Isolation and Characterization of Novel Endogenous Digitalis-like Factors in the Ovary of the Giant Toad, *Bufo marinus*

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We have previously described the structures of four novel unconjugated bufadienolides in the ovary of the toad, *Bufo marinus*. In this study, we report the separation and characterization of three novel bufadienolide conjugates. These compounds were purified by HPLC, and their structures were determined to be 11α,19-dihydroxytelocinobufagin-3-(12-hydroxydodecanoic acid) ester, 11α,19-dihydroxytelocinobufagin-3-(14-hydroxy-7-tetradecenoic acid) ester, and 11α,19-dihydroxytelocinobufagin-3-(14-hydroxytetradecanoic acid) ester on the basis of NMR and MS data. Numerous dicarboxylic acid esters of bufadienolides have previously been described, but the three bufadienolide conjugates described in this report differ from previously described esters in that they contain hydroxylated monocarboxylic acids. The function of these three conjugates is not known but they are, like bufotoxins, potent inhibitors of Na^+,K^+-ATPase and may play a developmental role in the differentiation of toad oocytes.

The most abundant known endogenous digitalis-like compounds (DLCs) of vertebrates are the bufadienolides found in free and conjugated forms in the tissues and body fluids of toads of the genus *Bufo*.[1−4] Bufadienolides and their conjugates resemble digitalis and related cardioactive glycosides of plant origin both in their chemical structures and in their potency as specific inhibitors of the digitalis-sensitive isoforms of Na^+,K^+-ATPase.[5] These endogenous toad DLCs have been detected in venom, skin,[1−4,6,7] plasma,[8−11] ovary,[12−14] bile,[15] brain, and other tissues,[16,17] but the biological role of these compounds is unknown. Toad DLCs are present in greatest abundance in skin and venom, where they have been found to consist of a heterogeneous group of compounds, including numerous relatively hydrophobic bufadienolides and their conjugates, the most abundant of which are arginyl dicarboxylic acid esters (collectively referred to as bufotoxins), dicarboxylic acid hemiesters, and 3-sulfates.[2−4] Concentrations of DLCs in other toad tissues and body fluids are much lower than their concentrations in skin and venom,[8−11,16,17] and, as a result, less is known about the chemical structure and biological properties of DLCs present in sites or fluids other than skin or venom. It has been demonstrated, however, that the DLC of toad plasma[10,11] and ovary[13,14] are chromatographically heterogeneous, and one potent bufadienolide has been identified in toad plasma.[10]

We have initiated chromatographic studies of the DLCs in the tissues and body fluids of the toad, *Bufo marinus*, with the goal of determining the physiological role of this potent group of endogenous compounds. We have found that the DLCs of toad ovaries, like the DLCs of skin, venom, plasma, and bile, are chromatographically heterogeneous. We have also found that the chromatographic patterns of DLC from ovaries differ markedly from the chromatographic profiles of DLC from skin, venom, plasma, and bile. We have initiated studies designed to characterize the chemical structure and Na^+,K^+-ATPase inhibitory activity of the DLC of toad ovaries. We have previously identified five active polar polyhydroxylated bufadienolides (four of them novel compounds) in ovaries.[14] The current report describes the isolation and characterization of three additional compounds, which represent a novel form of bufadienolide conjugate, from the ovaries of *B. marinus*.

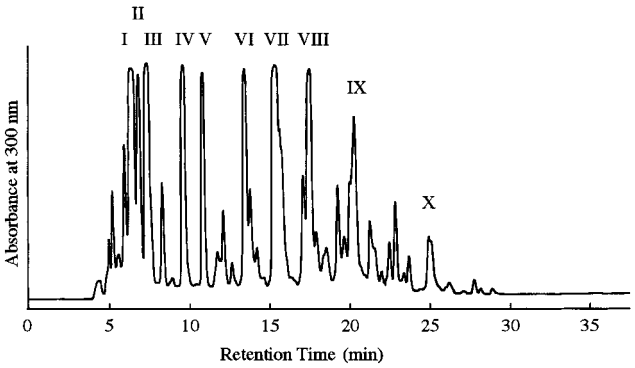

Figure 1. Preparative HPLC of ether extract prepared from EtOH extract of toad ovaries.

