A Novel Bufadienolide, Marinosin, in the Skin of the Giant Toad, *Bufo marinus*

Motomi MATSUKAWA,[a] Toshifumi AKIZAWA,*[,a] Masaaki OHIGASHI,[a] Jennifer F. MORRIS,[b] Vincent P. BUTLER, Jr.,[b] and Masanori YOSHIOKA[a]

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Setsunan University,[a] 45–1 Nagaotoge-cho, Hirakata, Osaka 573–01, Japan and Department of Medicine, College of Physicians & Surgeons of Columbia University,[b] 630 West 168th Street, New York, N.Y. 10032, U. S. A. Received July 4, 1996; accepted October 11, 1996

We have identified a novel cardiac steroid, 11,19-epoxy-19-methoxytelocinobufagin, named marinosin (1), in the skin of the toad, *Bufo marinus* (L.) SCHNEIDER. The treatment of compound 1 with 50% CH_3CN containing 0.1% trifluoroacetic acid yielded a 11α-hydroxyhellebrigenin (2), which has not previously been isolated from animals or plants. The structures of both compounds were established from spectral data obtained by NMR and MS, which were compared with those of a reference bufadienolide, 11α-hydroxytelocinobufagin. Compounds 1 and 2 have A/B *cis* and C/D *cis* configuration, which is characteristic of bufadienolides such as bufalin and marinobufagin. However, the stereo-structure of compound 1 was characterized by a boat form of the B ring, which is different from the chair form in typical bufadienolides such as compound 2. Compounds 1 and 2 both exhibited biological activity, as demonstrated by inhibition of Na^+,K^+-ATPase enzymatic activity and by inhibition of the binding of [^{3}H]ouabain to Na^+, K^+-ATPase; however, marinosin (1) was a less effective inhibitor than 2, 11α-hydroxyhellebrigenin. We have identified compound 2 in toad venom, but not in the skin.

Key words marinosin; 11α-hydroxyhellebrigenin; bufadienolide; Na^+,K^+-ATPase; [^{3}H]ouabain binding; *Bufo marinus*

The cutaneous glands and venom of toads of the amphibian genus *Bufo* are of particular interest because of the presence of large quantities of a group of endogenous compounds[1–3] which, like digitalis glycosides of plant origin, are potent inhibitors of Na^+,K^+-ATPase.[4] Many researchers have attempted to identify the endogenous digitalis-like substance (EDLS) in mammalians,[5] but so far without success. Lichtstein *et al.*[6] identified the bufadienolides as the EDLS in toad plasma. Recently, Yoshida *et al.*[7] reported an endogenous bufalin-like compound in human plasma. The EDLS of toad skin and venom are very heterogeneous, with the skin and venom of any single toad species usually containing at least four to six bufadienolides,[3,8,9] not only in unconjugated form but also in the form of C3 conjugates, including 3-sulfates, multiple dicarboxylic acid hemiesters and numerous arginyl dicarboxylic acid esters[3,8,10]; the arginyl dicarboxylic acid esters are usually referred to as bufotoxins.

The biological function of bufadienolides in toad is unknown. However, the fact that bufadienolides are present not only in toad skin and venom, but also in other toad tissues and body fluids, suggests that these compounds may play an important physiological role in toads. The isolation of EDLS from locations other than skin and venom has been hindered by the fact that the concentrations of toad EDLS are considerably lower in other tissues and body fluids than in skin and venom[6,11–13]; thus, there have been relatively few studies of the chemical structure and biological activity of EDLS from toad tissues and body fluids.[6,14] In order to study the role of EDLS in various toad tissues, we have initiated studies of EDLS in toad plasma,[15] bile[16] and eggs.[17] To enable us to compare the structures and activities of EDLS from other tissues and body fluids with the bufadienolides of skin and venom, it has been necessary to isolate known reference compounds from the skin and venom of the toad, *Bufo marinus*. In the course of these studies, we isolated and determined the structure of a novel bufadienolide-like compound, which was named marinoic acid. We report here a novel bufadienolide which shares certain structural features with other toad bufadienolides, but which contains a B ring in the boat form, rather than in the chair form present in typical toad bufadienolides. It is the purpose of this report to describe the isolation and characterization of this novel skin compound, which we have called marinosin.

