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New cytotoxic bufadienolides from the biotransformation of resibufogenin by *Mucor polymorphosporus*

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Abstract—Resibufogenin is a cytotoxic steroid isolated from the Chinese drug ChanSu. The biotransformation of resibufogenin by *Mucor polymorphosporus* afforded 22 products, and 15 of them were new. The transformation reactions involved hydroxylations at C-1β, C-5, C-7α, C-7β, C-12α, C-12β and C-16α, as well as epimerization or dehydrogenation of 3-OH. Hydroxylations at C-12α, C-12β and C-16α were the major reactions, each giving products in >5% yields, whereas the other products were obtained in fairly low yields. Some of the products showed decreased but still potent cytotoxicities. This investigation provided a useful approach to prepare new bufadienolides and most of them were difficult to obtain by chemical means.

1. Introduction

Biotransformation is an important tool in the structural modification of organic compounds, especially natural products, due to its significant regio- and stereo-selectivities.[1,2] Filamentous fungi have frequently been used to catalyze selective hydroxylation reactions, which are usually difficult to achieve by chemical means.[3] In the past several years, we have studied the biotransformation of several natural bufadienolides, including bufalin, cinobufagin and resibufogenin, and obtained more than 30 products, 22 of them being new compounds.[4–8] These substrates are the major effective constituents of the Chinese drug ChanSu,[9] and possess significant anticancer activities.[10–13] Our prospective aim is to discover new bufadienolide derivatives with more potent bioactivities and improved physico-chemical properties as drug candidates. Herein we report the biotransformation of resibufogenin by the filamentous fungus *Mucor polymorphosporus*.

2. Results and discussion

In the screening test, resibufogenin (**1**) was completely metabolized in the cultures of *M. polymorphosporus*. A number of new peaks were observed by HPLC in the incubation mixture, whereas no corresponding peak was found in control tests. The peaks showed UV absorption maxima at 294–298 nm, which is characteristic for the α-pyrone ring of bufadienolides. Thus, they should be the biotransformed products of resibufogenin. In the preparative biotransformation, a total amount of 800 mg of resibufogenin was fed to the microbial cultures. After 6 days incubation, the products were recovered by extracting with ethyl acetate. The obtained extract was subjected to ODS column chromatography and preparative HPLC to afford 22 pure compounds, which were structurally characterized by MS and extensive NMR techniques, including ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC, ^{1}H–^{1}H COSY and NOESY. These products were 7β-hydroxyl resibufogenin (**2**),[14] 3-*epi*-7β-hydroxyl resibufogenin (**3**), marinobufagin (5-hydroxyl resibufogenin, **4**),[15] 5,7β-dihydroxyl resibufogenin (**5**), 7α-hydroxyl resibufogenin (**6**), 12β-hydroxyl resibufogenin (**7**),[15] 3-*epi*-12β-hydroxyl resibufogenin (**8**), 5,12β-dihydroxyl resibufogenin (**9**), 12α-hydroxyl resibufogenin (**10**),[14] 3-*epi*-12α-hydroxyl resibufogenin (**11**), 5,12α-dihydroxyl resibufogenin (**12**), 1β,12α-dihydroxyl resibufogenin (**13**), 3-oxo-12α-hydroxyl resibufogenin (**14**), 12-oxo-resibufogenin (**15**), 16α-hydroxyl resibufogenin (**16**),[16] 3-*epi*-16α-hydroxyl resibufogenin (**17**), 3-oxo-16α-hydroxyl resibufogenin (**18**),[17] 7β,16α-dihydroxyl resibufogenin (**19**), 12β,16α-dihydroxyl resibufogenin (**20**), 1β,16α-dihydroxyl resibufogenin (**21**), 12α,16α-dihydroxyl resibufogenin (**22**), and 3-oxo-Δ^{4}-resibufogenin (**23**).[18]

Keywords: Biotransformation; Bufadienolide; Cytotoxicity; *Mucor polymorphosporus*; Resibufogenin.
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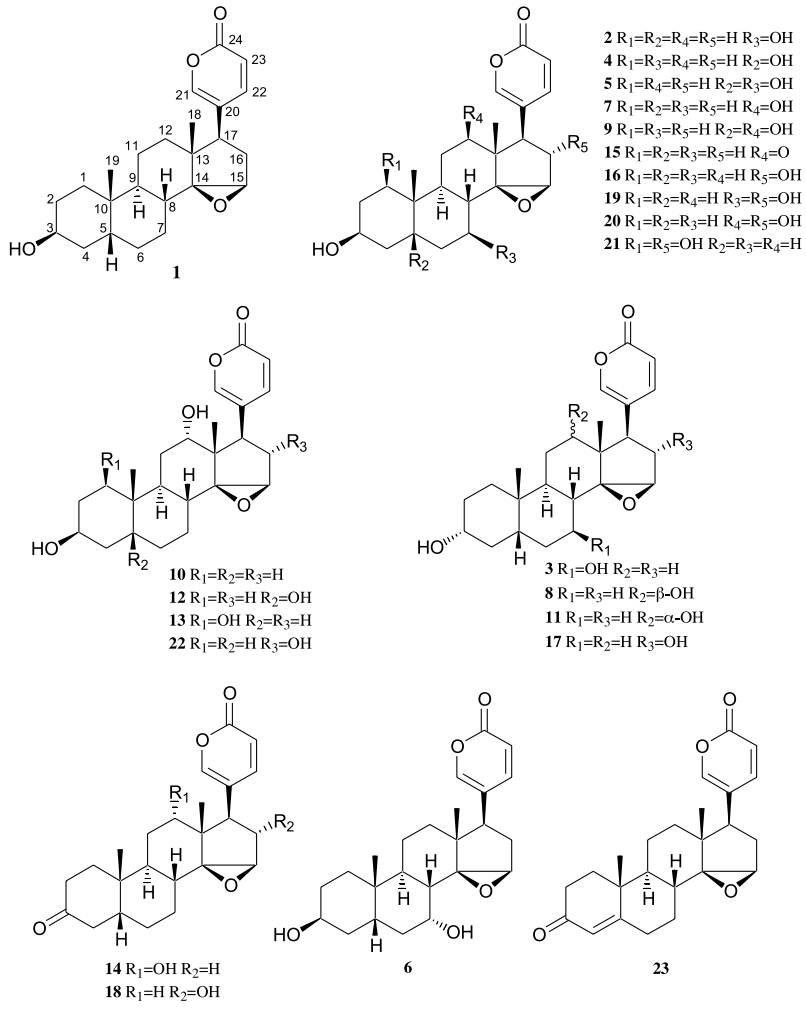

Figure 1. Chemical structures of compounds **1**–**23**.

