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# Occurrence of Bufadienolides in the Skin of *Bufo viridis* LAUR.[1)]

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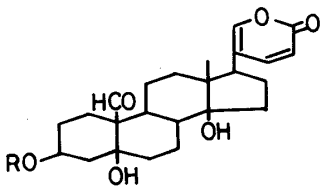
The occurrence of a new bufotoxin, hellebritoxin, together with known five bufogenins and three bufotoxins in the skin of the green toad, *Bufo viridis* LAUR., is reported. The structures were elucidated by degradative means and/or direct comparison with authentic samples. These bufadienolides were assayed for inhibitory activity towards guinea pig heart $Na^+, K^+$-adenosine triphosphatase.

**Keywords**——*Bufo viridis*; toad venom; cardiac steroid; bufogenin; bufotoxin; hellebritoxin; sodium–potassium-activated-ATPase inhibition

The green toad, *Bufo viridis* LAUR., is found widely in southern Europe. Erspamer disclosed the existence of indolealkylamines, such as bufoviridine, in the skin of this toad.[2)] In addition, Krylov *et al.*[3)] and Shimoni *et al.*[4)] examined the biological activity of the toad venom, but its constituents have not been clarified. In a series of studies on the toad venom,[5)] we characterized the cardiac steroids in the skin of the green toad and assayed them for $Na^+$, $K^+$-adenosine triphosphatase ($Na^+$, $K^+$-ATPase; EC 3.6.1.3) inhibitory activity.[6)]

Eighteen toads were sacrificed by freezing in dry ice, and the skins were immediately flayed off and extracted with ethanol. The ethanolic extract was concentrated *in vacuo* and the residue was subjected to column chromatography on silica gel. Subsequent reversed-phase column chromatography with methanol–water as a mobile phase separated a bufotoxin (bufogenin 3-suberoylarginine ester) mixture from indoleamines and amino acids. Preparative thin-layer chromatography (TLC) and /or high-performance liquid chromatography (HPLC) using a reversed-phase column were effective for the separation of the bufogenin and bufotoxin mixtures.

A new bufotoxin, mp 195—197 °C, $[\alpha]_D^{12} -20.0°$, was isolated as a colorless amorphous substance. The compound gave negative ninhydrin and positive Sakaguchi tests. Upon hydrolysis with 6 N hydrochloric acid, arginine was produced and identified by TLC. When subjected to enzymic hydrolysis with a hog pancreas lipase preparation followed by methylation with diazomethane, the bufotoxin afforded hellebrigenin 3-hemisuberate methyl ester (**2**), which was unequivocally characterized by direct comparison with a synthetic sample obtained from hellebrigenin (**1**) (Chart 1). These findings together with the elemental analyses supported the assignment of the structure hellebrigenin 3-suberoyl-L-arginine ester (**3**) for the




**1**: R = H
**2**: $R = CO(CH_2)_6COOCH_3$
**3**: $R = CO(CH_2)_6CONHCH(CH_2)_3NHCNH_2$ (COOH; NH)

Chart 1

TABLE I. Inhibition of Guinea Pig Heart $Na^+$, $K^+$-ATPase by Bufogenins and Bufotoxins

| Bufogenin | $I_{50}$ | Bufotoxin | $I_{50}$ |
|---|---|---|---|
| Marinobufagin | $1420 \pm 130$[a] (1.00) | Marinobufotoxin | $427 \pm 46$ (3.33)[b] |
| Hellebrigenin | $107 \pm 9.70$ (1.00) | Hellebritoxin | $56.4 \pm 3.33$ (1.90) |
| Arenobufagin | $54.0 \pm 6.67$ (1.00) | Arenobufotoxin | $88.6 \pm 10.2$ (0.61) |
| Telocinobufagin | $50.1 \pm 7.85$ (1.00) | Telocinobufotoxin | $33.9 \pm 6.25$ (1.48) |
| Bufotalinin | $561 \pm 88.0$ | | |
| Ouabain[c] | $157 \pm 16.3$ | | |

*a*) Mean ± S.E. ($\times 10^{-8}$ M; $n=3$). *b*) Potency relative to the respective genin. *c*) Reference compound.

new bufotoxin, which was named hellebritoxin. The absolute configuration of arginine was defined by the substrate specificity of the enzyme used.[5] Marinobufagin, hellebrigenin, arenobufagin, telocinobufagin, bufotalinin, marinobufotoxin, arenobufotoxin, and telocinobufotoxin were also isolated and identified by direct comparison with authentic samples. This is the first report of the isolation of cardiac steroids, including the new bufotoxin, from this toad venom.

