MARINOIC ACID, A NOVEL BUFADIENOLIDE-RELATED SUBSTANCE IN THE SKIN OF THE GIANT TOAD, *Bufo marinus*

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We found a novel substance, 3β-hydroxy-11,12-seco-5β,14β-bufa-20,22-dienolide-11,14-olide-12-oic acid (**1**), which we called marinoic acid, in the skin of the toad, *Bufo marinus*. The structure was established from NMR and MS data. Like bufadienolides, marinoic acid contained an A/B ring structure in the *cis* configuration and a D/α-pyrone ring structure, but the structure of the C ring differed considerably from that of bufadienolides. Marinoic acid exhibited biological activity, as demonstrated by inhibition of Na^+, K^+-ATPase enzymatic activity, and by inhibition of [^{3}H]ouabain binding to the digitalis receptor site on Na^+, K^+-ATPase, although marinoic acid was a less effective inhibitor than typical bufadienolides. Although marinoic acid cannot be classified as a bufadienolide, its chemical structure and its Na^+, K^+-ATPase inhibitory activity suggest that it is bufadienolide-related.

KEY WORDS marinoic acid; bufadienolide; Na^+, K^+-ATPase; ouabain binding; skin; *Bufo marinus*

Many bufadienolides have been isolated from toads of the genus *Bufo*, and the structures of these bufadienolides have been determined.[1] Shimada *et al.* examined the structure-activity relationships of bufadienolides and their derivatives,[2] and, recently, Lichtstein *et al.* discussed the possible physiological role of endogenous digitalis-like substances (EDLSs) in the toad.[3] We have instituted studies of EDLSs in the tissues of *Bufo marinus* and have described novel bufadienolides in the eggs[4] and the bile[5] of this toad. The current report describes a novel bufadienolide-related compound, the structure of which is considerably different from the structure of typical bufadienolides found in the skin of this toad.

One hundred eighty-two toads were sacrificed according to a procedure approved by the Institutional Animal Care and Use Committee. The skins were extracted with ethanol, and the extract was concentrated to 200 ml. The solution was subjected to column chromatography on silica-ODS, employing stepwise increasing concentrations of CH_3CN in water, including 0.1% trifluoroacetic acid in the eluant. The compounds which were eluted with 30% CH_3CN were purified by repetitive preparative HPLC with a reversed phase column (CAPCELL PAK C18, 15×250 mm, Shiseido Co.) employing a water-50% CH_3CN gradient method with/without TFA. All compounds were lyophilized and subjected to analytical HPLC (CAPCELL PAK C18, 4.6×250 mm, Shiseido Co.) with a multichannel detector, and to ^{1}H-NMR spectrometry. Many known bufadienolides and bufotoxins were isolated and identified, as well as a novel compound described here.

A novel compound was purified, possessing an α-pyrone ring characteristic of bufadienolides. This compound was stable in 50% CH_3CN. The chemical structure was determined on the basis of two-dimensional NMR spectrometry and MS. The structure of this compound was determined to be 3β-hydroxy-11,12-seco-5β,14β-bufa-20,22-dienolide-11,14-olide-12-oic acid (**1**), as shown in Fig. 1. We propose to call this novel compound **1** marinoic acid.

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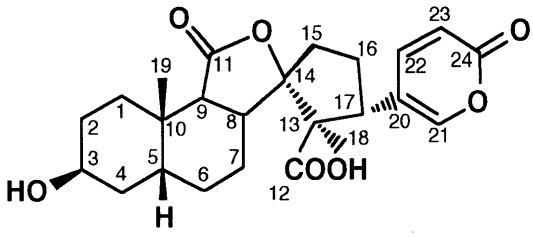
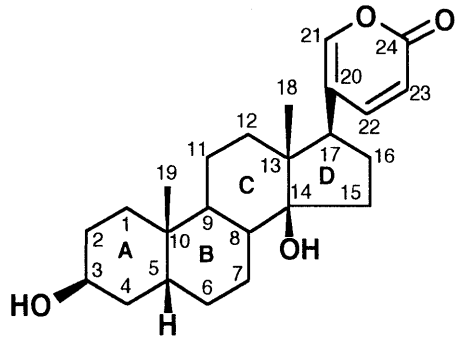
TABLE 1. ^{1}H- and ^{13}C- NMR Spectral Data for Marinoic Acid
(in Methanol-d_4, δ Values, J=Hz)