Table 1. ^{13}C NMR spectral data for compounds **1**–**12** (125 MHz, DMSO-d_6)

C	1	2	3	4	5	6	7	8	9	10	11	12
1	29.4t	29.0t	30.2t	24.8t	24.6t	29.4t	29.4t	30.3t	24.9t	29.3t	30.3t	24.7t
2	27.5t	27.4t	34.3t	27.3t	27.1t	27.6t	27.4t	34.7t	27.2t	27.5t	34.8t	27.3t
3	64.5d	64.2d	69.6d	66.4d	66.2d	64.4d	64.5d	69.7d	66.4d	64.5d	69.8d	66.4d
4	33.0t	34.0t	37.0t	36.5t	37.7t	34.4t	32.9t	36.0t	36.5t	33.1t	36.1t	36.7t
5	35.5d	36.2d	41.5d	74.1s	73.2s	35.4d	35.6d	41.1d	73.4s	35.6d	41.2d	73.5s
6	25.6t	34.1t	34.5t	34.0t	42.2t	36.5t	25.5t	26.0t	34.0t	25.7t	26.2t	34.1t
7	20.2t	66.2d	66.3d	22.4t	67.1d	63.6d	20.4t	20.6t	22.4t	20.2t	20.5t	22.5t
8	33.1d	40.3d	40.4d	32.2d	39.8d	36.5d	32.4d	32.6d	31.5d	33.0d	33.2d	32.1d
9	38.5d	37.3d	38.0d	41.6d	40.0d	31.8d	33.8d	34.6d	36.9d	30.8d	31.6d	34.2d
10	35.0s	34.6s	34.1s	40.4s	40.4s	35.9s	34.7s	34.2s	40.0s	34.6s	34.1s	40.0s
11	20.6t	21.0t	20.7t	21.1t	21.3t	20.2t	29.4t	29.2t	29.9t	28.4t	28.3t	29.0t
12	38.2t	38.2t	38.2t	38.3t	38.1t	37.4t	72.9d	72.8d	72.8d	74.0d	74.0d	74.2d
13	44.6s	45.1s	45.1s	44.4s	44.9s	45.2s	50.7s	50.7s	50.5s	48.9s	48.8s	48.7s
14	74.0s	76.2s	76.2s	73.4s	76.1s	72.2s	73.5s	73.5s	73.5s	72.5s	72.5s	72.6s
15	59.3d	61.1d	61.1d	59.4d	61.1d	63.3d	59.2d	59.3d	59.3d	61.2d	61.2d	61.3d
16	31.6t	31.2t	31.2t	31.5t	31.1t	31.7t	31.6t	31.6t	31.6t	34.8t	34.8t	34.8t
17	46.1d	45.8d	45.7d	46.0d	45.6d	45.3d	41.7d	41.7d	41.6d	42.7d	42.7d	42.7d
18	16.5q	16.3q	16.3q	16.4q	16.3q	16.3q	11.4q	11.4q	11.3q	17.3q	17.3q	17.2q
19	23.7q	23.6q	23.1q	16.7q	16.7q	23.3q	23.6q	23.0q	16.7q	23.4q	22.8q	16.5q
20	122.0s	121.5s	121.5s	121.9s	121.5s	122.2s	122.1s	122.1s	122.1s	122.8s	122.7s	122.7s
21	150.5d	150.8d	150.8d	150.5d	150.8d	150.4d	150.5d	150.5d	150.5d	150.1d	150.1d	150.1d
22	147.4d	147.2d	147.2d	147.3d	147.2d	147.4d	147.5d	147.5d	147.4d	147.7d	147.7d	147.7d
23	114.1d	114.2d	114.2d	114.1d	114.2d	114.1d	114.2d	114.2d	114.2d	114.3d	114.3d	114.3d
24	161.0s	161.0s	161.0s	161.0s	161.0s	161.0s	161.0s	161.0s	161.0s	161.1s	161.1s	161.1s

Table 2. ^{13}C NMR spectral data for compounds **13–23** (125 MHz, DMSO-d_6)

C	13	14	15	16	17	18	19	20	21	22	23
1	71.8d	35.9t	29.1t	29.4t	30.4t	35.9t	29.0t	29.4t	71.8d	29.3t	34.9t
2	32.0t	36.7t	27.3t	27.6t	34.8t	36.7t	27.5t	27.5t	32.1t	27.5t	33.5t
3	66.7d	211.6s	64.4d	64.4d	69.7d	211.5s	64.1d	64.4d	66.6d	64.4d	197.9s
4	33.0t	41.6t	32.8t	33.0t	36.0t	41.6t	34.0t	33.0t	32.9t	33.1t	123.2d
5	30.1d	43.0d	35.7d	35.4d	41.0d	42.9d	36.1d	35.6d	29.9d	35.6d	170.1s
6	25.2t	25.5t	25.2t	25.6t	26.0t	25.4t	34.2t	25.5t	25.0t	25.6t	31.3t
7	19.9t	19.7t	20.4t	20.2t	20.4t	19.6t	66.2d	20.4t	19.9t	20.1t	26.3t
8	33.2d	32.8d	32.5d	33.1d	33.3d	32.9d	40.4d	32.4d	33.3d	32.8d	33.0d
9	32.6d	31.6d	34.4d	38.5d	39.2d	38.5d	37.5d	33.8d	40.2d	30.9d	51.9d
10	40.0s	34.3s	35.0s	35.0s	34.5s	34.8s	34.5s	34.7s	39.5s	34.6s	38.3s
11	28.3t	28.4t	37.3t	20.6t	20.4t	20.7t	21.1t	29.4t	20.5t	28.1t	20.6t
12	73.9d	74.0d	210.5s	39.0t	39.0t	39.0t	39.7t	73.8d	38.9t	73.1d	37.8t
13	48.7s	48.9s	59.8s	43.9s	43.9s	43.9s	44.6s	50.0s	43.8s	48.0s	44.4s
14	72.4s	72.3s	73.4s	74.9s	74.9s	74.8s	77.0s	74.4s	74.8s	72.7s	73.4s
15	61.2d	61.2d	59.6d	61.4d	61.4d	61.4d	62.3d	61.2d	61.3d	61.9d	59.4d
16	34.8t	34.8t	32.1t	75.6d	75.6d	75.6d	75.2d	75.6d	75.5d	75.3d	31.4t
17	42.7d	42.7d	37.6d	58.4d	58.3d	58.5d	58.0d	54.5d	58.3d	53.8d	45.9d
18	17.3q	17.2q	17.5q	17.0q	17.0q	17.0q	16.9q	11.7q	17.0q	17.5q	16.4q
19	18.4q	21.8q	23.0q	23.7q	23.1q	22.1q	23.7q	23.6q	18.7q	23.5q	17.1q
20	122.7s	122.7s	120.8s	120.2s	120.2s	120.2s	119.8s	120.4s	120.1s	120.2s	121.8s
21	150.1d	150.1d	151.1d	150.4d	150.4d	150.5d	150.7d	150.3d	150.5d	150.5d	150.6d
22	147.7d	147.7d	114.5d	147.1d	147.1d	147.1d	147.0d	147.3d	147.1d	147.1d	147.3d
23	114.3d	114.3d	147.3d	114.3d	114.3d	114.3d	114.4d	114.3d	114.3d	114.4d	114.2d
24	161.1s	161.1s	160.9s	160.9s	160.9s	160.9s	160.8s	160.9s	160.9s	160.8s	161.0s

Fifteen of them (**3**, **5**, **6**, **8**, **9**, **11–15**, **17**, **19–22**) were new compounds. The chemical structures were illustrated in Figure 1. Compounds **2**, **7**, **10**, **16** and **23** had been obtained previously by chemical or enzymatic means, and **18** had been reported as a metabolite of cinobufagin in rat liver microsomes. However, their NMR data were determined and assigned here for the first time.

