Microbial Transformation of 20(*S*)-Protopanaxatriol by *Mucor spinosus*

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Microbial biotransformation of 20(*S*)-protopanaxatriol (**1**) by the fungus *Mucor spinosus* (AS 3.3450) yielded four new metabolites, the structures of which were determined to be 12-oxo-15α-hydroxyl-20(*S*)-protopanaxatriol (**2**), 27-hydroxyl-20(*S*)-protopanaxatriol (**3**), 12-oxo-26-hydroxyl-20(*S*)-protopanaxatriol (**4**), and 12-oxo-27-hydroxyl-20(*S*)-protopanaxatriol (**5**), respectively, on the basis of their chemical and spectroscopic data. All the new metabolites, as well as the substrate, had significant cytotoxic effects on HL-60 cells (human leukemia cells).

Ginsenosides, the major active components in *Panax ginseng* C. A. Meyer (Araliaceae), have been reported to exhibit antitumor effects,[1] particularly the inhibition of tumor-induced angiogenesis[2] and tumor invasion and metastasis[3,4] and the control of phenotypic expression and differentiation of tumor cells.[5,6] The aglycones of most ginsenosides were the dammaranes 20(*S*)-protopanaxatriol (PT, **1**) and 20(*S*)-protopanaxadiol (PD). PT has been reported to have strong cytotoxic activity against human leukemia cells (THP-1), by inducing DNA fragmentation and apoptosis.[7] These results suggest that the aglycones of ginsenosides are responsible for the antitumor effects. With our continuous investigation on the biotransformation of antitumor natural products,[8] we are especially interested in the various modified structures of PT transformed by microbes in order to find more active compounds. We report here the structures of four new metabolites produced by the fungus *Mucor spinosus* (AS 3.3450) and their cytotoxic effects against tumor cells.

Results and Discussion

20(*S*)-Protopanaxatriol (PT, **1**) was obtained by using the Smith degradation according to the literature reaction of a ginsenoside extract.[9] Incubation of **1** with *Mucor spinosus* (AS 3.3450) yielded four metabolites, **2**, **3**, **4**, and **5**.

Compound **2** was obtained as a white amorphous powder. Its molecular formula, $C_{30}H_{50}O_5$, was established via HRMS, ^{13}C NMR, and DEPT spectral data. Comparing with **1**, two new groups, one carbonyl [ν_{max} 1701 cm^{-1}; δ_C 211.3 (s)] and one secondary hydroxyl [ν_{max} 3409 cm^{-1}, δ_C 73.0 (t)], were found, and two signals of compound **1** (C-12, C-15) were missing. These data suggested that **2** should be a hydroxylated and oxidized product of **1**. In its HMBC spectrum, correlation signals between δ_C 211.3 and H-13 [δ_H 3.45 (1H, d, J = 9.5 Hz)], H-17 [δ_H 2.83 (1H, td, J = 4.0, 15.0, 9.5 Hz)], H-11 [δ_H 2.42 (1H, dd, J = 3.5, 12.5 Hz)] were observed, which indicated the position of the carbonyl group (C-12). Correlations between δ_C 73.0 and H-13, H-16 were also observed in the HMBC, which demonstrated the hydroxyl group to be at C-15 (Figure 1). A significant NOE correlation between H-15 [δ_H 4.48 (1H, d, J = 8.5 Hz)] and H-18 [8β-Me] was clearly observed in the NOESY spectrum, suggesting the 15-OH to be α-oriented. Thus, **2** was elucidated as 12-oxo-15α-hydroxyl-20(*S*)-protopanaxatriol.

Compound **3** was obtained as a white amorphous powder. Its molecular formula, $C_{30}H_{52}O_5$, was also established from

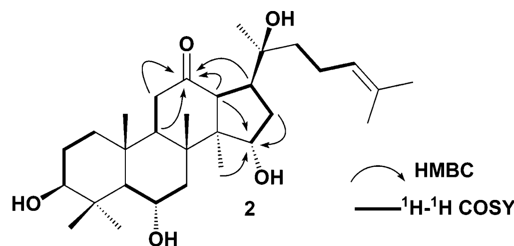

Figure 1. Key HMBC and ^{1}H−^{1}H COSY correlations in metabolite **2**.

