Microbial Transformation of Cinobufagin by *Syncephalastrum racemosum*

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Received January 9, 2008

Microbial transformation of a cytotoxic bufadienolide, cinobufagin (**1**), was performed by *Syncephalastrum racemosum*. The six metabolites obtained were identified as 7β-hydroxycinobufagin (**2**), 12β-hydroxycinobufagin (**3**), cinobufotalin (**4**), 5,12β-dihydroxycinobufagin (**5**), 4β,11α-dihydroxycinobufagin (**6**), and 4β,12α-dihydroxycinobufagin (**7**), respectively, on the basis of spectroscopic studies. Metabolites **2** and **5**−**7** were characterized as new compounds, and **2**−**7** proved to be cytotoxic against Bel-7402 human hepatoma cells.

The traditional Chinese medicine "Chan-Su", also called "toad poison" or "toad venom", is a product of the skin secretions of local toads (*Bufo bufo gararizans* Cantor and *Bufo bufo melanostictus* Schneider).[1] Cinobufagin (**1**) is one of the major bufadienolides with antitumor activity and potent cytotoxicity.[2–5] It has steroidal A/B *cis* and C/D *cis* ring junctions with a characteristic α-pyrone ring at C-17 and a 14β,15β-epoxide. Cinobufagin (**1**) induces apoptosis and elevated intracellular Ca^{2+} levels. Its IC_{50} value is 10^{-8} M by the MTT cytotoxicity method. Compound **1** is poorly soluble in water and toxic to humans.[6] Several attempts to modify **1** by biotransformation, to improve its solubility and activity, have been reported.[7–10] The major biotransformation reactions involved hydroxylation at 1β, 5, and 12β, and dehydrogenation occurs at C-3. In this paper, we report the structures of four new metabolites (**2**, **5**−**7**) transformed by the fungus *Syncephalastrum racemosum* and the biological activity of all compounds obtained against Bel-7402 cells.

Biotransformation of **1** (400 mg) was carried out by *S. racemosum* at 27 °C and 180 rpm for 4 days. The culture liquid was extracted with EtOAc five times to obtain an EtOAc extract (0.9 g). The extract was subjected to column chromatography over silica gel and further purified by preparative liquid chromatography to afford compounds **2** (4.0 mg), **3** (7.0 mg), **4** (5.0 mg), **5** (4.0 mg), **6** (3.0 mg), and **7** (5.0 mg). Among these, compounds **2** and **5**−**7** are new, and compounds **3**[8] and **4**[11] were reported earlier.

The HRMS of **2** showed a $[M + H]^+$ ion peak at *m*/*z* 459.2380, indicating the molecular formula $C_{26}H_{34}O_7$. When compared to **1**, the carbon signal at δ 20.0 (C-7) shifted downfield to δ 65.8 and showed HMBC correlations with H-8 (δ 1.87), H-9 (δ 1.78), and H-6 (δ 1.28), suggesting the introduction of a hydroxyl group at C-7. In the NOESY spectrum, the NOE enhancement between HO-7 (δ 3.45) and CH_3-19 (δ 0.90) supported the β-orientation of HO-7. Therefore, compound **2** was assigned as 7β-hydroxycinobufagin.

The HRMS of **5** gave a $[M + H]^+$ ion peak at *m*/*z* 475.2327, indicating the molecular formula $C_{26}H_{34}O_8$. The ^{13}C NMR spectrum showed two additional oxygen-bearing carbon signals at δ 73.2 and 73.6, suggesting that **5** is a dihydroxylated product of **1**. The signal at δ 73.6 exhibited HMBC correlations with CH_3-19 (δ 0.91) and H-9 (δ 2.08). Thus, metabolite **5** was hydroxylated at C-5. In addition, the C-11 and C-13 signals shifted downfield to δ 29.0 and 50.6, respectively, and H-12 (δ 3.45) gave correlations with C-11 and C-14. Correlations of a proton signal at δ 3.45 (H-12) with signals at δ 1.24 and 1.59 (H_2-11) were observed in the $^1H-^1H$ COSY spectrum. These data supported hydroxylation at C-12. By comparing with compound **1**, HO-5 was assigned with a β-orientation. The NOE enhancement of H-12 with H-16 (δ 5.42) was observed in the NOESY spectrum, and the C-18 signal of **5** resonated at δ 11.6 due to a γ-gauche effect.[12] All this evidence supported the β-orientation of HO-12. Therefore, compound **5** was characterized as 5,12β-dihydroxycinobufagin.