The products were fully characterized by comparing their NMR spectra with the substrate or related compounds. The general NMR rules of bufadienolides we previously reported were extensively utilized in this procedure.[7] The NMR data are given in Tables 1–5.

All the bufadienolides gave significant $[M+H]^+$ ions in the

Table 3. ^{1}H NMR spectral data for compounds **2–9** (500 MHz, DMSO-d_6, J in Hz)

H	2	3	4	5	6	7	8	9
1	1.42m	1.50m	1.68m	1.70m	1.42m	1.43m	1.52m	1.70m
	1.35m	1.18m	1.25m	1.25m	1.38m	1.35m	1.22m	1.24m
2	1.42m	1.68m	1.52m	1.50m	1.44m	1.45m	1.69m	1.54m
	1.32m	0.97m	1.46m	1.42m	1.31m	1.35m	0.97m	1.45m
3	3.83br s	3.31br s	3.99br s	3.95br s	3.78br s	3.87br s	3.38br s	3.99br s
4	1.82m	1.43m	2.08br d (11.5)	1.92m	1.76m	1.78m	1.57m	2.07m
	1.28m	1.41m	1.29m	1.40m	1.25m	1.16m	1.33m	1.28m
5	1.77m	1.38m			1.65m	1.72m	1.32m	
6	1.60m	1.58m	1.52m	1.42m	2.42m	1.72m	1.71m	1.52m
	1.25m	1.37m	1.14m	1.38m	1.29m	1.08m	1.16m	1.16m
7	3.54br s	3.57br s	1.42m	3.40br s	3.78br s	1.27m	1.29m	1.38m
			0.92m			0.94m	0.96m	0.90m
8	1.82m	1.82t (10.0)	1.86m	1.80t (10.0)	1.94br d (11.5)	1.80m	1.81m	1.80m
9	1.68m	1.72m	1.54m	1.45m	2.05m	1.65m	1.63m	1.55m
11	1.46m	1.45m	1.48m	1.51m	1.50m	1.58m	1.57m	1.60m
	1.18m	1.20m	1.24m	1.22m	1.28m	1.18m	1.17m	1.25m
12	1.63m	1.64m	1.60m	1.62m	1.57m	3.28m	3.28br s	3.30m
	1.44m	1.42m	1.41m	1.45m	1.40m			
15	4.08s	4.11s	3.58s	4.06s	4.17s	3.55s	3.58s	3.55s
16	2.44dd	2.46m	2.33dd	2.41dd	2.35dd	2.30dd	2.32dd	2.29dd
	(15.0,10.5)		(15.0,10.5)	(15.0,10.0)	(15.0,11.5)	(15.0,10.5)	(15.0,10.5)	(15.0,11.0)
	1.90d (15.0)	1.92d (15.5)	1.80m	1.90m	1.77m	1.82m	1.84m	1.82m
17	2.55d (10.0)	2.56d (10.0)	2.52d (10.5)	2.56d (10.0)	2.45d (11.5)	3.02d (10.5)	3.03d (10.5)	3.02d (10.5)
18	0.71 (3H,s)	0.71 (3H,s)	0.66 (3H,s)	0.70 (3H,s)	0.65 (3H,s)	0.56 (3H,s)	0.56 (3H,s)	0.56 (3H,s)
19	0.90 (3H,s)	0.88 (3H,s)	0.85 (3H,s)	0.86 (3H,s)	0.88 (3H,s)	0.88 (3H,s)	0.87 (3H,s)	0.84 (3H,s)
21	7.54d (2.0)	7.54d (2.5)	7.52d (2.5)	7.54d (2.5)	7.52d (2.5)	7.43s	7.44d (2.0)	7.43s
22	7.70dd	7.70dd	7.75dd	7.70dd	7.77dd	7.67d	7.67dd	7.66d
	(10.0,2.0)	(9.5,2.5)	(10.0,2.5)	(9.5,2.5)	(9.5,2.5)	(10.0)	(10.0,2.0)	(10.0)
23	6.26d (10.0)	6.26d (9.5)	6.25d (10.0)	6.26d (9.5)	6.25d (9.5)	6.25d (10.0)	6.25d (10.0)	6.25d (10.0)
3-OH	4.22d (2.5)	4.53d (4.0)	5.21d (3.5)	5.25br s	4.03d (3.0)	4.21br s	4.50d (4.0)	5.22d (3.5)
5-OH			4.80s	4.91s				4.82s
7-OH	3.64s	3.67s		3.62s	4.26d (2.5)			
12-OH						4.77d (4.5)	4.79d (5.0)	4.80d (5.0)

Table 4. ^{1}H NMR spectral data for compounds **10–15** and **23** (500 MHz, DMSO-d_6, J in Hz)

H	**10**	**11**	**12**	**13**	**14**	**15**	**23**
1	1.39m	1.47m	1.70m	3.58br s	1.94m	1.44m	2.02m
	1.32m	1.28m	1.22m		1.35m	1.27m	1.62m
2	1.50m	1.66m	1.55m	1.72m	2.42m	1.42m	2.38m
	1.31m	0.94m	1.36m	1.68m	1.98m	1.34m	2.16m
3	3.88br s	3.37br s	4.01br s	4.01br s		3.86br s	
4	1.85m	1.62m	2.10m	1.88m	2.70t (14.0)	1.78m	5.63s
	1.17m	1.33m	1.32m	1.29m	1.85br d (15.0)	1.16m	
5	1.68m	1.31m		1.94m	1.74m	1.75m	
6	1.75m	1.72m	1.55m	1.70m	1.76m	1.82m	2.35m
	1.06m	1.17m	1.12m	1.16m	1.18m	1.11m	2.23m
7	1.36m	1.34m	1.42m	1.42m	1.38m	1.40m	1.74m
	1.02m	1.03m	0.95m	1.35m	1.12m	1.07m	0.93m
8	1.80m	1.85brt (12.0)	1.84m	1.90m	1.96m	2.30dt (3.5,13.5)	2.06m
9	2.18dt (2.0,11.0)	2.26brt (11.0)	2.08m	2.04m	2.36m	1.92dt (3.5,13.5)	1.24m
11	1.55m	1.55m	1.58m	1.45m	1.62m	2.52t (14.0)	1.57m
	1.42m	1.40m	1.40m	1.42m	1.56m	2.19dd (14.0,3.5)	1.39m
12	3.57br s	3.57br s	3.54m	3.54br s	3.60br s		1.67dt (3.0,11.5)
							1.42m
15	3.57s	3.58s	3.57s	3.57s	3.63s	3.64s	3.54s
16	2.32dd	2.34brt (14.5)	2.32m	2.32m	2.32m	2.08dd	2.30dd
	(14.0,11.0)					(15.0,10.0)	(15.0,10.0)
	1.68m	1.70m	1.71m	1.68m	1.71m	1.84m	1.82d (15.0)
17	3.08d (10.0)	3.07d (10.0)	3.08d (10.0)	3.07d (10.5)	3.11br d (10.5)	3.80d (10.0)	2.55d (9.5)
18	0.61 (3H,s)	0.61 (3H,s)	0.61 (3H,s)	0.61 (3H,s)	0.64 (3H,s)	0.90 (3H,s)	0.73 (3H,s)
19	0.88 (3H,s)	0.86 (3H,s)	0.84 (3H,s)	0.97 (3H,s)	0.96 (3H,s)	0.95 (3H,s)	1.16 (3H,s)
21	7.47d (2.0)	7.48s	7.48s	7.47s	7.50s	7.54d (2.5)	7.54d (2.5)
22	7.74dd (10.0,2.0)	7.74d (10.0)	7.74d (10.0)	7.54d (9.5)	7.75d (9.5)	7.65dd (10.0,2.5)	7.75dd (9.5,2.5)
23	6.25d (10.0)	6.25d (10.0)	6.25d (10.0)	6.25d (9.5)	6.26d (9.5)	6.27d (10.0)	6.26d (9.5)
1-OH				4.82d (7.5)			
3-OH	4.18d (2.5)	4.49d (4.0)	5.19d (3.5)	5.17d (3.5)		4.23d (3.0)	
5-OH			4.79s				
12-OH	4.72d (4.5)	4.79d (3.5)	4.75d (4.0)	4.72d(3.0)	4.74d (4.5)		