HRMS, ^{13}C NMR, and DEPT data. Comparing with **1**, it was suggested that a hydroxyl group had been introduced into the substrate molecule. In the DEPT spectrum, the number of methyl carbons was reduced from 8 (**1**) to 7; meanwhile a new CH_2 signal at δ_C 60.8 (t) was found, suggesting that one methyl group had been oxidized. In the ^{13}C NMR, the chemical shift of Me-26 was upfield shifted about 3.8 ppm (δ_C 25.7 to 21.9), suggesting that the Me-27 was hydroxylated. The HMBC correlations between δ_C 60.8 (t), H-25 [δ_H 5.47 (1H, dd, J = 6.5, 7.0 Hz)], and Me-26 [δ_H 1.98 (3H, s)] confirmed the hydroxylation position at C-27. Thus, **3** was characterized as 27-hydroxyl-20(*S*)-protopanaxatriol.

Compounds **4** and **5** were also obtained as white amorphous powder. Their molecular formulas were established as $C_{30}H_{50}O_5$, on the basis of the combined analysis of HRMS and DEPT data. The ^{13}C NMR and DEPT spectral data of **4** indicated a quaternary carbon signal at δ_C 211.7 (s) and a new CH_2 signal at δ_C 68.1 (t), suggesting it to be the oxidized and hydroxylated product of **1**. The HMBC correlations between δ_C 211.7 (s) and H-11, H-13 indicated the carbonyl to be at C-12. In the ^{13}C NMR, the chemical shift of Me-27 was upfield shifted about 3.8 ppm (δ_C 17.6 to 13.9), indicating that Me-26 was hydroxylated. HMBC correlations between δ_C 68.1 (t) and Me-27, H-24 confirmed that the hydroxyl group was at C-26. Therefore, **4** was characterized as 12-oxo-26-hydroxyl-20(*S*)-protopanaxatriol. The ^{13}C NMR and DEPT data of **5** were very similar to those of **4** except for the new CH_2 signal at δ_C 60.8 (t), which suggested that the hydroxylation position was at C-27 instead of C-26. This assignment was confirmed by the HMBC correlations between δ_C 60.8 (t) and H-24, Me-26. Thus, **5** was characterized as 12-oxo-27-hydroxyl-20-(*S*)-protopanaxatriol. To our knowledge, all of the transformed products (**2**, **3**, **4**, and **5**) are new compounds.

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test (MTT) showed that four biotransformed products and PT were cytotoxic to HL-60 cell lines, but not

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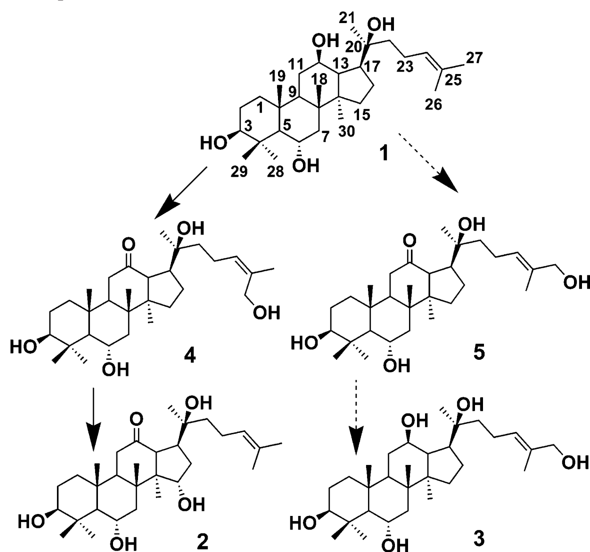
Scheme 1. Biotransformation of 20(*S*)-Protopanaxatriol by *Mucor spinosus*



to human gastric cancer cells (BGC) (IC_{50} > 100 μM), with IC_{50}'s vs HL-60 of 16.0, 15.0, 12.3, 10.8, and 16.3 μM for PT, **2**, **3**, **4**, and **5**, respectively.