The HRMS of **6** showed a $[M + H]^+$ ion peak at *m*/*z* 475.2328, consistent with a molecular formula of $C_{26}H_{34}O_8$. The ^{13}C NMR spectrum showed two additional oxygenated methine signals at δ 75.5 and 65.0, suggesting that **6** is also a dihydroxylated product of cinobufagin (**1**). The carbon signal of δ 75.5 had HMBC correlations with H-2 (δ 1.81) and H-6 (δ 1.88), indicating a hydroxyl group was substituted at C-4. Similarly, the signal at δ 65.0 showed long-range correlations with H-9 and H-12, confirming the additional hydroxylation at C-11. The NOE enhancement of H-11 (δ 4.05) with CH_3-19 (δ 1.06) proved the α-orientation of HO-11. In addition, the NOE enhancement between H-4 (δ 3.49) and H_α-2 (δ 1.22) supported the β-orientation of HO-4. Therefore, compound **6** was assigned as 4β,11α-dihydroxycinobufagin.

The HRMS of **7** also gave a $[M + H]^+$ ion peak at *m*/*z* 475.2328, suggesting a molecular formula of $C_{26}H_{34}O_8$. Two additional oxygen-bearing carbons appeared at δ 75.8 and 73.9 in the ^{13}C NMR spectrum. Similar to **6**, H-4 (δ 3.49) gave HMBC correlations with C-5 (δ 39.0), C-10 (δ 34.9), and C-3 (δ 69.9), revealing a hydroxyl group to be at C-4. In addition, C-11 (δ 28.9) and C-13 (δ 50.8) shifted downfield by 9.3 and 6.2 ppm, respectively, indicating an additional hydroxyl group at C-12. The H-12 signal (δ 3.30) gave a NOE enhancement with CH_3-18 (δ 0.63), and C-18 resonated at δ 18.7 due to a γ-*trans* effect,[12] all of which suggested that HO-12 is in an α-orientation. The NOE enhancement of H-4 (δ 3.49) with H_α-2 (δ 1.23) supported a β-orientation of HO-4. Therefore, compound **7** was assigned as 4β,12α-dihydroxycinobufagin. Although a number of hydroxylated products have been reported before,[7–10] this is the first report of 4β-hydroxylation of a bufadienolide.

The MTT bioassay showed that compounds **1**−**7** are cytotoxic to Bel-7402 cells in vitro, with IC_{50} values of 0.10 ± 0.03 (**1**), 0.54 ± 0.07 (**2**), 0.37 ± 0.04 (**3**), 1.21 ± 0.20 (**4**), 0.66 ± 0.08 (**5**), 5.25 ± 0.63 (**6**), and 0.34 ± 0.02 (**7**) μM. These data showed that all products had less potent activities than **1**, showing that 5β-, 7β-, and 12β-hydroxylation reduced cytotoxicity. In addition, it was noted that acetylation at C-16 was evident in metabolites **2**−**7**, and they exhibited more activity than derivatives of desacetycinobufagin (such as 12β-hydroxydeacetylcinobufagin, with an IC_{50} value of 56.4 μM).[8] This suggest that a C-16 acetyl group is important for mediating the cytotoxic activity of bufadienolides. These results

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10.1021/np800210a CCC: $40.75
Published on Web 06/18/2008

