Isolation and Characterization of New Bufotoxins from the Skin of Bufo melanostictus SCHNEIDER

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Isolation and Characterization of New Bufotoxins from the Skin of *Bufo melanostictus* SCHNEIDER[1)]

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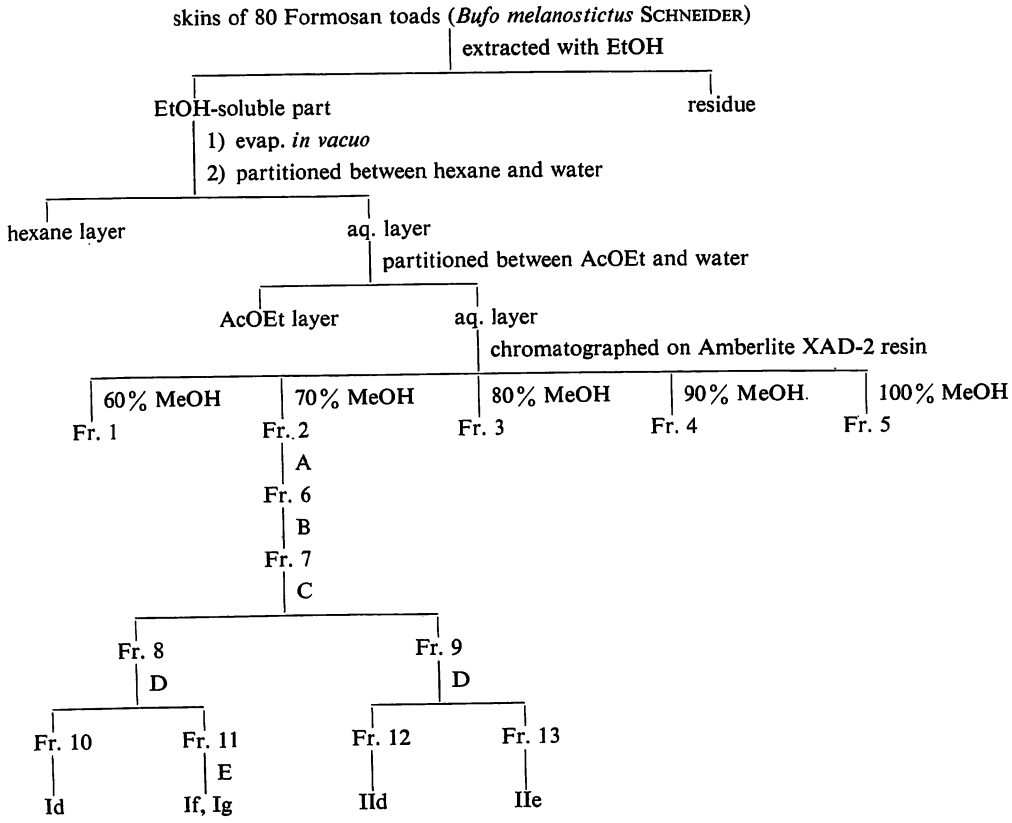
The occurrence of new bufotoxins, in which L-histidine, L-3-methylhistidine, or L-1-methylhistidine replaces the arginine residue of hitherto known "bufotoxin," in the skin of the Formosan toad, *Bufo melanostictus* SCHNEIDER, is reported. Five novel bufotoxins, bufotalin 3-suberoyl-L-histidine, -3-methylhistidine and -1-methylhistidine esters and 19-hydroxybufalin 3-suberoyl-L-histidine and -3-methylhistidine esters, were separated by usual chromatographic methods including reversed phase high-performance liquid chromatography, and their structures were elucidated by degradative and synthetic means.

