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# Biotransformation of cinobufagin by cell suspension cultures of *Catharanthus roseus* and *Platycodon grandiflorum*

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**Abstract**

The biotransformations of cinobufagin (**1**), an animal-originated bufadienolide, by cell suspension cultures of *Catharanthus roseus* and *Platycodon grandiflorum* were investigated. Incubation for 6 days of **1** with *C. roseus* yielded four products, desacetylcinobufotalin (**2**), 3-epi-desacetylcinobufagin (**3**), 1β-hydroxyl desacetylcinobufagin (**4**) and 3-epi-desacetylcinobufotalin (**5**), among which **4** is a new compound. Time course investigation revealed that the biotransformation rates of two major products, **2** and **3**, reached their highest levels of 44.7 and 16.5%, respectively, on the third day after substrate administration. Compounds **2**–**5** showed more potent cytotoxic activities against HL-60 cell lines than the parent compound **1**. A plausible biotransformation pathway is proposed to account for the formation of the observed products. From the culture supernatant of *P. grandiflorum*, **2** was isolated in 37.8% yield after 8 days of incubation with **1**. Cinobufotalin (**6**) and desacetylcinobufagin (**7**) were also obtained as minor products. The two plant suspension cultures exhibited similar transformation patterns on compound **1**.


*Keywords:* Cinobufagin; Bufadienolide; Biotransformation; *Catharanthus roseus*; *Platycodon grandiflorum*

## 1. Introduction

Chan'su or toad venom is a traditional medicine in China which has been used as a cardiotonic drug for centuries. Cinobufagin (**1**), a bufadienolide with 14β, 15β-epoxy ring, is one of its major active constituents (ca. 4–6% dry weight) [1,2]. As a rule, the oxirane ring is only found in bufadienolides from animal sources although steroids of this type are also widely distributed in plants such as *Urginea* species [3]. This character can be considered as a significant taxonomic mark. It might result from the great difference of enzymes involved in the bufadienolide biosynthetic pathway for plants and animals. But what will happen if the animal-originated bufadienolides are encountered with enzymes from plant systems?

Recently, bufadienolides were reported to exhibit cytotoxic effects and differentiation-inducing activities on human myeloid leukemia cell lines [4–6]. These results indicated their great potentiality as new antitumor agents. Cinobufagin was among the most intensively investigated due to its natural abundance in toad venom. However, it only showed moderate cytotoxicities ($IC_{50}$ $7.4 \times 10^{-4}$ mg/ml against the liver carcinoma cells PLC/PRF/5), and its clinical use is greatly limited due to its poor solubility in water. Therefore, structural modification of **1** is needed to improve its activities. In an endeavor to find new chemical entities

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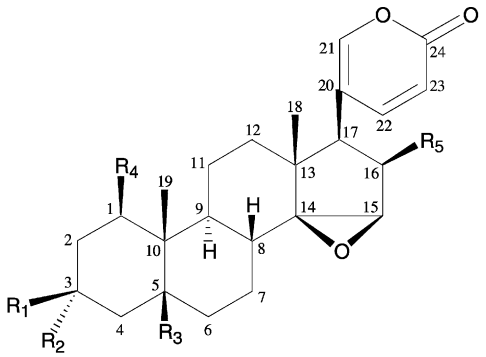
with improved cytotoxic activities, and being interested in the biotransformation of animal-originated natural products by plant culture systems, we examined plant cell suspension cultures for their capabilities to transform **1** [7]. In this paper, site-specific hydroxylation, epimerization, as well as deacetylation of **1** by *Catharanthus roseus* and *Platycodon grandiflorum* are reported.

## 2. Results and discussion

After 6 days of incubation, compound **1** was almost completely transformed by *C. roseus*. Four pure products of higher polarity were isolated from the culture supernatant. Their structures were respectively identified as desacetylcinobufotalin (**2**), 3-epi-desacetylcinobufagin (**3**), 1β-hydroxyl desacetylcinobufagin (**4**) and 3-epi-desacetylcinobufotalin (**5**) on the basis of their MS, $^{1}$H, $^{13}$C, DEPT, HSQC, HMBC and NOESY spectra [8] (Fig. 1). Among them **4** is a new compound. The $^{13}$C-NMR spectral data are given in Table 1.

