STRUCTURES OF NOVEL BUFADIENOLIDES IN THE EGGS OF A TOAD, *Bufo marinus*

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In this paper, we report chemical structures of five compounds including four novel polyhydroxylated cardiac steroids in the eggs of a toad, *Bufo marinus*. These cardiac steroids were purified by high-performance liquid chromatography, and their structures were determined to be 11α,19-dihydroxytelocinobufagin (I), 11α-hydroxytelocinobufagin (II), 11α,19-dihydroxymarinobufagin (III), 11α-hydroxymarinobufagin (IV) and 19-hydroxytelocinobufagin (V) on the basis of spectral data of nuclear magnetic resonance and mass spectroscopy. All the five compounds showed biological activity, as tested by inhibition of Na^+,K^+-ATPase activity and of [^{3}H]ouabain binding to the receptor on Na^+,K^+-ATPase. This is the first finding of bufadienolides as cardiac steroids in animal eggs.

KEYWORDS 11α,19-dihydroxytelocinobufagin; 11α-hydroxytelocinobufagin; 11α,19-dihydroxymarinobufagin; 11α-hydroxymarinobufagin; 19-hydroxytelocinobufagin; bufadienolide

Toads belonging to the Bufo family have many kinds of bufadienolides in their bodies and are good resources for the study of endogenous digitalis-like compounds (DLCs). In 1922 Wieland, *et al.* separated from the venom the so-called "bufotoxin" whose structure was subsequently assigned to bufotalin 14-suberoylarginine ester.[1] In 1970 Meyer and his co-workers disclosed the existence of cardenolides and their 3-hemisuberates in Ch'an Su.[2] Cardiotonic steroids were isolated in the skin of a Japanease toad, *Bufo vulgaris formosus*, and the structure-activity relationship was examined by Shimada *et al.*[3,4] Recently, Lichtstein reported the interesting and important suggestion of the role of DLCs as possible regulators of the Na^+,K^+-ATPase implicated in water and salt homeostasis in the toad.[5] We found evidence of the existence of DLCs in the eggs of the toad *Bufo marinus* by biological activity, as tested by inhibition of Na^+,K^+-ATPase activity and [^{3}H]ouabain binding to the receptor on Na^+,K^+-ATPase. This paper deals with the isolation and characterization of novel polyhydroxylated bufadienolides in the eggs of *Bufo marinus*.

One hundred eighty-two toads were sacrificed according to a procedure approved by the Institutional Animal Care and Use Committee. Eggs were extracted with alchohol and the extract was concentrated. DLCs in the concentrate were partitioned between ethyl acetate and water. The ethyl acetate layer was concentrated *in vacuo* to give a white powder. This was redissolved in methanol and analyzed by high-performance liquid chromatography (HPLC) with a multi-channel detector. UV spectra of major peaks showed a typical spectrum due to α-pyrone ring which is a common moiety of bufadienolides. Purification was achieved with HPLC by using a reverse phase column (Capcell pak C18, 15 x 250 mm, Shiseido Co.). Fourteen peaks were collected, and each peak was analyzed by reverse phase HPLC (L-column ODS, 4.6 x 150 mm, Chemical Inspection & Testing Institute), by UV spectrometry, and by nuclear magnetic resonance (NMR) spectrometry. Five compounds (I, II, III, IV and V) were identified as pure compounds, and their chemical structures were determined on the basis of ^{1}H-, ^{1}H-^{1}H-cosy-, ^{1}H-^{1}H-noesy and ^{13}C-^{1}H-cosy NMR spectra measured by an Omega 600 MHz NMR spectrometer. All protons were assigned as shown in Table I by ^{1}H-^{1}H-cosy spectra. Assignments of carbon signals (Table II) were performed on the basis of ^{13}C-^{1}H cosy spectra.