Results

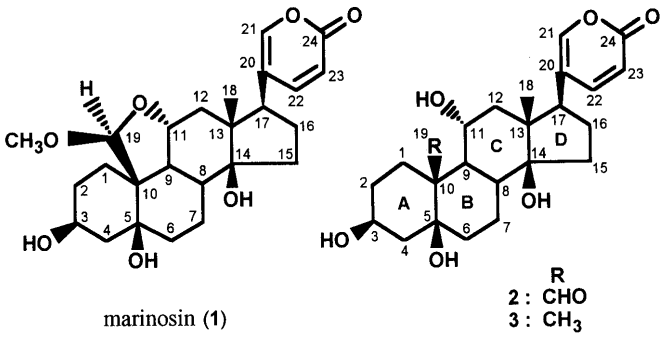
The skins of 182 toads were extracted with ethanol, and the extract was concentrated to approximately 200 ml. The solution was subjected to chromatography on a silica–octadecyl silica (ODS) column, employing stepwise increasing concentrations of CH_3CN in water containing 0.1% trifluoroacetic acid (TFA) as an eluent, as described in Experimental. The fraction which was eluted with 30% CH_3CN/0.1% TFA was subjected repetitively to preparative HPLC with a reverse-phase column. Each compound was eluted with CH_3CN at various concentrations without 0.1%TFA in the final purification. All compounds in this fraction were analyzed by analytical HPLC with a multichannel detector, and by ^{1}H-NMR spectroscopy.

Eight compounds were purified, each possessing an α-pyrone ring characteristic of bufadienolides such as bufalin. Their chemical structures were determined on the basis of two-dimensional NMR and MS data. Two compounds, telocinobufagin and telocinobufotoxin, had previously been described in toad skin.[10] Four compounds had not previously been found in toad skin, but had been isolated from the eggs of *Bufo marinus*; these compounds were 11α,19-dihydroxytelocinobufagin, 11α-hydroxytelocinobufagin (**3**), 11α-hydroxymarinobufagin and 19-hydroxytelocinobufagin.[17] A bufadienolide-related compound called marinoic acid was isolated.[18] The structure of the last compound (**1**) was judged to be novel by

* To whom correspondence should be addressed.

^{1}H-NMR analysis and, as described below, its structure was determined to be that shown in Fig. 1.

Compound **1** was not stable under acidic conditions. In the presence of 50% CH_3CN/0.1% TFA at room temperature, compound **1** was converted gradually to the previously undescribed compound **2**. The degradation product **2** was stable and was not converted to any other compound under these conditions. Both compounds exhibited a UV λ_{max} at 300 nm (data not shown), which suggested the presence of an α-pyrone ring. This suggested that the change in chromatographic behavior was due to a structural change in the steroidal portion of the molecule. The structures of compounds **1** and **2** were determined to be 11,19-epoxy-19-methoxytelocinobufagin (**1**) and 11α-hydroxyhellebrigenin (**2**), respectively. We propose to call the novel compound **1** marinosin.



marinosin (**1**)

Fig. 1. Structures of Compounds **1**, **2**, and **3**

For the structural study of compounds **1** and **2**, the assignment of proton and carbon signals in dimethyl sulfoxide (DMSO)-d_6 was performed by comparison with the signals of compound **3**, which had previously been isolated from the eggs of *Bufo marinus*[17] and which was used as a reference compound. The protons of compounds **1** and **2** were assigned on the basis of ^{1}H–^{1}H correlation spectroscopy (COSY) and rotating frame nuclear Overhauser effect spectroscopy (ROESY) spectra, as shown in Table 1. The assignments of carbon signals were determined from the ^{13}C–^{1}H COSY and heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra, as listed in Table 2.

Marinosin (**1**) was obtained as a white powder (5.2 mg), mp 233—236 °C, $[\alpha]_D^{20}$ +13.7° (c=0.45, MeOH). The molecular formula, $C_{25}H_{34}O_7$ was established from the peaks in positive ion FAB-MS at m/z 447 $(M+H)^+$ and negative ion FAB-MS at m/z 445 $(M-H)^-$. The ^{1}H- and ^{13}C-NMR spectra of compound 1, analyzed with the aid of in ^{1}H–^{1}H COSY (Fig. 3) and ^{13}C–^{1}H COSY, exhibited the presence of a tertiary methyl group (δ_H 0.70; δ_C 19.85), a methoxyl group (δ_H 3.37; δ_C 55.21), an α-pyrone ring (δ_H 7.53, 7.77, 6.31; δ_C 121.84, 149.17, 146.99, 114.24, 161.16), a lactone group (δ_H 4.99, 3.72, 0.96; δ_C 114.35, 79.47, 54.03), a secondary hydroxyl group (δ_H 3.52; δ_C 66.60), two tertiary hydroxyl groups (δ_C 74.77, 84.26), and two sp^3 quaternary carbons (δ_C 49.24, 51.43). In the ^{1}H–^{1}H COSY spectrum of compound **1**, as shown in Fig. 2, two hydroxy methine proton signals appearing at 3.52