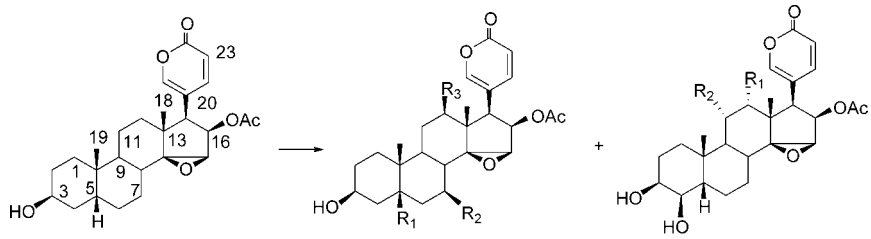

Figure 1. Biotransformation of cinobufagin (**1**) by *Syncephalastrum racemosum*.

will provide some guidance for the synthesis of bufadienolides of pharmaceutical interest.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 243B polarimeter. UV spectra were detected on a YV-1091 UV−vis spectrophotometer. IR spectra were obtained on an Avatar 360 FT-TR spectrometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 with TMS as internal standard. HRMS were obtained on a Bruker APEXII Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. Silica gel (200−300 mesh) was purchased from Qingdao Marine Chemical Group, Qingdao, People's Republic of China. A SpectraSeries HPLC apparatus (Thermo Quest) with a 100 μL loop was used for preparative liquid chromatography. Samples were separated on a YMC ODS-A column (5 μm, Φ 10 × 250 mm). The flow rate was 2.0 mL/min, and the detection wavelength was 296 nm. Cinobufagin (**1**) was isolated from "Chan-Su" by one of the authors (X-C.M.) and identified unambiguously by NMR and MS techniques.[13] The purity was above 98%, as determined by HPLC.

Microorganisms and Culture Media. *Syncephalastrum racemosum* was purchased from China General Microbiological Culture Collection Center in Beijing, People's Republic of China. All culture and biotransformation experiments using filamentous fungi were performed in potato medium, which was prepared as following: 200 g of husked potato were cut into pieces, boiled in deionized water for 0.5 h, and filtered. The filtrate was combined with water to 1 L. Then, 20 g of glucose was added. The potato medium was sterilized before use.[9]

Biotransformation of Cinobufagin (1) by *S. racemosum*. Mycelia from agar slants (1 cm^2) were transferred to 250 mL Erlenmeyer flasks containing 100 mL of medium and cultured at 27 °C and 180 rpm for 36 h to make a stock inoculum. Then, a 5 mL volume of the inoculum was added to a 1000 mL flask containing 400 mL of potato medium, and the flasks were placed on a rotary shaker operating at 180 rpm at 27 °C. Compound **1** was dissolved in acetone to reach a concentration of 20 mg/mL. After 36 h of preculture, 0.5 mL of each solution was added into each flask, and these flasks were maintained under the fermentation conditions for 4 days. Culture controls consisted of fermentation blanks in which microorganisms were grown without substrate but fed with the same amount of acetone. In total, 400 mg of compound **1** was used for preparative biotransformation. Substrate controls contained the sterile medium with the same amount of substrate and were incubated under the above conditions.

The culture was filtered and the filtrate was extracted with the same volume of EtOAc five times. The organic phase was collected and concentrated in vacuo. The residues (0.9 g) were applied to a silica gel column and eluted with petroleum ether−acetone (in a gradient manner from 100:3 to 1:1, at a flow rate of 1.5 mL/min). Fraction 2 was purified to yield **2** (4.0 mg) and **3** (7.0 mg). Fraction 3 was purified to yield **4** (5.0 mg). Fractions 4−6 were subjected to preparative liquid chromatography and eluted with MeOH−H_2O (40:60) to obtain **5** (4.0 mg), **6** (3.0 mg), and **7** (5.0 mg).

7*β*-Hydroxycinobufagin (2): white powder (MeOH); $[\alpha]^{22}_D$ +46.1 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (3.65), 295 (3.50) nm; IR ν_{max} (KBr) 3402, 2935, 1722, 1240, 1028 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Tables 1 and 2; HRFTICRMS *m/z* 459.2380 [M + H]$^+$ (calcd for $C_{26}H_{35}O_7$, 459.2377).