Results and Discussion

The ovaries from 182 toads were extracted by refluxing in 2 L of 100% HPLC grade EtOH for 2 h. After filtration with cotton and storage at 4 °C for 1 week, an oily layer formed at the bottom of the EtOH extract. After the oily layer had been removed and discarded, the alcohol extract was concentrated and lyophilized. This extract was partitioned with ether-H_2O, and then with EtOAc−H_2O. The ether layer was concentrated *in vacuo*. The residue did not dissolve completely in 1 mL of 50% MeCN containing 0.1% TFA, but dissolved after the addition of an additional 2 mL 95% MeCN containing 0.1% TFA. This solution was analyzed by HPLC. The major peaks of the chromatogram had UV absorption maxima at or near 300 nm, suggesting the existence of α-pyrone rings, characteristic of bufadienolides and their conjugates. Purification of the compounds in the major peaks was achieved by using reversed-phase preparative HPLC (Figure 1). The fractions in each

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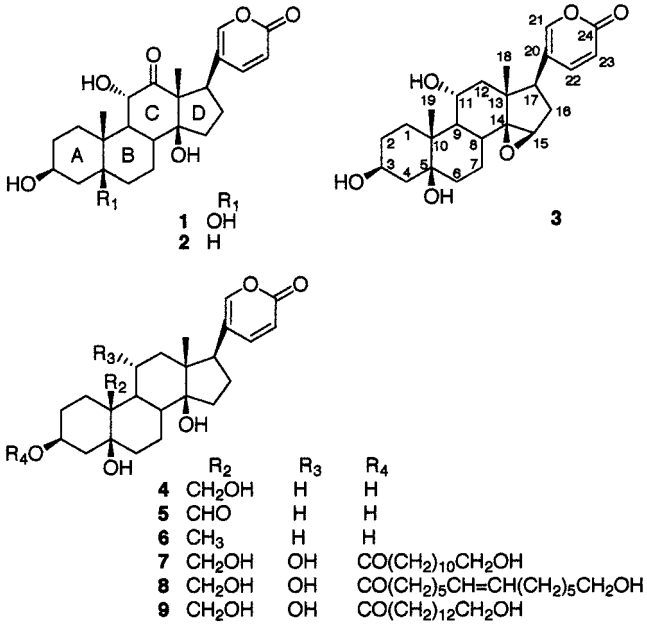
Table 1. ^{1}H-NMR Spectral Data for Compounds **7**−**9**[a]

proton	7	8	9
H-1	2.25 (1H, br dd, J = 15.7, 4.0)	2.25 (1H, m)	2.22 (1H, m)
	2.19 (1H, m)	2.18 (1H, m)	2.18 (1H, m)
H-2	2.01 (1H, m)	2.01 (1H, m)	2.01 (1H, m)
	1.68 (1H, m)	1.67 (1H, m)	1.67 (1H, m)
H-3	5.11 (1H, br s, $W_{1/2}$ = 11)	5.11 (1H, br s, $W_{1/2}$ = 12)	5.11 (1H, br s, $W_{1/2}$ = 11)
H-4	2.25 (1H, br dd, *J* = 15.7, 4.0)	2.25 (1H, br dd, *J* = 15.7, 4.0)	2.25 (1H, m)
	1.54 (1H, m)	1.54 (1H, m)	1.54 (1H, m)
H-6	1.87 (1H, br td, *J* = 13.5, 4.4)	1.88 (1H, m)	1.88 (1H, m)
	1.51 (1H, m)	1.51 (1H, m)	1.51 (1H, m)
H-7	2.03 (1H, m)	2.03 (1H, m)	2.03 (1H, m)
	1.27 (1H, m)	1.30 (1H, m)	1.30 (1H, m)
H-8	1.86 (1H, m)	1.86 (1H, m)	1.86 (1H, m)
H-9	1.75 (1H, m)	1.75 (1H, m)	1.76 (1H, m)
H-11	3.88 (1H, td, *J* = 10.4, 4.0)	3.88 (1H, br td, *J* = 10.5, 4.0)	3.88 (1H, br td, *J* = 10.1, 3.2)
H-12	1.66 (1H, br dd, *J* = 13.3, 4.0)	1.67 (1H, m)	1.66 (1H, m)
	1.54 (1H, m)	1.53 (1H, m)	1.53 (1H, m)
H-15	2.14 (1H, m)	2.12 (1H, m)	2.14 (1H, m)
	1.74 (1H, m)	1.73 (1H, m)	1.74 (1H, m)
H-16	2.20 (1H, m)	2.21 (1H, m)	2.19 (1H, m)
	1.77 (1H, m)	1.77 (1H, m)	1.76 (1H, m)
H-17	2.62 (1H, dd, *J* = 9.2, 6.5)	2.62 (1H, br dd, *J* = 9.3, 6.5)	2.62 (1H, br dd, *J* = 9.5, 6.7)
H-18	0.75 (3H, s)	0.75 (3H, s)	0.75 (3H, s)
H-19	4.22 (1H, d, *J* = 11.3)	4.21 (1H, d, *J* = 11.1)	4.20 (1H, d, *J* = 10.9)
	3.84 (1H, d, *J* = 11.3)	3.84 (1H, d, *J* = 11.1)	3.84 (1H, d, *J* = 11.3)
H-21	7.44 (1H, dd, *J* = 2.4, 0.8)	7.44 (1H, br dd, *J* = 2.4, 0.8)	7.44 (1H, br d, *J* = 2.0)
H-22	7.94 (1H, dd, *J* = 9.7, 2.4)	7.94 (1H, dd, *J* = 9.7, 2.4)	7.94 (1H, dd, *J* = 9.7, 2.4)
H-23	6.27 (1H, dd, *J* = 9.7, 0.8)	6.27 (1H, dd, *J* = 9.7, 0.8)	6.27 (1H, d, *J* = 9.7)
−(CH$_2$)−			
H-2′	2.33 (2H, td, *J* = 7.3, 4.8)	2.34 (2H, td, *J* = 7.3, 4.0)	2.33 (2H, td, *J* = 7.3, 4.4)
H-3′	1.62 (2H, t, *J* = 7.3)	1.63 (2H, m)	1.63 (2H, m)
H-4′	1.32 (2H, m)	1.34 (2H, m)	1.33 (2H, m)
H-5′	1.31 (2H, m)	1.37 (2H, m)	1.29 (2H, m)
H-6′	1.31 (2H, m)	2.05 (2H, m)	1.29 (2H, m)
H-7′	1.31 (2H, m)	5.36 (1H, m)	1.29 (2H, m)
H-8′	1.31 (2H, m)	5.36 (1H, m)	1.29 (2H, m)
H-9′	1.31 (2H, m)	2.05 (2H, m)	1.29 (2H, m)
H-10′	1.31 (2H, m)	1.37 (2H, m)	1.29 (2H, m)
H-11′	1.52 (2H, m)	1.35 (2H, m)	1.29 (2H, m)
H-12′	3.54 (2H, t, *J* = 6.9)	1.35 (2H, m)	1.34 (2H, m)
H-13′		1.54 (2H, m)	1.53 (2H, m)
H-14′		3.54 (2H, td, *J* = 6.4, 4.0)	3.53 (2H, t, *J* = 6.5)