Table 1. ^{1}H-NMR Spectral Data for Compounds **1**, **2**, and **3**

Proton	**1**	**2**	**3**
1α	1.17 (1H, m)	2.50 (1H, m)	2.23 (1H, br dt, J=13.3, 2.6)
1β	1.24 (1H, br s)	2.00 (1H, m)	1.62 (1H, td, J=13.5, 3.6)
2α	1.70 (1H, br d, J=7.3)	1.79 (1H, m)	1.82 (1H, tt, J=13.7, 3.2)
2β	1.17 (1H, m)	1.58 (1H, m)	1.38 (1H, m)
3α	3.52 (1H, m, $W_{1/2}$=22)	4.04 (1H, br s, $W_{1/2}$=8)	4.00 (1H, br s, $W_{1/2}$=10)
4α	1.70 (1H, br d, J=11.9)	2.03 (1H, dd, J=11.9, 2.7)	2.01 (1H, dd, J=14.3, 3.1)
4β	1.48 (1H, br t, J=11.9)	1.33 (1H, br d, J=11.9)	1.31 (1H, br dt, J=14.3, 2.4)
6α	1.89 (1H, m)	1.54 (1H, m)	1.21 (1H, br dt, J=12.9, 3.1)
6β	1.36 (1H, m)	1.81 (1H, br td, J=12.8, 3.5)	1.51 (1H, m)
7α	0.99 (1H, m)	1.16 (1H, br qd, J=13.6, 3.3)	1.06 (1H, br qd, J=14.1, 4.0)
7β	1.84 (1H, br q, J=12.5)	2.03 (1H, m)	1.86 (1H, br dq, J=14.1, 3.0)
8β	2.32 (1H, br td, J=12.5, 2.6)	1.81 (1H, br td, J=12.8, 3.5)	1.51 (1H, m)
9α	0.96 (1H, br t, J=12.5)	1.60 (1H, m)	1.53 (1H, m)
11β	3.72 (1H, br td, J=12.5, 3.3)	3.64 (1H, m, $W_{1/2}$=22)	3.56 (1H, m, $W_{1/2}$=20)
12α	1.48 (1H, br t, J=12.5)	1.32 (1H, br t, J=12.2)	1.39 (1H, br t, J=12.3)
12β	1.90 (1H, dd, J=12.5, 3.3)	1.54 (1H, br dd, J=12.0, 3.7)	1.55 (1H, m)
15α	1.95 (1H, m)	1.99 (1H, m)	1.98 (1H, m)
15β	1.48 (1H, m)	1.55 (1H, m)	1.60 (1H, m)
16α	2.10 (1H, m)	2.03 (1H, m)	2.04 (1H, m)
16β	1.71 (1H, m)	1.63 (1H, m)	1.60 (1H, m)
17α	2.62 (1H, br t, J=8.5)	2.50 (1H, m)	2.52 (1H, dd, J=9.1, 6.0)
18	0.70 (3H, s)	0.58 (3H, s)	0.61 (3H, s)
19	4.99 (1H, s)	9.97 (1H, s)	0.92 (3H, s)
21	7.53 (1H, br d, J=2.6)	7.53 (1H, dd, J=2.4, 1.0)	7.54 (1H, dd, J=2.6, 0.8)
22	7.77 (1H, dd, J=9.7, 2.6)	7.86 (1H, dd, J=9.5, 2.4)	7.87 (1H, dd, J=9.7, 2.6)
23	6.31 (1H, dd, J=9.7, 0.4)	6.27 (1H, dd, J=9.5, 1.0)	6.28 (1H, dd, J=9.7, 0.8)
19-OMe	3.37 (3H, s)		
3-OH	4.51 (br d, J=4.5)	5.36 (br s)	5.13 (d, J=3.6)
5-OH	4.30 (br s)	*a*)	4.89 (s)
14-OH	4.34 (s)	4.23 (s)	4.12 (s)
11-OH		4.22 (d, J=6.0)	4.07 (d, J=6.1)

In DMSO-d_6, at 30 °C, 600 MHz. δ in ppm from TMS. (J=Hz) *a*) Signal could not be determined.

and 3.72 ppm were assigned to H-3 and H-11, respectively. The H-3 proton ($W_{1/2}=22$ Hz) was in an axial orientation, judging from the large coupling constant (Table 1).

Table 2. ^{13}C-NMR Spectral Data for Compounds **1**, **2**, and **3**

Carbon	Compound **1**	**2**	**3**
1	35.58	18.97	26.26
2	32.73	27.19	28.36
3	66.60	65.86	66.79
4	47.70	37.37	36.99
5	74.77	74.19	73.81
6	32.77	36.81	35.19
7	19.43	23.78	23.31
8	37.95	40.52	39.60
9	54.03	44.27	43.96
10	49.24	54.90	41.31
11	79.47	66.38	67.05
12	45.76	49.72	50.27
13	51.48	48.12	48.25
14	84.26	82.56	82.80
15	30.95	31.38	32.12
16	29.73	27.86	28.08
17	49.67	49.39	49.66
18	19.85	17.42	17.66
19	114.35	209.53	17.04
20	121.84	121.98	122.15
21	149.17	149.37	149.28
22	146.99	147.09	147.16
23	114.24	114.09	114.09
24	161.16	161.21	161.22
19-OMe	55.21		

In DMSO-d_6, at 30 °C, 150 MHz. δ in ppm from TMS.