Position	H	C
1	1.57 (1H,m)	30.15
	2.22 (1H,m)	
2	1.62 (1H,br t,J=14.5)	29.49
	1.57 (1H,m)	
3	4.04 (1H,br s,1/2J=8)	67.78
4	1.83 (1H,br t,J=14.1)	35.27
	1.37 (1H,br d,J=14.1)	
5	1.76 (1H,br d,J=14.1)	37.63
6	1.32 (1H,br d,J=14.1)	29.42
	1.82 (1H,br t,J=13.6)	
7	2.15 (1H,br qd,J=14.1,3.3)	22.38
	1.76 (1H,br d,J=14.1)	
8	2.45 (1H,td,J=14.1,3.3)	50.53
9	3.13 (1H,d,J=14.1)	42.19
10		36.90
11		178.41
12		178.92
13		58.19
14		96.49
15	2.22 (2H,m)	39.00
16	1.94 (1H,m)	26.45
	2.02 (1H,m)	
17	3.67 (1H,br t,J=9.5)	46.22
18	1.08 (3H,s)	18.57
19	1.01 (3H,s)	23.57
20		120.65
21	7.58 (1H,br d,J=2.5)	153.67
22	7.72 (1H,dd,J=9.8,2.5)	148.85
23	6.28 (1H,dd,J=9.8,0.9)	116.44
24		164.89

δ in ppm from tetramethylsilane (TMS).



marinoic acid (**1**)



bufalin

Fig. 1. Structures of Marinoic Acid and Bufalin

Marinoic acid (**1**) was obtained as a white powder (5.2 mg), mp 97.5 - 99.5 ℃, $[\alpha]_D^{20}$ +22.76° (c=0.50, MeOH). **1** showed IR absorption at 3408 cm^{-1} (OH), 2700-2500 cm^{-1} (br, COOH), 1714 cm^{-1} (COO), 1740cm^{-1} (α-pyrone). The molecular formula, $C_{24}H_{30}O_7$ was established from positive ion FAB-MS at m/z 431 $(M+H)^+$ and negative ion FAB-MS at m/z 429 $(M-H)^-$. The electron impact (EI)-MS of **1** exhibited the decarboxylate ion peak at m/z 386 ($C_{23}H_{30}O_5$) based on high-resolution EI-MS (Found, 386.20978; Calcd for 386.20940), and the fragment ion peak at m/z 175 ($C_{11}H_{11}O_2$).

All proton and carbon signals of **1** were compared with the signals of reference bufadienolides[4-6] and assigned as shown in Table I by the ^{1}H-^{1}H-cosy spectrum, the ^{13}C-^{1}H-cosy spectrum, the ^{1}H-detected heteronuclear multiple-quantum coherence (HMBC) spectrum, and the nuclear Overhauser and exchange spectroscopy (NOESY) spectrum. In the ^{1}H-^{1}H cosy and ^{13}C-^{1}H cosy spectra of **1**, correlations of protons at the moieties of the A/B ring and the D/α-pyrone ring were the same as those of bufalin. In the HMBC spectrum, the H-9 proton was correlated with C-8, C-10 and C-19, and the H-17 proton was correlated with C-16, C-20, C-21, C-22, C-13, C-18, as shown in Fig. 2(a). Moreover, the configuration of the H-3 proton was in an equatorial orientation, judging from coupling constants (1/2J = 8 Hz). In the same manner, the relationship between H-8 and H-9 was *trans* diaxial ($J_{8,9}$ = 14.1 Hz). In the NOESY spectrum [Fig. 2(b)], cross peaks were observed between H-2α, H-4α and H-9 of the A/B ring. H-18 was correlated with H-21 and H-22 of the D/α-pyrone ring. Hence the structures of the moieties of the A/B ring and the D/α-pyrone ring of **1** were similar to those of bufalin. However, proton signals due to H-11 and H-12 of the C ring of bufalin were not detected, while two carbonyl carbons (178.41 ppm, 178.92 ppm) appeared. The C-14 signal was shifted to a lower field than that of bufalin. The hydroxy proton signal at the C-14 position did not appear in DMSO-d_6. These data suggested that the structure of the C ring was completely different from that of bufalin. In the HMBC spectrum, the H-15 proton was correlated with the C-8 and