[a] Spectra obtained in CD_3OD at 600 MHz. Chemical shifts are expressed in ppm and *J* values are in Hz.

peak were collected, lyophilized, and subjected to reversed-phase analytical HPLC. Compounds **6** and **7** were isolated as purified compounds from fractions IV and VI, respectively. Another eight fractions (I, II, III, V, VII, VIII, IX, and X) were again subjected to preparative HPLC, resulting in the isolation of **1**, **2**, **3**, **4**, **5**, **8**, and **9** as pure compounds, as verified by analytical HPLC and ^{1}H NMR spectrometry. The ^{1}H NMR spectra suggested that all compounds had α-pyrone rings in their structures. On the basis of NMR and MS, 11α-hydroxymarinobufagin (**3**), 19-hydroxytelocinobufagin (**4**), and hellebrigenin (**5**) were identified in fractions I, II, and III, respectively; compounds **3**−**5** were present in greater abundance in the EtOAc layer, as decribed previously.[14] Compound **1** from fraction I was 5-hydroxyarenobufagin, a bufadienolide previously isolated from a Japanese snake, *Rhabdophis tigrinus*.[18] Compound **2** from fraction III, and compound **6** from fraction IV were arenobufagin and telocinobufagin, respectively. Another three compounds **7**, **8**, and **9** from fractions VI, VII, and VIII, respectively, were novel. Compounds **1**, **2**, and **6**−**9** were isolated mainly from the ether extract. The chemical structures of **7**−**9** were determined on the basis of data obtained by 2D NMR and by mass spectrometery.



Compound **7**, which was purified from the ether layer, was obtained as a white powder (21.3 mg). The molecular formula, $C_{36}H_{56}O_9$, was established from positive ion FABMS at *m*/*z* 633 $[M + H]^+$ and negative ion FABMS at *m*/*z* 631 $[M - H]^-$. Negative ion FABMS of **7** also showed the appearance of a fragment ion peak at *m*/*z* 433 $[C_{24}H_{33}O_7]^-$ indicating the presence of a bufadienolide. The ^{1}H NMR and ^{13}C NMR spectra of **7** (Tables 1 and 2) showed signal patterns characteristic of a bufadienolide. The ^{13}C NMR spectrum of **7**, however, exhibited the presence of a