In the same manner, the relationships between H-8 and H-9 ($J_{8,9}=12.5$ Hz) and between H-9 and H-11 ($J_{9,11}=12.5$ Hz) were established to be *trans* diaxial. The methyl proton signal at 0.70 ppm was assigned to H-18 because of long-range coupling to the H-12α proton signal at 1.48 ppm. This was also supported by the NOEs in the ROESY spectrum, as shown in Fig. 2. In the HMBC spectrum of compound **1**, the methine proton signal at 4.99 ppm, which was correlated with C-1 (35.58 ppm), C-10 (49.24 ppm), C-9 (37.95 ppm), C-11 (79.47 ppm), and C-OMe (55.21 ppm), was assigned to H-19, and the methoxy group was placed at the C-19 position [Figs. 3 and 4a]. The methine carbon signal at C-19 (114.35 ppm), which was correlated with the proton at 4.99 ppm by the ^{13}C–^{1}H COSY spectrum, appeared at a lower field, and the hydroxyl proton signal at the C-11 position did not appear in DMSO-d_6. Thus, compound **1** had an acetal linkage between the C-11 and C-19 positions. The proton at H-19 showed connectivities with those at H-1β and H-2β in the ROESY spectrum, and therefore the absolute configuration at C-19 was assumed to be *S*. Furthermore, the stereochemistry of compound **1** was confirmed to be as shown in Fig. 2 by the observation of NOEs between H-3α and H-6α, and between H-1α and H-6α, and between H-1α and H-3α in the ROESY spectrum. Marinosin has the A/B *cis* and C/D *cis* configuration that is characteristic of all toad and plant bufadienolides. However, the boat form of the B ring [Fig. 4a] is different from the chair form found in typical toad bufadienolides as shown in Fig. 4b, though the boat form has previously been