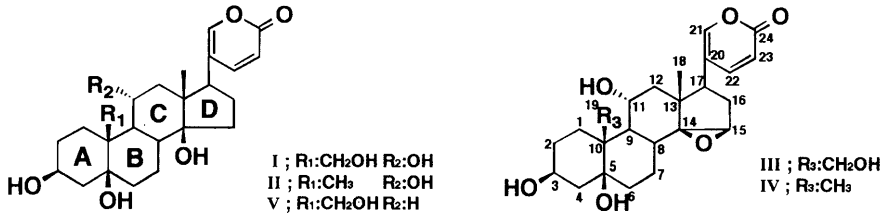

Fig. 1. Structures of Compound **I, II, III, IV,** and **V**

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TABLE I. ^{1}H-NMR Spectral Data for Compound **I, II, III, IV,** and **V** (600 MHz, Methanol-d4, δ-Values, J = Hz)

Proton	I	II	III	IV	V
H-1	2.35 (1H,br d,J=14.3)	2.27 (1H,br dt,J=14.1,3.6)	2.37 (1H,br d,J=14.4)	2.31 (1H,m)	2.34 (1H,td,J=14.2,4.7)
	2.23 (1H,td,J=14.3,3.6)	1.78 (1H,td,J=14.1,3.6)	2.18 (1H,br td,J=14.4,3.6)	1.81 (1H,br td,J=14.1,3.1)	1.41 (1H,m)
H-2	1.94 (1H,br tt,J=14.3,3.2)	1.91 (1H,br tt,J=14.1,3.0)	1.93 (1H,m)	1.90 (1H,m)	1.71 (2H,m)
	1.67 (1H,br dt,J=14.3,2.6)	1.55 (1H,br dt,J=13.7,2.8)	1.67 (1H,m)	1.55 (1H,m)	
H-3	4.10 (1H,br t,J=2.2,1/2J=8)	4.13 (1H,br t,J=2.8,1/2J=7.5)	4.09 (1H,br s,1/2J=9.0)	4.11 (1H,br s,1/2J=10.5)	4.09 (1H,br s,1/2J=9.0)
H-4	2.15 (1H,dd,J=14.7,3.2)	2.16 (1H,dd,J=14.5,2.8)	2.16 (1H,br dd,J=14.7,3.0)	2.16 (1H,dd,J=14.7,3.1)	2.21 (1H,dd,J=15.0,3.3)
	1.43 (1H,br dt,J=14.7,2.4)	1.48 (1H,br dt,J=14.5,2.8)	1.41 (1H,m)	1.46 (1H,br dt,J=14.7,2.6)	1.43 (1H,br dd,J=14.7,2.6)
H-6	1.83 (1H,td,J=13.7,4.5)	1.69 (1H,m)	1.84 (1H,br dd,J=14.0,4.9)	1.66 (1H,td,J=13.7,4.5)	1.89 (1H,td,J=13.7,4.6)
	1.48 (1H,br dt,J=13.7,3.6)	1.36 (1H,br dt,J=13.3,3.4)	1.41 (1H,m)	1.30 (1H,br dt,J=13.0,3.1)	1.52(1H,br d,J=13.4)
H-7	2.00 (1H,br dq,J=13.7,4.2)	1.95 (1H,m)	1.64 (1H,m)	1.61 (1H,m)	2.00 (1H,br dq,J=12.8,4.4)
	1.30 (1H,br qd,J=13.7,4.2)	1.26 (1H,m)	1.01 (1H,qd,J=13.0,4.4)	0.98 (1H,m)	1.28 (1H,m)
H-8	1.80 (1H,m)	1.68 (1H,m)	2.23 (1H,br td,J=12.7,3.6)	2.02 (1H,br td,J=11.7,3.9)	1.72 (1H,m)
H-9	1.80 (1H,m)	1.68 (1H,m)	1.78 (1H,br t,J=11.4)	1.72 (1H,dd,J=11.5,10.2)	1.70 (1H,m)
H-11	3.81 (1H,m)	3.74 (1H,ddd,J=10.9,9.3,4.4)	3.98 (1H,br td,J=10.7,4.0)	3.86 (1H,td,J=10.5,4.3)	1.51 (1H,br d,J=11.6)
					1.28 (1H,m)
H-12	1.65 (1H,dd,J=13.2,4.2)	1.67 (1H,dd,J=13.5,4.4)	1.87 (1H,dd,J=12.9,4.0)	1.88 (1H,dd,J=12.9,4.3)	1.67 (1H,m)
	1.51 (1H,dd,J=13.2,11.6)	1.54 (1H,dd,J=13.5,10.9)	1.52 (1H,br t,J=12.2)	1.57 (1H,m)	1.40 (1H,m)
H-15	2.11 (1H,m)	2.11 (1H,m)	3.61 (1H,br s)	3.60 (1H,br s)	2.05 (1H,m)
	1.71 (1H,m)	1.73 (1H,m)			1.68 (1H,m)
H-16	2.18 (1H,m)	2.19 (1H,m)	2.45 (1H,ddd,J=15.5,10.3,1.2)	2.42 (1H,ddd,J=15.6,10.2,1.3)	2.19 (1H,m)
	1.74 (1H,m)	1.75 (1H,m)	1.96 (1H,br d,J=15.5)	1.95 (1H,br d,J=15.6)	1.73 (1H,m)
H-17	2.61 (1H,dd,J=9.4,6.5)	2.61 (1H,dd,J=9.3,6.4)	2.61 (1H,br d,J=9.9)	2.60 (1H,br d,J=9.4)	2.54 (1H,dd,J=9.8,6.3)
H-18	0.74 (3H,s)	0.73 (3H,s)	0.82 (3H,s)	0.80 (3H,s)	0.70 (3H,s)
H-19	4.23 (1H,d,J=11.1)	1.04 (3H,s)	4.19 (1H,d,J=11.1)	1.08 (3H,s)	4.25 (1H,d,J=11.2)
	3.81 (1H,d,J=11.1)		3.88 (1H,d,J=11.1)		3.55 (1H,d,J=11.2)
H-21	7.43 (1H,dd,J=2.5,0.8)	7.44 (1H,dd,J=2.6,0.8)	7.45 (1H,dd,J=2.6,0.9)	7.45 (1H,dd,J=2.6,0.9)	7.41 (1H,dd,J=2.6,0.7)
H-22	7.93 (1H,dd,J=9.7,2.5)	7.94 (1H,dd,J=9.7,2.8)	7.85 (1H,dd,J=9.7,2.6)	7.86 (1H,dd,J=9.7,2.6)	7.97 (1H,dd,J=9.7,2.6)
H-23	6.27 (1H,dd,J=9.7,0.8)	6.26 (1H,dd,J=9.7,0.8)	6.25 (1H,dd,J=9.7,0.9)	6.25 (1H,dd,J=9.7,0.9)	6.26 (1H,dd,J=9.7,0.7)