Table 2. ^{13}C-NMR Spectral Data for Compounds **7**−**9**[a]

carbon	7	8	9
C-1	22.99	23.09	22.65
C-2	26.38	26.09	28.18
C-3	72.12	72.13	72.15
C-4	37.57	37.54	37.42
C-5	76.46	76.45	76.48
C-6	36.96	36.97	36.92
C-7	25.15	25.14	25.15
C-8	41.24	41.24	41.26
C-9	45.71	45.76	45.94
C-10	44.72	44.71	44.75
C-11	69.27	69.27	69.29
C-12	51.65	51.65	51.67
C-13	50.06	50.06	50.08
C-14	85.53	85.53	85.56
C-15	33.18	33.18	33.19
C-16	29.60	29.59	29.61
C-17	51.97	51.96	52.00
C-18	18.34	18.32	18.34
C-19	65.56	65.56	65.58
C-20	124.47	124.47	124.50
C-21	150.68	150.68	150.71
C-22	149.15	149.15	148.18
C-23	115.53	115.53	115.54
C-24	164.72	164.72	164.76
C-1′	174.92	174.89	174.97
C-2′	35.64	35.61	35.66
C-3′	26.11	25.99	26.13
C-4′	30.22[b]	29.81[b]	30.24[b]
C-5′	30.40[b]	30.22[b]	30.42[b]
C-6′	30.59[b]	28.15	30.62[b]
C-7′	30.62[b]	131.07[c]	30.65[b]
C-8′	30.66[b]	130.68[c]	30.73[b]
C-9′	30.73[b]	28.02	30.76[b]
C-10′	26.97	30.52[b]	30.76[b]
C-11′	33.70	30.87[b]	30.78[b]
C-12′	63.06	26.87	27.00
C-13′		33.69	33.73
C-14′		63.04	63.09

[a] Spectra obtained in CD_3OD at 150 MHz. Chemical shifts are expressed. [b,c]Assignments may be interchanged in each column.

tertiary carbon signal at 174.92 ppm and a hydroxy methylene signal at 63.06 ppm, together with 10 additional methylene carbon signals between 26 and 36 ppm in the DEPT spectrum. These NMR and MS data suggested that **7** was a new conjugated bufadienolide. For the steroidal moiety, the ^{1}H and ^{13}C NMR spectra of **7** in the ^{1}H−^{1}H-correlation spectroscopy (COSY) and ^{13}C−^{1}H COSY exhibited the presence of an α-pyrone ring (δ_H 7.44, 7.94, and 6.27; δ_C 124.47, 150.68, 149.15, 115.53, and 164.72), a tertiary methyl group (δ_H 0.75; δ_C 18.34), a pair of protons of a hydroxymethyl group (δ_H 3.84, 4.22; δ_C 65.56), two secondary hydroxyl groups (δ_H 5.11; δ_C 72.12, δ_H 3.98; δ_C 69.27), and five quaternary carbons, including two oxgenated carbons (δ_C 76.46, 85.53). Furthermore, the connectivities of rings A, B, C, and D, the tertiary methyl group, and the hydroxymethyl group through to the α-pyrone ring were revealed by interpretation of the HMBC spectrum. Two hydroxy methine proton signals appearing at 5.11 and 3.98 ppm, which correlated with carbon signals at 72.12 and 69.27 ppm, were assigned to H-3 and H-11, respectively. The configuration of the H-3 proton ($W_{1/2}$ = 11 Hz) was equatorial, judging from the coupling constant. H-11 had an axial configuration, as shown by the large coupling constant (J = 10.4 Hz) assigned to $J_{9,11}$ and $J_{11,12\alpha}$ with the equatorial $J_{11,12\beta}$ = 4.0 Hz. The methyl proton signal at 0.75 ppm was assigned to H-18 because a cross peak was observed to exhibit long-range coupling between an H-18 proton signal and an H-12α proton signal at 1.54 ppm.

In the ^{1}H−^{1}H NOESY study of **7**, a proton at the H-17 position was correlated with a proton at H-21 in the α-pyrone ring. In addition, a proton at H-22 was correlated with protons at H-16 and H-18. This resulted in the conclusion that the α-pyrone ring was in a β configuration and fixed.

These NMR studies showed that the genin of **7** was 11α,19-dihydroxytelocinobufagin, a structure previously identified after isolation from the EtOAc layer.[14]

In the ^{13}C NMR spectrum, 12 signals were observed other than those of the genin. In the ^{1}H−^{1}H COSY and ^{13}C−^{1}H COSY spectra, a hydroxy methylene proton signal at 3.54 ppm (H-12′) was correlated with a methylene proton signal at 1.52 ppm (H-11′), which was, in turn, correlated with a methylene signal at 1.31 ppm (H-10′). Methylene protons next to the ester carbonyl at 2.33 ppm (H-2′) were correlated with a methylene proton signal at 1.62 ppm (H-3′), which was correlated with a signal at 1.32 ppm (H-4′). Five carbon signals appearing between 30 and 31 ppm were correlated with the methylene proton signals at 1.31 ppm. These data were consistent with the side chain of compound **7** being the ester of 12-hydroxydodecanoic acid. Moreover, the proton at the C-3 position (5.11 ppm) was shifted to a lower field than that of the genin (4.10 ppm) (data not shown). Therefore, the structure of compound **7** was assigned as 11α,19-dihydroxytelocinobufagin-3-(12-hydroxydodecanoic acid) ester.