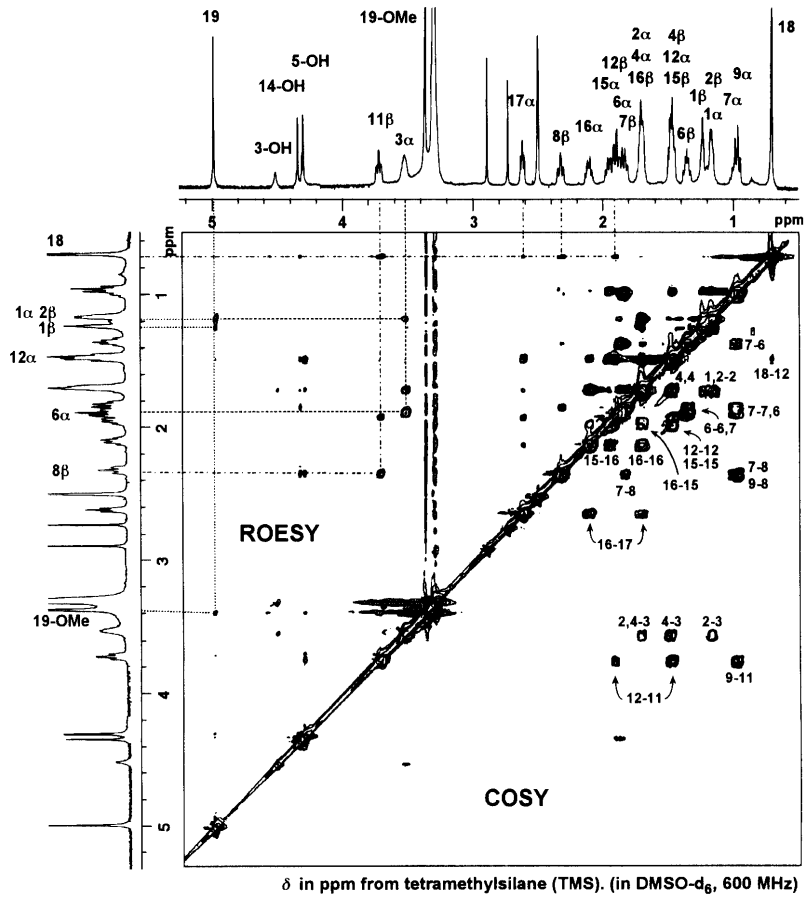


Fig. 2. ^{1}H–^{1}H COSY and ROESY Spectra of Compound **1**

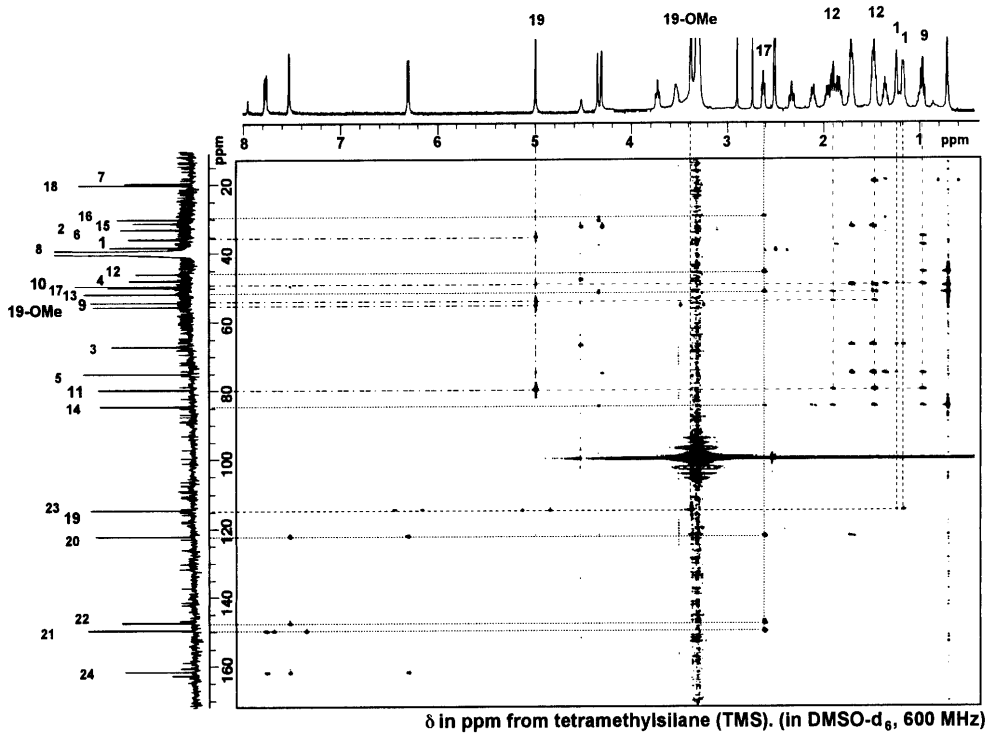


Fig. 3. HMBC Spectrum of Compound **1**

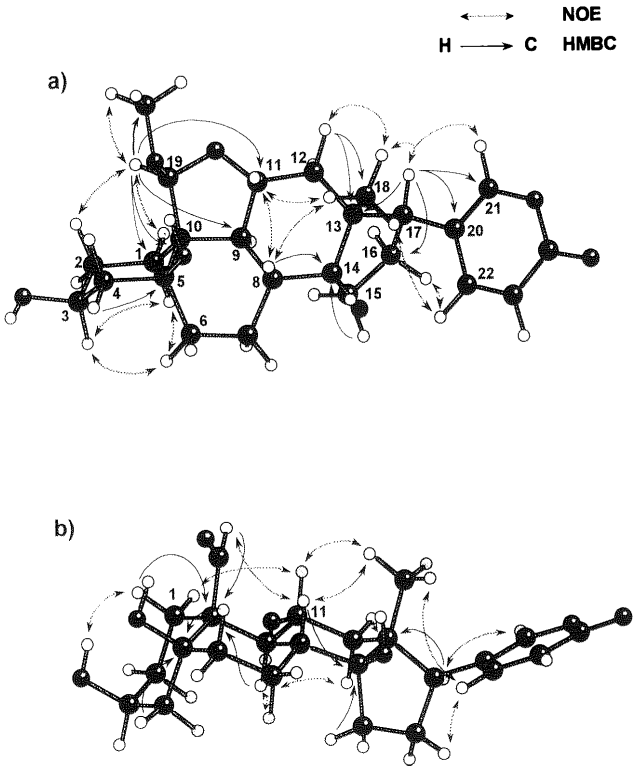


Fig. 4. HMBC and NOE Correlations for Compounds **1** (a) and **2** (b)

described in a plant bufadienolide, bryophyllin B.[19]