δ in ppm from tetramethylsilane (TMS). All the chemical shifts are based on the COSY spectral data.

Compound I: white powder (28.0 mg), mp. 161 - 164 °C (dec.), FAB-MS m/z : 435 $[M(C_{24}H_{34}O_7) + H]^+$, $[\alpha]_D$ +7.1° (c=2.80, methanol). Two protons appearing at 4.10 and 3.81 ppm were assigned to H-3α and H-11β judging from coupling constants including H-12, respectively. Two protons at 4.23 and 3.81 ppm were assigned to geminal H-19 judging from coupling constants and noesy spectrum. Typical signals depending on the α-pyrone rings were found in the lower field as shown in Table I. The assignment of the chemical shifts of ^{13}C-NMR spectra was performed on the basis of ^{13}C-^{1}H cosy spectra, and these data strongly supported this structure. Two signals, 69.83 and 70.24 ppm, were assigned to hydroxy carbons at C-3 and C-11, respectively, by ^{13}C-^{1}H cosy spectrum. On the basis of ^{1}H-detected heteronuclear multiple-bond multiple-quantum coherence (HMBC) spectrum, two signals of 79.47 and 86.26 ppm were assigned to C-5 and C-14, respectively. The signal at 66.71 ppm was assigned to C-19.

Compound II: white powder (2.0 mg), mp. 175.5 - 177.5 °C (dec.), FAB-MS m/z : 419 $[M(C_{24}H_{34}O_6) + H]^+$, $[\alpha]_D$ +6.9° (c=0.20, methanol). Two protons appearing at 4.13 and 3.74 ppm were assigned to H-3α and H-11β, respectively. Two methyl protons appearing at 0.73 and 1.04 ppm were assigned to H-18 and H-19, respectively, by the data of noesy spectrum. On the basis of ^{13}C-^{1}H cosy spectrum, the assignment of carbon signals is listed in Table II. This compound was the same as a bufadienolide previously isolated from a Japanese snake, *Rhabdophis tigrinus*.[6]

