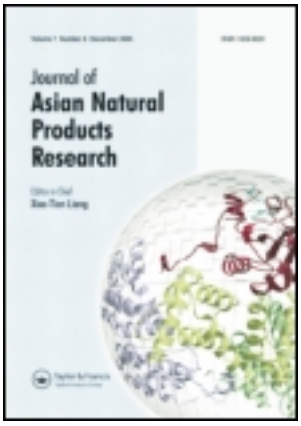

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Novel microbial transformation of resibufogenin by *Fusarium solani*

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In this paper, microbial transformation of resibufogenin by *Fusarium solani* AS 3.1829 was investigated, and five transformed products were isolated and identified as 3-ketone-resibufogenin (**2**), 3-one-cyclic 3-(1,2-dimethyl-1,2-ethanediylacetal)-resibufogenin (**3**), 3-dimethoxyl-resibufogenin (**4**), 3-epi-resibufogenin (**5**), and 3-epi-15α-hydroxy-7βH-bufalin (**6**), respectively. Among them, **3**, **4**, and **6** are new compounds, and the rare double oxidization of C-3 was reported. In addition, the cytotoxicities of transformed products were also investigated.

Keywords: biotransformation; bufadienolides; *Fusarium solani*; resibufogenin

1. Introduction

Chan Su is a traditional Chinese medicine derived from the skin secretions of giant toads including *Bufo bufo melanostictus* Schneider and *B. bufo gargarizans* Cantor [1–3]. The major active constituents of Chan Su are bufadienolides having the blood pressure-stimulating, strong cardiac, antiviral, and local anesthetic activities. Resibufogenin is one of the main bioactive bufadienolides in the crude drug with the content of 3%. 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. It has been reported to exhibit significant inhibitory activities against various human cancer cells including human cervical carcinoma cells (Hela) and human hepatoma cells (Bel7402) with IC_{50} values of 1–10 nM, respectively [4]. However, the poor water solubility limited its clinical use.

Microbial transformation is a useful technique in the structural modification of natural products having the advantages of the significant stereo- and regio-selectivity [5–9]. A large number of bufadienolide derivatives by microbial transformation have been reported in the previous researches [10–12]. Meantime, microbial transformation could also be used as the *in-vitro* model to simulate the drug metabolism in mammals [13].

To find new bufadienolide derivatives with more potent bioactivities and improve water solubility, we investigated microbial transformation of resibufogenin (**1**) by *Fusarium solani* AS 3.1829 in this paper. Totally, five derivatives of **1** were isolated and identified by spectral methods including 2D NMR. Among them, products **3**, **4** and **6** are new, and compound **5** is the major metabolite of rats *in vivo*. Some novel region-specific oxidation and

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hydrolysis reactions of **1** were observed. In addition, the cytotoxicities of transformed products against Hela cells were also investigated.

2. Results and discussion

2.1 Preliminary screening for biotransformation

The screening test of biotransformation showed that substrate (**1**) could be completely transformed by *F. solani* AS 3.1829 rapidly. And some new peaks appeared in HPLC of the incubation extract; meanwhile, no corresponding peaks were observed in control tests. The UV absorption maxima of these new peaks were at 294–298 nm, which is characteristic for a-pyrone ring of bufadienolides. This evidence suggested that they were the biotransformed products of **1**. In the preparative biotransformation, five products were isolated, and their chemical structures were determined by the extensive spectral techniques. The chemical structures and possible biotransformation pathway are illustrated in Figure 1.

2.2 Characterization of biotransformed products

Compounds **2** and **5** were identified as 3-ketone-resibufogenin and 3-epi-resibufogenin, respectively. Their ^{1}H and ^{13}C NMR spectral data were in agreement with those reported in the literatures [14–16].

Compound **3** was obtained as a white powder in methanol. Its HR-MS showed an $[M + H]^+$ ion peak at *m/z* 455.2427, indicating the molecular formula of $C_{28}H_{38}O_5$. Compared with the molecular weight of **1**, the molecular weight of **3** is added for *m/z* 70, suggesting that a new side chain was substituted in the molecule of resibufogenin. Meanwhile, the signals of H-3 (δ 4.08) and C-3 (δ 64.5) of **1** were not observed [1] and an oxygen-bearing quarternary carbon signal at δ 108.4 appeared in the ^{13}C NMR spectrum of **3**, suggesting that C-3 was substituted by two oxygen atoms. In HMBC spectrum, the long-range correlations of the carbon signal at δ 108.4 with H_a-4 (δ 1.95) and H_a-2 (δ 1.53) were observed. This evidence indicated that the dioxygen