TOF-MS of **4** showed an $[M+H]^{+}$ ion peak at *m*/*z* 417, indicating the molecular formula of $C_{24}H_{32}O_6$. The disappearance of signals corresponding to the acetyl group revealed that **4** is a deacetylated derivative of **1**. Accordingly, H-16 resonated at a significantly higher field ($\delta$4.60, whereas $\delta$5.46 for **1**). When compared to that of **1**, the $^{13}$C-NMR spectrum of **4** exhibited an additional oxygen-bearing methine signal at $\delta$71.7, suggesting the introduction of a hydroxyl group in the molecule. Its location was deduced to be at C-1 by the long-range coupling between C-1 and 19-methyl protons ($\delta$0.97) in the HMBC spectrum. In accordance, the signal for C-19 significantly shifted upfield ($\Delta\delta - 5.0$ ppm) when compared to that of compound **1**. The 3-OH was concluded to be in the β-configuration by comparing the $^{1}$H-NMR spectrum with that of **1**. Instead of unusually broad singlets for bufadienolides with a 3α-hydroxyl group due to the great couplings between axial protons (H-3β and H-2α, H-3β and H-4α), the signal for H-3 in **4** appeared as a much narrower one ($W_{1/2} = 6.5$ Hz, whereas $W_{1/2} = 24.0$ and 20.2 Hz for **3** and **5**, respectively) [9]. In the $^{1}$H-NMR spectrum, an additional signal assigned to H-1 was observed at $\delta$3.60 (1H, brs). It showed a W-type long-range coupling with




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ |
|---|---|---|---|---|---|
| **1** | OH | H | H | H | OAc |
| **2** | OH | H | OH | H | OH |
| **3** | H | OH | H | H | OH |
| **4** | OH | H | H | OH | OH |
| **5** | H | OH | OH | H | OH |
| **6** | OH | H | OH | H | OAc |
| **7** | OH | H | H | H | OH |

Fig. 1. Chemical structures of **1**–**7**.

H-3 ($\delta$3.99, 1H, brs) in the $^{1}$H–$^{1}$H COSY spectrum, which strongly revealed that both H-1 and H-3 had the α-configuration [10]. As a result, no NOE enhancement between H-1 and H-5 ($\delta$1.89) for H-1β derivatives was observed in the NOESY spectrum [11]. On the basis of the above analysis, **4** was identified as 1β-hydroxyl desacetylcinobufagin. All the $^{1}$H- and $^{13}$C-NMR spectral data were unambiguously assigned by 2D-NMR spectra. The spectral data for **3** and **5** were reported and assigned here for the first time although their structures had been presented once in 1987 [12].

Compound **4** is the first example of a bufadienolide containing both 14β, 15β-epoxy ring and 1β-hydroxyl group. It is of great interest to note that 1-hydroxylated bufadienolides have only been isolated from the plant kingdom while never from animal sources. And all the 1-hydroxyl groups are located in the β-configuration with no exception [13]. The isolation of **4** as the

Table 1
$^{13}$C-NMR spectral data for **1**–**7** (DMSO-$d_6$, 125 MHz)

