (35 cm × 1.5 cm i.d.) using MeOH as a solvent. Treatment of the eluate with MeOH–ether gave IX (10 mg) as a colorless amorphous substance. mp 203°. $[\alpha]_D^{27}$ +9.4° (c=0.05 in MeOH). *Anal.* Calcd for $C_{37}H_{54}N_2O_{10}\cdot 3H_2O$: C, 59.98; H, 8.16; N, 3.78. Found: C, 59.81; H, 7.79; N, 4.15.

Synthesis of Telocinobufagin 3-Suberoyl-D-glutamine Ester (X)——D-Glutamine (5 mg) in H_2O (1 ml) was added to a solution of VIII (5 mg) in pyridine (1 ml) and the solution was treated in the manner described for IX. Treatment with MeOH–ether gave X (2 mg) as a colorless amorphous substance. mp 190—193°. $[\alpha]_D^{27}$ −8.2° (c=0.06 in MeOH). An analytical sample was not obtained because the insufficient starting material was available.

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