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ORIGINAL ARTICLE

Novonestmycins A and B, two new 32-membered bioactive macrolides from *Streptomyces phytohabitans* HBERC-20821

Zhongyi Wan[1], Wei Fang[2], Liqiao Shi[2], Kaimei Wang[2], Yani Zhang[2], Zhigang Zhang[2], Zhaoyuan Wu[2], Ziwen Yang[1,2] and Yucheng Gu[3]

Two new 32-membered macrolide compounds, named Novonestmycins A (1) and B (2), were isolated from the soil strain *Streptomyces phytohabitans* HBERC-20821. Their structures were elucidated by using spectroscopic methods, including 1D, 2D-NMR and MS spectrometry. The two compounds showed strong activities against the phytophathogenic fungi *Corynespora cassiicola*, *Rhizoctonia solani* and *Septoria nodorum*, with MIC values of 0.78, 0.39 and 0.78 μg ml^{-1}, respectively. In addition, the two compounds exhibited potent inhibitory activities against four different human tumor cell lines as well as one 5-FU-resistant human hepatocellular carcinoma cell line, with IC_{50} of 0.15–0.48 μg ml^{-1} and 0.24–1.34 μg ml^{-1}, respectively.
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INTRODUCTION

Macrolides are an effective and safe class of antibiotics, mainly produced by actinomycetes and fungi. Their structures and biological activities are highly diverse. 12-, 14- and 16-membered macrolides are potent antibacterial agents that inhibit protein biosynthesis, larger macrolides (>16-membered), often containing fused-ring systems and linked with sugar derivatives, and that exhibit various interesting biological activities such as antifungal, antitumor, immunosuppressive, insecticide and anthelmintic action.[1] In the course of our screening for new biological antibiotics, the strain HBERC-20821 was isolated from a soil sample collected at Wawushan Hill, Sichuan province, PR China, and it showed excellent antifungal activities against some phytopathogenic fungi. It was found to produce new 32-membered macrolide antibiotics Novonestmycins A and B, which showed strong antifungal and cytotoxic activity. In this report, we describe the taxonomy and fermentation of strain HBERC-20821, and the isolation, structural elucidation and biological activity of Novonestmycins A and B.

RESULTS

Taxonomy of strain HBERC-20821

The vegetative mycelia grew abundantly on yeast extract–malt extract agar, but grew poorly on glycerol–asparagine agar, inorganic salt–starch agar and sucrose–nitrate agar. The color of vegetative mycelia was light yellow to light brown and the aerial mycelia grew abundantly and appeared as straight spore chains on ISP-2 agar. The spores have smooth surface and are 0.6–0.8 × 1–1.5 μm in size (Figure 1). D-glucose and D-mannitol were used as the sole carbon source and D-fructose, melibions, raffinose, sucrose and xylose were not used. No soluble pigment was found in test medium. The 16S rRNA sequence (1392 bp) of the strain HBERC-20821 was submitted to Gen Bank under the accession number KF765441, which showed the highest similarity (99%) to that of *Streptomyces phytohabitans* KLBMP 4601.[2] The phylogenetic tree generated by the neighbor-joining method clearly revealed the evolutionary relationship of the strain HBERC-20821 with *Streptomyces* (Supplementary Figure S1). On the basis of the 16S rRNA gene sequence analysis, the strain HBERC-20821 was designated as a species of *S. phytohabitans* HBERC-20821.

Structure elucidation of novonestmycins A and B

The physico-chemical properties of Novonestmycins A (**1**) and B (**2**) are summarized in Table 1. Compound **1** was isolated as a white, amorphous powder with the molecular formula $C_{62}H_{100}O_{24}$, determined on the basis of NMR and HRESIMS data (*m/z* 1251.6504 $[M+Na]^+$, calcd for $C_{62}H_{100}O_{24}Na$, 1251.6497). Detailed analysis of the NMR data including the DEPT spectrum and the HSQC spectrum showed the presence of 8 quaternary carbons (including a hemiacetal carbon at δ_c 99.9 and four carbonyl carbons at δ_c 208.5, 173.9, 168.9, 167.8), 8 aromatic/olefinic carbons, 18 oxygenated methines (including one anomeric carbon at δ_c 98.5), 7 aliphatic methines, 12 methylenes and 11 methyl carbons (including a methoxy carbon at δ_c 61.4). Except for the four olefinic double bonds and four carbonyls,

