

Microbial transformation of three bufadienolides by *Nocardia* sp. and some insight for the cytotoxic structure–activity relationship (SAR)

Jian Zhang,^{a,b} Yang Sun,^b Ji-Hua Liu,^a Bo-Yang Yu^{a,*} and Qiang Xu^b

^aDepartment of Complex Prescription of TCM, China Pharmaceutical University, Nanjing 210038, People's Republic of China

^bState Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, People's Republic of China

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Abstract—Resibufogenin, cinobufagin, and bufalin are cytotoxic steroids isolated from the Chinese drug Chan'su. Biotransformation of these three bufadienolides by *Nocardia* sp. NRRL 5646 was investigated. Notably, resibufogenin was converted to 3-acetyl 15 β -hydroxyl bufotalin, via an unprecedented 14 β ,15 β -epoxy ring cleavage and a regio-selective acetoxylation. This product showed significantly increased cytotoxic activity. The regio-selective acetylation of the 3-OH was also involved in the other reactions. The structures of metabolites were established by ESI-LC/MS and 2D NMR techniques. The in vitro cytotoxic activities against human cancer cell lines of the substrates and the transformed products were determined by the MTT method and their structure–activity relationship (SAR) was discussed. This investigation provided a useful approach to prepare new bufadienolides and the SAR research.

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Chan'su, also called toad venom or toad poison, which is made of the skin secretions of giant toads, including *Bufo gargarizans* and *B. melanostictus*, is a popular Chinese Traditional Medicine (TCM). It has been used extensively in clinics, either alone or in combination with other herbal ingredients in the forms of brand complex formula such as *Liu-Shen-Wan* and *She-Xian-Bao-Xin-Wan*. Bufadienolides, the major active constituents of Chan'su, are C-24 steroids, the characteristic structural feature of which is a doubly unsaturated six membered lactone ring (α -pyrone) on position 17 β . Furthermore, these compounds are characterized by the *trans*-junction of rings B and C and usually the *cis*-junction of rings C and D.^{1,2} Resibufogenin, cinobufagin, and bufalin are the three major components of Chan'su. And their contents in the crude drug could be as high as 5–10% of the dry weight. They have been reported to exhibit significant inhibitory activities against human myeloid leukemia cells (K562, U937,

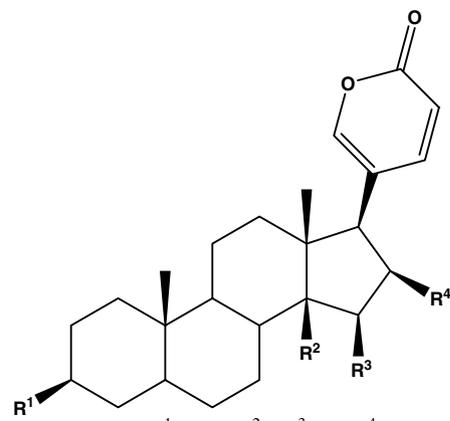
ML1, and HL60), human hepatoma cells (SMMC7221), and prostate cancer cells (LNCaP, DU105, and PC3). The activities are mediated by induction of cell apoptosis and cell differentiation, and the regulations of a variety of genes and proteins are involved in the process.^{3–5}

Biotransformation is an alternative tool in the structural modification of complex natural products due to its great capabilities to catalyze novel reactions and its regio- and stereo-selectivity.^{6,7} Microorganisms, especially actinomycetes, are well known as efficient and selective catalysts. In previous papers we reported the biotransformation of some complex natural products and obtained a series of new products.^{8–11} Therefore, it is envisioned that biotransformation of bufadienolides may provide some analogues which could be utilized for screening for new activities or better activity/side effects profile. By studying the cytotoxicities of the obtained products, we can explore the structure and activity relationship (SAR) of such compounds (Fig. 1).

Nocardia sp. NRRL 5646, one of the commonly used microbes, for biotransformation because of its broad-spectrum of enzymatic capability such as carboxylic acid and aldehyde reduction, phenol methylation, and skele-

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*Corresponding author. Tel.: +86 25 83313080; fax: +86 25 85391042; e-mail: boyangyu59@163.com

Compound 1 R¹=OH, R²=R³=O, R⁴=HCompound 2 R¹=OAc, R²=R³=O, R⁴=HCompound 3 R¹=OAc R²=R³=OH, R⁴=OAcCompound 4 R¹=OH, R²=R³=O, R⁴=OAcCompound 5 R¹=OAc, R²=R³=O, R⁴=OAcCompound 6 R¹=OH, R²=OH, R³=R⁴=HCompound 7 R¹=OAc, R²=OH, R³=R⁴=H**Figure 1.** Structures of resibufogenin (**1**), cinobufagin (**4**), and bufalin (**6**) and related biotransformation products.

