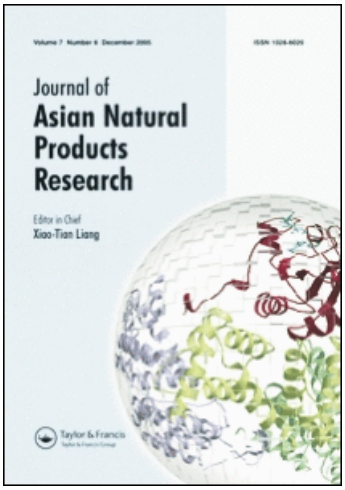

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Microbial hydroxylation of cinobufagin by *Mucor spinosus*

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Mucor spinosus has been employed for the biotransformation of cinobufagin (**1**) to afford three metabolites. On the basis of their physico-chemical data, the structures of the transformation products have been characterized as 1β-hydroxy-cinobufagin (**2**), 12β-hydroxy-cinobufagin (**3**) and 1β,12β-dihydroxy-cinobufagin (**4**), of which metabolites **2** and **4** are new compounds. *In vitro* cytotoxic activities of the biotransformation products and the substrate-cinobufagin have been assayed against four tumor cell lines of Bel 7420, BGC 823, HeLa and HL 60; they all showed cytotoxic activities.

Keywords: Biotransformation; Cinobufagin; *Mucor spinosus*

1. Introduction

Chan'su, or toad venom, is a traditional Chinese medicine that has been used as a cardiotonic drug for centuries. Bufadienolides are the major active constituents in Chan'su. The structural features of bufadienolides represent a type of steroid with A/B *cis* and C/D *cis* skeleton and an α-pyrone ring at the 17-position. Pharmacological investigations have shown that bufadienolides exhibit a variety of biological activities, such as cardiotonic, blood pressure stimulation, respiration and antineoplastic activities [1–3]. Cinobufagin (**1**), a bufadienolide with a 14β,15β-epoxy ring, is one of the major active constituents in toad venom (*ca.* 4–6% dry weight) [4]. Recently, our laboratory has focused on the structure optimization of some important active natural products by biological systems and the utilization of microbes as a model to mimic mammalian metabolism. Several of these compounds, such as taxanes, artemisinin, *Tripterygium wilfordii* diterpenoids–triptolide and triptonide, have been scrutinized using plant cell suspension cultures and microbes [5–13]. In continuation of our effort to this end, the biotransformation of cinobufagin (**1**) by *Mucor spinosus* has been conducted and three transformation products, including two new compounds, obtained. Here, we report the structure characterization and cytotoxic activity assays of the three transformation products.

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2. Results and discussion

Incubation of cinobufagin (**1**) with *Mucor spinosus* yielded three compounds (**2**–**4**, figure 1). TLC analyses showed that the R_fs of **2**–**4** were all smaller than that of the substrate, suggesting that they are more polar than the substrate cinobufagin. From their IR and ^{1}H and ^{13}C NMR spectra, one could speculate that these transformed compounds might be hydroxylated products on different positions of cinobufagin.

The FAB mass spectrum of **2** showed a quasi-molecular ion peak $[M + 1]^+$ at m/z 459, which is consistent with the molecular formula $C_{26}H_{34}O_7$. ^{1}H and ^{13}C NMR spectra of **2** show a new proton signal at δ 3.75 and a new carbon signal at δ 73.37. DEPT spectra reveal that the number of the secondary carbons has changed from 7 to 6, while the number of the tertiary carbons has increased from 6 to 7. These data further support the hydroxylation of a secondary carbon. Compared with cinobufagin, the signal of C-19 was shifted upfield to δ 19.21, suggesting that the hydroxyl is at C-1. The signal of C-5 shifted significantly to δ29.9 ($\Delta\delta$ 5.6) as a result of γ-gauche effect, suggesting the β-orientation of 1-OH. Furthermore, H-3 resonated at a relatively lower field at δ 4.09, resulting from the hydrogen bonding between 1-OH and 3-OH. Therefore, compound **2** was identified as 1β-hydroxy-cinobufagin, which is a new compound.

^{1}H and ^{13}C NMR spectra of **4** show new proton signals at δ 3.75, 3.61, and new carbon signals at δ 70.49 and 75.49. The FAB mass spectrum shows the [M + 1] ion at m/z 475.0. These evidences suggest that **4** is a dihydroxylated product. DEPT spectra reveal that the number of the secondary carbons changed from 7 to 5, and that the tertiary carbons increased from 6 to 8. These data support the idea that two secondary carbons have been hydroxylated. In the HMBC spectrum of **4**, long-range correlations occur between the proton at δ 3.75 and the 19-carbon at δ 19.08; proton at δ 3.61 and 18-carbon at δ 12.10. This suggests that the two hydroxyl groups are at C-1 and C-12. The signal of H-12 (δ 3.53) is a double doublet, which results from coupling between H-12 and H-11β ($^3Jaa = 11.4$ Hz), as well as that between H-12 and H-11α ($^3Jae = 4.2$ Hz). Thus, 12-OH is deduced to be in the β-configuration. The stereochemistry of 1-OH was determined to be in the β-form for the same reasons as depicted for compound **2**. Based on the above, **4** is characterized as 1β,12β-dihydroxy-cinobufagin, which is also a new compound.

