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

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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 15β-hydroxyl bufotalin, via an unprecedented 14β,15β-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.

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 17β. 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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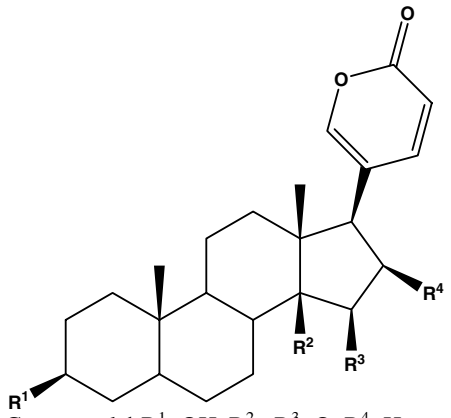

Compound **1** R^1=OH, R^2= R^3=O, R^4=H

Compound **2** R^1=OAc, R^2= R^3=O, R^4=H

Compound **3** R^1=OAc R^2= R^3=OH, R^4=OAc

Compound **4** R^1=OH, R^2= R^3=O, R^4=OAc

Compound **5** R^1=OAc, R^2= R^3=O, R^4=OAc

Compound **6** R^1=OH, R^2=OH, R^3=R^4=H

Compound **7** R^1=OAc, R^2=OH, R^3=R^4=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** ($C_{24}H_{32}O_4$, 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 Na_2SO_4, 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 $[M\text{-}H]^-$ ion peak at 501.2, suggesting the molecular formula $C_{28}H_{38}O_8$. 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. ^{13}C 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 derivates,[14–18] we confirmed that compound **3** was a hydroxylated bufotalin derivate. 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

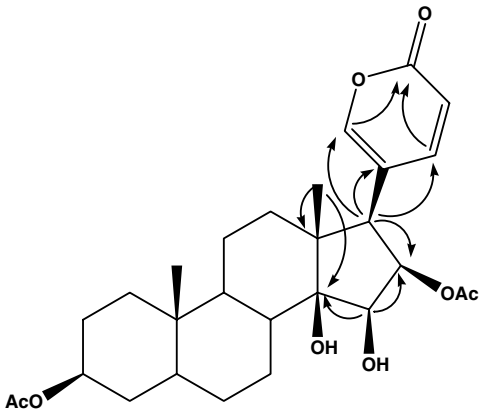

Figure 2. Key HMBC correlations for 3-acetyl 15β-hydroxyl bufotalin (**3**).