ton re-arrangement of quinovic acid glycosides via methyl migration,⁸ was found to convert the substrates efficiently to less polar metabolites. In this report, we describe the unique biotransformation results of resibufogenin, cinobufagin, and bufalin upon microbe *Nocardia* sp. and the cytotoxic activity of the related compounds. The substrates were incubated at a preparative-scale with microbe *Nocardia* sp. according to the standard two-stage fermentation protocol. The work-up and isolation of metabolites were carried out similarly as described previously.¹²

A 100 mg sample of **1** (C₂₄H₃₂O₄, M_r = 384) was used for the preparative-scale incubation for 120 h. The incubation cultures were filtered and the broth was extracted with EtOAc. The pooled EtOAc solution was dried over anhydrous Na₂SO₄, and the solvent was removed in a vacuum. The resulting residue was subjected to silica gel chromatographic separation which afforded two metabolites, M-1 (**2**, 12% yield based on the starting material **1**) and M-2 (**3**, 29%).

M-1 (**2**) was obtained as white powder. Extensive mass and NMR spectroscopic studies revealed it to be 3-acetyl resibufogenin on the basis of its NMR data (Table 1) with literature values.¹³

The ESI-MS spectrum of M-2 (**3**) showed the [M-H]⁻ ion peak at 501.2, suggesting the molecular formula C₂₈H₃₈O₈. Comparing the NMR spectrum with that of compound **2**, there was one additional acetoxyl group signals and the signal at δ 75.1 was assigned as a tertiary

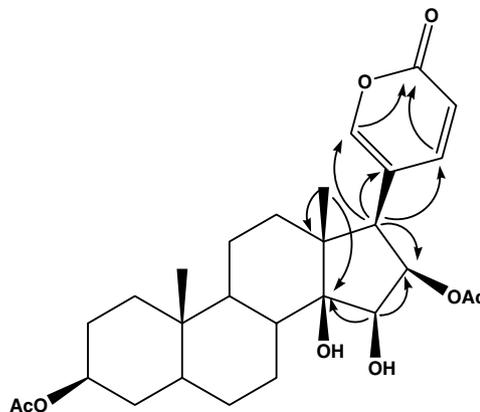
Table 1. ¹³C NMR spectral data of compounds **1–3**^a

C	1	2	3	C	1	2	3
1	29.5	30.6	30.5	14	74.7	74.5	85.1
2	27.9	26.1	26.0	15	60.0	60.0	74.2
3	66.7	70.6	70.6	16	32.4	32.5	75.1
4	33.3	30.8	30.7	17	47.8	47.7	47.7
5	36.0	37.3	37.2	18	16.9	17.0	16.8
6	25.8	25.2	25.2	19	23.8	23.8	23.7
7	21.1	21.0	21.1	20	122.3	122.5	121.5
8	39.3	39.2	39.3	21	149.6	150.7	150.6
9	33.6	33.9	33.6	22	147.0	147.3	148.7
10	35.5	35.5	35.4	23	115.2	115.2	115.2
11	20.8	21.3	21.1	24	162.0	161.7	160.3
12	39.4	39.5	39.5	C=O		170.3	170.3
13	45.3	45.3	47.7	CH ₃		21.3	21.3

^a Recorded in pyridine-d₅.

carbon by the DEPT experiments, and the disappearance of one signal at δ 32.5 (C-16) of one secondary carbon showed that the structure of **3** was very similar to that of cinobufagin, which contains one acetoxyl group at C-16. When compared that to resibufogenin, the C-14 and C-15 epoxy ring signals shifted downfield by 10.4 ppm and 14.2 ppm, this suggested the cleavage of the epoxy ring. By the literature study of the NMR data of the bufalin, cinobufagin, bufotalin, and their derivatives,^{14–18} we confirmed that compound **3** was a hydroxylated bufotalin derivative. In accordance, the HMBC spectrum showed the long-range correlation between H-15 (δ 3.57) and C-14 (δ 85.1). The stereochemistry of 15-OH was established as β-configuration by comparing the carbon signals with those in 15β-hydroxyl bufalin (the chemical shift of the C-15 with the α-configuration hydroxy group is 78.2 ppm).¹⁹ In the NOESY spectrum, a correlation between H-15 and H-7 (δ 0.94) suggested that H-15 was α-oriented and the hydroxyl group was β, thereby compound **3** was further identified as 3-acetyl 15β-hydroxyl bufotalin (Figs. 2 and 3).