| Carbon | 1[a] | 2[a] | 3 | 4 | 5 | 6[b] | 7[b] |
|---|---|---|---|---|---|---|---|
| 1 | 29.3t | 24.8t | 30.2t | 71.7d | 29.3t | 24.7t | 29.5t |
| 2 | 27.4t | 27.2t | 34.6t | 32.0t | 30.0t | 27.9t | 27.9t |
| 3 | 64.5d | 66.5d | 69.7d | 66.6d | 65.8d | 68.0d | 66.7d |
| 4 | 32.9t | 36.5t | 35.9t | 32.8t | 41.8t | 36.9t | 33.3t |
| 5 | 35.5d | 73.4s | 41.0d | 29.9d | 72.8s | 74.3s | 35.9d |
| 6 | 25.4t | 33.9t | 25.9t | 24.9t | 35.1t | 34.4t | 25.6t |
| 7 | 20.0t | 22.3t | 20.3t | 19.7t | 22.6t | 23.1t | 20.9t |
| 8 | 32.6d | 31.8d | 32.8d | 32.8d | 32.0d | 32.1d | 33.1d |
| 9 | 38.0d | 41.4d | 39.0d | 39.9d | 41.6d | 42.7d | 39.3d |
| 10 | 34.9s | 40.4s | 34.4s | 40.0s | 40.0s | 40.9s | 35.5s |
| 11 | 20.5s | 21.0t | 20.2t | 20.4t | 20.5t | 21.3t | 20.7t |
| 12 | 38.7t | 38.6t | 38.8t | 38.7t | 39.0t | 40.0t | 40.1t |
| 13 | 44.6s | 44.2s | 44.3s | 44.2s | 44.1s | 45.0s | 45.3s |
| 14 | 71.9s | 71.7s | 71.5s | 71.4s | 71.7s | 72.3s | 72.9s |
| 15 | 59.3d | 62.0d | 61.9d | 61.9d | 61.9d | 59.4d | 61.8d |
| 16 | 74.5d | 70.4d | 70.5d | 70.4d | 70.4d | 74.6d | 72.7d |
| 17 | 48.9d | 50.7d | 50.7d | 50.7d | 50.6d | 50.2d | 52.4d |
| 18 | 16.9q | 16.7q | 16.9q | 16.9q | 16.0q | 17.1q | 17.3q |
| 19 | 23.5q | 17.0q | 22.9q | 18.5q | 16.8q | 16.7q | 23.7q |
| 20 | 116.0s | 117.8s | 117.7s | 117.7s | 117.7s | 116.2s | 116.7s |
| 21 | 152.1d | 151.1d | 151.0d | 151.0d | 151.0d | 151.4d | 151.0d |
| 22 | 148.4d | 149.9d | 149.8d | 149.8d | 149.8d | 148.1d | 148.2d |
| 23 | 112.8d | 112.0d | 111.8d | 111.8d | 111.8d | 113.9d | 114.3d |
| 24 | 160.7s | 161.2s | 161.1s | 161.1s | 161.1s | 161.5s | 161.8s |
| $CH_3\underline{C}O$ | 169.2s | | | | | 170.1s | |
| $\underline{C}H_3CO$ | 20.1q | | | | | 20.4q | |

[a] 75 MHz.
[b] In $CDCl_3$.

biotransformation product by plant cultures suggested that plant species showed higher homogeneity of their enzyme systems.

The time course of the biotransformation of **1** by *C. roseus* was investigated. The results are illustrated in Fig. 2. The substrate was almost completely metabolized by the plant cells 48 h after administration. The maximum concentrations in the culture supernatant for two major products, **2** and **3**, appeared on the third day and the biotransformation rates were determined by HPLC to be 44.7 and 16.5%, respectively. Then the concentrations decreased gradually, indicating the formation of other secondary products. Accordingly, some new peaks were observed in the HPLC chromatograms for samples harvested on the fifth and sixth days. A possible biotransformation pathway is illustrated in Fig. 3.

Compound **1** was also actively metabolized by *P. grandiflorum*. After 8 days incubation, three metabolites were isolated from the culture supernatant. Their structures were respectively identified as cinobufotalin (**6**), desacetylcinobufagin (**7**) and **2** by comparison with literature spectral data [8]. The isolated yield of the major product **2** was 37.8%. Interestingly, **6** is also a major constituent of toad venom. Our result suggested that **1** might be an intermediate in the biosynthetic pathway of **6**.