Keywords——*Bufo melanostictus*; toad venom; bufotalin 3-suberoyl-L-histidine ester, 19-hydroxybufalin; bufotalin 3-suberoyl-L-3-methylhistidine ester, 19-hydroxybufalin; bufotalin 3-suberoyl-L-1-methylhistidine ester; 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 occurrence of three new types of bufotoxins, in which the succinoyl, adipoyl, and pimeloyl groups replace the suberoyl residue of "bufotoxin," in the Japanese toad, *Bufo vulgaris formosus* BOULENGER[2)] and the tropical toad, *Bufo marinus* (L.) SCHNEIDER.[3)] The existence of bufogenin 3-sulfates[2-4)] and analogous conjugates of cardenolide named cardenobufotoxin[2,5)] was also demonstrated. In addition, we isolated a new bufotoxin having L-glutamine instead of arginine as an amino acid component from the skin of the North American toad, *Bufo americanus*.[6)] The present paper deals with the isolation and characterization of new bufotoxins having L-histidine, L-3-methylhistidine, or L-1-methylhistidine as an amino acid component from the skin of the Formosan toad, *Bufo melanostictus* SCHNEIDER.

Eighty toads were sacrificed by freezing in dry ice, and the skins were immediately flayed off and extracted with ethanol. The ethanolic extract was partitioned with the hexane-water system and then with the ethyl acetate-water system.[2,3)] 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 stepwise-increasing concentrations of aqueous methanol as shown in Chart 1. Each eluate was subjected to dry column chromatography on silica gel employing ethyl acetate with stepwise-increasing concentrations of methanol and then chloroform–methanol–water (80 : 20 : 2.5) as eluents. Further purification was performed by high-performance liquid chromatography (HPLC) on a reversed phase column and then, in certain cases, preparative thin-layer chromatography (TLC).

A new bufotoxin (Id), mp 193—193.5 °C (dec.), $[\alpha]_D^{20}$ −8.1 °, was isolated as a colorless amorphous substance. This compound gave negative ninhydrin and Sakaguchi tests. Upon hydrolysis of Id with 6 N hydrochloric acid, histidine was separated and identified on an amino acid analyzer. Upon enzymic hydrolysis with a hog pancreas lipase preparation[7)] followed by methylation with diazomethane, Id afforded bufotalin 3-hemisuberate methyl ester (Ib), mp 171—173 °C, as colorless needles; this product was identified by direct comparison with an



A: chromatography on silica gel; eluent, AcOEt–MeOH
B: chromatography on silica gel; eluent, $CHCl_3$–MeOH–H_2O
C: HPLC; eluent, MeOH–0.3% $(NH_4)_2CO_3$
D: HPLC; eluent, CH_3CN–0.3% $(NH_4)_2CO_3$
E: preparative TLC on silica gel

Chart 1. Separation of Bufogenin Conjugates

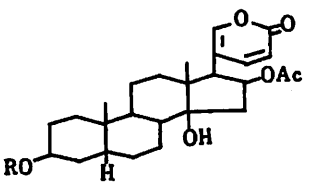
authentic sample.[8] In order to clarify the complete structure, bufotalin 3-suberoyl-L- and -D-histidine esters were synthesized from bufotalin 3-hemisuberate (Ia) by the active ester method.[6] The proton nuclear magnetic resonance (^{1}H-NMR) spectra and chromatographic behavior (HPLC, TLC) of these synthetic conjugates were identical with those of the natural product. However, the diastereoisomers could not be differentiated from each other by physical methods. Upon enzymic hydrolysis with the above enzyme preparation, the synthetic L-histidine ester (Id) provided bufotalin 3-hemisuberate (Ia) while the D-isomer ester (Ie) gave only the genin (bufotalin) instead of Ia even after incubation for a prolonged time. A similar result was obtained for enzymic hydrolysis of epimeric marinobufagin 3-suberoylglutamine esters.[6] These results supported the assignment of the structure bufotalin 3-suberoyl-L-histidine ester to the new bufotoxin (Id).