Table 5. ^{1}H NMR spectral data for compounds **16–22** (500 MHz, DMSO-d_6, J in Hz)

H	**16**	**17**	**18**	**19**	**20**	**21**	**22**
1	1.42m	1.70m	1.98m	1.44m	1.44m	3.62br d (7.5)	1.43m
	1.42m	1.52m	1.34m	1.38m	1.35m		1.35m
2	1.44m	1.72m	2.34m	1.42m	1.43m	1.72m	1.52m
	1.35m	0.96m	2.01m	1.36m	1.32m	1.66m	1.34m
3	3.89br s	3.39br s		3.86br s	3.89br s	4.01br s	3.90br s
4	1.80m	1.65m	2.71m	1.65m	1.75m	1.88m	1.80m
	1.18m	1.32m	1.84m	1.32m	1.18m	1.32m	1.18m
5	1.70m	1.80m	1.72m	1.78m	1.72m	1.93m	1.69m
6	1.75m	1.74m	1.78m	1.60m	1.72m	1.70m	1.76m
	1.08m	1.21m	1.21m	1.28m	1.08m	1.18m	1.07m
7	1.32m	1.30m	1.38m	3.55br s	1.30m	1.38m	1.32m
	1.02m	1.02m	1.12m		0.92m	1.04m	1.02m
8	1.91m	1.88m	1.96m	1.88t (10.0)	1.82m	1.96m	1.91m
9	1.68m	1.68m	1.80m	1.72m	1.65m	1.54m	2.17m
11	1.46m	1.46m	1.54m	1.42m	1.58m	1.30m	1.65m
	1.15m	1.15m	1.31m	1.20m	1.18m	1.21m	1.47m
12	1.76m	1.74m	1.84m	1.79m	3.62br s	1.72m	3.65br s
	1.65m	1.62m	1.65m	1.68m		1.63m	
15	3.44s	3.46s	3.52s	3.92s	3.42s	3.45s	3.41s
16	4.00d (3.5)	4.01br s	4.01br s	4.08br s	4.03d (4.5)	3.99d (4.5)	3.87d (11.0)
17	2.43s	2.44s	2.46 (overlapped)	2.49s	2.92s	2.42s	2.89s
18	0.63 (3H,s)	0.63 (3H,s)	0.67 (3H,s)	0.69 (3H,s)	0.53 (3H,s)	0.63 (3H,s)	0.64 (3H,s)
19	0.90 (3H,s)	0.88 (3H,s)	0.98 (3H,s)	0.92 (3H,s)	0.89 (3H,s)	0.99 (3H,s)	0.90 (3H,s)
21	7.54d (2.0)	7.55d (2.0)	7.56s	7.57s	7.44s	7.54d (2.5)	7.56d (2.5)
22	7.60dd	7.60dd	7.61d (10.0)	7.59d (10.0)	7.51d (10.0)	7.60dd	7.61dd
	(10.0,2.0)	(10.0,2.0)				(10.0, 2.5)	(10.0,2.5)
23	6.25d (10.0)	6.25d (10.0)	6.26d (10.0)	6.27d (10.0)	6.26d (10.0)	6.25d (10.0)	6.26d (10.0)
1-OH						4.82d (7.5)	
3-OH	4.22d (2.5)	4.54d (4.5)		4.25d (2.5)	4.24d (3.0)	5.18d (4.5)	4.21d (3.0)
7-OH				3.53s			
12-OH					4.75d (5.0)		5.94d (2.5)
16-OH	5.47d (4.0)	5.53d (3.0)	5.46d (4.5)	5.62br s	5.46d (4.5)	5.45d (4.5)	5.21d (11.0)

atmospheric pressure chemical ionization (APCI) mass spectra. The mono- or dihydroxylation of resibufogenin could be deduced by the $[M+H]^+$ ions at *m/z* 401 or *m/z* 417, respectively. And a 2-unit decrease of $[M+H]^+$ indicated the presence of a carbonyl group. The exact position and stereo-configuration of the hydroxyl or carbonyl groups, however, were determined by NMR spectroscopy. Any substitution led to stable and characteristic changes in the ^1H and ^{13}C NMR spectra, as we had summarized.[7] Upon the introduction of 1β-OH, C-19 was shifted upfield to δ 17–18 due to γ-*gauche* effect, and C-10 was shifted downfield by 4–5 ppm. The 1β- and 3β-hydroxyl groups were both resonated at much lower fields (δ 4.8–5.2) because of their intra-molecular hydrogen bonding. The W-type long-range coupling between H-1α and H-3α could be observed in the ^1H–^1H COSY spectrum. When 3-OH was epimerized into α-configuration, H-3β appeared as a very broad peak ($W_{1/2}$ = 20–25 Hz) due to its $^3J_{aa}$ couplings with H-2α and H-4α. Similar to 1β-OH, the introduction of 5-OH led to the upfield shift of C-19 to δ 16–17, and the downfield shift of 3-OH. When a 7β-OH was present, H-7α appeared as a very broad peak due to its $^3J_{aa}$ couplings with H-6β and H-8. When 7-OH was in the α-configuration, however, the H-7β signal was much narrower, and C-9 was shifted upfield to δ 31–33 due to the γ-*gauche* effect of 7α-OH. Both 12α-OH and 12β-OH resulted in 3–4 ppm upfield shift of C-17, 4–6 ppm downfield shift of C-13, and 0.5 ppm downfield shift of H-17. The epimers could be differentiated by the chemical shift of C-18, which was shifted upfield to δ 10–12 at the presence of a 12β-OH due to γ-*gauche* effect, while was hardly affected by 12α-OH. The 16α-OH could be characterized by the 12–14 ppm downfield shift of C-17. In addition, H-17 appeared as a sharp singlet since the dihedral angle of H-17 and H-16β was approximately 90°. This feature was different from 16β-OH bufadienolides, where H-17 appeared as a doublet (J = 8.5–9.5 Hz). The above rules were applied to the structural elucidation of biotransformation products.