The transformation products of **1** by *C. roseus* and *P. grandiflorum* were compared by HPLC. From Fig. 4, it can be seen that the two plant suspension cultures showed similar biotransformation patterns on compound **1**. The 5-hydroxylated and 16-*O*-deacetylated product **2** was the major metabolite in either system. The three epimerized products, **3** and **5**, were also detected in both cultures though in different yields. However, compound **4**, the new product with 1β-hydroxyl group, was only observed in the *Catharanthus* culture.

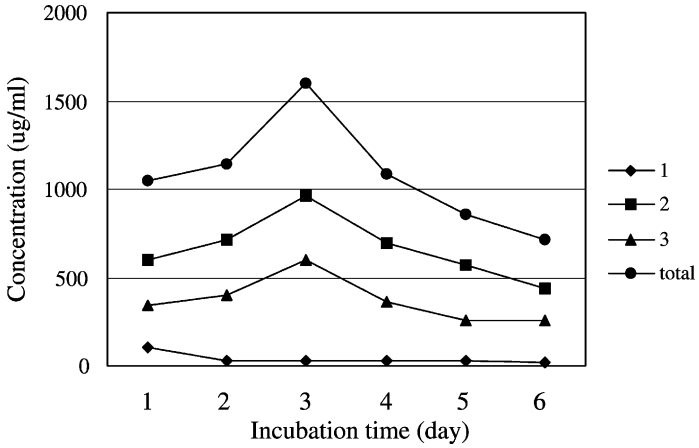



Fig. 2. Time course of the biotransformation of **1** by *Catharanthus roseus*. The results represent the mean values of duplicate determinations. For **1**–**3**, see Fig. 1. Total means the total amount of **1**–**3**.

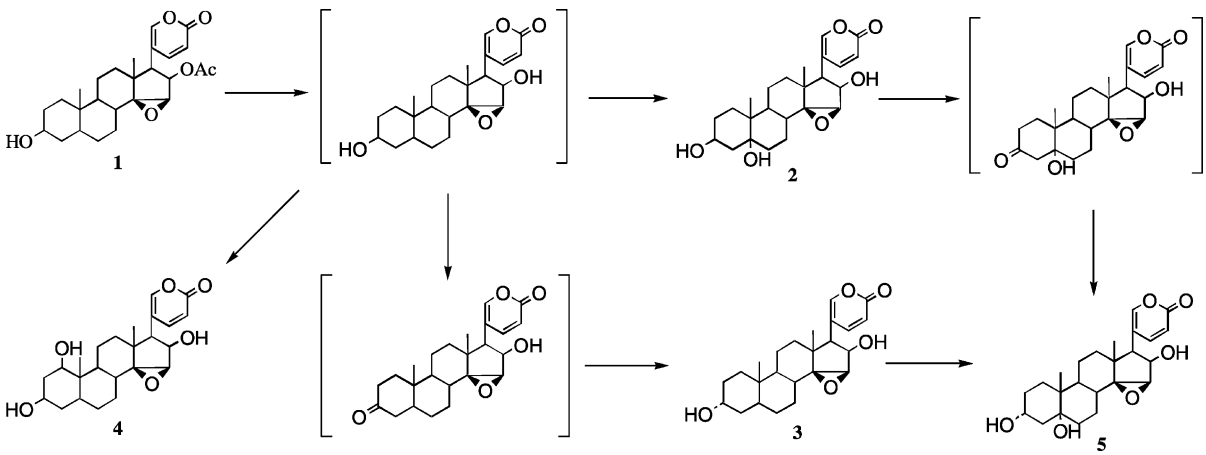



Fig. 3. A proposed biotransformation pathway of cinobufagin (**1**) by *Catharanthus roseus*.

In 1991, Zhang et al. investigated the metabolism of **1** in rat liver microsomes. Six metabolites were detected and identified by HPLC–MS [14,15]. It is noteworthy that deacetylation at the 16-position and epimerization at the 3-position were also found to be the major metabolic routes. In the research and development of new drugs, microorganisms have lately been used as models for drug metabolism studies due to the similarity between microbial metabolites and those obtained from mammalian systems [16]. From our result, it can be deduced that biotransformation by plant cultures can also be of great help in the study of drug metabolism.