In order to clarify the structure–activity relationship, the isolated bufadienolides were tested for inhibitory activity towards $Na^+$, $K^+$-ATPase. The molar concentrations of these compounds giving half-maximal inhibition ($I_{50}$) of $Na^+$, $K^+$-ATPase from guinea pig heart are listed in Table I. Marinobufotoxin, hellebritoxin and telocinobufotoxin exhibited nearly equivalent or higher activities as compared with the respective genins while the activity of arenobufotoxin was somewhat lower than that of arenobufagin. These results are compatible with those obtained with other bufogenins and bufotoxins.[6]

Further studies on cardiac steroids in toad venoms are being conducted in these laboratories, and the details will be reported elsewhere.

## 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 field desorption (FD)-mass spectral (MS) measurements were run on Hitachi M-52G and JEOL JMS-01SG-2 spectrometers, respectively. Proton nuclear magnetic resonance ($^1$H-NMR) spectra were recorded using tetramethylsilane as an internal standard on a JEOL FX-100 spectrometer at 100 MHz. Abbreviations: s = singlet, d = doublet, and dd = doublet of doublets. Silica gel $HF_{254}$ and Silica gel 60 (70—230 mesh) (E. Merck AG, Darmstadt) were used for preparative TLC and column chromatography, respectively. The packing material of a Sep-pak $C_{18}$ cartridge (Waters Assoc., Milford, MA) was used for reversed-phase column chromatography. 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 Toyo Soda 803A chromatograph equipped with a ultraviolet (UV) detector (280 nm) and a TSKgel ODS-80TM column (5 $\mu$m: 15 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**——Eighteen toads (*Bufo viridis* LAUR.) obtained from Vivarium Co. (Tokyo) were sacrificed by freezing in dry ice. The skins were immediately flayed off and extracted with EtOH (1 l) for 3 months. After removal of insoluble materials by filtration through a Celite layer, the filtrate was concentrated *in vacuo* below 50 °C to give a brown oily residue (2.15 g).

**Isolation of Bufogenins and Bufotoxins**——The residue obtained above was chromatographed on Silica gel 60

(23 cm × 2 cm i.d.) employing benzene–AcOEt (1 : 2) as an eluent. Further purification of the dried eluate by preparative TLC (benzene–AcOEt (1 : 2)) and HPLC ($CH_3CN$–$H_2O$ (7 : 10)) gave the following bufogenins: marinobufagin (3 mg), hellebrigenin (5 mg), arenobufagin (12 mg), telocinobufagin (1 mg), and bufotalinin (0.6 mg). These bufogenins were identified by direct comparison with authentic samples (MS, $^1$H-NMR spectra and chromatographic behavior[7,8]).

Further chromatography on silica gel with AcOEt–MeOH (1 : 1) as described above gave a crude bufotoxin mixture, which in turn was purified by reversed-phase chromatography (15 cm × 2 cm i.d.) using MeOH–$H_2O$ (1 : 3) as an eluent. Subsequent separation by HPLC with $CH_3CN$–$H_2O$ (1 : 2; $t_R$ 5 min) and recrystallization of the dried eluate from MeOH–ether gave a new bufotoxin (**3**; 3.5 mg) as a colorless amorphous substance. Three known bufotoxins were also obtained by reversed-phase chromatography and HPLC[8]: marinobufotoxin (7 mg), arenobufotoxin (17.5 mg), and telocinobufotoxin (11.5 mg). These bufotoxins were identical with authentic samples in terms of $^1$H-NMR spectra and chromatographic behavior.[8-10]

