CONFORMATIONAL PREFERENCE OF TWO TOAD POISON BUFADIENOLIDES, CINOBUFAGIN AND BUFOTALIN

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Dedicated to Dr Jan Fajkos on the occasion of his 75th birthday.

The solution state conformations of pyran-2-one ring (E ring) of two toad poison bufadienolides, cinobufagin and bufotalin, were analyzed by using NOE experiments at low temperatures and molecular dynamics simulation study. The rotation of E ring of the two bufadienolides, was found to be affected by the 14β,15β-epoxy and 16β-acetoxy groups and to be restricted at low temperatures. In the experimental, cinobufagin and bufotalin were isolated from Ch'an Su by the new chromatographic methods using the hydrophobic gel sephadex LH-20 and HP-cellulofine as carriers.

Key words: Steroids; Bufadienolides; Cinobufagin; Bufotalin; Conformation analysis; NMR spectroscopy; Semiempirical calculations; *Ab initio* calculations; Molecular mechanics.

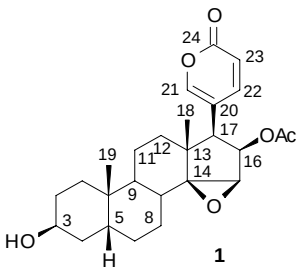
The Chinese drug Ch'an Su, called "Senso" in Japan, is a product of the skin gland of toads, *Bufo bufo gargarizans*, and related species, has been traditionally used as a cardiotonic, diuretic, anodyne and hemostatic agent. Its major effective components, generally called, "toad poison" or "bufadienolides", are steroids that have steroidal A/B *cis* and C/D *cis* structure with a pyran-2-one ring (E ring) at C17-position and exhibit a range of biological activities, such as cardiotonic, blood pressure stimulating, respiration, and antineoplastic activities. Of the bufadienolides, resibufogenin is now being used as a cardiotonic drug, and bufalin has recently been reported to have strong surface anesthetic activity[1] and cytotoxic and differentitation-apoptosis activity on murine

leukemia HL-60 cells[2]. On the other hand, the isolation of these bufadienolides from natural sources in our laboratory was very simplified by the use of hydrophobic gel such as sephadex LH-20 or HP-cellulofine as the carrier of chromatography[3].

Also, recently, the 3D structural features that are common to the active bufadienolides were characterized so that studies of 3D quantitative structure–activity relationships (QSAR) by the Comparative Molecular Field Analysis (CoMFA) and of correlation of steric and electrostatic fields of molecules with their activities[4] are now made possible.

Reports on conformational analysis of steroids, such as bufadienolides and cardenolides are few. Previously, we reported on the ring conformations of two bufadienolides, bufarenogin and Ψ-bufarenogin[5]. As regards the stereochemistry of D and E ring linkage of bufadienolides and cardenolides, if E ring rotates freely, with two energy minima at torsion angles differing about 180° as the lactone ring is planar[6].

In the present study, conformational analysis of the lactone ring (E ring) of two bufadienolides, cinobufagin[7] (**1**, 16β-acetyloxy-14,15β-epoxy-3β-hydroxy-5β,14β-bufa-20,22-dienolide) and bufotalin[7] (**2**, 16β-acetoxy-3β,14-dihydroxy-5β,14β-bufa-20,22-dienolide), was performed by using the temperature-varying NMR and computational methods. The stereochemistry of E ring is considered to be closely related to their biological activities.



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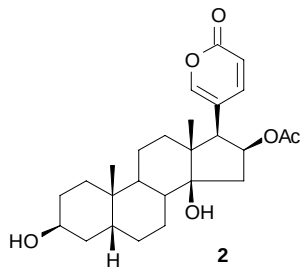

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EXPERIMENTAL

General Details. Optical rotations were measured with a JASCO DIP-4 polarimeter and the $[\alpha]_D$ values are given in 10^{-1} deg cm^2 g^{-1}. Mass, UV and IR spectra were taken with a VG-Aotospec spectrometer, a Hitachi 557 spectrophotometer and a JASCO A-302 spectrophotometer, respectively. TLC was performed on precoated Kieselgel 60 P254 (Merck). NMR spectra were recorded on a Bruker DRX-5(X) spectrometer. For the homo- and hetero-nuclear NMR measurements solutions of 5 mg of bufadienolides in 0.5 ml of $CDCl_3$ (degassed) was used. The spectra were recorded at 300 K. Phase sensitive NOESY experiments were made with a mixing time of 1 s. The value of the delay used to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 ms and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 ms. NMR coupling constants (*J*) are given in Hz.