To confirm the structure of the side-chain moiety, compound **7** and 12-hydroxydodecanoic acid were each hydrolyzed with 6N HCl, and then derivatized with *n*-butyl alcohol containing 10% HCl, and analyzed by GC. The chromatogram of the butyl derivative of the hydrolysate of **7** contained two peaks (at 71.85 and 72.27 min, respectively), which corresponded with two peaks (at 71.60 and 72.13 min) in the chromatogram of 12-hydroxydodecanoic acid, which had been processed in a similar manner; these peaks were not present in treated specimens containing only 11α,19-dihydroxytelocinobufagin. A single peak (71.62 min), which was found in the chromatogram of the nonhydrolyzed butyl derivative of **7** was the same as that observed with 12-hydroxydodecanoic acid, but was not present in the case of 11α,19-dihydroxytelocinobufagin. To confirm these data, the butyl derivative of the hydrolysate of **7** and 12-hydroxydodecanoic acid were run together. The chromatogram of the reaction mixture revealed the coincidence of the peaks. When the reaction mixture of the nonhydrolyzed butyl derivatives was analyzed in the same mannner, a single peak was observed. These data suggested that the structure of the side-chain moiety of **7** was 12-hydroxydodecanoic acid.

Compound **8** was obtained as a white powder (16.5 mg). The molecular formula, $C_{38}H_{58}O_9$, was established from positive ion FABMS at *m/z* 659 $[M + H]^+$ and negative ion FABMS at *m/z* 657 $[M - H]^-$. Negative ion FABMS of **8** showed the appearance of a fragment peak at *m/z* 433 $[M - 225]^-$, which was identical with that observed for compound **7**, again indicating the presence of a bufadienolide genin in compound **8**. ^{1}H and ^{13}C NMR spectra also indicated that this compound was a bufadienolide derivative. The ^{1}H−^{1}H COSY and ^{13}C−^{1}H COSY spectra of compound **8** provided evidence that the structure of the genin moiety in **8** was identical with that of **7**: 11α,19-dihydroxytelocinobufagin. The chemical shifts of protons and carbons are listed in Tables 1 and 2. The signal of the proton on H-3 was shifted to a lower field (5.11 ppm) than that of the genin, as had been observed with compound **7**.

In the ^{13}C NMR spectrum, 14 signals were observed in addition to those of the genin and were identified as emanating from a side chain conjugated to the C-3 position. A carbon appearing at 174.89 ppm was assigned to an ester carbon, and two carbon signals appearing at 130.68 and 131.07 ppm were assigned to methine signals (−CH═CH−), while the other 11 carbons appeared as methylene signals in the DEPT spectrum.

In the $^{13}C-^{1}H$ COSY spectrum, a hydroxy methylene signal appearing at 63.04 ppm was correlated with the signal of two protons appearing at 3.54 ppm (H-14′, td). This proton signal was correlated with the signal of two protons appearing at 1.54 ppm (H-13′), and the H-13′ protons were correlated with the two protons at 1.35 ppm (H-12′) in the $^{1}H-^{1}H$ COSY spectrum. These results suggested a partial structure of $-C^{12'}H_2-C^{13'}H_2-C^{14'}H_2$-OH. The methylene proton signal at 2.34 ppm (H-2′), which was correlated with an ester carbon signal at 174.89 ppm (C-1′) in the HMBC spectrum, was correlated with a methylene proton signal at 1.63 ppm (H-3′), and the H-3′ proton signal was correlated with a methylene signal at 1.34 ppm (H-4′). These data suggested that a partial structure of the side chain was ($-OC^{1'}O-C^{2'}H_2-C^{3'}H_2-C^{4'}H_2-$). Moreover, the signal of two methine protons at 5.36 ppm was correlated with the signal of two pairs of methylene protons at 2.05 ppm, which were, in turn, correlated with the signal from two pairs of methylene protons at 1.37 ppm. These data suggested the presence of ($-CH_2-CH_2-CH═CH-CH_2-CH_2-$) in the side chain. The remaining methylene proton signal at 1.35 ppm was assigned to H-11′.

These NMR data, together with the MS data, suggested that the structure of the side-chain moiety of **8** was an ester of 14-hydroxytetradecenoic acid and that the position of the double bond was between C-7′ and C-8′ or between C-8′ and C-9′. To determine the position of the double bond, compound **8** was derivatized with dimethyl disulfide (DMDS). The EIMS spectrum of the DMDS derivatives of **8** showed the appearance of fragment ion peaks at *m/z* 336, 175, 161, and 157. Thus, the position of the double bond was determined to be between C-7′ and C-8′. Therefore, the structure of compound **8** was determined to be 11α,19-dihydroxytelocinobufagin-3-(14-hydroxy-7-tetradecenoic acid) ester. The stereostructure between C-7′ and C-8′ was determined from the chemical shift of C-6′ and C-9′ in the HMBC spectrum. The signals at 28.15 ppm (C-6′) and 28.02 ppm (C-9′) were correlated with the two methine protons at 5.36 ppm (H-7′,H-8′), suggesting the *Z* configuration.