The ^{13}C NMR spectrum of **2** showed an additional oxygenated methine signal at δ 66.2, and C-8 appeared at a lower field at δ 40.3, suggesting the hydroxylation at C-7. According to the HMQC spectrum, the signal of H-7 (δ 3.54) appeared as a very broad peak, indicating the β-configuration of 7-OH. Thus, compound **2** was identified as 7β-hydroxyl resibufogenin. The presence of 7β-OH in compound **3** was established by comparing its NMR data with **2**. In addition, the HMBC spectrum showed a long-range coupling of H-8 (δ 1.82) with C-7 (δ 66.3). However, the A-ring carbon signals were very different from those of **2**, but similar to 3-*epi*-7β-hydroxyl bufalin,[7] indicating that the 3-OH should be in the α-configuration. Thus, compound **3** was identified as 3-*epi*-7β-hydroxyl resibufogenin. A comparison of the NMR data of **5** with those of **2** and 5-hydroxyl resibufogenin (**4**) suggested that compound **5** was hydroxylated both at C-5 and C-7β. The quaternary carbon signal at δ 73.2 should thus be assigned to C-5. In accordance, C-19 was shifted upfield to δ 16.7, and 3-OH was shifted downfield to δ 5.25 because of its hydrogen bonding with 5-OH. The signal of H-8 (δ 1.80) appeared as a triplet (J = 10.0 Hz) due to its $^3J_{aa}$ couplings with H-7α and H-9, and confirmed the β-configuration of 7-OH. Therefore, compound **5** was identified as 5,7β-dihydroxyl resibufogenin.

The ^{13}C NMR spectrum of **6** showed an extra oxygenated methine signal at δ 63.6. Its HMBC correlation with H-8 (δ 1.94) indicated that C-7 was hydroxylated. When compared to **2**, the signal of H-7 (δ 3.78) appeared as a much narrower peak, suggesting the α-configuration of 7-OH. In accordance, C-9 (δ 31.8) resonated at a much higher field than that of **2** ($\delta_{C\text{-}9}$37.3), due to the γ-*gauche* effect of 7α-OH. The NOE enhancement of 7-OH (δ 4.26) with H-4α (δ 1.25) was also observed. Therefore, compound **6** was identified as 7α-hydroxyl resibufogenin.

Compounds **7** and **8** were both hydroxylated at 12β-position since their C-18 signals shifted upfield to δ 11.4, and C-13 downfield to δ 50.7. In addition, the NOESY spectra showed NOE enhancements of H-12 (δ 3.28) with H-16α (δ 2.32) and H-17 (δ 3.03). However, their A-ring carbon signals were very different. The H-3 signal (δ 3.38) of **8** appeared as a very broad peak, suggesting that its 3-OH should be in α-configuration. Thus, they were identified as 12β-hydroxyl resibufogenin (**7**) and 3-*epi*-12β-hydroxyl resibufogenin (**8**), respectively. The presence of 12β-OH in compound **9** was established in a similar manner, and was confirmed by the HMBC correlation of H-18 (δ 0.56) with C-12 (δ 72.8). In addition, a new quaternary carbon appeared at δ 73.4, and C-10 was shifted downfield to δ 40.0, while C-19 upfield to δ 16.7. All evidences supported the presence of 5-OH. The HMBC correlation of H-19 (δ 0.84) with C-5 was also observed. Therefore, compound **9** was identified as 5,12β-dihydroxyl resibufogenin.

Compounds **10**–**14** were identified as 12α-hydroxylated derivatives of resibufogenin, since their C-13 signals were shifted downfield to δ 48.7–48.9, C-17 shifted upfield to δ 42.7, and C-18 appeared at δ 17.2–17.3. The chemical shift of C-18 could hardly be affected by 12α-OH since they were in γ-*trans* positions. In the HMBC spectrum of compound **11**, H-18 (δ 0.61) had a long-range coupling with C-12 (δ 74.0). The signal of C-9 was shifted upfield by 6.9 ppm when compared to resibufogenin, due to the γ-*gauche* effect of 12α-OH. The NOE enhancement of 12α-OH (δ 4.79) with H-17 (δ 3.07) was also observed in the NOESY spectrum (Fig. 2). The 3α-OH of **11** was established by the broad peak of H-3 (δ 3.37). Therefore, compound **11** was identified as 3-*epi*-12α-hydroxyl resibufogenin. A carbonyl signal appeared at δ 211.6 in the ^{13}C NMR spectrum of **14**, and C-2 (δ 36.7) and C-4 (δ 41.6) were significantly shifted downfield, suggesting the presence of C-3 carbonyl group. The HMBC spectrum showed long-range couplings of C-3 with H-2 and H-4. Thus, compound **14** was identified as 3-oxo-12α-hydroxyl resibufogenin. It could be considered as an intermediate in the epimerization from 12α-hydroxyl resibufogenin (**10**) to **11**. For compound **12**, a quaternary carbon appeared at δ 73.5, C-10 shifted downfield to δ 40.0, and C-19 upfield to δ 16.5, suggesting the hydroxylation at C-5. Both 3-OH (δ 5.19) and 5-OH (δ 4.79) shifted remarkably downfield due to intra-molecular hydrogen bonding effect. Thus, the structure of **12** should be 5,12α-dihydroxyl resibufogenin. The NMR spectra of **13** was very similar to that of **12**, with C-10 shifted downfield to δ 40.0, C-19 upfield to δ 18.4, and 3-OH downfield to δ 5.17.

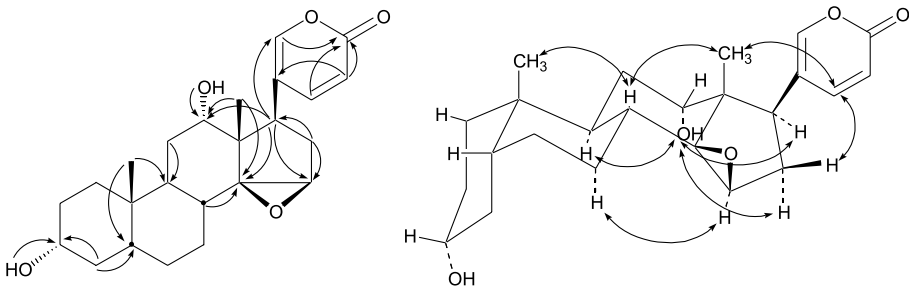

Figure 2. Key HMBC (left) and NOESY (right) correlations of compound **11**.

