CONFORMATIONAL PREFERENCE OF TWO TOAD POISON BUFADIENOLIDES, BUFARENOGIN AND Ψ-BUFARENOGIN

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Abstract - The solution state conformations of two toad poison bufadienolides, bufarenogin and ψ-bufarenogin, which are the epimers at C-12, were analyzed by using NOE experiments and molecular dynamics simulation study. The C-ring of bufarenogin with 12β-hydroxy function was shown to take a chair form, whereas that of ψ-bufarenogin with 12α-hydroxy function, a boat form.

The Chinese drug Ch'an Su, called "Senso" in Japan, is a product of the skin gland of toad *Bufo bufo gargarizans*, and related species, and has been used traditionally as a cardiotonic, diuretic, anodyne and hemostatic agent.[1] Its major effective components, generally called "the toad poison" or "bufadienolides" are steroids having a characteristic steroidal A/B *cis* and C/D *cis* structure with an α-pyrone ring at C17-position and exhibiting a range of biological activities, such as cardiotic, blood pressure stimulating, respiration, and antineoplastic activities. Of the bufadienolides, resibufogenin is now clinically used as a cardiotonic drug, and bufalin has recently been reported to have a strong surface anesthetic activity [2] and a cytotoxic and differentiation-apoptosis activities on murine leukemia HL-60 cells.[3] Recently, the 3 dimensional structural features that are common to the active bufadienolides were characterized so that studies on the 3D quantitative structure-activity relationships (QSAR) by using Comparative Molecular Field Analysis (CoMFA) and of correlation of the steric and electrostatic fields of the molecules to their activities [4] may now be conducted.

In the present work, conformational preference of C ring of the two bufadienolides, bufarenogin (**1**)[5] and ψ-bufarenogin (**2**)[5] (Figure 1) was studied by using NMR and computational methods. The C-ring conformation of these bufadienolides may be related to their biological activities.

NOE ENHANCEMENTS

In solution, steroids such as bufadienolides and cardenolides are known to take all chair ring conformation. In the case of cardenolide, ouabain, with mannopyranoside at C-3 of A ring, a conformational exchange process involving a chair/twist-boat interconversion of A ring has been observed at a low temperature.[6]

To determine the conformation of the two of bufadrenogins in solution, we conducted first homo- and heteronuclear NMR experiments, such as COSY, HMQC, HMBC and NOESY, of bufarenogin and ψ-bufarenogin to obtain a complete assignment of the ^{1}H (Table 1) and ^{13}C (see Experimental) NMR signals. Chair conformation of all of the six-membered rings of the steroid portion of bufarenogin was demonstrated by the strong NOE correlations between H-8 and H-19, H-9 and H-12, H-12 and H-15, and H-12 and H-17 (Figure 2).

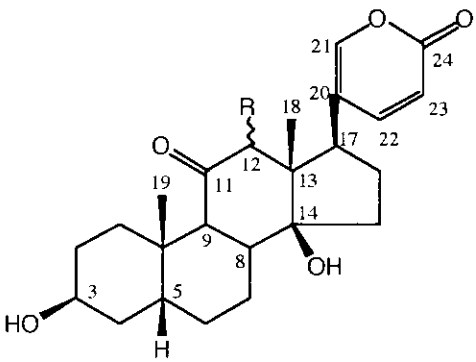
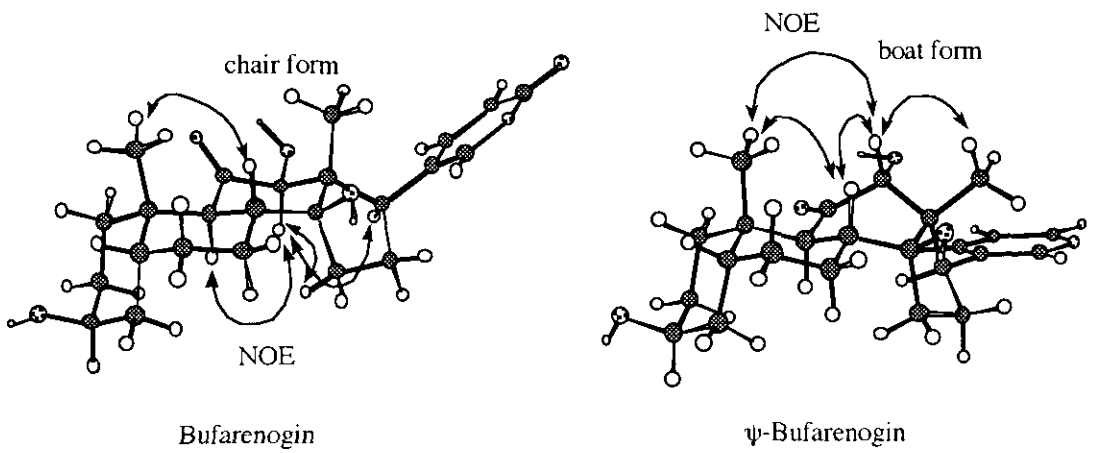


Figure 1. Bufarenogin: R=β-OH
ψ-Bufarenogin: R=α-OH

In the case of ψ-bufarenogin, NOEs were observed between H-8 and H-12, H-12 and H-19, and H-12 and H-18, which indicated a boat conformation of C ring.