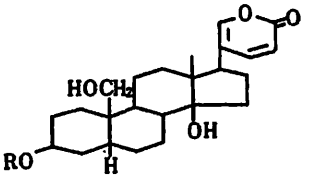
The second new bufotoxin (If), mp 170—174 °C (dec.), $[\alpha]_D^{18}$ +5.6°, gave a negative ninhydrin test. Hydrolytic cleavage with 6 N hydrochloric acid furnished solely 3-methylhistidine as an amino acid component. Enzymic hydrolysis and subsequent methylation with diazomethane yielded bufotalin 3-hemisuberate methyl ester (Ib) as described above. In order to establish the complete structure, bufotalin 3-suberoyl-L-3-methylhistidine ester was synthesized as described above. The synthetic sample proved to be identical with the natural product. The absolute configuration of the amino acid was considered to be L- as judged from

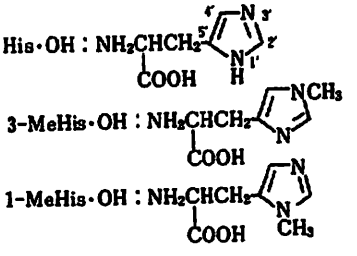
the specificity of the enzyme used. The above result lends support to the assignment of the structure bufotalin 3-suberoyl-L-3-methylhistidine ester to If.

The third new bufotoxin (Ig) which was obtained as a colorless oil in a small amount (2 mg) was unequivocally characterized as bufotalin 3-suberoyl-L-1-methylhistidine ester by degradative means and direct comparison with a synthetic sample.

The fourth new bufotoxin (IId), $[\alpha]_D^{15}$ +3.0°, isolated as a colorless oil, gave a negative ninhydrin test. Inspection of the ^{1}H-NMR spectrum (300 MHz) revealed that the 3β-hydroxyl group was esterified with a carboxylic acid having an amino acid moiety. Hydrolysis with 6 N hydrochloric acid yielded histidine, which was identified on an amino acid analyzer. When incubated with hog pancreas lipase preparation for 4 d, IId underwent enzymic hydrolysis to afford 19-hydroxybufalin (IIa) and 19-hydroxybufalin-3-hemisuberate (IIb). Compound IIa was identical with an authentic specimen.[9] Treatment of IIb with diazomethane in the usual manner yielded the methyl ester (IIc). In the mass spectrum (MS), IIc exhibited fragment ion peaks at *m/z* 189 and 171, indicating the presence of suberic acid as a dicarboxylic acid component.[2] These results permitted us to assign the structure 19-hydroxybufalin 3-suberoyl-L-histidine ester to IId. The fifth new bufotoxin (IIe), $[\alpha]_D^{15}$ +4.8°, obtained as a colorless oil, was characterized as 19-hydroxybufalin 3-suberoyl-L-3-methylhistidine ester on the basis of similar criteria.







Ia: R=CO(CH$_2$)$_6$COOH
Ib: R=CO(CH$_2$)$_6$COOMe
Ic: R=CO(CH$_2$)$_6$COO-(C$_6$H$_4$)-NO$_2$
Id: R=CO(CH$_2$)$_6$CO-L-His·OH
Ie: R=CO(CH$_2$)$_6$CO-D-His·OH
If: R=CO(CH$_2$)$_6$CO-L-3-MeHis·OH
Ig: R=CO(CH$_2$)$_6$CO-L-1-MeHis·OH

IIa: R=H
IIb: R=CO(CH$_2$)$_6$COOH
IIc: R=CO(CH$_2$)$_6$COOMe
IId: R=CO(CH$_2$)$_6$CO-L-His·OH
IIe: R=CO(CH$_2$)$_6$CO-L-3-MeHis·OH

Chart 2

Recently Verpoorte *et al.* obtained several bufogenins from the venom of this toad, but no investigation has been done on "bufotoxin."[9] Instead of so-called "bufotoxin," we isolated new bufotoxins which possess L-histidine, L-3-methylhistidine, and L-1-methylhistidine as an amino acid component. Pharmacological tests of these new bufotoxins are being conducted, and the results will be reported elsewhere in the near future.