However, the additional oxygenated signal at δ 71.8 was assigned as a methine carbon by the DEPT experiment, and suggested the hydroxylation at C-1β. Compound **13** was thus identified as 1β,12α-dihydroxyl resibufogenin. In the ^{13}C NMR spectrum of **15**, a carbonyl signal appeared at δ 210.5. It showed HMBC correlations with H-18 (δ 0.90) and H-17 (δ 3.80), which allowed its assignment to C-12. In accordance, C-11 (δ 37.3) and C-13 (δ 59.8) were shifted downfield remarkably by 16.7 and 15.2 ppm, respectively, when compared to resibufogenin. Thus, compound **15** was identified as 12-oxo-resibufogenin. It could be considered as the transformation intermediate from **7** to **10**.

Compounds **16**–**22** were characterized as 16α-hydroxylated derivatives of resibufogenin. In the ^{13}C NMR spectrum of compound **17**, a new oxygenated methine signal appeared at δ 75.6, and C-17 shifted downfield to δ 58.3, suggesting the presence of 16-OH. It was confirmed by the HMBC correlations of H-16 (δ 4.01) with C-20 (δ 120.2) and C-13 (δ 43.9). The signal of H-17 appeared as a singlet, consistent with the α-configuration of 16-OH. In the NOESY spectrum, the NOE enhancements of H-16β (δ 4.01) with H-21 (δ 7.55) and H-22 (δ 7.60) were observed. The 3α-OH of **17** was established by the broad peak of H-3 (δ 3.39). Thus, compound **17** was identified as 3-*epi*-16α-hydroxyl resibufogenin. In the ^{13}C NMR spectrum of **18**, a carbonyl signal appeared at δ 211.5, and C-2 (δ 36.7) and C-4 (δ 41.6) resonated at relatively lower fields. Its structure was established as 3-oxo-16α-hydroxyl resibufogenin. Compounds **19**–**22** were dihydroxylated derivatives of resibufogenin, giving $[M+H]^+$ ions at *m/z* 417. Their structures were identified by comparing with structurally related compounds and confirmed by 2D NMR spectra. The additional carbon signal of **19** at δ 66.2 showed long-range correlation with H-8 (δ 1.88), and its corresponding proton signal at δ 3.55 appeared as a very broad peak, suggesting the hydroxylation at C-7β. Thus, compound **19** was identified as 7β,16α-dihydroxyl resibufogenin. The signal of C-18 in compound **20** was shifted upfield to δ 11.7, and C-13 shifted downfield to δ 50.0, suggesting the hydroxylation at C-12β, and allowed the identification of compound **20** as 12β,16α-dihydroxyl resibufogenin. In the ^{13}C NMR spectrum of **21**, an additional methine signal appeared at δ 71.8, the signal of C-10 was shifted downfield to δ 39.5 and C-19 upfield to δ 18.7, suggesting that C-1 was hydroxylated. The ^{1}H–^{1}H COSY spectrum showed a W-type long-range coupling of H-1 (δ 3.62) with H-3 (δ 4.01), suggesting the β-configuration of 1-OH. Both 1-OH (δ 4.82) and 3-OH (δ 5.18) were resonated at much lower fields due to intra-molecular hydrogen bonding, and each showed NOE enhancements with H-5 (δ 1.93). In addition, the signal of C-17 was shifted downfield to δ 58.3, suggesting the presence of 16-OH. The NOESY spectrum showed NOE enhancements of H-16 (δ 3.99) with H-22 (δ 7.60), and 16-OH (δ 5.45) with H-17 (δ 2.42), and established the α-configuration of 16-OH. In accordance, the signal of H-17 appeared as a sharp singlet. Thus, compound **21** was identified as 1β,16α-dihydroxyl resibufogenin. The C-13 signal of **22** was shifted downfield to δ 48.0 and C-18 appeared at δ 17.5, suggesting the hydroxylation at C-12α. In addition, downfielded C-17 (δ 53.8) and a sharp singlet for H-17 (δ 2.89) suggested the presence of 16α-OH. They were confirmed by the HMBC correlations of H-18 (δ 0.64) with C-12 (δ 73.1), and H-16 (δ 3.87) with C-20 (δ 120.2), respectively. Thus, compound **22** was identified as 12α,16α-dihydroxyl resibufogenin.

Most of the biotransformed products of resibufogenin by *M. polymorphosporus* were mono- or dihydroxylated derivatives at various positions, including C-1β, C-5, C-7α, C-7β, C-12α, C-12β and C-16α. The epimerization and dehydrogenation at C-3 were also observed. Hydroxylations at C-12α, C-12β and C-16α were found to be the major reactions, each giving products in $>5\%$ isolated yields, while the other products were only obtained in fairly low yields. Previously, we had reported the biotransformation of bufalin by *M. spinosus*, where the major reactions involved hydroxylations at C-7β, C-12β and C-16α, and 7β-hydroxylated products constituted more than 50% of the total metabolites.[7] Since the structures of resibufogenin and bufalin only differed in the 14β,15β-epoxy ring, this ring appeared to inhibit 7β-hydroxylation of bufadienolides due to its close spatial position with C-7. This assumption could be evidenced by our latest results from the substrate specificity investigation of bufadienolide 12β-hydroxylation by *Alternaria alternata*.[19]

For natural bufadienolides, hydroxyl substitutions usually occur at C-5, C-11α or C-12β, and bufadienolides with a hydroxyl group at C-1β, C-7α, C-7β or C-16α obtained in this study are rarely seen.[20] Our result obviously indicated the great potential of biotransformation in the synthesis of natural product-derived new compounds.

Mucor species are of the most frequently used microbial catalysts, and have been reported to catalyze the hydroxylation of a variety of natural products, including steroids, diterpenes and sesquiterpenes, leading to arrays of hydroxylated products in most of the reactions.[21–27] The potent hydroxylation capabilities of *Mucor* species should

Table 6. Cytotoxic activities of the biotransformed products against human cancer cell lines ($n=3$)

Compound	IC_{50} (μmol/L)		
	Bel-7402	BGC-823	HeLa
Taxol	3.4×10^{-1}	1.0	1.5×10^{-2}
1	1.3×10^{-1}	1.1×10^{-1}	1.0×10^{-2}
2	2.7	2.2	5.0×10^{-1}
3	28.7	14.0	3.9
4	4.3	10.8	1.4
6	4.9	3.7	2.1
7	3.5	3.9	8.0×10^{-1}
8	45.9	33.4	37.0
9	16.8	17.8	7.2
10	14.4	8.2	3.4
11	35.7	57.0	19.1
12	97.3	53.8	78.4
13	41.8	30.5	5.8
14	17.3	30.5	4.2
15	5.4	19.0	4.6
16	>100	>100	44.6
17	38.1	>100	34.7
19	>100	>100	55.4
20	25.2	10.0	6.7
21	18.5	20.3	2.9
22	14.4	4.3	4.5
23	5.8	5.6	3.6

be due to their cytochrome P450 enzyme systems.[1,2] Obviously, there could be a family of monohydroxylases responsible for the hydroxylation reactions. Their properties warrant further investigation so that the biotransformation process could be monitored more directly and efficiently.