Materials. Ch'an Su was obtained in a Hong Kong folk-medicinal market. Cinobufagin and bufotalin were isolated from Ch'an Su by the following procedure.

Isolation of Cinobufagin and Bufotalin

Ch'an Su (50 g) was three times extracted with CH_2Cl_2 (100 ml) at room temperature for three days each to give 11.5 g of the crude extract. The extract was chromatographed on Sephadex LH-20 column (850 mm × 50 mm) with hexane/CH_2Cl_2/MeOH (4 : 5 : 1) by the careful slow dropping elution using a fraction collector (1 000 drops for each fraction). The chromatography provided both the cinobufagin rich fraction (480 mg) and the bufotalin rich fraction (670 mg). The former was chromatographed on HP-cellulofine column (500 mm × 19 mm) with hexane/AcOEt/MeOH (4 : 5 : 1) by the similar technique as above to give cinobufagin[8] (**1**, 332 mg, m.p. 215–217 °C) and resibufogenin[9] (144 mg, an amorphous solid), respectively. The latter was chromatographed on HP-cellulofine column (600 mm × 24 mm) with hexane/toluene/MeOH (3 : 2 : 1) to give bufotalin[10,11] (**2**, 227 mg, m.p. 221–223 °C) and bufalin[10] (431 mg, m.p. 238–241 °C), respectively. These bufadienolides were found identical with the authentic specimens.

Molecular Simulation

Computer modeling and all calculations were carried out by using the MACROMODEL program (Version 4.5) on an IRIS 4D computer. Force field evaluation was performed with the MM2* force field[12]. The dielectric constant (ε) was assumed to be equal to the interatomic distances (r) as $\varepsilon = kr$. Solvent molecules were not included in the calculations. The extended cut off distances employed were ±8 for van der Waals, ±20 for charge/electrostatics and ±10 for charge/multipole electrostatics. Each MC search was carried out by using the Pseudo Monte Carlo routine in MACROMODEL. The structures, which were obtained within 10 kcal/mol of the lowest energy conformer, were minimized by the use of molecular mechanics calculation. The each energy minimum conformer corresponding to **1** and **2** form at E ring of cinobufagin and bufotalin was finally geometry minimized by PM3 method[13], followed by HF/STO-3G level. These calculations were conducted by using a program package SPARTAN 4.0 (Wavefunction Inc. California).

RESULTS AND DISCUSSION

*Assignments of ^1H and ^{13}C NMR Signals of Cinobufagin **1** and Bufotalin **2***

^1H NMR spectra, of bufadienolides are complex and a complete assignment of signals is extremely difficult, because many signals appear in a narrow range of high magnetic field. However, for the conformational analysis of bufadienolides, assignment of signals is the first step to be cleared, which is to be made by the recently developed 2D NMR techniques (Table I). The definite assignment of signals allowed us to perform various conformational analysis of the two bufadienolides, cinobufagin **1** and bufotalin **2**.

*Temperature Dependence of NMR Spectra of Cinobufagin **1** and Bufotalin **2***

In the ^1H NMR spectrum of cinobufagin (**1**) in $CDCl_3$ at room temperature H-21 and H-22 gave broad signals. On the other hand, the other protons gave sharp signals (Table I).

TABLE I
^1H and ^{13}C NMR signal assignments (δ in ppm, *J* in Hz) of cinobufagin (**1**) and bufotalin (**2**) in $CDCl_3$ at 300 K