Compound **9** was obtained as a white powder (3.7 mg). The molecular formula, $C_{38}H_{60}O_9$, was established from positive ion FABMS at *m/z* 661 $[M + H]^+$ and negative ion FABMS at *m/z* 659 $[M - H]^-$. The ^{1}H NMR and ^{13}C NMR spectra of **9** were similar to those of **7**. Based on the $^{1}H-^{1}H$ COSY and $^{13}C-^{1}H$ COSY and HMBC spectra of **9**, the genin was determined to be 11α,19-dihydroxytelocinobufagin, as had been determined with **7** and **8**. The signal of the proton on H-3 was shifted to a lower field (5.11 ppm), as had been the case with compound **7**. However, in the ^{13}C NMR spectrum, 14 signals were observed other than those of the genin. From these data and from data obtained by MS, the structure of **9** was that of another novel bufadienolide conjugate. The chemical shifts of protons and carbons were similar to those of **7**, as listed in Tables 1 and 2. In the $^{13}C-^{1}H$ COSY spectra, a tertiary carbon signal at 174.97 ppm and a hydroxy methylene signal at 63.09 ppm were observed, as seen in **7**. In the $^{1}H-^{1}H$ COSY spectrum, the relationship between the pairs of signals at 2.33 ppm (H-2′) and 1.63 ppm (H-3′), 1.63 ppm (H-3′) and 1.33 ppm (H-4′), 3.53 ppm (H-14′) and 1.53 ppm (H-13′), and 1.53 ppm (H-13′) and 1.34 ppm (H-12′), were the same as those of in **7**. In contrast with **7**, however, eight carbon signals appearing between 30 and 31 ppm were correlated with the methylene proton signal at 1.29 ppm. These data suggested that the side chain of compound **9** was the ester of 14-hydroxytetradecanoic acid. Therefore, the structure of compound **9** was determined to be 11α,19-dihydroxytelocinobufagin-3-(14-hydroxytetradecanoic acid) ester.

The abilities of **7**−**9** to inhibit the enzymatic activity of dog kidney Na^+,K^+-ATPase were measured. The 50% inhibitory concentrations (IC_{50} values) of compounds **7**, **8**, and **9** were 0.12 μM, 0.10 μM, and 0.10 μM, respectively. The IC_{50} value of 11α,19-dihydroxytelocinobufagin, which was the common genin of **7**−**9**, was 0.12 μM. These results indicate that the three novel bufadienolide esters described in this report are potent inhibitors of Na^+,K^+-ATPase, exhibiting inhibitory potencies comparable to that of their common genin.

We have previously demonstrated the presence of novel unconjugated bufadienolides in the ovaries[14] (and, to a considerably lesser extent, in the skin)[7] of *B. marinus*. These bufadienolides are potent inhibitors of Na^+,K^+-ATPase[14] but differ from marinobufagin and telocinobufagin, the major unconjugated bufadienolides of *B. marinus* skin and venom,[19] in that the ovarian bufadienolides are polyhydroxylated.[14] In the current study, we have demonstrated that at least one of the polyhydroxylated bufadienolides of *B. marinus* ovary is also present in conjugated form. In addition, we have demonstrated that the bufadienolide conjugates of *B. marinus* ovary are also novel compounds, that is, esters of hydroxylated monocarboxylic acid. In contrast, the well-characterized bufadienolide esters present in toad skin and venom are principally arginyl suberate esters (bufotoxins), with smaller amounts of bufadienolide dicarboxylic acid hemiesters (largely hemisuberates).[3,4] Like the previously reported unconjugated, polyhydroxylated ovarian bufadienolides,[14] the hydroxylated carboxylic acid esters identified in this study were potent inhibitors of Na^+,K^+-ATPase ($IC_{50} = 0.1$ μM).

The localization of the bufadienolide fatty acid esters in the toad ovary was not determined in the current study, and, thus, it is not known whether these compounds are present in the oocytes and/or the ovarian follicles. Analysis of extruded eggs has not yet been possible because of our inability to obtain ovulating female toads during the most recent annual toad breeding season that followed the identification of the esterified compounds. Fatty acid esters of steroid hormones, such as estradiol, androsterone, pregnenolone, and corticosterone have been detected in numerous mammalian tissues and body fluids,[20−22] including ovarian follicular fluid,[21,23] but the function of these fatty acid esters, like the function of bufadienolide fatty acid esters, remains unknown.[22]