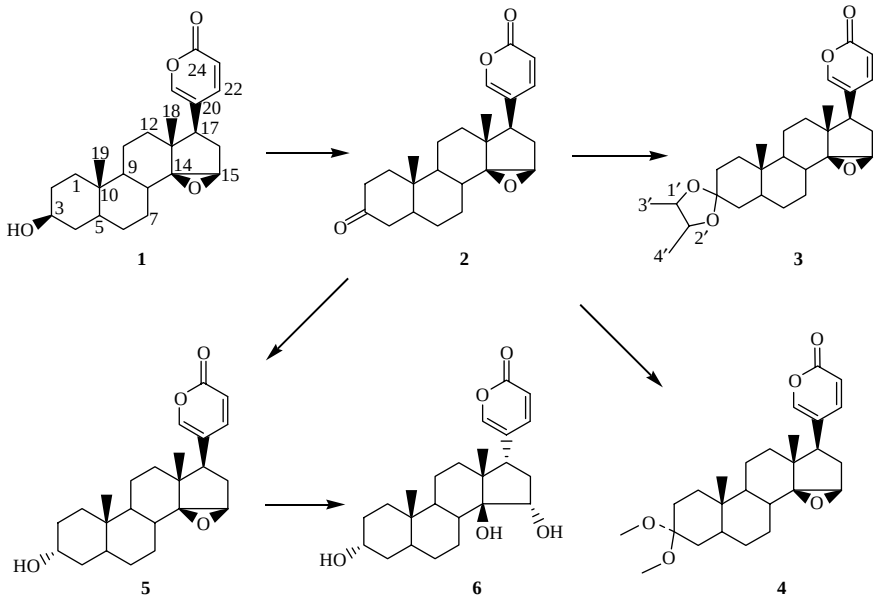

Figure 1. A possible biotransformation pathway of **1** by *F. solani* AS 3.1829.

substitution should be located at C-3. The proton signal at δ 1.15 showed the HMBC correlations with C-1′ (δ 77.6) and C-2′ (δ 77.4). And the ^{1}H–^{1}H COSY correlation between H-1′ (δ 3.53) and CH_3-3′ (δ 1.15) was observed. In the NOESY spectrum, the NOE enhancements of H-1′ with H-2 (δ 1.40) and H_b-4 (δ 1.25) were observed. This evidence indicated that two oxygen-bearing tertiary carbons connected with the two oxygens of C-3 to form a new five-membered ring at C-3 position. On the basis of the above analysis, compound **1** was determined as 3-one-cyclic 3-(1,2-dimethyl-1,2-ethanediyl acetal)-resibufogenin. All ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

Compound **4** was obtained as a white powder. Its HR-MS showed an $[M + H]^+$ ion peak at *m/z* 429.2566, indicating the molecular formula of $C_{26}H_{36}O_5$. The ^{1}H NMR spectrum showed two new methyl groups at δ 3.07 and 3.01. Comparison of its ^{13}C NMR spectrum of **4** with that of **1** suggested the disappearance of C-3 at δ 64.5 in compound **1** and the appearance of an additional carbon signal at δ 100.2 in the chemical structure of **4**. In HMBC spectrum, the carbon signal at δ 100.2 had long-range correlations with H-1 (δ 1.68) and H-5 (δ 1.61), confirming that C-3 was substituted by two oxygens. Meantime, the methyl groups at δ 3.07 and 3.01 displayed the HMBC correlations with C-3 (δ 100.2), implying two methoxyls located at C-3. On the basis of the above analysis, compound **4** was determined to be 3-dimethoxyl-resibufogenin. All ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

Compound **6** was obtained as a white powder in methanol. Its HR-MS showed an $[M + H]^+$ ion peak at *m/z* 403.2452, indicating the molecular formula of $C_{24}H_{34}O_5$. The carbon signals of **6** were almost the same with those of 15β-hydroxyl-bufalin [17]. In NOESY spectrum, 18-Me had NOE with H-17 (δ 2.78), suggesting that the 17-pyrone ring should be α-oriented. And the chemical shift of C-12 changed to δ 33.5, due to the *γ-gauche* effect. In the NOESY spectrum, H_a-16 (δ 2.31) showed correlations with H-15 (δ 4.06) and H-17 (δ 2.78), suggesting that 15-OH should be in α-configuration. On the basis of the above analysis, compound **6** was determined as 3-epi-15α-hydroxy-17βH- bufalin. All ^{1}H and ^{13}C NMR spectral data were unambiguously assigned by 2D NMR spectra (Table 1).