Position	Cinobufagin		Bufotalin	
	^1H	^{13}C	^1H	^{13}C
1	1.51 (2 H, m)	29.48	1.48 (2 H, m)	29.55
2	1.54 (2 H, m)	27.90	1.51 (2 H, m)	27.87
3	4.14 (1 H, s)	66.70	4.13 (1 H, s)	66.74
4	1.87 (2 H, m) 1.34	32.23	1.88 (2 H, m) 1.36	33.23
5	1.78 (1 H, brd)	35.89	1.77 (1 H, m)	35.87
6	1.22 (1 H, brd) 1.86	25.64	1.27 (1 H, brd) 1.89	26.37
7	0.93 (1 H, qd, 13.1, 3.9) 1.48	20.62	1.32 (1 H, m) 1.77	21.52
8	2.04	33.06	1.56	35.54
9	1.60	39.27	1.54	42.26
10	–	35.51	–	35.27
11	1.56 1.32 (1 H, qd, 13.7, 2.9)	20.93	1.42 1.19	21.06
12	1.40 (1 H, brt) 1.76 (1 H, dt)	40.05	1.28 1.56	40.40
13	–	45.21	–	49.40
14	–	72.50	–	84.37
15	3.64 (1 H, d, 0.7)	59.46	2.63 (1 H, dd, 15.6, 9.6) 1.83 (1 H, brd)	40.79
16	5.45 (1 H, dd, 9.3, 1.4)	74.75	5.52 (1 H, td, 9.0, 1.8)	73.59
17	2.79 (1 H, d, 9.3)	50.38	2.86 (1 H, d, 8.9)	57.15
18	0.81 (3 H, s)	17.26	0.77 (3 H, s)	16.46
19	0.98 (3 H, s)	23.71	0.94 (3 H, s)	23.70
20	–	116.22	–	116.92
21	7.16 (1 H, brs)	151.39	7.24 (1 H, dd, 2.5, 0.8)	150.97
22	7.91 (1 H, brs)	148.34	8.03 (1 H, dd, 9.8, 2.6)	149.23
23	6.21 (1 H, dd, 9.8, 0.7)	113.88	6.18 (1 H, dd, 9.8, 0.9)	113.07
24	–	161.74	–	162.02
1′	1.88 (3 H, s)	20.53	1.86 (3 H, s)	20.94
2′	–	170.19	–	170.06

The broad signals of H-21 and H-22 indicated that the rotation of pyran-2-one ring around the C(17)–C(20) single bond is restricted and that the rate of conformational changes in the NMR time scale due to rotation was slow enough to give signals of intermediate conformations. At 330 K, the signals of H-21 and H-22 appeared sharp, demonstrating that the conformational change of the pyran-2-one ring were rapid enough to form an equilibrium state. At 230 K, the signals of H-22 again appeared as a sharp one, indicating that one of the conformations was now predominant. Table II shows a relatively high temperature-dependence of chemical shift of H-22: the difference in the chemical shifts of H-22 at 230 and 330 K is as large as 0.18 ppm. Such large chemical shifts of H-22 signal strongly support the assumption that the rotation of pyran-2-one ring is significantly restricted and one rotamer is predominant at 230 K.

TABLE II

Chemical shift (ppm), multiplicity and coupling constants (Hz) of cinobufagin (**1**) ^{1}H NMR signals at various temperatures (K)

Temperature	H-15	H-16	H-17	H-21	H-22	H-23
330	3.63 (d, 0.7)	5.46 (dd, 9.3, 1.4)	2.78 (d, 9.3)	7.16 (d, 1.9)	7.87 (dd, 9.8, 2.2)	6.19 (dd, 9.8, 0.8)
300	3.64 (d, 0.7)	5.45 (dd, 9.3, 1.3)	2.78 (d, 9.3)	7.16 (brs)	7.89 (brs)	6.20 (dd, 9.8, 0.7)
270	3.65 (brs)	5.44 (dd, 9.3, 0.8)	2.79 (d, 9.8)	7.14 (brs)	7.99 (brs)	6.22 (dd, 9.8, 0.5)
230	3.67 (brs)	5.44 (brd, 9.3)	2.80 (brd, 9.3)	7.11 (brs)	8.05 (dd, 9.8, 1.9)	6.24 (brd, 9.8)

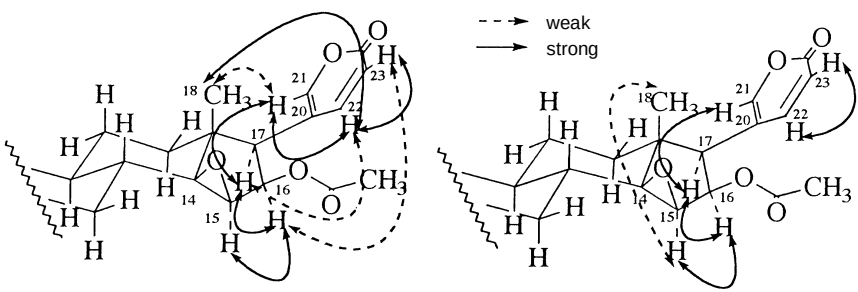

FIG. 1

Proposed conformations of cinobufagin (**1**) in $CDCl_3$ at 230 K (left) and 330 K (right). Arrows show the selected NOE relationship. At high temperature, pyran-2-one ring is considered to rotate freely

This stable conformation of cinobufagin elucidated by the NOE relationships at 230 K is shown in Fig. 1. The strong NOE enhancements between H-21 and H-17, and between H-22 and H-18 indicated that the two protons, H-21 and H-17, are on the same side of the C(17)–C(20)–C(21) plane with the conformation of C(21) and C(16) being almost antiperiplanar with O(21) and C(16) on the other sides of the C(17)–C(20)–C(21) plane. There was no NOE enhancement observed between H-22 and any of the steroidal skeleton protons at 330 K; suggesting that the pyran-2-one ring rotated freely at this temperature.