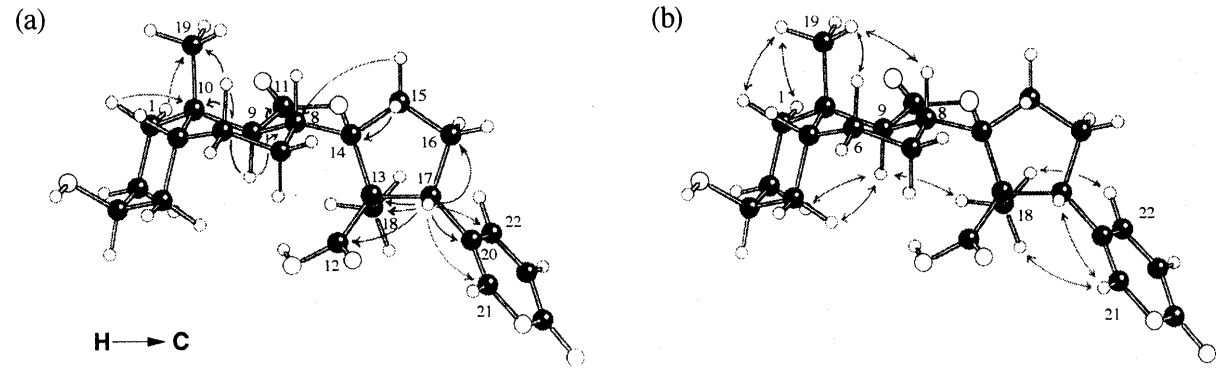


Fig. 2. HMBC (a) and NOESY (b) Correlations for Marinoic Acid

C-14 carbons; therefore, the B and D rings of **1** were connected between the C-8 and C-14 positions. The H-9 and the H-17 protons were correlated with one carbonyl carbon at 178.41 ppm (C-11) and with the other carbonyl carbon (C-12), respectively, as shown in Fig. 2(a). In addition, the NOE between H-18 and H-9, which was present in **1**, as shown in Fig. 2(b), but which is not observed in bufalin, strongly supported the proposed structure. On the basis of these data, the structure of **1** was determined to be a γ-lactone ring between C-11 and C-14, which replaces the C ring present in typical bufadienolides such as bufalin, as shown in Fig. 1. Comparing the stereostructure of **1** with that of bufalin, the D and α-pyrone rings of **1** were rotated to a right angle to the plane of the B and γ-lactone (C) rings. Thus, this novel bufadienolide-related compound can be classified into a different category from that of a typical bufadienolide.

The ability of **1** to inhibit the enzymatic activity of sheep kidney Na^+, K^+-ATPase[7] (IC_{50} = 3.7 μM) was somewhat less than that of one bufadienolide, marinobufagin (IC_{50} = 1.0 μM), and was considerably less than that of the more potent bufadienolide, bufalin (IC_{50} = 16 nM). The relative abilities of these three compounds to inhibit the binding of [^{3}H]ouabain to Na^+, K^+-ATPase[7] were in the same order of potency (marinoic acid: IC_{50} = 3.3 μM, K_D = 0.3 μM; marinobufagin: IC_{50} = 0.8 μM, K_D = 0.06 μM; bufalin: IC_{50} = 15 nM, K_D = 1.2 nM). Although a *cis* C/D ring configuration is present in bufalin and other potent bufadienolides, the fact that **1**, which lacks a steroidal ring structure, is an effective inhibitor of Na^+, K^+-ATPase suggests the possibility that the stereochemistry of the C/D ring junction may be of less critical importance than the stereochemistry of the *cis* A/B ring junction or of the δ-lactone ring.[5,8] Further studies of the relationship between biological activity and the stereochemistry of the C/D ring structure may be useful in the design of new synthetic derivatives of cardiotonic steroids.

This study has provided no information concerning the biosynthetic origin or metabolic fate of marinoic acid. Studies of the biosynthesis and metabolism of marinoic acid will be required to determine the relationship of marinoic acid to other toad EDLSs. To confirm the structure of marinoic acid, crystallization for X ray analysis is now in progress.

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