Table 1. ^{1}H NMR Spectroscopic Data (500 MHz, DMSO-d_6, *J* in Hz) for Compounds **1**, **2**, and **5**−**7**

position	1	2	5	6	7
1	1.38 (m)	1.37 (m)	1.25 (m)	1.42 (m)	1.39 (m)
	1.73 (m)	1.43 (m)	1.70 (m)	1.57 (m)	1.42 (m)
2	1.35 (m)	1.31 (m)	1.48 (m)	1.22 (m)	1.23 (m)
	1.46 (m)	1.46 (m)	1.59 (m)	1.81 (m)	1.66 (m)
3	3.89 (brs)	3.84 (brs)	3.99 (brs)	3.51 (brs)	3.51 (brs)
4	1.20 (m)	1.32 (m)	1.20 (m)	3.49 (brs)	3.49 (brs)
	1.81 (m)	1.69 (m)	1.67 (m)		
5	1.69 (m)	1.75 (m)		1.31 (m)	1.46 (m)
6	1.08 (m)	1.28 (m)	1.17 (m)	1.34 (m)	1.36 (m)
	1.76 (m)	1.60 (m)	1.54 (m)	1.88 (m)	1.69 (m)
7	0.98 (m)	3.49 (brs)	0.88 (m)	1.36 (m)	1.12 (m)
	1.33 (m)		1.39 (m)	1.63 (m)	1.31 (m)
8	1.90 (m)	1.87 (brt, 9.5, 11.5)	1.88 (m)	2.40 (dt, 5.0, 12.0)	1.89 (dt, 2.5, 12.0)
9	1.69 (m)	1.78 (m)	2.08 (dd, 14.5, 3.0)	2.31 (br dd, 2.0, 12.0)	2.10 (m)
11	1.28 (m)	1.20 (m)	1.24 (m)	4.05 (brs)	1.20 (m)
	1.52 (m)	1.45 (m)	1.59 (m)		1.62 (m)
12	1.55 (m)	1.55 (m)	3.45 (m)	1.88 (m)	3.30 (dd, 11.5, 4.0)
	1.70 (m)	1.72 (m)		1.47 (m)	
15	3.78 (s)	4.20 (s)	3.75 (s)	3.57 (s)	3.54 (s)
16	5.46 (d, 9.5)	5.50 (d, 9.5)	5.42 (d, 9.5)	5.34 (d, 9.5)	5.35 (d, 9.5)
17	2.88 (d, 9.5)	2.91 (brd, 9.5)	3.35 (d, 9.5)	2.80 (d, 9.5)	3.36 (d, 9.5)
18	0.72 (s)	0.74 (s)	0.62 (s)	0.95 (s)	0.63 (s)
19	0.88 (s)	0.90 (s)	0.91 (s)	1.06 (s)	0.89 (s)
21	7.47 (s)	7.48 (s)	7.35 (s)	7.46 (s)	7.36 (s)
22	7.80 (brs)	7.81 (brs)	7.80 (brs)	7.85 (brs)	7.81 (brs)
23	6.24 (d, 9.5)	6.24 (d, 9.5)	6.23 (d, 9.5)	6.21 (d, 9.5)	6.22 (d, 9.5)
26	1.80 (s)	1.80 (s)	1.81 (s)	1.80 (s)	1.81 (s)

5,12*β*-Dihydroxycinobufagin (5): white powder (MeOH); $[\alpha]^{22}_D$ +12.1 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (3.63), 296 (3.46) nm; IR ν_{max} (KBr) 3400, 2944, 1723, 1243, 1029 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Tables 1 and 2; HRFTICRMS *m/z* 475.2327 [M + H]$^+$ (calcd for $C_{26}H_{35}O_8$, 475.2326).