Structurally, it is particularly noteworthy that compound **3**, a bufotalin derivative, does not have the 14β,15β-epoxy group in the skeleton. As we all know, the presence of a 14β hydroxy group or a 14β,15β-epoxy

**Figure 2.** Key HMBC correlations for 3-acetyl 15β-hydroxyl bufotalin (**3**).

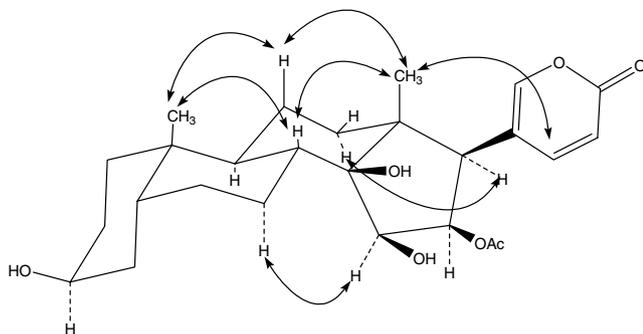


Figure 3. Selected NOESY correlations for 3-acetyl 15 β -hydroxyl bufotalin (**3**).

group is assumed to be an integral part of the natural genins. The large variety of known genin is chiefly due to the number and position of the substituents.² This is the first successful direct conversion of the resibufogenin to a bufotalin derivative, a reaction which should be very difficult to conduct through organic synthesis. From the point view of SAR research, the 16-acetoxy group and 14 β -hydroxyl group are essentially important for the activities of bufadienolides, thus the disappearance of 14 β , 15 β -epoxy ring would increase the cytotoxicity. So it is envisioned that compound **3** would be more active than corresponding resibufogenin analogues. The cytotoxic activities of the biotransformed products were evaluated by the MTT method. Compound **3** showed strong inhibitory activities against human hepatoma HepG2 cells, human gastric cancer BGC-823 cells, and human cervical carcinoma HeLa cells with IC₅₀ values of 2.8, 0.50, and 3.1 μ mol/L, respectively. Its cytotoxicity increased as high as bufalin which is one of the most active bufadienolides isolated from Chan'su. The results were in agreement with the structure–activity relationships previously proposed by Ye and Kamano very well.^{19–21} During the past years, there were many reports on the biotransformation of bufadienolides, the metabolites were mostly hydroxylated products,^{18,19,22–27} the epoxide hydrolase catalyzed epoxy ring cleavage was first observed. It was very helpful to study the biosynthesis pathways of the bufadienolides with or without epoxy ring. The product with enhanced cytotoxicity also encouraged us to explore the structure diversity and the SAR of bufadienolides.

To investigate the capability and selectivity of the enzyme system in *Nocardia* sp. to hydrolyze the 14 β ,15 β -epoxy ring in the bufadienolides, cinobufagin (**4**) was employed for parallel biotransformation under the same conditions. It was found that compound **4** can only be metabolized to 3-acetyl cinobufagin (**5**). It was interesting to note that the minor structure difference between compounds **1** and **4** is an acetoxy group at position of C-16, but the biotransformation result showed great difference. The explanation for this observation could be either the blockade of the access of the substrate to the active domain of the hydrolase enzyme due to the 16-acetoxy group, or more likely, the hydrolysis, hydroxylation, and acetylation are consecutive processes with the assumption that the catalytic active domains are proximate. The biotransformation of bufalin (**6**)

Table 2. Cytotoxic activities of the biotransformation products against human cancer cell lines ($n = 3$)

Compound	IC ₅₀ ^a (μ mol/L)		
	HepG2	HeLa	BGC-823
Taxol	0.82 \pm 0.06	0.02 \pm 0.01	1.15 \pm 0.14
1	>50	>50	7.89 \pm 0.21
2	>50	>50	19.80 \pm 1.37
3	3.10 \pm 0.44	0.45 \pm 0.06	3.37 \pm 0.25
4	5.20 \pm 0.17	0.76 \pm 0.06	8.50 \pm 0.12
5	6.44 \pm 0.41	1.09 \pm 0.11	4.11 \pm 0.55
6	0.64 \pm 0.09	0.73 \pm 0.07	0.99 \pm 0.36
7	0.74 \pm 0.06	1.14 \pm 0.32	0.70 \pm 0.06

^aData were expressed as means \pm SD of three experiments and each includes triplicate sets.

which has two hydroxyl groups on C-3 and C-14 was also studied to explore the regio-selectivity of the acetylation. Only one metabolite 3-acetyl bufalin (**7**) was found. It showed great regio-selectivity of the biotransformation of bufadienolides by *Nocardia* sp. The acetylated products of compounds **5** and **7** showed little weaker but still potent cytotoxicities than the corresponding substrates. These results provided guidance for the future directed synthesis of bufadienolides of pharmaceutical interest (Table 2).

Nocardia sp. NRRL 5646 contains an astonishing array of interesting and useful enzymes, many of which are similar to those found in mammals. A soluble form of nitric oxide synthase(NOS_{NOC}), carboxylic acid reductase, aryl aldehyde oxidoreductase, aldehyde reductase, guanylate cyclase, and ornithine transcarbamoylase, have been characterized in this organism.^{27–30} And the unique catalytic capability of *Nocardia* sp. to the bufadienolides deserves further exploration to enhance the chemical diversity of bufadienolides. Such studies could provide new platforms for combinatorial synthesis^{31–33} and the development of new bufadienolide antitumor drugs.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2007.09.065](https://doi.org/10.1016/j.bmcl.2007.09.065).

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