Table 2
Cytotoxic activities of compounds **1**–**5** against human cancer cell lines

| Compound | $IC_{50}$ (μmol/l) | | | |
|---|---|---|---|---|
| | Bel-7402 | Hela | HL-60 | MCF-7 |
| **1** | 0.60 | 3.11 | 20.29 | 14.56 |
| **2** | 7.41 | 24.26 | 11.00 | – |
| **3** | – | – | 4.90 | 74.03 |
| **4** | – | 60.20 | 0.02 | – |
| **5** | – | – | 3.10 | – |

No effect (–).

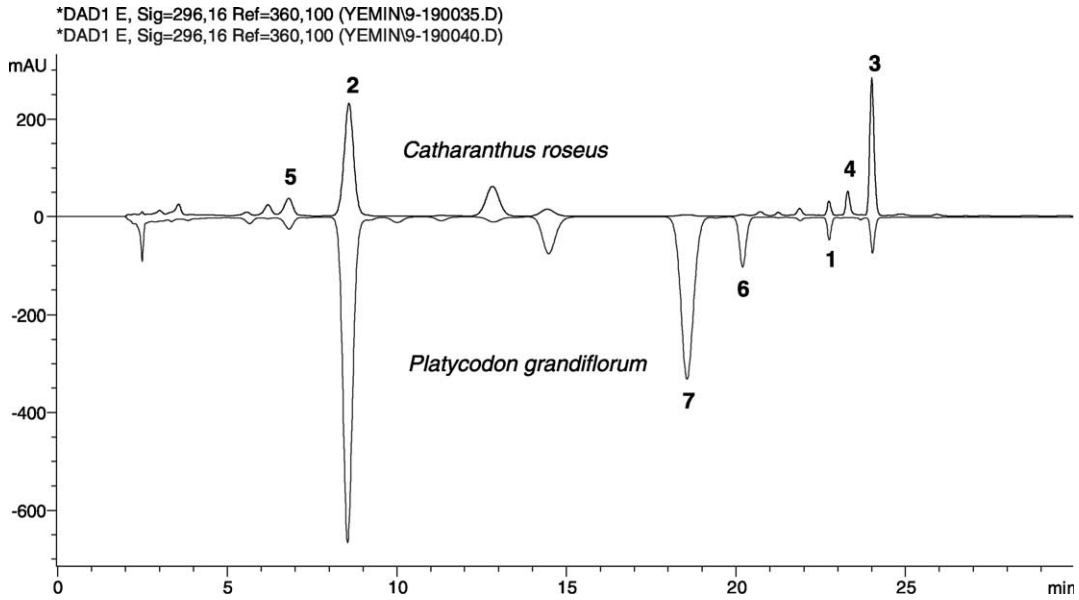



Fig. 4. HPLC chromatograms of the biotransformation products of **1** by *Catharanthus roseus* and *Platycodon grandiflorum*. The eluent was MeOH–$H_2O$ (53:47 (v/v)), held for 15 min, gradient to (75:25 (v/v)) in 5 min, and then held for 10 min. The flow rate was 1.0 ml/min. The analysis was monitored at 296 nm. Column temperature was 25 °C. For **1**–**7**, see Table 1.

In vitro cytotoxic activities of **1**–**5** were measured on four human cancer cell lines (Table 2). All the products showed more potent cytotoxic activities against HL-60 cells than the parent compound. The $IC_{50}$ values for **3**–**5** were 4.90, 0.02 and 3.10 μmol/l, respectively. It seems that 1β-hydroxylation and 3-epimerization could improve the cytotoxicities of cinobufagin derivatives.