11α-Hydroxyhellebrigenin (**2**) was obtained as a white powder (1.5 mg), mp 242—244 °C. The molecular formula, $C_{24}H_{32}O_7$, was established from the peaks in the positive ion FAB-MS at *m/z* 433 and negative ion FAB-MS at *m/z* 431. The ^{1}H-NMR spectrum was similar to that of compound **3**, except for the disappearance of a methyl signal at 0.92 ppm (17.04 ppm in ^{13}C-NMR spectrum) and for the appearance of an aldehyde signal at 9.97 ppm (209.53 ppm in the ^{13}C-NMR spectrum). A carbon signal at 18.97 ppm, which was assigned to C-1 on the basis of ^{1}H–^{1}H- and ^{13}C–^{1}H-COSY spectra, was shifted to a higher field than that of compound **3**, while the signal at 54.90 ppm (C-10) was shifted to lower field. These data strongly support the structure illustrated in Fig. 1. In the ROESY spectrum of compound **2**, a cross peak was observed between H-1α and H-11β which was not present in the ROESY spectrum of compound **1**. Therefore, compound **2** was assumed to take the chair form typical of bufadienolides, as shown in Fig. 4b. This compound was identified in the venom by a similar purification procedure without TFA.

Table 3. Inhibition of Na^+,K^+-ATPase Enzymatic Activity and of [^{3}H]Ouabain Binding to Na^+,K^+-ATPase

Inhibitor	Inhibition of Na^+,K^+-ATPase enzymatic activity IC_{50} (μM)	Inhibition of [^{3}H]ouabain binding IC_{50} (μM)
Compound **1**	18.2	3.4
2	0.18	0.022
3	0.089	0.041
Marinobufagin	1.0	0.78
Bufalin	0.016	0.015

IC_{50}, the concentration of each compound which produced 50% inhibition.

The novel bufadienolide, marinosin (**1**), was less potent than the bufadienolide studied in this report as an inhibitor of Na^+, K^+-ATPase enzymatic activity and as an inhibitor of the binding of [^{3}H]ouabain to Na^+, K^+-ATPase. Compounds **2** and **3** were more potent inhibitors than marinobufagin, but were less potent than another reference bufadienolide, bufalin, in both of these assay systems (Table 3).

Discussion

This study presents further evidence for the biochemical heterogeneity of the EDLS of toad skin. We have isolated six cardioactive steroids which had not previously been identified in toad skin. Four of these six compounds are typical bufadienolides that have recently been isolated from the eggs of *Bufo marinus*: 11α,19-dihydroxytelocinobufagin, 11α-hydroxytelocinobufagin (**3**), 11α-hydroxymarinobufagin and 19-hydroxytelocinobufagin.[17] The fifth compound, 3β-hydroxy-11,12-seco-5β,14β-bufa-20,22-dienolide-11,14-olide-12-oic acid, is a bufadienolide-related compound which we named marinoic acid.[18] The sixth novel compound, 11,19-epoxy-19-methoxytelocinobufagin (**1**) named marinosin, has a structural feature previously described in a plant bufadienolide, *i.e.*, the B ring of compound **1** was in the boat form rather than the chair form present in all other toad bufadienolides.

Marinosin, marinoic acid and the four bufadienolides isolated from toad skin in this study were all in unconjugated form. We did not identify conjugates of any of these compounds in the current study, but the possibility exists that some or all of these compounds, like many toad skin bufadienolides, may also be present in the form of bufotoxins or other C3 conjugates.

Although somewhat less potent than marinobufagin, marinosin was an effective inhibitor of Na^+,K^+-ATPase. This finding suggests that marinosin may play a biological role in the toad, but the biological function of marinosin, like that of all toad EDLS, is not currently known. The fact that marinosin is considerably less potent than most bufadienolides suggests that the stereochemistry of the B ring is important in the interaction of bufadienolides with Na^+,K^+-ATPase.

The biosynthetic origin and metabolic fate of marinosin are also unknown. Presumably, marinosin, marinoic acid and bufadienolides are derived from similar precursor molecules but, in view of the difference from other bufadienolides in the B ring structure, it is not known whether marinosin is further modified by the same enzymes which modify skin bufadienolides. Marinosin (**1**) was, however, readily converted *in vitro* to 11α-hydroxyhellebrigenin (**2**), and, although **2** was not detected in toad skin, it is present in toad venom. In addition, this structural transformation increased the inhibitory activity. These observations suggest the possibility that there may be another pathway to reduce or increase the biological activity of bufadienolides in addition to the structural transformation of the lactone-ring.[16] Marinosin may be an important compound for understanding the biosynthesis of bufadienolides in the toad. We have not yet attempted to identify marinosin or compound **2** in other toad organs or body fluids, so it is possible that these compounds may be present, and play a role, in body fluids and tissues other than skin.