These results demonstrated that 20(*S*)-protopanaxatriol could be selectively carbonylated at position C-12 by *M. spinosus* (AS 3.3450). Studies on the biotransformation kinetics showed that after 3 h of addition of PT **4** was largely produced, but after 72 h compound **2** was the major product while **4** virtually disappeared from the reaction mixture (Scheme 1). This process provides a useful means for chemists to produce interesting new derivatives of PT for investigation of structure−activity relationships.

Experimental Section

General Experimental Procedures. Optical rotations were performed with a Perkin-Elmer PE324B digital polarimeter in MeOH at 25 °C. IR spectra were carried out on a Nicolet Avatar FTIR spectrometer in KBr disks. HR-FT-ICR-MS was conducted with a Bruker APEX FT-ICR mass spectrometer. The NMR experiments were carried out on a Varian INOVA 500 spectrometer using standard Varian sequences for 1D and 2D NMR experiments in pyridine-d_5, and chemical shifts are expressed in δ (ppm) referred to tetramethylsilane (TMS).

Silica gel (200−300 mesh, Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) and ODS (Chromatorex, 100−200 mesh, Fujisylisia Co. Ltd., Japan) were used for open column chromatography. Analytical HPLC was performed on an Agilent 1100 HPLC connected to a diode array detector (DAD), using a YMC PAK ODS-A column (250 mm × 4.6 mm i.d, 5 μm, YMC Co. Ltd, Japan) with MeCN−H_2O linear gradient elution (0 min 30:70, 30 min 100:0, v/v) as a mobile phase at 0.7 mL/min. Preparative HPLC was performed using a TSP P100 pump connected with a TSP UV 100 detector (at 210 nm), using a YMC Pak ODS-A column (250 mm × 20 mm i.d, 5 μm, YMC Co. Ltd., Japan) with MeCN−H_2O (40:60, v/v) as a mobile phase at 1.0 mL/min. Thin-layer chromatography (TLC) was performed on precoated 0.25 mm thick silica gel F254 glass-backed plates (Qingdao Haiyang Chemical Co. Ltd., Qingdao, China) with visualization under UV light (254 nm) and by dipping the plates into a solution of 10% (v/v) H_2SO_4 in EtOH and heating.

Preparation of PT. Ginseng extract was kindly donated by Jiuhui Modern Chinese Herbs Co. Ltd (Changsha, China), and the total content of ginsenoside was more than 80% w/w. Ginseng extract powder (20 g) was dissolved in H_2O (1700 mL), and $NaIO_4$ (86.7 g) was added to the solution. The mixture was stirred for 5 h at room temperature and filtered using vacuum. The filtrate was dissolved in 70% (v/v) EtOH, and $NaBH_4$ (7.0 g) was added. The solution was then allowed to stand for 17 h at room temperature. After dilution with water (300 mL), the solution was acidified to pH 1.8−2.0 by adding 2 N H_2SO_4 and then kept at room temperature overnight. The reaction mixture was extracted three times with equal volumes of ether, and the ether layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The residue (2.37 g) was chromatographed on a silica gel column (120 g) eluted with $CHCl_3$−EtOAc (12:1−2:1), which yield fractions A−E. Fraction C was purified by using HPLC to yield PT (**1**, 350 mg, 1.75%), the structure of which was confirmed by comparison with the literature values.[10]

Microorganisms and Culture Conditions. All microorganisms screened in our experiments were obtained from the China General Microbiology Culture Center (CGMCC), China. They were as follows: *Cunninghamella blakeslaena* AS 3.790, *C. elegans* AS 3.1207, *Aspergillus niger* AS 3.795, *A. niger* AS 3.1858, *A. avernaceus* AS 3.4454, *Mucor spinosus* AS 3.3450, *M. spinosus* AS 3.2450, *M. subtilissimus* AS 3.2454, *M. polymorphosporus* AS 3.3443, *Alternaria alternata* AS 3.577, *A. alternata* AS 3.4578, *A. longipes* AS 3.2875, *Absidia coerulea* AS 3.3389, *Trichoderma viride* AS 3.2942.