## 3. Experimental

### 3.1. General experimental procedures

Melting points were determined on an XT4A apparatus and uncorrected. Optical rotations were measured with a Perkin-Elmer 243B polarimeter. UV spectra were detected on a TU-1901 UV-Vis spectrophotometer. IR spectra were obtained on an Avatar 360 FT–IR spectrophotometer. 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 the internal standard. MALDI-TOF mass spectra were measured on a Bruker BIFLEX III mass spectrometer. Silica gel (200–300 mesh) was purchased from Qingdao Marine Chemical Group, China. Bufadienolides were detected on TLC by spraying with 10% $H_2SO_4$ and heating at 120 °C to produce yellowish fluorescent spots. Cinobufagin (**1**) was purchased from Chinese Institute for Control of Pharmaceutical and Biological Products, China. The purity was above 98% determined by HPLC.

### 3.2. Plant cell cultures

Cell suspension cultures of *C. roseus* (L.) G. Don and *P. grandiflorum* (Jacq.) A.D.C. were established from young leaves of the plants. The cultures were maintained in MS medium supplemented with 0.5 mg/l 6-BA, 0.5 mg/l NAA, 0.2 mg/l 2,4-D and 3% (w/v) sucrose on a rotary shaker (110 rpm) at 25 °C in darkness [17].

### 3.3. Biotransformation of *1* by Catharanthus roseus

The substrate (**1**, 20 mg) in 1 ml EtOH was added to 400 ml of suspension cells pre-cultured for 7 days

in a 1000 ml Erlenmeyer flask. In total, 560 mg substrate was used. The incubation was continued for another period of 6 days. After incubation, the culture was filtered and the filtrate was extracted with the same volume of EtOAc for five times. The organic phase was collected and concentrated in vacuo to dryness. The residue (1.2 g) was applied to a silica gel column ($\phi$2.0 cm × 55 cm) and eluted with petroleum ether–acetone (a gradient from 400:115 to 1:1, 1.0 ml/min) to yield **2** (25.6 mg), **3** (16.4 mg), **4** (15.4 mg), and **5** (18.9 mg), respectively.

*Desacetylcinobufotalin* (**2**): colorless needles; $C_{24}H_{32}O_6$; mp 275–277 °C; $[\alpha]_D^{25}$ +24.02° (*c* 0.066, MeOH); UV $\lambda_{max}$ (MeOH): 204.0, 295.0 nm; FAB-MS (*m*/*z*): 417 $[M+H]^+$ (28), 401 (25), 282 (16), 155 (11), 133 (20), 109 (24), 93 (43), 57 (100); $^1$H-NMR (DMSO-$d_6$, 500 MHz): 7.91 (1H, d, $J$ = 9.8 Hz, H-22), 7.44 (1H, brs, H-21), 6.15 (1H, d, $J$ = 9.8 Hz, H-23), 4.60 (1H, dd, $J$ = 4.2 Hz, 9.0 Hz, H-16), 3.99 (1H, brs, H-3), 3.50 (1H, s, H-15), 2.58 (1H, d, $J$ = 9.0 Hz, H-17), 0.84 (3H, s, 19-$CH_3$), 0.64 (3H, s, 18-$CH_3$).

*3-epi-Desacetylcinobufagin* (**3**): white powders; $C_{24}H_{32}O_5$; mp 278–279 °C; $[\alpha]_D^{25}$ + 20.58° (*c* 0.012, MeOH); UV $\lambda_{max}$ (MeOH): 207.0, 294.0 nm; TOF-MS (*m*/*z*): 423 $[M+Na]^+$, 401 $[M+H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): 7.91 (1H, d, $J$ = 9.5 Hz, H-22), 7.43 (1H, s, H-21), 6.15 (1H, d, $J$ = 9.5 Hz, H-23), 4.63 (1H, dd, $J$ = 4.0, 9.5 Hz, H-16), 3.51 (1H, s, H-15), 3.39 (1H, brs, H-3), 2.59 (1H, d, $J$ = 9.5 Hz, H-17), 0.86 (3H, s, 19-$CH_3$), 0.65 (3H, s, 18-$CH_3$).