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. Mass spectral measurements were run on a Hitachi M-52G spectrometer. ^{1}H-NMR spectra were recorded using tetramethylsilane as an internal standard on JEOL FX-100 and Bruker CXP-300 spectrometers at 100 and 300 MHz, respectively. Abbreviations: s=singlet, d=doublet, dd=doublet of doublets, and m=multiplet. For preparative TLC, Silica gel HF_{254} (E. Merck AG, Darmstadt) was used as an adsorbent. Silica gel 60 (70—230 mesh) and Silica gel H (E. Merck AG) were used for column chromatography with AcOEt–MeOH and $CHCl_3$–MeOH–H_2O, respectively, as eluents. Amberlite XAD-2 and XAD-4 resins were purchased from Rohm and Haas Co. (Philadelphia, PA). L-3-Methylhistidine was kindly donated by Ajinomoto Co.

(Kawasaki). L-1-Methylhistidine, hog pancreas lipase preparation and other reagents were purchased from Calbiochem-Behring Co. (La Jolla, CA), 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 (280 nm) and a μBondapak C_{18} column (30 cm × 0.39 cm i.d.) (Waters Assoc., Milford, MA) at a flow rate of 1 ml/min unless otherwise stated.

Extraction of Steroidal Components——Eighty toads (*Bufo melanostictus* SCHNEIDER) obtained from Vivarium Co. (Tokyo) were sacrificed by freezing in dry-ice. The skins were immediately flayed off and extracted with EtOH (16 l) for a week. 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 and then AcOEt–H_2O systems 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.[2,3] 19-Hydroxybufalin (10 mg)[9] was obtained from the AcOEt layer by column chromatography.

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 (50 cm × 3 cm i.d.) to adsorb bufogenin conjugates. The column was washed with H_2O (15 l), and then eluted successively with 60% MeOH (500 ml), 70% MeOH (500 ml), 80% MeOH (500 ml), 90% MeOH (500 ml), and anhydrous MeOH (1 l).[2,3]

Isolation of Bufotalin 3-Suberoyl-L-histidine Ester (Id)——Fr. 2 (see Chart 1) was concentrated *in vacuo* to afford a brown residue (250 mg), which was chromatographed on silica gel (45 cm × 2 cm i.d.) and eluted successively with AcOEt (100 ml), AcOEt–MeOH (2 : 1) (200 ml), AcOEt–MeOH (1 : 1) (300 ml), AcOEt–MeOH (1 : 2) (250 ml), and anhydrous MeOH (100 ml). Fr. 6 (120 mg) was rechromatographed on silica gel (40 cm × 1.5 cm i.d.) using $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) as a solvent to give Fr. 7 (70 mg), which was separated by HPLC using MeOH–0.3% $(NH_4)_2CO_3$ (5 : 4) as a mobile phase to give Fr. 8 and Fr. 9. Fr. 8 was further purified by HPLC using CH_3CN–0.3% $(NH_4)_2CO_3$ (1 : 2) as a mobile phase to give Fr. 10 and Fr. 11. Fr. 3 (200 mg) was also processed in a similar fashion. Treatment of dried Fr. 10 combined with the corresponding fraction derived from Fr. 3 with MeOH–ether gave Id (3 mg) as a colorless amorphous substance. mp 193—193.5 °C (dec.). $[\alpha]_D^{20}$ −8.1° (c=0.09, MeOH). ^{1}H-NMR ($CD_3OD/CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 1.85 (3H, s, $OCOCH_3$), 5.06 (1H, m, 3α-H), 5.50 (1H, m, 16α-H), 6.18 (1H, d, J=10 Hz, 23-H), 7.00 (1H, br s, 4′-H), 7.30 (1H, d, J=3 Hz, 21-H), 8.16 (2H, m, 22-H and 2′-H). Compound Id showed the same chromatogaphic behavior with a synthetic sample. HPLC [CH_3CN–0.3% $(NH_4)_2CO_3$ (1 : 2); t_R 12.5 min], TLC [$CHCl_3$–MeOH–H_2O (80 : 20 : 2.5); *Rf* 0.14].