The cytotoxic activities of the biotransformed products were evaluated by the MTT method (Table 6). The results were in agreement with the structure-activity relationships previously proposed by Ye et al. and Kamano et al.[7,28,29] Resibufogenin showed strong inhibitory activities against human hepatoma Bel-7402 cells, human gastric cancer BGC-823 cells and human cervical carcinoma HeLa cells, with IC_{50} values of 0.13, 0.11, and 0.01 μmol/L, respectively. All the products showed less potent activities. The 7β- or 12β-hydroxylations reduced the activities by less than 10 folds, and the resultant products still showed significant cytotoxic effects. However, the epimerization or dehydrogenation of 3-OH, and 12α-hydroxylation remarkably reduced the activities. All the 16α-hydroxylated products showed very weak cytotoxic activities, with IC_{50} values >10 μmol/L. These results provided guidance for the future directed synthesis of bufadienolides of pharmaceutical interest.

3. Experimental

3.1. General

Melting points were determined with an XT4A apparatus (uncorrected). Optical rotations were measured with a Perkin-Elmer 243B polarimeter. UV spectra were detected with a TU-1901 UV–vis spectrophotometer. IR spectra were recorded in KBr with an Avatar 360 FT-IR spectrophotometer. ^{1}H and ^{13}C 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 at ambient temperature with tetramethylsilane (TMS) as the internal standard. The chemical shifts (δ values) were given in parts per million (ppm) relative to TMS at 0 ppm. The coupling constants (J values) were reported in hertz (Hz). Mass spectra were measured on a Finnigan LCQ Advantage mass spectrometer equipped with an APCI source. High-resolution mass spectra (HR-MS) were obtained on a Bruker APEX II Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer.

3.2. Chemicals

Resibufogenin (**1**) was isolated from the Chinese drug ChanSu, and unambiguously identified by NMR and MS techniques. The purity was determined to be >99.5% by HPLC analysis. Chromatorex ODS (100–200 mesh) for column chromatography was purchased from Fuji Silysia Chemical Ltd., Japan. All chemical solvents used for products isolation were of analytical grade or higher.

3.3. Microorganism and culture media

The fungal strain of *Mucor polymorphosporus* AS 3.3443 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 a potato medium consisting of 20 g of potato extract, 20 g of glucose, and 1000 mL of distilled H_2O. The media were sterilized in an autoclave at 121 °C and 1.06 kg/cm^2 for 30 min.

3.4. Preparative HPLC conditions

For the isolation of biotransformation products, a Spectra-SERIES HPLC apparatus (Thermo Quest) with a 100 μL loop was used. Samples were separated on a YMC ODS-A column (5 μm, ∅10×250 mm). The flow rate was 2.0 mL/min, and the detection wavelength was 296 nm.

3.5. Biotransformation procedure

Mycelia of *M. polymorphosporus* from agar slants were aseptically transferred to 250 mL Erlenmeyer flasks containing 80 mL of liquid potato medium. The fungus was incubated at 25 °C on a rotary shaker (180 rpm) in the dark for 24 h to make a stock inoculum. An amount of 8 mL of the inoculum was then added to each of the 40 1-L flasks containing 350 mL of potato medium. After 24 h incubation, a total amount of 800 mg of resibufogenin dissolved in 40 mL of ethanol was distributed equally among the 40 flasks. The incubation was allowed to continue for additional 6 days on the shaker. The cultures were then pooled and filtered in vacuo. The filtrate was extracted with 10 L of ethyl acetate for three times. The organic extract was evaporated to dryness in a rotary evaporator under reduced pressure at 60 °C to yield 2.7 g of a brownish solid.

3.6. Isolation and purification of biotransformation products

The sample was separated on an ODS open column (∅3 cm by 30 cm) and eluted with MeOH–H_2O (10:90–80:20, v/v) to afford five fractions (I–V). Fraction I was subjected to preparative HPLC and eluted with MeOH–H_2O (45:55, v/v) to yield **9** (7.5 mg). Fraction II was separated by preparative HPLC and eluted with MeOH–H_2O (47.5:52.5, v/v) to give **12** (28.9 mg), **19** (8.4 mg), and **20** (25.8 mg). Fraction III was subjected to preparative HPLC and eluted with MeOH–H_2O (50:50, v/v) to yield **5** (1.6 mg), **7** (48.0 mg), and **13** (14.0 mg). Fraction IV was separated by preparative HPLC and eluted with MeOH–H_2O (58:42, v/v) to yield **2** (10.7 mg), **3** (3.4 mg), **4** (2.1 mg), **6** (2.0 mg), **8** (43.7 mg), **10** (39.7 mg), **11** (5.9 mg), **14** (3.0 mg), **15** (2.8 mg), **16** (58.6 mg), **21** (3.2 mg), **22** (3.8 mg), and **23** (2.8 mg). Fraction V was subjected to preparative HPLC and eluted with MeOH–H_2O (75:25, v/v) to yield **17** (7.1 mg) and **18** (1.0 mg). Purities of the above products were >95%, determined by HPLC-UV means.

3.6.1. 3-*epi*-7β-Hydroxyl resibufogenin (3). White powder; $C_{24}H_{32}O_5$; mp 249–251 °C; $[\alpha]_D^{25}$ +5.2 (*c* 0.2, MeOH); UVλ_{max}(MeOH): 204.0, 298.0 nm; APCI-MS (*m/z*): 401 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_5$ $[M+H]^+$, 401.2328, found 401.2319; ^{13}C and ^1H NMR data, see Tables 1 and 3.

3.6.2. 5,7β-Dihydroxyl resibufogenin (5). White powder; $C_{24}H_{32}O_6$; mp 224–227 °C; $[\alpha]_D^{25}$ +32.1 (*c* 0.1, MeOH); UVλ_{max}(MeOH): 206.0, 299.0 nm; APCI-MS (*m/z*): 417 $[M+H]^+$; ^{13}C and ^1H NMR data, see Tables 1 and 3.

3.6.3. 7α-Hydroxyl resibufogenin (6). White powder; $C_{24}H_{32}O_5$; mp 138–140 °C; $[\alpha]_D^{25}$ +11.7 (*c* 0.1, MeOH); UVλ_{max}(MeOH): 204.0, 299.0 nm; IRν_{max}(KBr): 3437, 2928, 1713, 1634, 1538, 1453, 1125, 1037 cm^{-1}; APCI-MS (*m/z*): 401 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_5$ $[M+H]^+$, 401.2328, found 401.2322; ^{13}C and ^1H NMR data, see Tables 1 and 3.

3.6.4. 3-*epi*-12β-Hydroxyl resibufogenin (8). White powder; $C_{24}H_{32}O_5$; mp 233–235 °C; $[\alpha]_D^{25}$ +12.1 (*c* 1.0, MeOH); UVλ_{max}(MeOH): 206.0, 299.0 nm; IRν_{max}(KBr): 3379, 2934, 2868, 1705, 1632, 1538, 1454, 1130, 1033 cm^{-1}; APCI-MS (*m/z*): 401 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_5$ $[M+H]^+$, 401.2328, found 401.2322; ^{13}C and ^1H NMR data, see Tables 1 and 3.