Experimental

Materials Female toads, *Bufo marinus* (L.), were purchased from National Reagents, Bridgeport, CT, and killed by pithing according to a procedure approved by the Institutional Animal Care and Use Committee. Sheep kidney Na^+,K^+-ATPase was prepared according to the method of Lane *et al.*[20] [^{3}H]Ouabain (specific activity 15.4 Ci/mmol) was purchased from New England Nuclear, Billerica, MA. Silica (120 Å)–ODS was a generous gift from Isekagaku Co., Ltd., (Tokyo). Bufalin, ouabain and pyruvate kinase (700 U/ml)/lactic dehydrogenase (1000 U/ml) were purchased from Sigma (St Louis, MO). Marinobufagin was isolated from the skin of *Bufo marinus* using an extraction method similar to the method employed for isolation of bufadienolides in the current study. All other reagents were of the highest chemical grade commercially available.

Preparation of Skin Extract Skins from 182 toads were collected and EDLS were extracted with 4 l of ethanol for 2 weeks. After filtration through cotton, the extract was concentrated to approximately 200 ml in an evaporator and stored at 4 °C until use.

Preparative Silica ODS Chromatography The initial preparative separation of the crude ethanol extract of toad skin was carried out on a silica (120 Å)–ODS column (6 cm i.d. × 10 cm), eluting stepwise with increasing concentrations of acetonitrile (CH_3CN): initially H_2O, followed successively by 30% CH_3CN, 50% CH_3CN, 70% CH_3CN and, finally, 100% CH_3CN.

Preparative HPLC A preparative HPLC system was employed, as described previously.[18] A reverse-phase column (Capcell Pak C18, 15 mm i.d. × 250 mm, Shiseido Co., Ltd., Tokyo) was used for separation, employing a 30 min linear gradient from water containing 0.1% TFA (A) to 50% CH_3CN, also containing 0.1% TFA (B) (initial; A/B = 90/10, final; A/B = 0/100). The flow rate was 6 ml/min. The UV absorption of the eluate was monitored at 300 nm. Further purification was performed using the same conditions, except for the purification of 11α,19-dihydroxytelocinobufagin and of 11α-hydroxytelocinobufagin, in which a gradient from water containing 0.1% TFA (A) to 30% CH_3CN, also containing 0.1% TFA (B) was employed.

Analytical HPLC An analytical HPLC system was employed as previously described.[21] The chromatographic system consisted of a JASCO HPLC system with a multichannel detector. A reverse-phase column (Capcell Pak C18, 4.6 mm i.d. × 250 mm, Shiseido Co.) was used for separation employing a 30 min linear gradient from water containing 0.1% TFA to 70% CH_3CN containing 0.1% TFA. The flow rate was 1.0 ml/min. The eluate was monitored with a multichannel detector.

NMR Analysis ^{1}H- and ^{13}C-NMR spectra were recorded on an Omega 600 spectrometer in DMSO-d_6, using tetramethylsilane (TMS) as an internal standard. The probe temperature was maintained at 30 °C. Chemical shifts are given in ppm and coupling constants (J values) in hertz (Hz). The following abbreviations are used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Chemical structures were determined by the study of NMR spectra, using ^{1}H–^{1}H COSY, ^{1}H–^{1}H ROESY, ^{13}C–^{1}H COSY and ^{1}H-detected HMBC spectra. Data processing was usually carried out on a SUN4 workstation (SPARC Station 330, C. Itoh Techno Science Co., Ltd., Tokyo).

Mass Spectroscopy FAB-MS were measured with a JEOL JMS-PX303 mass spectrometer (JEOL, Tokyo) with a glycerine matrix at 3 kV.

Inhibition of Na^+,K^+-ATPase Enzymatic Activity Inhibitory activity was determined by a microtiter plate modification[15] of the method of Wallick *et al.*[22] Twenty microliters aliquots of inhibitor solution in ethanol, at multiple concentrations, were added in triplicate to individual wells of a 96-well flat-bottomed microtiter plate (Immulon 2, Dynatech Laboratories, Chantilly, VA). After removal of ethanol by evaporation in a Speed Vac concentrator (Savant Instruments, Hicksville, NY), specimens were resuspended at 37 °C in a final volume of 250 μl of a reaction mixture containing 25 mM histidine (pH 7.4), 5 mM Na_2ATP, 25 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 0.4 mM β-NADH and 1 mM phosphoenolpyruvate along with 2 μl of pyruvate kinase/lactic dehydrogenase and 0.2—0.6 μg of sheep kidney Na^+,K^+-ATPase. After a 10 min equilibration period at 37 °C, ATPase activity was determined in a Thermomax Microplate Reader (Molecular Devices, Menlo Park, CA) by monitoring the oxidation of β-NADH, as reflected in a decrease in 340 nm absorbance measured at 30 s intervals over a 30 min period, during which time enzymatic activity, in the absence of inhibitors, was linear. Ouabain and bufalin at final concentrations of 1×10^{-4} M completely inhibited ATPase activity, so that ouabain- and bufalin-insensitive activity was essentially zero. Results were calculated as the concentrations which produced 50% inhibition of enzymatic activity (IC_{50}), as measured during the final 15 min of incubation with the enzyme.