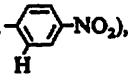
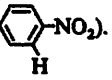
Isolation of Bufotalin 3-Suberoyl-L-3-methylhistidine (If) and -1-methylhistidine (Ig) Esters——Fr. 11 together with the corresponding fraction from Fr. 3 was subjected to preparative TLC using $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) as a developing solvent. Elution of the adsorbent corresponding to the spot of *Rf* 0.18 with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) and recrystallization of the dried eluate from acetone gave If (3 mg) as a colorless amorphous substance. mp 170—174 °C (dec.). $[\alpha]_D^{18}$ +5.6° (c=0.07, MeOH). ^{1}H-NMR ($CD_3OD/CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 1.86 (3H, s, $OCOCH_3$), 3.65 (3H, s, N–CH_3), 5.06 (1H, m, 3α-H), 5.50 (1H, m, 16α-H), 6.18 (1H, d, J=10 Hz, 23-H), 7.00 (1H, br s, 4′-H), 7.32 (1H, d, J=3 Hz, 21-H), 8.00 (1H, br s, 2′-H), 8.20 (1H, dd, J=10, 3 Hz, 22-H). Elution of the adsorbent corresponding to the spot of *Rf* 0.12 with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) gave Ig (2 mg) as a colorless oil. ^{1}H-NMR ($CD_3OD/CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 1.86 (3H, s, $OCOCH_3$), 3.80 (3H, s, N–CH_3), 5.06 (1H, m, 3α-H), 5.50 (1H, m, 16α-H), 6.18 (1H, d, J=10 Hz, 23-H), 7.00 (1H, br s, 4′-H), 7.32 (1H, d, J=3 Hz, 21-H), 8.08 (1H, br s, 2′-H), 8.20 (1H, dd, J=10, 3 Hz, 22-H). Compounds If and Ig showed the same HPLC chromatographic behavior as the corresponding synthetic samples on two different columns (μBondapak C_{18}, TSKgel 120T (Toyo Soda Co., Tokyo)) with 3 solvent systems (MeOH–0.3% $(NH_4)_2CO_3$ (5 : 3), CH_3CN–0.3% $(NH_4)_2CO_3$ (1 : 2), tetrahydrofuran–0.3% $(NH_4)_2CO_3$ (2 : 5)).

Isolation of 19-Hydroxybufalin 3-Suberoyl-L-histidine (IId) and -3-methylhistidine (IIe) Esters——Fr. 9 was further purified by HPLC using CH_3CN–0.3% $(NH_4)_2CO_3$ (3 : 7) as a mobile phase to give Fr. 12 and Fr. 13. The same fractions were also obtained from Fr. 3 by almost the same purification procedures. Fr. 12 together with the corresponding fraction from Fr. 3 was evaporated *in vacuo* to give IId (8 mg) as a colorless oil. $[\alpha]_D^{15}$ +3.0° (c=0.17, MeOH). ^{1}H-NMR (pyridine-d_5/$CDCl_3$) δ: 0.79 (3H, s, 18-CH_3), 3.56 and 4.02 (each 1H, each d, J=12 Hz, 19-$C\underline{H}_2OH$), 5.21 (1H, m, $W_{1/2}$=11 Hz, 3α-H), 6.24 (1H, d, J=12 Hz, 23-H), 7.04 (1H, br s, 4′-H), 7.29 (1H, s, 21-H), 7.76 (1H, br s, 2′-H), 8.11 (1H, d, J=12 Hz, 22-H). Fr. 13 together with the corresponding fraction from Fr. 3 was evaporated *in vacuo* to give IIe (10 mg) as a colorless oil. $[\alpha]_D^{15}$ +4.8° (c=0.26, MeOH). ^{1}H-NMR (pyridine-d_5/$CDCl_3$) δ: 0.84 (3H, s, 18-CH_3), 3.62 and 4.09 (each 1H, each d, J=12 Hz, 19-$C\underline{H}_2OH$), 5.28 (1H, m, $W_{1/2}$=11 Hz, 3α-H), 6.25 (1H, d, J=12 Hz, 23-H), 6.83 (1H, br s, 4′-H), 7.35 (1H, s, 21-H), 7.45 (1H, br s, 2′-H), 8.17 (1H, d, J=12 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 110 °C for 8 h. A portion of the resulting solution was applied to the amino acid analyzer.