2.3 Cytotoxic activity

The cytotoxicities of metabolites against Hela cells were determined by the MTT method. The IC_{50} values of metabolites **1**–**6** were 4.5×10^{-2}, 2.9, 0.89, 0.17, 8.1, and 85.2 çM, respectively. Our results suggested that the biotransformation reactions such as 3-isomerization, 3-dioxidation, and hydrolysis of C-14 and C-15 would obviously decrease the cytotoxic activities of resibufogenin.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a PerkinElmer 243B polarimeter. 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 an internal standard. HR-MS were measured on a Bruker APEXII Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer. The HPLC equipments used were the Dionex 3000 HPLC and Agilent 1100 series HPLC system equipped with diode-array detector. The analysis was accomplished with Agilent Extend-C18 column (5 μm, φ 4.6 × 250 mm), and preparative chromatographic isolation was carried out with a YMC-Pack ODS-A (5 μm, φ 10 × 250 mm). HPLC grade acetonitrile (from Tedia, OH, USA) was

Table 1. ^{1}H NMR (500 MHz, DMSO-d_6) and ^{13}C NMR (125 MHz, DMSO-d_6) spectral data of compounds **3**, **4**, and **6**.

	3		**4**		**6**	
	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR	^{1}H NMR	^{13}C NMR
1	1.63 m	33.9	1.68 m	27.0	1.54 m	30.9
	1.22 m		1.26 m		1.30 m	
2	1.53 m	31.2	1.61 m	32.4	1.55 m	36.6
	1.40 m		1.60 m		1.09 m	
3	–	108.4	–	100.2	3.36 br s	70.0
4	1.95 m	37.3	1.65 m	32.6	1.64 m	36.6
	1.25 m		1.50 m		1.33 m	
5	1.49 m	39.2	1.61 m	38.7	1.32 m	40.9
6	1.72 m	25.7	1.72 m	25.5	1.86 m	26.8
	1.15 m		1.18 m		1.79 m	
7	1.32 m	20.2	1.34 m	20.2	1.68 m	29.2
	0.96 m		0.96 m		1.23 m	
8	1.83 m	33.3	1.87 m	33.1	1.72 m	42.3
9	1.62 m	38.6	1.62 m	38.8	1.97 m	42.4
10	–	34.6	–	34.7	–	34.8
11	1.45 m	20.9	1.44 m	20.6	1.43 m	23.7
	1.19 m		1.18 m		0.99 m	
12	1.62 m	38.4	1.61 m	38.1	1.35 m	33.5
	1.45 m		1.46 m		0.99 m	
13	–	44.8	–	44.6	–	59.9
14	–	74.2	–	73.9	–	85.5
15	3.60 s	59.6	3.60 s	59.4	4.06 d (4.5)	79.3
16	2.37 dd (14.5, 10.5)	31.8	2.40 dd (14.5, 10.5)	31.5	2.31 dd (15.0, 10.5)	38.5
	1.80 d (14.5)		1.84 d (14.5)		1.51 m	
17	2.53 d (10.5)	46.4	2.52 d (10.5)	46.2	2.78 dd (12.0, 10.5)	46.0 d
18	0.67 (3H, s)	16.8	0.67 (3H, s)	16.5	0.92 (3H, s)	20.0 q
19	0.89 (3H, s)	23.1	0.89 (3H, s)	23.0	0.87 (3H, s)	22.5 q
20	–	122.2	—	122.0	—	119.5 s
21	7.52 s	150.8	7.51 s	150.5	7.54 s	149.4 d
22	7.75 dd (10.0, 2.5)	147.6	7.75 dd (10.0, 2.5)	147.3	7.55 dd (10.5, 3.0)	147.8 d

1	1.63 m	33.9	1.68 m	27.0	1.54 m	30.9
23	6.25 d (10.0)	114.3	6.24 d (10.0)	114.1	6.30 d (10.5)	114.2 d
24	–	161.2	–	161.0		161.9 s
1′	3.53 br s	77.6	3.07 s	46.7		
2′	3.53 br s	77.4	3.01 s	46.7		
3′	1.15 d (3H, 5.5)	17.1				
4′	1.15 d (3H, 5.5)	16.9				

used. Other solvents were of AR grade (from Beijing Chemical Company, Beijing, China). Silica gels for column chromatography and silica gel plate for thin-layer chromatography (TLC) were products of Qingdao Marine Chemical Factory (Qingdao, China). Resibufogenin (**1**) was isolated from the crude material of Chan Su, and was identified by NMR and MS techniques. Its purity was above 98% by HPLC analysis.