Inhibition of [^{3}H]Ouabain Binding Inhibition of the binding of [^{3}H]ouabain to Na^+,K^+-ATPase was determined by a nitrocellulose

membrane filtration method.[22] Inhibitors were usually evaporated to dryness in a Speed Vac, and the residue was added to the reaction mixture (final concentration: 50 mM Tris–HCl buffer pH 7.4, 0.1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 5 mM ATP [Tris salt, vanadium-free], 25 nM [^{3}H]ouabain). The whole was incubated for 2 h at 37 °C, then chilled water was added and the mixture was passed through a Millipore membrane filter. Membranes were washed with water, and counted in a liquid scintillation counter. Specific binding was calculated by subtracting the binding observed in the absence of unlabeled ouabain, and represented 93% of the total binding under control conditions. Results are expressed as the concentration of each inhibitor which produced 50% inhibition of [^{3}H]ouabain binding (IC_{50}).

Acknowledgements These studies were supported by research grant HL 10608 from the National Institutes of Health. The authors are grateful to Dr. Masatoshi Nishi and Mr. Shoji Yamaguchi of the Faculty of Pharmaceutical Sciences, Setsunan University for NMR and MS measurements.

References

1) Abel J. J., Macht D. I., *J. Pharmacol. Exp. Ther.*, **3**, 319—377 (1912).
2) Chen K. K., Chen A. L., *Arch. Internat. Pharmacodynam Ther.*, **47**, 297—317 (1934).
3) Shimada K., Fujii Y., Yamashita E., Niizaki Y., Sato Y., Nambara T., *Chem. Pharm. Bull.*, **25**, 714—730 (1977).
4) Shimada K., Ohishi K., Fukunaga H., Ro J. S., Nambara T., *J. Pharmacobio-Dyn.*, **8**, 1054—1059 (1985).
5) Goto A., Ishiguro T., Yamada K., Ishii M., Yoshioka M., Eguchi C., Shimora M., Sugimoto T., *Biochem. Biophys. Res. Commun.*, **173**, 1093—1101 (1990).
6) Lichtstein D., Kachalsky S., Deutsch J., *Life Sci.*, **38**, 1261—1270 (1986).
7) Numazawa S., Honma Y., Yamamoto T., Yoshida T., Kuroiwa Y., *Leukemia Res.*, **19**, 945—953 (1995).
8) Fieser L. F., Fieser M., "Steroids," Reinhold Publishing Corp., New York, 1959, pp. 727—809.
9) Barbier M., Schröter H., Meyer K., Schindler O., Reichstein T., *Helv. Chim. Acta*, **42**, 2486—2505 (1959).
10) Shimada K., Nambara T., *Chem. Pharm. Bull.*, **27**, 1881—1886 (1979).
11) Flier J. S., Maratos-Flier E., Pallotta J. A., McIsaac D., *Nature* (London), **279**, 341—343 (1979).
12) Lichtstein D., Gati I., Haver E., Katz U., *Life Sci.*, **51**, 119—128 (1992).
13) Lichtstein D., Gati I., Babila T., Haver E., Katz U., *Biochim. Biophys. Acta*, **1073**, 65—68 (1991).
14) Ohno S., Ohmoto T., *Yakugaku Zasshi*, **81**, 1341—1345 (1961).
15) Butler V. P., Jr., Morris J. F., Akizawa T., Matsukawa M., Keating P., Hardart A., Furman I., *Am. J. Physiol.*, **271**, 325—332 (1996).
16) Lee S-S, Derguini F., Bruening R. C., Nakanishi K., Wallick E. T., Akizawa T., Rosenbaum C. S., Butler V. P. Jr., *Heterocycles*, **39**, 669—686 (1994).
17) Akizawa T., Mukai T., Matsukawa M., Yoshioka M., Morris J. F., Butler V. P., Jr., *Chem. Pharm. Bull.*, **42**, 754—756 (1994).
18) Matsukawa M., Akizawa T., Morris J. F., Butler, V. P., Jr., Yoshioka M., *Chem. Pharm. Bull.*, **44**, 225—257 (1996).
19) Yamagishi T., Haruna M., Yan X. Z., Chang J. J., Lee K. H., *J. Nat. Prod.*, **52**, 1071—1079 (1989).
20) Lane L. K., Potter J. D., Collins J. H., *Prep. Biochem.*, **9**, 157—170 (1979).
21) Chiba T., Akizawa T., Matsukawa M., Pan-Hou H., Yoshioka M., *Chem. Pharm. Bull.*, **42**, 1864—1869 (1994).
22) Wallick E.T., Pitts B. J. R., Lane L. K., Schwartz A., *Arch. Biochem. Biophys.*, **202**, 442—449 (1980).