**Structure Elucidation of Compound 3**——Compound **3** showed the following physical and chemical properties: ninhydrin test, negative; Sakaguchi test, positive. mp 195—197 °C, $[\alpha]_D^{12}$ −20.0° ($c$=0.13, MeOH). *Anal.* Calcd for $C_{38}H_{56}N_4O_{10}\cdot 2H_2O$: C, 59.67; H, 7.91; N, 7.32. Found: C, 59.62; H, 7.62; N, 7.08. $^1$H-NMR ($CD_3OD/CDCl_3$) $\delta$: 0.68 (3H, s, 18-$CH_3$), 5.16 (1H, s, 3α-H), 6.26 (1H, d, $J$=12 Hz, 23-H), 7.28 (1H, d, $J$=4 Hz, 21-H), 7.92 (1H, dd, $J$=12, 4 Hz, 22-H), 10.08 (1H, s, 19-CHO).

Compound **3** (<1 mg) 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) using $CHCl_3$–MeOH–17% $NH_4OH$ (2 : 2 : 1) and phenol–$H_2O$ (3 : 1) as developing solvents, and arginine was detected by ninhydrin test.

Compound **3** (1 mg) 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 extracted with AcOEt. The organic phase was washed with $H_2O$, dried over anhydrous $Na_2SO_4$, and evaporated down *in vacuo*. The residue was redissolved 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 using benzene–AcOEt (1 : 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.13 was eluted with AcOEt to give hellebrigenin (**1**) (<1 mg) as a colorless oily substance. MS *m/z*: 416 ($M^+$). HPLC: μBondapak $C_{18}$ (Waters Assoc.), $CH_3CN$–$H_2O$ (7 : 10; $t_R$ 4.5 min). TLC: benzene–AcOEt (1 : 1; *Rf* 0.13). These data were consistent with those of the authentic sample. The adsorbent corresponding to the spot of *Rf* 0.32 was eluted with AcOEt to give **2** (<1 mg) as a colorless oily substance. The product was identical with synthetic hellebrigenin 3-hemisuberate methyl ester (**2**) in terms of MS and chromatographic behavior. MS *m/z*: 189, 171.[8] FD-MS *m/z*: 586 ($M^+$). HPLC: μBondapak $C_{18}$, $CH_3CN$–$H_2O$ (2 : 1; $t_R$ 3.0 min), MeOH–$H_2O$ (5 : 2; $t_R$ 6.5 min). TLC: benzene–AcOEt (1 : 1; *Rf* 0.32).

**Synthesis of Hellebrigenin 3-Hemisuberate Methyl Ester (2)**——Compound **1** (5 mg) was refluxed with suberic anhydride (15 mg) in pyridine (2 ml) for 3 h and then extracted with AcOEt. The organic phase was washed with 5% HCl and $H_2O$, and dried over anhydrous $Na_2SO_4$. After evaporation of the solvent, the residue was redissolved in MeOH (0.1 ml) and treated with $CH_2N_2$ in the usual manner. The crude product was purified by preparative TLC using benzene–AcOEt (1 : 1) as a developing solvent. The adsorbent corresponding to the spot of *Rf* 0.32 was extracted with AcOEt. The dried extract was recrystallized from ether to give **2** as colorless leaflets (1.5 mg), mp 165—168 °C. $^1$H-NMR ($CDCl_3$) $\delta$: 0.86 (3H, s, 18-$CH_3$), 3.64 (3H, s, $COOCH_3$), 5.15 (1H, s, 3α-H), 6.26 (1H, d, $J$=12 Hz, 23-H), 7.20 (1H, d, $J$=4 Hz, 21-H), 7.76 (1H, dd, $J$=12, 4 Hz, 22-H), 10.04 (1H, s, 19-CHO). FD-MS *m/z*: 586 ($M^+$).

**Assay for the Inhibition of $Na^+$, $K^+$-ATPase**——The samples were tested for inhibitory activity towards $Na^+$, $K^+$-ATPase (10 μmol Pi/mg protein·h) from guinea pig heart by the method described in the previous paper.[6] The concentration of a compound required for 50% inhibition was defined as the $I_{50}$ value.

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