3.6.5. 5,12β-Dihydroxyl resibufogenin (9). White powder; $C_{24}H_{32}O_6$; mp 149–152 °C; $[\alpha]_D^{25}$ +7.9 (*c* 0.4, MeOH); UVλ_{max}(MeOH): 205.0, 297.0 nm; IRν_{max}(KBr): 3408, 2938, 1712, 1631, 1537, 1452, 1040 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2268; ^{13}C and ^1H NMR data, see Tables 1 and 3.

3.6.6. 3-*epi*-12α-Hydroxyl resibufogenin (11). White powder; $C_{24}H_{32}O_5$; mp 139–140 °C; $[\alpha]_D^{25}$ +3.7 (*c* 0.4, MeOH); UVλ_{max}(MeOH): 204.0, 300.0 nm; IRν_{max}(KBr): 3427, 2936, 2867, 1712, 1631, 1537, 1453, 1127, 1046 cm^{-1}; APCI-MS (*m/z*): 401 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_5$ $[M+H]^+$, 401.2328, found 401.2323; ^{13}C and ^1H NMR data, see Tables 1 and 4.

3.6.7. 5,12α-Dihydroxyl resibufogenin (12). White powder; $C_{24}H_{32}O_6$; mp 146–148 °C; $[\alpha]_D^{25}$ +23.4 (*c* 1.2, MeOH); UVλ_{max}(MeOH): 204.0, 300.0 nm; IRν_{max}(KBr): 3420, 2936, 1712, 1632, 1538, 1452, 1377, 1223, 1128, 1040 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2268; ^{13}C and ^1H NMR data, see Tables 1 and 4.

3.6.8. 1β,12α-Dihydroxyl resibufogenin (13). White powder; $C_{24}H_{32}O_6$; mp 164–166 °C; $[\alpha]_D^{25}$ +5.2 (*c* 0.6, MeOH); UVλ_{max}(MeOH): 205.0, 299.0 nm; IRν_{max}(KBr): 3410, 2935, 1714, 1633, 1537, 1451, 1377, 1228, 1134, 1051 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2267; ^{13}C and ^1H NMR data, see Tables 2 and 4.

3.6.9. 3-Oxo-12α-hydroxyl resibufogenin (14). White powder; $C_{24}H_{30}O_5$; mp 131–133 °C; $[\alpha]_D^{25}$ +6.2 (*c* 0.2, MeOH); UVλ_{max}(MeOH): 206.0, 298.0 nm; IRν_{max}(KBr): 3444, 2937, 1707, 1635, 1538, 1454, 1378, 1119, 1051 cm^{-1}; APCI-MS (*m/z*): 399 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{31}O_5$ $[M+H]^+$, 399.2171, found 399.2164; ^{13}C and ^1H NMR data, see Tables 2 and 4.

3.6.10. 12-Oxo-resibufogenin (15). White powder; $C_{24}H_{30}O_5$; mp 209–210 °C; $[\alpha]_D^{25}$ +35.1 (*c* 0.2, MeOH); UVλ_{max}(MeOH): 204.0, 298.0 nm; IRν_{max}(KBr): 3492, 2930, 1713, 1539, 1453, 1373, 1126, 1038 cm^{-1}; APCI-MS (*m/z*): 399 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{31}O_5$ $[M+H]^+$, 399.2171, found 399.2163; ^{13}C and ^1H NMR data, see Tables 2 and 4.

3.6.11. 3-*epi*-16α-Hydroxyl resibufogenin (17). White powder; $C_{24}H_{32}O_5$; mp 140–142 °C; $[\alpha]_D^{25}$ −24.1 (*c* 0.4, MeOH); UVλ_{max}(MeOH): 205.0, 299.0 nm; IRν_{max}(KBr): 3417, 2934, 2867, 1712, 1631, 1536, 1454, 1374, 1241, 1128, 1040 cm^{-1}; APCI-MS (*m/z*): 401 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_5$ $[M+H]^+$, 401.2328, found 401.2321; ^{13}C and ^1H NMR data, see Tables 2 and 5.

3.6.12. 7β,16α-Dihydroxyl resibufogenin (19). White powder; $C_{24}H_{32}O_6$; mp 127–130 °C; $[\alpha]_D^{25}$ −33.4 (*c* 0.5,

MeOH); UVλ_{max}(MeOH): 206.0, 298.0 nm; IRν_{max}(KBr): 3492, 3284, 2932, 1708, 1630, 1539, 1453, 1261, 1141, 1036 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2265; ^{13}C and ^1H NMR data, see Tables 2 and 5.

3.6.13. 12β,16α-Dihydroxyl resibufogenin (20). White powder; $C_{24}H_{32}O_6$; mp 159–160 °C; $[\alpha]_D^{25}$ −3.2 (*c* 1.1, MeOH); UVλ_{max}(MeOH): 203.0, 299.0 nm; IRν_{max}(KBr): 3419, 2936, 2879, 1711, 1632, 1537, 1453, 1237, 1134, 1035 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2267; ^{13}C and ^1H NMR data, see Tables 2 and 5.

3.6.14. 1β,16α-Dihydroxyl resibufogenin (21). White powder; $C_{24}H_{32}O_6$; mp 188–190 °C; $[\alpha]_D^{25}$ −20.7 (*c* 0.2, MeOH); UVλ_{max}(MeOH): 205.0, 296.0 nm; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2269; ^{13}C and ^1H NMR data, see Tables 2 and 5.

3.6.15. 12α,16α-Dihydroxyl resibufogenin (22). White powder; $C_{24}H_{32}O_6$; mp 136–140 °C; $[\alpha]_D^{25}$ −42.1 (*c* 0.2, MeOH); UVλ_{max}(MeOH): 205.0, 298.0 nm; IRν_{max}(KBr): 3400, 2934, 1714, 1631, 1536, 1452, 1237, 1132, 1034 cm^{-1}; APCI-MS (*m/z*): 417 $[M+H]^+$; HR-FT-ICRMS *m/z* calcd for $C_{24}H_{33}O_6$ $[M+H]^+$, 417.2276, found 417.2269; ^{13}C and ^1H NMR data, see Tables 2 and 5.

3.7. Bioassay

Human hepatoma Bel-7402 cells, human gastric cancer BGC-823 cells and human cervical carcinoma HeLa cells were maintained in RPMI 1640 medium (GIBCO/BRL, Maryland, USA) supplemented with 10% (v/v) fetal bovine serum and cultured in 96-well microtiter plates for the assay. Appropriate dilutions (10^{-3}–10^2 μmol/L) of the test compounds were added to the cultures. After incubation at 37 °C, 5% CO_2 for 72 h, the survival rates of the cancer cells were evaluated by the MTT method.[30] The activity was shown as the IC_{50} value, which is the concentration (μmol/L) of test compound to give 50% inhibition of cell growth. Results were expressed as the mean value of triplicate determinations. Taxol™ was selected as the positive control.

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