Compounds **2**–**4**, together with their substrate cinobufagin (**1**), were tested for their *in vitro* cytotoxic activities against the human tumor cell lines of Bel 7420, BGC 823, HeLa and HL 60. The IC_{50} s of **1**–**4** on the tumor cells of Bel 7420, BGC 823, HeLa, and HL 60 are given in table 2.

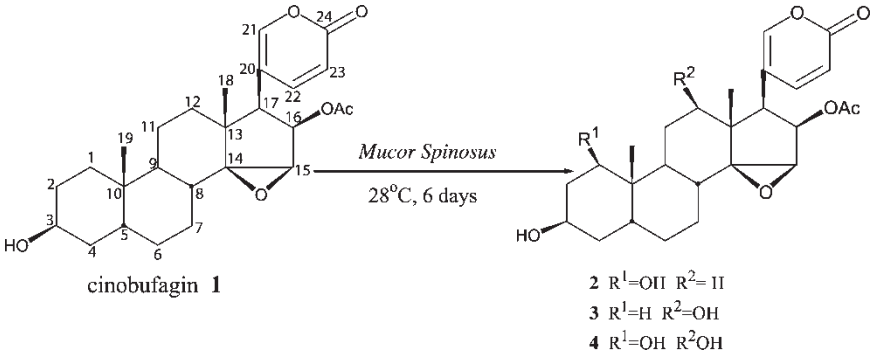

Figure 1. Biotransformation of cinobufagin by *Mucor spinosus*.

3. Experimental

3.1 General experimental procedure

Melting points were measured on a XT_{4A} micro-melting point apparatus and are uncorrected. UV spectra were measured on a Cary 300 spectrophotometer. IR spectra were recorded on a NEXUS-470 FTIR (Nicolet) spectrophotometer in KBr pellets. ^{1}H and ^{13}C NMR spectra were measured with an INOVA-500 spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR) in DMSO-d_6 with TMS as internal standard. FAB-MS spectra were recorded on a VG-EAB-HS mass spectrometer (UK). TLC analyses were performed on silica gel G. All chemicals were purchased from the Beijing Chemical Factory. Silica gels were produced by Qingdao Haiyang Chemical Group Co., China.

3.2 Microorganisms and medium

The *Mucor spinosus* strain was purchased from the China General Microbiological Culture Collection Center and was maintained on PDA slant. Fungus was incubated in potato medium, prepared as follows: 200 g husked potato was cut into pieces, boiled in deionized water for 0.5 h and filtered. The filtrate was then added to water (1 L), and glucose (20 g) was added. The potato medium was sterilized before use.

3.3 Biotransformation procedure

Substrates were each dissolved in ethanol as 10 mg mL^{-1} solutions. For screening analysis, the microbial strain was incubated in 250 mL conical flasks, each containing 50 mL potato medium. The culture medium was incubated for 48 h at 150 rpm (27°C). Subsequently 3 mg of substrate was fed into the system. Fermentation was allowed to proceed for 6 days. The mycelium was then filtered under vacuum and the filtrate extracted with diethyl ether (4 ×). The extract was then evaporated to give a residue that was dissolved with methanol for TLC analysis using chloroform–acetone–cyclohexane (3:3:4) as eluting solvent. To prepare the biotransformed products, *Mucor spinosus* was incubated in 1 L conical flasks, each containing 250 mL of potato medium. The culture medium was incubated for 48 h at 150 rpm (27°C). Cinobufagin (400 mg) was then fed to the *Mucor spinosus* culture system.

3.4 Extraction and isolation

About 1200 mg of extract was obtained from the incubation of the substrate with *Mucor spinosus*. The extract was chromatographed on a silica gel column eluted gradiently with cyclohexane–acetone. Three transformed products, **2** (5 mg), **3** (10 mg) and **4** (3 mg), were obtained (figure 1).

1β-Hydroxy-cinobufagin (**2**): white amorphous powder; $C_{26}H_{34}O_7$; mp 223–225°C; UV λ_{max} (MeOH) (nm): 203.0, 293.0; IR ν_{max} (KBr) (cm^{-1}): 3428, 2929, 1724, 1637, 1538, 1229, 1045; FAB-MS*m/z* 457.0 [M − 1]; ^{1}H NMR δ(ppm): 7.93 (1H d, $J = 9.3$ Hz, H-22), 7.39 (1H, brs, H-21), 6.15 (1H, d, $J = 9.9$ Hz, H-23), 5.47 (1H, dd, $J = 9.4$ Hz, 1.5 Hz, H-16), 4.48 (1H, d, $J = 5.4$ Hz, 3-OH), 4.43 (1H, d, $J = 7.8$ Hz,1-OH), 4.08 (1H, brs, H-3), 3.78 (1H, brs, H-1), 3.75 (1H, s, H-15), 2.88 (1H, d, $J = 9.4$ Hz, H-17), 0.82 (3H, S, 18-CH_3), 0.97 (3H, S, 19-CH_3), 1.88 (3H, s, 26-CH_3); for ^{13}C NMR data, see table 1.