As in the case of cinobufagin (**1**), the NMR signals of all of the pyran-2-one ring protons in bufotalin (**2**) appeared as sharp signals at 330 K (data not shown), and at 230 K, the signals ascribable to H-21 and H-22 both appeared as broad singlets, which indicated the presence of a steric hindrance preventing the free rotation of pyran-2-one ring in this case.

Such a steric hindrance is considered to be caused by the acetyl substituent at C(16), because in the case of the bufadienolides having no acetyl group at C(16) the corresponding signal broadening was not observed even at 230 K. In cinobufagin (**1**), the epoxide group may also contribute to preventing puckering of the five-membered D ring.

Molecular Simulation

To obtain the conformation of cinobufagin (**1**) at 230 K, conformational search was conducted by using of systematic pseudo Monte Carlo (MC) simulation[13]. Each conformation generated by the MC calculation was minimized by the use of molecular mechanics calculation of MM2* force field implemented in MacroModel/Batchmin (Version 4.5)[14]. The MC procedure of Still and Goodman[13] was performed in a pseudosystematic way, by changing the torsion angles of the C–C and C–O bonds in the five- and six-membered rings, acetyl group and E ring chain in the range of 0–180°. A total of 5 000 MC steps were performed to produce 26 conformers. After the MC conformational search, each of the resulting conformations was subjected to the energy-minimization calculation to reduce the gradient rms to less than ±0.001 kcal/Å± mol. To eliminate possible duplicate conformations, a comparison was performed on the heavy atoms only.

Of the resulting 26 conformations, 6 appeared to be within a range of energy-deviation of 1 kcal/mol. The low energy level conformers obtained were found to belong to either of the two families differing in the stereochemistry direction of pyran-2-one ring linkage (type *I* with O(21) *exo* to C(16) and type *II* O(21) *endo* to C(16), see Fig. 2). As the lactone ring is planar, two energy minima were obtained as expected at torsion angles differing by about 180°. In each example, the difference between the two energy minima was relatively small, presumably due to the asymmetry of the lactone ring. In addition, one of the low energy conformers was almost the same as super imposable one derived from the NOE relationships reported above for the pyran-2-one ring. The geometry of the global minimum conformers in each of two groups were optimized by

the semi-empirical molecular orbital calculation (PM3) method[12], and then by HF/STO-3G level. The calculated heat of formation of each conformer is shown in Table III, indicating that concerning the energy the type *I* form of cinobufagin is more stable than the type *II*. In the case of bufarenogin, almost the same results were obtained: the type *I* form is energetically more stable than the type *II*.

These results imply that the lactone ring is not allowed to rotate freely both in cinobufagin and bufotalin. We are interested in the relation between the direction of the lactone ring and biological activity. Attempts to obtain information based on these conformational aspects are currently being made in our laboratories.

TABLE III
Heat of formation (a.u.) of stable conformers *I* and *II* of cinobufagin and bufotalin *I* and *II* forms at E ring calculated by HF/STO-3G

Conformer	Cinobufagin (**1**)	Bufotalin (**2**)
I	–1 435.5460223	–1 455.1429867
II	–1 435.5270508	–1 455.1218675

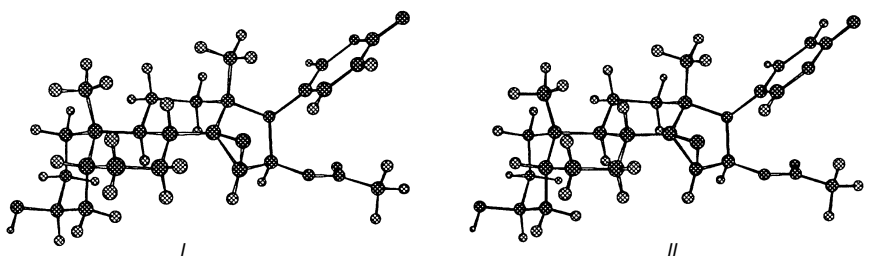

FIG. 2
Stable conformers of cinobufagin (**1**) of type *I* and type *II* forms obtained by MC and subsequent semiempirical calculations

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