Table 1. ^{1}H NMR Signal Assignments of bufarenogin and ψ-bufarenogin in $CDCl_3$ at 300 K.

position	δ_H [int. mult, J(Hz)]	
	bufarenogin	ψ-bufarenogin
1	1.23 and 2.46 (each 1H, m)	1.95 and 2.14 (each 1H, m)
2	1.58 (2H, m)	1.27 (2H, m)
3	4.34 (1H, s)	4.42 (1H, s)
4	1.65 (2H, m)	1.74 (2H, m)
5	2.22 (1H, m)	2.18 (1H, m)
6	1.45 and 1.92 (each 1H, m)	1.35 and 1.99 (each 1H, m)
7	1.77 and 2.35 (each 1H, m)	2.26 (2H, m)
8	2.43 (1H, m)	2.69 (2H, d, 3.2)
9	3.04 (1H, d, 12.6)	2.93 (1H, s)
12	4.46 (1H, s)	4.54 (1H, s)
15	2.27 and 2.47 (2H, m)	1.68 and 2.03 (2H, m)
16	1.63 and 2.35 (2H, m)	2.22 (2H, m)
17	3.58 (1H, dd, 7.2, 9.7)	2.89 (1H, s)
18	0.98 (3H, s)	1.49 (3H, s)
19	1.45 (3H, s)	1.06 (3H, s)
21	7.66 (1H, d, 1.9)	7.70 (1H, d, 1.8)
22	8.17 (1H, dd, 2.6, 9.7)	7.90 (1H, dd, 2.6, 9.5)
23	6.36 (1H, dd, 9.9)	6.32 (1H, d, 9.7)



Bufarenogin ψ-Bufarenogin

Figure 2. Possible predominant conformers of bufarenogin and ψ-bufarenogin in $CDCl_3$; arrows show selected strong NOE correlations in phase sensitive NOESY spectra.

MOLECULAR DYNAMICS SIMULATION

Molecular dynamics (MD) and molecular mechanics (MM) simulations can provide further additional information about the conformational aspects derived from the NMR studies. The MD calculation overcomes the local minima problem and previously we reported the presence of *cis/trans* forms in a cyclic peptides, RA-VII, at high-temperature by the MD calculation.[7] However, MD calculation cannot cover all the possible conformers partitioned off by various energy barriers at low temperature.

For the simulation study, an initial structure of ψ-bufarenogin was first constructed, in which all six-membered rings of the steroid portion were of the chair conformation and then the energy was minimized. By using this chair conformer as the starting structure in MD the conformational changes in the C ring were monitored by measuring the H-8 and H-12 distance (Figure 3). This calculation demonstrated a slow interconversion (50-200 psec.) of C-ring between the chair form and boat form. No conformational changes of the other steroid rings were observed. When the starting structure included C-ring of boat form, conformers having C-ring of chair form were not obtained during a 500 psec simulation. Thus, the MD calculations indicated that the boat conformation was the preferred structure for the C ring of ψ-bufarenogin, which agreed with the observation in the NMR study. In addition, the global minimum structures of the two conformers each including chair form C-ring or boat form C-ring of ψ-bufarenogin were minimized by the semi-empirical molecular orbital calculation (pm3) method.[8] The calculated heat of formation of each conformer is shown in Table 2, which indicated that the conformer with boat form C-ring of ψ-bufarenogin was energetically more stable than the conformer with chair form C-ring. In the case of bufarenogin, the reverse was observed: the conformer with chair form C ring was more stable than that with boat form C-ring.

Table 2. Heat of formation (kcal/mol) calculated by pm3 method of two stable conformers of bufarenogin and ψ-bufarenogin having C-ring of chair and boat forms.

	Bufarenogin	ψ-Bufarenogin
chair form	-251.149	-245.142
boat form	-248.990	-252.459

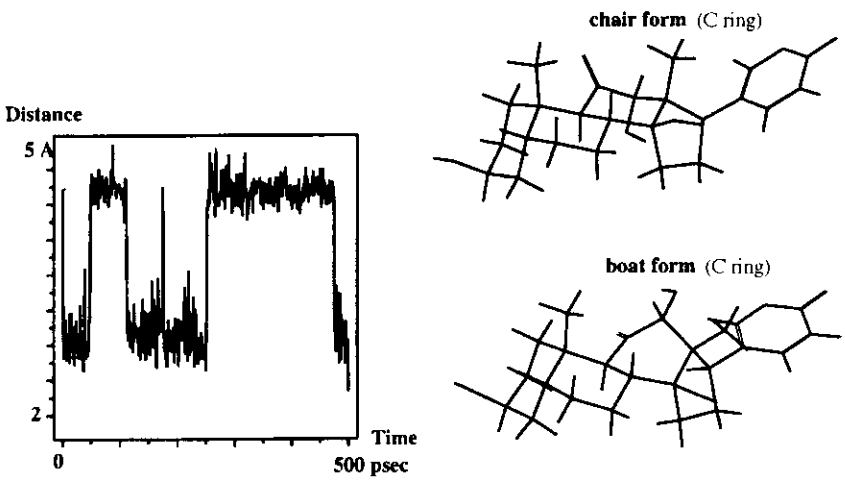


Figure 3. Monitoring plot of the distance between H-8 and H-12 during MD run (1000 K), and two conformers of ψ-bufarenogin one having C-ring of chair form and the other boat form.