Table 1. ^{13}C NMR spectral data of **1**–**4** (125 MHz, in DMSO-d_6).

C	*1*	*2*	*3*	*4*
1	29.3	71.8	29.4	73.4
2	27.4	31.9	27.4	34.0
3	64.5	66.7	64.5	68.5
4	32.9	32.7	32.9	33.5
5	35.5	29.9	35.6	32.9
6	25.4	24.9	25.4	26.6
7	20.0	19.6	20.2	21.1
8	32.6	32.7	31.9	31.4
9	38.0	39.9	33.3	36.6
10	34.9	40.8	34.7	34.7
11	20.5	20.4	30.7	30.7
12	38.7	38.7	73.4	75.2
13	44.6	44.4	50.8	52.0
14	71.9	71.8	71.0	71.8
15	59.3	59.2	59.2	60.3
16	74.5	74.5	74.6	75.7
17	48.9	48.8	44.7	46.4
18	16.9	16.9	11.7	12.1
19	23.5	18.6	23.5	19.1
20	116.0	115.9	116.2	113.9
21	152.1	152.1	152.0	154.4
22	148.4	148.4	148.6	149.0
23	112.8	112.8	112.9	113.9
24	160.7	160.7	160.7	161.7
$OCOCH_3$	169.2	169.2	169.3	170.3
$OCOCH_3$	20.1	20.1	20.2	20.4

12β-Hydroxy-cinobufagin (**3**): flaky crystals (cyclohexane-acetone); $C_{26}H_{34}O_7$; mp 254–256°C; UV λ_{max} (MeOH) (nm): 202.0, 295.0; IR ν_{max} (KBr) (cm^{-1}): 3427, 2932, 1716, 1635, 1448, 1243, 1031; FAB-MS *m/z* 457.5 [M − 1]; ^1H NMR δ(ppm): 7.99 (1H, br. s, H-22), 7.36 (1H, br. s, H-21), 6.23 (1H, d, $J = 8.5$ Hz, H-23), 5.45 (1H, d, $J = 9.0$ Hz, H-16), 4.48 (br. s, OH), 4.04 (1H, br. s, 3-H), 3.73 (1H, br. s, 15-H), 3.49 (1H, d, $J = 4.5$ Hz, 12-OH), 3.47 (1H, d, $J = 4.5$ Hz, 17-H), 1.88 (3H, s, 26-CH_3), 1.00 (3H, s, 19-CH_3), 0.76 (3H, s, 18-CH_3); for ^{13}C NMR data, see table 1.

1β,12β-Dihydroxy-cinobufagin (**4**): white amorphous powder; $C_{26}H_{34}O_8$; mp 179–181°C; UV λ_{max} (MeOH) (nm): 207.0, 294.0; IR ν_{max} (KBr) (cm^{-1}): 3400, 2933, 1719, 1635, 1538, 1449, 1242, 1045; FAB-MS *m/z* 475.4 [M + 1]; ^1H NMR δ(ppm): 7.98 (1H, br. s, H-22), 7.33 (1H, brs, H-21), 6.16 (1H, d, $J = 9.6$ Hz, H-23), 5.44 (1H, dd, $J = 9.6$ Hz/1.2 Hz,H-16), 4.07 (1H, brs, 3-H), 3.74 (2H, brs, 15-H, 1-H), 3.53 (1H, dd, $J = 11.4$ Hz, 4.2 Hz, 12-H), 3.41 (1H, d, $J = 9.6$ Hz, 17-H), 1.91 (3H, s, 26-CH_3), 1.09 (3H, s, 19-CH_3), 0.86 (3H, s, 18-CH_3); for ^{13}C NMR data, see table 1.

Table 2. IC_{50}s of **1**–**4** (μM) against four tumor cell lines.

Compound	*Bel 7402*	*BGC 823*	*HeLa*	*HL 60*
1	0.04	0.05	0.04	0.04
2	0.22	0.29	0.12	0.20
3	0.24	0.20	0.24	0.37
4	4.34	4.80	10.70	5.68
Paclitaxel	5.40	6.50	0.01	3.5×10^{-4}

3.5 Cytotoxicity assay

The *in vitro* cytotoxic activities of **1**–**4** against the human tumor cells of Bel 7420, BGC 823, HeLa and HL 60 were tested by the MTT method. Their IC_{50}s are given in table 2.

Acknowledgements

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