Enzymic Hydrolysis of Id, If, and Ig——Id, If, or Ig (1 mg) was dissolved in 10% MeOH in 1% NaCl (2.5 ml) and incubated with hog pancreas lipase preparation (1 mg) at 37 °C for 2 h. The incubation mixture was concentrated *in vacuo* and extracted with AcOEt. The extract was subjected to preparative TLC using AcOEt as a developing solvent.

Elution of the adsorbent corresponding to the spot of *Rf* 0.30 with AcOEt gave Ia (< 1 mg). A solution of Ia in MeOH was 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* 0.38 was eluted with AcOEt and the dried eluate was recrystallized from ether–hexane to give Ib (< 1 mg) as colorless needles; this product was identical with an authentic sample[8] in MS and chromatographic behavior. MS *m/z*: 614 (M^+), 189, 171.[8] HPLC [μPorasil column (Waters Assoc.); hexane–tetrahydrofuran (5 : 3); t_R 6.5 min],TLC (AcOEt; *Rf* 0.38).

Synthesis of Bufotalin 3-Hemisuberate *p*-Nitrophenyl Ester (Ic)——*N, N'*-Dicyclohexylcarbodiimide (25 mg) was added to a solution of bufotalin 3-hemisuberate (Ia)[2] (40 mg) and *p*-nitrophenol (25 mg) in AcOEt (3 ml), and the solution was allowed to stand at room temperature for 12 h. After removal of the precipitate by filtration, the filtrate was evaporated down *in vacuo*. The residue was purified by preparative TLC using benzene, benzene–AcOEt (9 : 1) twice, and finally benzene–AcOEt (4 : 1) as developing solvents. The adsorbent corresponding to the spot of *Rf* 0.19 was eluted with AcOEt and the dried eluate was recrystallized from ether to give Ic (16 mg) as a colorless amorphous substance. mp 153.5—155 °C. $[\alpha]_D^{20}$ +7.1 ° (*c* = 0.42, CH_2Cl_2). *Anal.* Calcd for $C_{40}H_{51}NO_{11} \cdot H_2O$: C, 64.93; H, 7.22; N, 1.89. Found: C, 64.67; H, 7.21; N, 1.99. ^{1}H-NMR ($CDCl_3$) δ: 0.78 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.86 (3H, s, $OCOCH_3$), 5.08 (1H, m, 3α-H), 5.50 (1H, m, 16α-H), 6.16 (1H, d, *J* = 10 Hz, 23-H), 7.24 (1H, d, *J* = 3 Hz, 21-H), 7.24 (2H, d, *J* = 10 Hz, ), 7.98 (1H, dd, *J* = 10, 3 Hz, 22-H), 8.22 (2H, d, *J* = 10 Hz, ).

Synthesis of Bufotalin 3-Suberoyl-L-histidine Ester (Id)——L-Histidine (20 mg) in H_2O (2 ml) was added to a solution of Ic (15 mg) in pyridine (4 ml), and the solution was allowed to stand at room temperature for 12 h. The resulting solution was diluted with H_2O (300 ml) and percolated through a column packed with Amberlite XAD-4 resin (40 cm × 1 cm i.d.), then eluted with MeOH (200 ml). The eluate 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* 0.14 was eluted with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5). Treatment of the dried eluate with MeOH–ether gave Id (7 mg) as a colorless amorphous substance. mp 193—193.5 °C (dec.). $[\alpha]_D^{18}$ −7.1 ° (*c* = 0.18, MeOH). *Anal.* Calcd for $C_{40}H_{55}N_3O_{10} \cdot 2H_2O$: C, 62.07; H, 7.68; N, 5.43. Found: C, 61.96; H, 7.22; N, 5.28.