It is interesting that the above two bufadienolides, bufarenogin and ψ-bufarenogin possess different solution conformations at C ring. The effect of the difference in the C-ring conformation on the biological activities is currently studied in our laboratory in the hope of obtaining futher information about the structure-activity relationships.

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}. MS, UV, and IR spectra were taken with a VG-Autospec spectrometer, a Hitachi 557 spectrophotometer and a JASCO A-302 spectrophotometer, respectively. HPLC was performed with an Inertsil PREP-ODS column (20 mm i.d. ×250 mm, GL Science Inc.) packed with 10 mm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck). NMR spectra were recorded on a Brucker DRX-500 spectrometer. For the homo- and hetero-nuclear measurements, a solution of 5 mg of sample in 0.5 mL of degassed $CDCl_3$ was used. The spectra were recorded at 300 K. Phase sensitive NOESY experiments were made with a mixing time of 1 sec. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 Hz and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec. The nmr coupling constants (J) are given in Hz.

Materials. - Ch'an Su was commercially obtained in a Hong Kong folk-medicinal market.

Isolation. - Ch'an Su (200 g) was extracted three times with MeOH (500 ml) for a week to give an extract (50 g), which was subjected to HP-20 column chromatography eluted by aq. MeOH. The fraction (4.5 g) eluted by MeOH containing 40% H_2O was subjected to ODS column chromatography with MeOH containing 42% H_2O, then to ODS HPLC with MeCN containing 83% H_2O to give two bufadienolides, bufarenogin (0.46 %) and ψ-bufarenogin (0.80 %).

Bufarenogin: Colorless powder, $[\alpha]_D$ +7.9° (c 0.08, MeOH), *m/z* : 416 $[M]^+$, ν_{max}(KBr)/cm^{-1}: 3340, 2900, and 1680, ^1H NMR ($CDCl_3$): listed in Table 1, ^{13}C NMR ($CDCl_3$): 29.96 (C1), 29.07 (C2), 65.56 (C3), 34.23 (C4), 37.49 (C5), 26.72 (C6), 23.27 (C7), 44.05 (C8), 44.86 (C9), 35.48 (C10), 211.00 (C11), 80.69 (C12), 60.15 (C13), 83.47 (C14), 33.41 (C15), 29.22 (C16), 46.93 (C17), 11.74 (C18), 24.27 (C19), 122.80 (C20), 150.30 (C21), 147.28 (C22), 115.30 (C23), and 161.98 (C24).

ψ-Bufarenogin: Colorless powder, $[\alpha]_D$ -32.1° (c 0.2, MeOH), *m/z* : 416 $[M]^+$, ν_{max}(KBr)/cm^{-1}: 3310, 2900, and 1700, ^1H NMR ($CDCl_3$): listed in Table 1, ^{13}C NMR ($CDCl_3$): 30.58 (C1), 29.34 (C2), 65.76 (C3), 34.87 (C4), 37.20 (C5), 27.79 (C6), 21.87 (C7), 40.72 (C8), 46.68 (C9), 38.15 (C10), 214.94 (C11), 83.62 (C12), 55.23 (C13), 83.12 (C14), 34.42 (C15), 28.42 (C16), 46.19 (C17), 19.53 (C18), 24.62 (C19), 120.44 (C20), 151.41 (C21), 148.20 (C22), 114.30 (C23), and 161.95 (C24).

Molecular Dynamics. - Computer modeling and all calculations were carried out by using the molecular-modeling software package SYBYL[9] ver. 6.3 (Tripos, Inc., St. Louis, MO) on an IRIS 4D computer. Force field was performed with the TRIPOS force field.[10] The dielectric constants (ε) were assumed to be equal to the interatomic distances (r) , as ε=r. Solvent molecules were not included in the calculations. In MD simulation, each system was equilibrated for 500 psec. with 1.0-fs time steps in a thermal bath set at 1000 K. Structures were sampled at 0.1-ps intervals. The sampled structures derived from the dynamics trajectories were then energy-minimized by using the MAXMIN program until the energy difference became less than 0.01 kcal·mol^{-2}·$Å^{-1}$. The energy-minimized conformers of bufarenogin and ψ-bufarenogin with chair form C-ring and boat form C ring , respectively, were finally geometry-minimized by the pm3 method.[8]

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