It is not known whether the ovarian bufadienolide fatty acid esters are synthesized in the ovary or elsewhere in the toad. Furthermore, the biological role of the fatty acid esters of bufadienolides, like the biological role of unconjugated bufadienolides and of other bufadienolide conjugates in other toad tissues and body fluids, is not known at this time. The developing amphibian embryo contains considerable Na^+,K^+-ATPase activity, and it has been suggested that this enzyme may play an important role in oocyte activation (during ovulation and fertilization) and

in the formation of blastocoele fluid.[24] Thus, it seems reasonable to speculate that the ovarian bufadienolide esters described in this report may be present in toad oocytes and may modulate embryonic Na^+,K^+-ATPase activity, perhaps until the developing embryo is able to synthesize its own bufadienolides. Further studies will be required to determine whether the Na^+,K^+-ATPase of the early toad embryo is sensitive to inhibition by bufadienolides and other DLCs. Most adult *B. marinus* tissues studied to date (skin, heart, bladder, and kidney) contain Na^+,K^+-ATPase molecules that are quite resistant to digitalis and related inhibitors (Ki = 35−1600 μM),[25−28] but we have recently described a digitalis-sensitive Na^+,K^+-ATPase (Ki = 0.02 μM) in the brains of adult toads.[29] Such a sensitive Na^+,K^+-ATPase may also be present in the toad oocyte or embryo, perhaps in the developing central nervous system. Thus, it seems possible that the bufadienolides and their fatty acid esters of toad ovary may play a useful biological role in the development of the toad oocyte or in the differentiation of the fertilized egg.

Experimental Section

General Experimental Procedures. Preparative HPLC was performed on an Applied Biosystem (Tokyo, Japan) 151A separation system, using a reversed-phase column (Capcell pak C18, 15 × 250 mm, Shiseido Co., Tokyo, Japan). Analytical HPLC was performed, on a JASCO (Tokyo, Japan) Intelligent gradient system, using reversed-phase C18 columns (Capcell pak C18, 4.6 × 250 mm, Shiseido Co., and an L-column ODS, 4.6 × 150 mm, Chemicals Inspection and Testing Institute, Japan). Melting points were obtained on a Yanagimoto micro melting point (MS−S3) apparatus. UV spectra were measuerd with a JASCO UVDEC-610C. Optical rotations were measured in MeOH with a JASCO DIP-140 digital polarimeter. The concentration units are expressed in g/mL. IR spectra were recorded on Herschel FT/IR-420+Micro20 IR spectrometer (JASCO, Tokyo). FABMS and EIMS were performed on a JEOL JMS−DX300 mass spectrometer (JEOL, Tokyo) with a glycerine matrix at 3 kV. HRFABMS were performed on a JMS HX 110A mass spectrometer (JEOL, Tokyo). NMR spectra were recorded using tetramethylsilane as an internal standard on a GE Omega 600 MHz NMR spectrometer (Shimazu, Kyoto) and on a JASCO GX 400 spectrometer. Abbreviations used: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad. Data processing was usually carried out on a SUN4 workstation (SPARC Station 330, C. Itoh Techno Science Co., Ltd., Tokyo). Gas chromatography was performed on a GC-14A instrument (Shimadzu, Kyoto) equipped with a capillary column (0.32 mm i.d. × 50 m, Gasukuro Kogyo Inc., Tokyo) coated with 100% silicone Neutrabond-1. ATPase activity was measured on 96-well flat-bottom microtiter plates (Iwaki Glass, Chiba, Japan), using an EL340 Bio Kinetics Reader (Bio-Tek Instruments, Winooski, VT). 12-Hydroxydodecanoic acid was obtained from the Aldrich Chemical Co. (Milwaukee, WI).

Female toads (*B. marinus*, *n* = 182) purchased from National Reagents (Bridgeport CT) were sacrificed by pithing according to a protocol approved by the Columbia University Health Sciences Institutional Animal Care and Use Committee. The ovaries were removed and stored at −80 °C until use. Na^+,K^+-ATPase from dog kidney (in 50% glycerol) was purchased fron the Sigma Chemical Company (St. Louis, MO).

Preparative reversed-phase HPLC was performed at room temperature on a 15 × 250 mm C18 column, employing a 30-min linear gradient elution from (A) 30% MeCN, containing 0.1% trifluoroacetic acid (TFA) to (B) 95% CH_3CN, also containing 0.1% TFA (A/B = 90/10 to 0/100). The flow rate was 6.0 mL/min. The UV absorbance of the effluent at 300 nm was monitored, and fractions with 300-nm absorbance were collected and lyophilized.

Analytical HPLC was performed on reversed-phase C18 columns, employing a linear gradient for 30 min from H_2O to 70% CH_3CN (both containing 0.1% TFA). The purity of compounds isolated by analytical HPLC was analyzed by determining that purified compounds were eluted as single peaks under different chromatographic conditions; purity was further confirmed by monitoring the 3D UV spectrum of column effluents, using a multichannel UV detector (JASCO).