*1β-Hydroxyl desacetylcinobufagin* (**4**): white crystalline powders; $C_{24}H_{32}O_6$; mp 177–180 °C; $[\alpha]_D^{25}$: −3.0° (*c* 0.033, MeOH); UV (MeOH) $\lambda_{max}$: 207.0, 294.0 nm; IR (KBr) $\nu_{max}$: 3401 (OH), 1714 (C=O), 1627 (C=C), 1537, 1453, 1132, 1047 cm$^{-1}$; TOF-MS (*m*/*z*): 417.2 $[M + H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): 7.90 (1H, d, $J$ = 9.5 Hz, H-22), 7.43 (1H, s, H-21), 6.14 (1H, d, $J$ = 10.0 Hz, H-23), 5.12 (1H, d, $J$ = 4.9 Hz, 3-OH), 4.99 (1H, d, $J$ = 4.5 Hz, 16-OH), 4.78 (1H, d, $J$ = 7.3 Hz, 1-OH), 4.60 (1H, dd, $J$ = 4.4, 8.6 Hz, H-16), 3.99 (1H, brs, H-3), 3.60 (1H, brs, H-1), 3.49 (1H, s, H-15), 2.56 (1H, d, $J$ = 9.3 Hz, H-17), 1.92 (1H, m, H-8), 1.91 (1H, m, H-4), 1.89 (1H, m, H-5), 1.70 (2H, m, H-2), 1.67 (1H, m, H-6), 1.60 (1H, m, H-12), 1.52 (1H, brt, $J$ = 10.0 Hz, H-9), 1.38 (1H, m, H-12), 1.35 (1H, m, H-7), 1.31 (1H, m, H-11), 1.27 (1H, m, H-4), 1.17 (1H, m, H-11), 1.15 (1H, m, H-6), 1.01 (1H, m, H-7), 0.97 (3H, s, 19-$CH_3$), 0.65 (3H, s, 18-$CH_3$); anal. calcd. for $C_{24}H_{32}O_6$ (%): C, 69.21; H, 7.74. Found: C, 69.52; H, 7.76.

*3-epi-Desacetylcinobufotalin* (**5**): white powders; $C_{24}H_{32}O_6$; mp 292–295 °C; $[\alpha]_D^{25}$ 16.8° (*c* 0.041,MeOH); UV $\lambda_{max}$ (MeOH): 207.0, 294.0 nm; TOF-MS (*m*/*z*): 439 $[M + Na]^+$, 417 $[M + H]^+$; $^1$H-NMR (DMSO-$d_6$, 500 MHz): 7.90 (1H, dd, $J$ = 1.6, 9.8 Hz, H-22), 7.43 (1H, d, $J$ = 1.6 Hz, H-21), 6.14 (1H, d, $J$ = 9.8 Hz, H-23), 4.62 (1H, dd, $J$ = 5.3, 9.5 Hz, H-16), 3.51 (1H, s, H-15), 3.75 (1H, brs, H-3), 2.59 (1H, d, $J$ = 9.5 Hz, H-17), 0.79 (3H, s, 19-$CH_3$), 0.65 (3H, s, 18-$CH_3$).

### *3.4. Biotransformation of **1** by Platycodon grandiflorum*

The substrate (**1**, 10 mg) in 1 ml EtOH was added to 400 ml of suspension cells pre-cultured for 7 days in a 1000 ml Erlenmeyer flask. In total 50 mg substrate was used. The incubation was continued for 6 days. Then the culture was filtered and the filtrate was extracted with the same volume of EtOAc for five times. The organic phase was collected and concentrated in vacuo to dryness. The residue was applied to a silica gel column and eluted with petroleum ether–acetone (a gradient from 4:1 to 3:2, 1 ml/min) to yield **6** (2.7 mg), **7** (6.7 mg) and **2** (18.9 mg), respectively.