Stock cultures of the fungi were stored on potato dextrose agar slants at 4 °C. Seed cultures were obtained by transferring fungi from stock cultures to potato dextrose broth. Preliminary screen experiments were conducted in 50 mL Erlenmeyer flasks containing 20 mL of potato medium. For each fungus, two flasks were inoculated with the seed culture and incubated on a rotary shaker (120 rpm) for 2 days at 25 °C. PT (**1**, 10 mg) in ethanol (0.2 mL) was added to one flask, including an ethanol control at the same concentration. The fermentation was carried out for 5 days, and the cultures were then pooled and filtered. The filtrates were extracted three times with equal volumes of ethyl acetate, and the extracts were evaporated in a vacuum and analyzed on TLC plates. Substrate controls were composed of sterile medium to which the substrate was added and incubated without the microorganism. The TLC analysis showed that *Mucor spinosus* (AS 3.3450) was able to transform PT into more products than the other microbes tested.

Preparative Scale Biotransformation. For preparative scale biotransformation by *M. spinosus*, sixteen 1000 mL flasks containing 400 mL of potato broth medium were used and the fungus was incubated for 3 days. A 100 mg/mL solution of **1** (0.2 mL) in ethanol was fed into each flask. Incubating conditions, extracting process, and chromatographic purification were the same as described above. On the basis of spectroscopic analysis, their structures were elucidated as 12-oxo-15α-hydroxyl-20(*S*)-protopanaxatriol (**2**, 15 mg, 4.69%), 27-hydroxyl-20(*S*)-protopanaxatriol (**3**, 10 mg, 3.12%), 12-oxo-26-hydroxyl-20(*S*)-protopanaxatriol (**4**, 20 mg, 6.25%), and 12-oxo-27-hydroxyl-20(*S*)-protopanaxatriol (**5**, 5 mg, 1.56%), respectively.

12-Oxo-15α-hydroxyl-20(*S*)-protopanaxatriol (2): white amorphous powder; $[\alpha]^{25}_{D}$ +29.9° (*c* 0.748, MeOH); IR (KBr) ν_{max} 3412(OH), 1701(>C=O) cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 2; HR-FT-ICR-MS *m/z* 491.3724 [M + H]$^+$ (calcd for $C_{30}H_{51}O_5$, 491.3731).

27-Hydroxyl-20(*S*)-protopanaxatriol (3): white amorphous powder; $[\alpha]^{25}_{D}$ +43.8° (*c* 0.315,MeOH); IR (KBr) ν_{max} 3347(OH) cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 2; HR-FT-ICR-MS *m/z* 493.3896 [M + H]$^+$ (calcd for $C_{30}H_{53}O_5$, 493.3887).

12-Oxo-26-hydroxyl-20(*S*)-protopanaxatriol (4): white amorphous powder; $C_{30}H_{50}O_5$; $[\alpha]^{25}_{D}$ +50.9° (*c* 0.334, MeOH); IR (KBr) ν_{max} 3407(OH), 1697(>C=O) cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 2; HR-FT-ICR-MS *m/z* 491.3731 [M + H]$^+$ (calcd for $C_{30}H_{50}O_5$, 491.3740).

12-Oxo-27-hydroxyl-20(*S*)-protopanaxatriol (5): white amorphous powder; $C_{30}H_{50}O_5$; $[\alpha]^{25}_{D}$ +21.3° (*c* 0.141, MeOH); IR (KBr) ν_{max} 3405(OH), 1697(>C=O) cm^{-1}; ^1H NMR (pyridine-d_5, 500 MHz), see Table 1; ^{13}C NMR (pyridine-d_5, 125 MHz), see Table 2; HR-FT-ICR-MS *m/z*491.3731 [M + H]$^+$ (calcd for $C_{30}H_{50}O_5$, 491.3740).

Table 1. ^{1}H NMR Data (δ) of Metabolites (500 MHz, in pyridine-d_5)