GC Analysis. Compounds were dissolved in 50 μL of 6N HCl in ampules, and samples were hydrolyzed at 110 °C for 10 h. The hydrolysates were transferred to vials and dried in a centrifugal evaporator *in vacuo* at room temperature. To each vial, 150 μL of *n*-butyl alcohol containing 10% HCl was added, and the specimen was heated at 80 °C for 1 h. After drying in the centrifugal evaporator in vacuo, butyl derivatives were dissolved in 100 μL EtOAc. The solution (1 μL) was injected by the splitless method into a gas chromatograph. The column was heated under the conditions of a linear gradient system from 50 °C to 185 °C from 0 to 30 min. After 3 min at 185 °C, the gradient was continued to 250 °C at 68 min and, finally, to 255 °C at 90 min. The pressure of the carrier gas, N_2, was 0.25 kg/cm^2, and air pressure was 0.5 kg/cm^2. The flow rate was 55 mL/min. The eluate was detected with a flame ionization detector at 290 °C.

DMDS Adducts. To determine the position of double bonds in hydroxylated fatty acids, DMDS adducts[30,31] were prepared as follows: 100 μL of hexane, 100 μL of DMDS, and 20 μL of iodine (59.1 mg/mL) in Et_2O were added to compound **8** (80 nmol) in a vial, and then the vial was sealed and heated at 50 °C for 48 h. After the reaction, 200 μL of hexane was added, followed by 200 μL of 50% sodium thiosulfate solution; the addition of sodium thiosulfate resulted in the yellowish color (due to iodine) being cleared into the aqueous phase.[31] The hexane layer was removed, taken to dryness in a centrifugal evaporator *in vacuo*, and then dissolved in MeOH and subjected to EIMS.

Isolation of Metabolites. 11α,19-Dihydroxytelocinobufagin-3-(12-hydroxydodecanoic acid) ester (7): mp ca. 250 °C (dec); $[\alpha]^{23}_D$ +3.0° (*c* 1.06, MeOH); UV (EtOH) λ_{max} 300 nm; IR (KBr) ν_{max} 3440, 2928, 1710, 1395 cm^{-1}; HRFABMS *m/z* 633.4001 (calcd for $C_{36}H_{57}O_9$, 633.4006); ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively.

11α,19-Dihydroxytelocinobufagin-3-(14-hydroxy-7-tetradecenoic acid) ester (8): mp 98−102 °C; $[\alpha]^{23}_D$ +3.2° (*c* 0.83, MeOH); UV (EtOH) λ_{max} 299 nm; IR (KBr) ν_{max} 3413, 2929, 1712, 1415 cm^{-1}; HRFABMS *m/z* 659.4169 (calcd for $C_{38}H_{59}O_9$, 659.4163); ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively. EIMS of DMDS derivatives *m/z* 336 $[HOOC(CH_2)_5CH(SCH_3)CH(SCH_3)(CH_2)_5CH_2OH]^+$, 175 $[HOOC(CH_2)_5CH{=}SCH_3]^+$, 161 $[H_3CSCH(CH_2)_5CH_2OH]^+$, 157 $[OCCH(CH_2)_4CH{=}SCH_3]^+$.

11α,19-Dihydroxytelocinobufagin-3-(14-hydroxytetradecanoic acid) ester (9): mp 158−163 °C; $[\alpha]^{23}_D$ −13.16° (*c* 0.19, MeOH); UV (EtOH) λ_{max} 300 nm; IR (KBr) ν_{max} 3357, 3070, 1665, 1348 cm^{-1}; HRFABMS *m/z* 661.4310 (calcd for $C_{38}H_{61}O_9$, 661.4319); ^{1}H and ^{13}C NMR data, see Tables 1 and 2, respectively.

Inhibition of Na^+,K^+-ATPase Enzymatic Activity. The Na^+,K^+-ATPase inhibitory activity of purified compounds was determined by a microtiter method, as described previously.[7] Multiple concentrations of inhibitors in 50% EtOH were added in triplicate to individual wells of 96-well microtiter plates. After removal of EtOH by evaporation, specimens were resuspended at 37 °C in a final volume of 250 μL of a reaction mixture, which was identical with that used previously,[11] except that the Na^+,K^+-ATPase employed (5 μg/250 μL) was derived from dog kidney. After a 10-min incubation at 37 °C, ATPase activity was determined in the ELISA reader by monitoring the oxidation of β-NADH, as reflected in a decrease in 340 nm absorbance measured at 3 min intervals over a 30-min period, during which time enzymatic activity was linear. Ouabain and bufalin at final concentrations of 10^{-4} M completely inhibited ATPase activity, so that ouabain- and bufalin-insensitive activity was essentially zero. Results were calculated as the concentrations that produced 50% inhibition of

enzymatic activity (IC_{50}), as measured during the final 15 min of incubation with enzyme.

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