*Cinobufotalin* (**6**): white powders; $C_{26}H_{34}O_7$; mp 258–260 °C; TOF-MS (*m*/*z*): 459.1 $[M + H]^+$; $^1$H-NMR ($CDCl_3$, 500 MHz): 7.89 (1H, d, $J$ = 10.0 Hz, H-22), 7.16 (1H, brs, H-21), 6.21 (1H, d, $J$ = 10.0 Hz, H-23), 5.44 (1H, d, $J$ = 9.3 Hz, H-16), 4.20 (1H, brs, H-3), 3.62 (1H, s, H-15), 2.79 (1H, d, $J$ = 9.3 Hz, H-17), 1.89 (3H, s, C$\underline{H}_3$COO), 0.98 (3H, s, 19-$CH_3$), 0.82 (3H, s, 18-$CH_3$).

*Desacetylcinobufagin* (**7**): white powders; $C_{24}H_{32}O_5$; mp 129–130 °C; FAB-MS (*m*/*z*): 401 $[M + H]^+$ (100), 282 (4), 159 (4), 109 (10), 95 (14); $^1$H-NMR ($CDCl_3$, 500 MHz): 7.95 (1H, d, $J$ = 10.0 Hz, H-22), 7.24 (1H, brs, H-21), 6.23 (1H, d, $J$ = 10.0 Hz, H-23), 4.73 (1H, d, $J$ = 9.0 Hz, H-16), 4.14 (1H, brs, H-3), 3.57 (1H, s, H-15), 2.60 (1H, d, $J$ = 9.0 Hz, H-17), 0.98 (3H, s, 19-$CH_3$), 0.81 (3H, s, 18-$CH_3$).

### 3.5. HPLC analysis

An Agilent 1100 liquid chromatograph system with a photodiode array detector was used. The column was Extend-$C_{18}$ (5 μm, $\phi$4.6 mm × 250 mm) packing. The eluent was MeOH-$H_2O$ (53:47 (v/v)), held for 15 min, linearly gradient to (75:25 (v/v)) in 5 min, and then held for 10 min. The flow rate was 1.0 ml/min. The analysis was monitored at 296 nm. Column temperature was 25 °C.

### 3.6. Time course of the biotransformation of *1* by *Catharanthus roseus*

Five grams (fresh weight) of *Catharanthus* cells was inoculated into 500 ml Erlenmeyer flasks containing 200 ml of culture medium. After 5 days incubation, 3.0 mg of **1** in 1 ml EtOH was added to the culture. The incubation was allowed to continue for further 1–6 days, respectively. After that, the culture was filtered and the supernatant was extracted with EtOAc. The organic phase was concentrated in vacuo and dissolved in 1 ml of MeOH. The sample was filtered through a 0.45 μm filter membrane just before use. A 10 μl of the solution was injected into the HPLC instrument for analysis. The major products, **2** and **3**, together with the substrate in the culture supernatant were quantitatively determined. The regression equations for **1**, **2**, and **3** were $Y = 7.93X - 43.67$ ($r = 0.9993$), $Y = 7.18X - 85.96$ ($r = 0.9993$), and $Y = 4.93X - 28.16$ ($r = 0.9992$), respectively. Here $Y$ represents peak area, $X$ the concentration (μg/ml), and $r$ the correlation coefficient.

### 3.7. Bioassay

The human cancer cell lines (Bel-7402, Hela, HL-60, and MCF-7) were maintained in RPMI medium 1640 (GIBCO/BRL) plus 10% fetal bovine serum and grown in 96-well microtiter plates for the assay. Appropriate dilutions of the test compounds ($10^{-3}$ to $10^2$ μmol/l) were added to the culture. After incubation at 37 °C, 5% $CO_2$ for 48 h, the survival rate of the cells in the cultures were evaluated by the MTT method. The effect was shown as $IC_{50}$ (which is the concentration of test compound (μmol/l)) to give 50% inhibition of the growth of the carcinoma cells. Results were expressed as the mean values for triplicate determinations.

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