position	**2**	**3**	**4**	**5**
1	1.43	1.71	0.95	1.40
	0.97	1.03	1.45	0.89
2	1.85	1.89	1.82	1.89
	1.84	1.84	1.84	1.86
3	3.49(brs)	3.52(brs)	3.50	3.49
4				
5	1.27	1.21(d,10.5)	1.22(d,10.5)	1.22(d,10.5)
6	4.46	4.39	4.44	4.42
7	2.71(dd,13,3.5)	1.89(dd,17.5,4.0)	1.94(dd,17.5,4)	1.92(dd,17.5,4)
	2.24(m)	1.85(dd,17.5,5.0)	1.93(dd,17.5,4)	1.87(dd,17.5,4)
8				
9	2.00(dd,11.5,3.5)	1.57	1.88(dd,11.5,3.5)	1.88(dd,11.5,3.5)
10				
11	2.42(dd,12.5,3.5)	1.54	2.33	2.30
	2.35(m)	2.14		2.32
12		3.91(brs)		
13	3.45(d,9.5)	2.04	3.32(d,9.5)	3.29(d,9.5)
14				
15	4.88(d,8.5)	1.55	1.89	1.90
		1.01	1.17	1.20
16	2.62(m)	1.82	2.04	2.00
	2.04(m)	1.31	1.88	1.76
17	2.83(td,4,15,9.5)	2.30	2.71	2.68
18	1.46(s)	1.10(s)	1.26(s)	1.25(s)
19	1.05(s)	1.00(s)	0.98(s)	0.98(s)
20				
21	1.40(s)	1.36(s)	1.42(s)	1.36(s)
22	1.76	1.67	1.80	1.77
	1.80	2.05	1.77	1.80
23	2.35	2.69	2.49	2.51
	2.24	2.43	2.37	2.38
24	5.22(dd,7,6.5)	5.47(dd,7,6.5)	5.77(d,7)	5.42(d,7)
25				
26	1.62(s)	1.98(s)	4.27(s)	1.98(s)
27	1.56(s)	4.53(d,12)	1.81(s)	4.47(s)
		4.45(d,12)		
28	1.98(s)	1.98(s)	1.92(s)	1.96(s)
29	1.43(s)	1.44(s)	1.42(s)	1.42(s)
30	1.25(s)	0.94(s)	0.88(s)	0.86(s)

Table 2. ^{13}C NMR Data (δ) of Metabolites (125 MHz, in pyridine-d_5)

position	**1** (PT)	**2**	**3**	**4**	**5**
1	39.3t	39.4t	39.3t	39.4t	39.4t
2	28.0t	28.0t	28.1t	27.9t	27.9t
3	78.3d	78.1d	78.4d	78.1d	78.1d
4	40.3s	39.9s	40.3s	40.0s	40.0s
5	61.7d	61.5d	61.8d	61.5d	61.5d
6	67.6d	67.7d	67.7d	67.6d	67.6d
7	47.4t	47.3t	47.5t	46.7t	46.7d
8	41.1s	42.4s	41.1s	41.7s	42.2s
9	50.0d	54.7d	50.1d	54.0d	54.0d
10	39.3s	39.1s	39.3s	38.9s	38.9s
11	31.9t	40.3t	32.0t	40.3t	40.3t
12	71.0s	211.3s	71.0d	211.7s	211.8s
13	48.2d	54.0d	48.2d	56.2d	56.2d
14	51.6s	56.5s	51.6s	55.5s	55.5s
15	31.3t	73.1d	31.3t	31.9t	31.9t
16	26.8t	34.3t	26.8t	24.5t	24.5t
17	54.7d	40.9d	54.7d	44.1d	44.1d
18	17.5q	17.5q	17.6q	17.4q	17.4q
19	17.4q	17.3q	17.5q	17.3q	17.3q
20	72.9s	73.0s	72.9s	73.2s	73.2s
21	27.0q	26.1q	27.0q	26.5q	26.5q
22	35.8t	42.4t	36.2t	41.8t	41.6t
23	22.9t	23.6t	22.5t	23.2t	23.2t
24	126.2d	125.7d	127.9d	125.2d	127.5d
25	130.7s	130.9s	136.2s	136.2s	136.3s
26	25.8q	25.7q	21.9q	68.1t	21.8q
27	17.6q	17.6q	60.8t	13.9q	60.8t
28	31.9q	31.7q	32.0q	31.8q	31.8q
29	16.4q	16.4q	16.5q	16.4q	16.4q
30	17.0q	11.4q	17.0q	17.3q	17.3q

Bioassay. Human gastric cancer BGC-823 cells and human leukemia HL-60 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in RPMI 1640 medium (Gibco RBL Co.) containing 10% fetal bovine serum supplemented with L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cell growth was evaluated by MTT assay procedure as previously reported.[11]

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