3.2 Microorganism and culture medium

F. solani AS 3.1829 was purchased from China General Microbiological Culture Collection Center (Beijing, China) and maintained on potato agar slants at 4°C. Fermentations of fungi were carried out in the potato medium. Potato medium was made by the following procedures: 200 g of husked potatoes was boiled in water for 30 min, then solution was filtered, and the filtrate was added with 1 L water after adding 20 g of glucose.

3.3 Culture and biotransformation procedures

Screening scale biotransformation of **1** by *F. solani* AS 3.1829 was carried out in 250 ml Erlenmeyer flasks containing 100 ml of liquid potato medium. Mycelia were transferred into the flasks from the slants. The flasks were placed on the rotary shakers, operating at 180 rpm at 30°C. The substrates were dissolved in EtOH to reach a concentration of 10 mg/ml, and 0.2 ml of the solution was added into each fermentation flask after 36 h. The incubation was allowed to continue for an additional 4 days. Substrate controls that consisted of liquid potato medium containing the same amount of substrate were incubated under the same conditions. Culture controls consisted of fermentation blanks in which microorganisms were grown only with the same amount of EtOH. And no

transformed products were observed, suggesting that the substrate was very stable.

Preparative scale biotransformation of **1** by *F. solani* AS 3.1829 was carried out in 1000 ml Erlenmeyer flasks containing 400 ml of liquid potato medium. A total amount of 1000 mg of **1** was used for preparative biotransformation. Other procedures were the same as screening scale biotransformation.

3.4 Extraction, purification, and identification of biotransformation products

The culture was filtered *in vacuo*. The filtrate was extracted with the same volume of EtOAc for three times to obtain 6 g extract. The residue of ethyl acetate extract was subjected to a silica gel column and eluted with petroleum ether–acetone (from 10:1 to 1:1, v/v) to afford six fractions (I–VI). Fraction I (0.5 g) was subjected to preparative HPLC and eluted with MeOH–H_2O (95:5, v/v; 2.0 ml/min; 298 nm) to yield **2** (10.0 mg). Fraction III (1.2 g) was crystallized in acetone to give **5** (605.5 mg). Fraction IV (0.6 g) was separated by preparative HPLC and eluted with MeOH–H_2O (85:15, v/v; 2.0 ml/min; 298 nm) to give **3** (16.8 mg) and **4** (6.1 mg). Fraction V (0.9 g) was subjected to preparative HPLC and eluted with MeOH–H_2O (65:35, v/v; 2.0 ml/min; 298 nm) to yield **6** (19.6 mg). Purities of all the transformed products were above 96% by RP-HPLC analysis.

*3.4.1 3-One-cyclic-3-(1,2-dimethyl-1,2-ethanediylacetal)-resibufogenin (**3**)*

White powder; $C_{28}H_{38}O_5$; $[\alpha]_D^{22}$ +10.1 ($c = 0.12$, MeOH); UV λ_{max} (MeOH): 297.0 nm. IR ν_{max} (KBr): 3400.4, 2948.3, 1723.4, 1230.8, 1015.5 cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) spectral data, see Table 1. HR-FT-ICR-MS: *m/z* 455.2427 $[M + H]^+$ (calcd for $C_{28}H_{39}O_5$, 455.2798).

*3.4.2 3-Dimethoxylresibufogenin (**4**)*

White powder; $C_{26}H_{36}O_5$; $[\alpha]_D^{22}$ +16.6 ($c = 0.08$, MeOH); UV λ_{max} (MeOH): 296.0 nm. IR ν_{max} (KBr): 3350, 2900.3, 1736.6, 1282 cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) spectral data, see Table 1. HR-FT-ICR-MS: *m/z* 429.2596 $[M + H]^+$ (calcd for $C_{26}H_{37}O_5$, 429.2641).

*3.4.3 3-Epi-15α-hydroxy-17βH-bufalin (**6**)*

White powder; $C_{24}H_{34}O_5$; $[\alpha]_D^{22}$ +14.1 ($c = 0.08$, MeOH); UV λ_{max} (MeOH): 298.0 nm. IR ν_{max} (KBr): 3376, 2942, 1711, 1257 cm^{-1}. ^1H NMR (DMSO-d_6, 500 MHz) and ^{13}C NMR (DMSO-d_6, 125 MHz) spectral data, see Table 1. HR-FT-ICR-MS: *m/z* 403.2452 $[M + H]^+$ (calcd for $C_{24}H_{35}O_5$, 403.2484).

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