Compound III: white powder (2.5 mg), mp. 132 - 136 °C (dec.), FAB-MS m/z : 433 $[M(C_{24}H_{32}O_7) + H]^+$, $[\alpha]_D$ +30.9° (c=0.25, methanol). The spectrum of ^{1}H-NMR was similar to compound I except for a proton at 3.61 ppm as a broad singlet. In a ^{1}H-^{1}H cosy spectrum, there was only one proton which had cross peaks to H-16. Carbon signals at 33.56 and 75.99 ppm were assigned to C-8 and C-14 which were shifted to higher fields than those of I, respectively. On the other hand, the signal at 61.79 ppm shifted to a lower field than that of I and was assigned to C-15. These data suggested the existence of the 14,15-epoxy group. Other chemical shifts were similar to those of I.

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Compound IV: white powder (3.0 mg), mp. 171 - 175 °C (dec.), FAB-MS m/z : 417 $[M(C_{24}H_{32}O_6) + H]^+$, $[\alpha]_D$ +20.4° (c=0.30, methanol). ^{1}H-NMR spectrum of A- and B-rings was very similar to II, and that of C- and D-rings was almost identical to III. In a ^{13}C-^{1}H cosy spectrum, all carbon signals were assigned as shown in Table II, and the structure of IV was identified as a reduced form at 19 position of III.

Compound V: white powder (5.7 mg), mp. 147 - 149 °C (dec.), FAB-MS m/z : 419 $[M(C_{24}H_{34}O_6) + H]^+$, $[\alpha]_D$ +15.0° (c=0.57 methanol). In a ^{1}H-^{1}H cosy spectrum, a proton appearing at 4.09 ppm was assigned to H-3α, and another two protons at 4.25 and 3.55 ppm were assigned to geminal protons at 19 position that were the same as I and III. There was no more signal due to a hydroxy group. The chemical shifts of C-3, C-5, C-14 and C-19 were so similar to those of I that the structure of V was determined as an 11-dehydroxy derivative of I.

TABLE II. ^{13}C-NMR Spectral Data for Compound **I, II, III, IV,** and **V** (150 MHz, Methanol-d_4, δ-Values)

Carbon	I	II	III	IV	V
C-1	22.62	28.49	22.78	28.36	20.90
C-2	30.00	30.25	29.98	30.29	29.05
C-3	69.83	70.20	69.80	70.20	69.59
C-4	39.26	38.89	39.31	38.89	38.76
C-5	79.47	77.32	79.09	77.23	79.61
C-6	37.60	37.03	36.80	36.25	37.57
C-7	25.61	25.60	24.50	24.52	25.81
C-8	42.08	42.21	33.56	33.86	42.63
C-9	46.37	46.69	49.73	49.94	40.93
C-10	45.60	43.75	45.90	43.89	44.41
C-11	70.24	70.09	69.99	69.71	23.76
C-12	52.58	52.47	51.18	51.12	42.90
C-13	50.87	50.84	46.30	46.79	50.39
C-14	86.26	86.02	75.99	75.76	86.90
C-15	33.89	34.15	61.79	61.84	33.66
C-16	30.42	30.38	33.84	33.56	30.51
C-17	52.70	52.63	49.13	49.11	52.89
C-18	19.17	19.03	18.81	18.67	18.02
C-19	66.71	18.41	66.58	18.32	66.74
C-20	125.23	125.23	124.80	124.78	125.75
C-21	151.44	151.44	152.70	152.70	151.30
C-22	149.90	149.93	150.15	150.16	150.11
C-23	116.30	116.28	116.24	116.23	116.24
C-24	165.49	165.51	165.15	165.16	165.57

δ in ppm from TMS. All the chemical shifts are based on the ^{13}C-^{1}H-cosy spectral data.

All five compounds inhibited Na^+,K^+-ATPase enzymatic activity, with relative potency of V > I, II > ouabain > III, IV, and their relative [^{3}H]ouabain binding activities to the enzyme[7] were in the same order. Animal eggs have not been exploited to find this kind of DLC. It is not expected that they are biosynthesized at such an early stage of ontogenesis. DLCs in the eggs of the toad are abundant, and may prove useful for chemical and biological studies. The serial study of chemical structure and bioactivity is desirable to elucidate not only the biosynthesis of DLCs, but also the potential roles of DLCs in the toad and in its fertilized eggs.

Further purification of the other DLCs is in progress now, and a detailed report including biological activities will be published soon.

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