4*β*,11α-Dihydroxycinobufagin (6): white powder (MeOH); $[\alpha]^{22}_D$ +22.4 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.68), 298 (3.51). IR ν_{max} (KBr) 3461, 2919, 1708, 1247, 1049 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Tables 1 and 2; HRFTICRMS *m/z* 475.2328 [M + H]$^+$ (calcd for $C_{26}H_{35}O_8$, 475.2326).

4*β*,12α-Dihydroxycinobufagin (7): white powder (MeOH); $[\alpha]^{22}_D$ −53.6 (*c* 0.06, MeOH); UV (MeOH) λ_{max} (log ϵ) 203 (3.59), 298 (3.50); IR ν_{max} (KBr) 3461, 2919, 1708, 1247, 1049 cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz), see Tables 1 and 2; HRFTICRMS *m/z* 475.2328 [M + H]$^+$ (calcd for $C_{26}H_{35}O_8$, 475.2326).

Bioassay. Human hepatoma (Bel-7402) cells were maintained in RPMI 1640 medium (Gibco/BRL, Rockville, MD) supplemented with 10% (v/v) fetal bovine serum and cultured in 96-well microtiter plates. Appropriate dilutions of the test compounds were added to the cultures. The cells were cultured at 37 °C, with 5% CO_2 for 72 h. The survival

Table 2. ^{13}C NMR Spectroscopic Data (125 MHz, DMSO-d_6) for Compounds **1**, **2**, and **5**−**7**

position	**1**	**2**	**5**	**6**	**7**
1	29.3 t	29.1 t	24.9 t	28.8 t	28.9 t
2	27.4 t	27.4 t	27.2 t	22.9 t	22.6 t
3	64.5 d	64.4 d	66.5 d	69.9 d	69.9 d
4	32.9 t	33.9 t	35.6 t	75.5 d	75.8 d
5	35.5 d	36.2 d	73.6 s	40.8 d	39.0 d
6	25.4 t	34.3 t	33.8 t	24.0 t	24.6 t
7	20.0 t	65.8 d	22.2 t	22.1 t	22.8 t
8	32.6 d	41.0 d	31.0 d	26.3 d	31.2 d
9	38.0 d	37.1 d	36.4 d	42.7 d	36.1 d
10	34.9 s	34.5 s	40.4 s	35.4 s	34.9 s
11	20.5 t	21.1 t	29.9 t	65.0 d	29.8 t
12	38.7 t	38.7 t	73.2 d	47.4 t	73.9 d
13	44.6 s	45.2 s	50.6 s	44.2 s	50.8 s
14	71.9 s	73.5 s	71.0 s	73.4 s	71.7 s
15	59.3 d	60.5 d	59.3 d	58.7 d	58.8 d
16	74.5 d	74.2 d	74.5 d	74.3 d	74.6 d
17	48.9 d	48.8 d	44.6 d	49.5 d	44.8 d
18	16.9 q	16.8 q	11.6 q	19.0 q	18.7 q
19	23.5 q	23.6 q	16.6 q	26.2 q	23.5 q
20	116.0 s	115.7 s	116.1 s	116.1 s	116.2 s
21	152.1 d	152.5 d	152.0 d	151.9 d	152.0 d
22	148.4 d	148.3 d	148.4 d	148.6 d	148.6 d
23	112.8 d	113.0 d	112.9 d	112.6 d	112.9 d
24	160.7 s	160.7 s	160.7 s	160.7 s	160.8 s
25	169.2 s	169.2 s	169.3 s	169.3 s	169.3 s
26	20.1 q	20.2 q	20.1 q	20.1 q	20.2 q

rates of the cancer cells were evaluated by the MTT method.[14] Activity was determined as IC_{50} values, the concentration of test compound to give 50% inhibition of cell growth. Results were expressed as the mean value of triplicate determinations.

Acknowledgment. We thank the National Natural Science Foundation of China (30701088), the Changjiang Scholars and Innovative Team in University (985-2-063-112), and PHR (IHLB) for financial support.

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NP800210A