Synthesis of Bufotalin 3-Suberoyl-D-histidine Ester (Ie)——D-Histidine (20 mg) in H_2O (2 ml) was added to a solution of Ic (15 mg) in pyridine (4 ml), and the solution was treated in the manner described for Id. Treatment of the dried eluate with MeOH–ether gave Ie (8 mg) as a colorless amorphous substance. mp 190—192 °C (dec.). $[\alpha]_D^{18}$ −10.6 ° (*c* = 0.21, MeOH). *Anal.* Calcd for $C_{40}H_{55}N_3O_{10} \cdot 3/2H_2O$: C, 62.81; H, 7.64; N, 5.49. Found: C, 62.45; H, 7.14; N, 5.26. The ^{1}H-NMR spectrum of Ie was identical with that of Id. Incubation of Ie with the hog pancreas lipase preparation for more than 5 d and subsequent treatment with CH_2N_2 gave bufotalin[2] but not Ib.

Synthesis of Bufotalin 3-Suberoyl-L-3-methylhistidine Ester (If)——L-3-Methylhistidine (35 mg) in H_2O (3 ml) was added to a solution of Ic (25 mg) in pyridine (5 ml), and the solution was treated in the manner described for Id. The adsorbent corresponding to the spot of *Rf* 0.18 was eluted with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5) and treatment of the dried eluate with MeOH–ether gave If (11 mg) as a colorless amorphous substance. mp 172—174 °C (dec.). $[\alpha]_D^{18}$ +4.5 ° (*c* = 0.28, MeOH). *Anal.* Calcd for $C_{41}H_{57}N_3O_{10} \cdot 3H_2O$: C, 61.09; H, 7.88; N, 5.21. Found: C, 61.09; H, 7.54; N, 5.04.

Synthesis of Bufotalin 3-Suberoyl-L-1-methylhistidine Ester (Ig)——L-1-Methylhistidine (35 mg) in H_2O (3 ml) was added to a solution of Ic (25 mg) in pyridine (5 ml), and the solution was treated in the manner described for Id. The adsorbent corresponding to the spot of *Rf* 0.12 was eluted with $CHCl_3$–MeOH–H_2O (80 : 20 : 2.5). The dried eluate was treated with MeOH–ether to give Ig (16 mg) as a colorless amorphous substance. mp 173—174.5 °C (dec.). $[\alpha]_D^{18}$ +6.3 ° (*c* = 0.40, MeOH). *Anal.* Calcd for $C_{41}H_{57}N_3O_{10} \cdot 2H_2O$: C, 62.49; H, 7.80; N, 5.33. Found: C, 62.55; H, 7.67; N, 5.31.

Enzymic Hydrolysis of IId and IIe——IId or IIe (2 mg) was dissolved in 10% MeOH in 1% NaCl (2.5 ml) and incubated with the hog pancreas lipase preparation (2 mg) at 37 °C for 4 d. The incubation mixture was concentrated and extracted with AcOEt. The extract was subjected to preparative TLC using $CHCl_3$–MeOH (11 : 2) as a developing solvent. Elution of the adsorbent corresponding to the spot of *Rf* 0.57 with AcOEt gave IIa (< 1 mg) as a colorless amorphous substance. MS *m/z*: 402 (M^+), 367, 335.[9] Elution of the adsorbent corresponding to the spot of *Rf* 0.45 with AcOEt gave IIb (< 1 mg). A solution of IIb in MeOH was treated with an ethereal solution of CH_2N_2. After usual work-up, the crude product obtained was purified by preparative TLC using AcOEt as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.68 was eluted with AcOEt to give IIc. MS *m/z*: 572 (M^+), 367, 335, 189, 171.

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