[1]College of Life Sciences, Wuhan University, Wuhan, PR China; [2]National Engineering Research Center for Bio-pesticide, Hubei Academy of Agricultural Sciences, Wuhan, PR China and [3]Syngenta Jealott's Hill International Research Centre, Bracknell, Berkshire, UK
Correspondence: Dr W Fang, National Engineering Research Center for Bio-pesticide, Hubei Academy of Agricultural Sciences, Wuhan 430064, PR China.
E-mail: fangw111@163.com
or Professor Z Yang, College of Life Sciences, Wuhan University, Wuhan 430072, PR China.
E-mail: lyk666888@126.com
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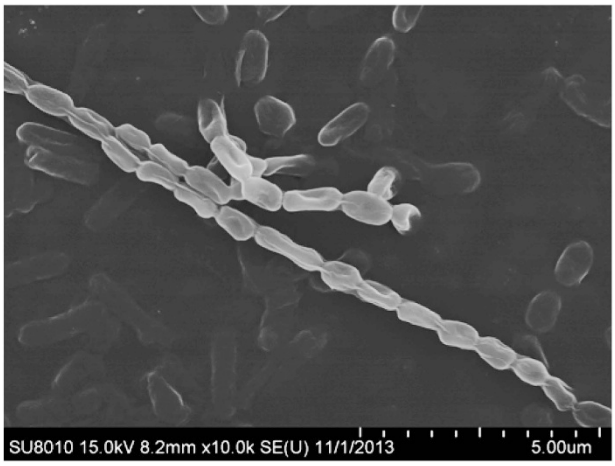

Figure 1 Scanning electron micrograph of strain HBERC-20821, showing spore chains after growth on ISP medium 2 agar at 28 °C for 4 weeks. Bar 5.0 μm.

Table 1 Physico-chemical properties of compounds 1 and 2

	1	*2*
Appearance	White powder	White powder
Molecular formula	$C_{62}H_{100}O_{24}$	$C_{68}H_{110}O_{26}$
$[\alpha]_D^{25}$	+70° (*c*=0.1, MeOH)	−100° (*c*=0.07, MeOH)
HRESI-MS (m/z)		
Found.	1251.6504 $[M+Na]^+$	1365.7174 $[M+Na]^+$
Calcd.	1251.6497 $[M+Na]^+$	1365.7178 $[M+Na]^+$
UV(MeOH) λ_{max}	222 (3.71), 260 (3.28),	222 (3.79), 260 (3.43),
nm (log ε)	295 (3.03)	290 (2.98, sh)
IR (KBr) ν_{max}	3435, 1711, 1647, 1294,	3433, 1711, 1647, 1288,
$(cm)^{-1}$	1288, 1085, 977	1294, 1085, 993

13 degrees of unsaturation deduced from the molecular formula revealed that **1** contains either the five ring systems. The ^{1}H-NMR spectrum of **1** (Supplementary Figure S3) showed three spin aromatic protons at δ_H 7.46 (1H, dd, J=9.0, 2.0 Hz, H-7″), 7.46 (1H, d, J=2.0 Hz, H-3″) and 6.82 (1H, d, J=9.0 Hz, H-6″), suggesting that **1** contains a 1,3,4-tri-substituted benzene ring. The ^{1}H−^{1}H COSY NMR spectrum of **1** showed a series of correlations involving three partial structural units: H-2 to H-13, H-16 to H-39, and H-1′ to H-6′.

That the structure of **1** (Figure 2) was similar to Notonesomycin A[3] was elucidated by HMBC data (Figure 3), which revealed the connectivities of structural units: HMBC correlations of H-13 to C-14 and H-16 to C-14 indicated that the ketone group was located on C-14 position;[4] HMBC correlations of H-16 and H-19 to a hemiketal carbon at C-15 (δ_C 99.9) revealed the presence of a tetrahydropyran ring. In addition, the HMBC correlations of H-2 to C-1 and H-31 to C-1 showed that **1** was a 32-membered macrolide; the HMBC correlation of H-1′ to C-5′ suggested the presence of a sugar moiety (C-1′–C-6′), which was linked to C-37 by HMBC correlations of H-1′ to C-37 and H-37 to C-1′; the ^{13}C-NMR data and the HMBC correlation of H-3″ to C-1″ and H-7″ to C-1″ revealed the presence of a protocatechuic acid unit, which was connected to C-3′ as deduced by the HMBC correlation of H-3′ to C-1″. Consequently, the structure of **1** was established and all the protons and carbons of **1** could be assigned by 1D and 2D-NMR data (Tables 2 and 3).

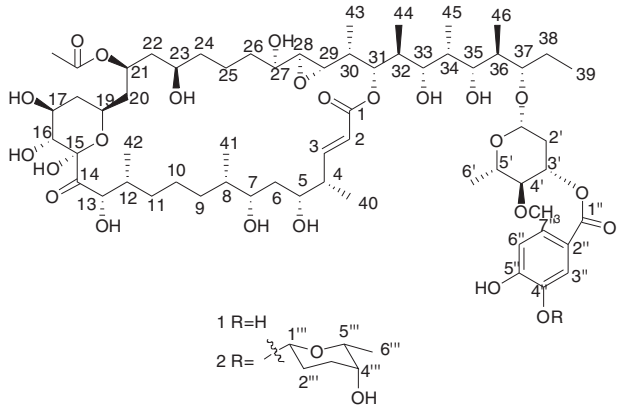

Figure 2 Structures of compounds **1** and **2**.

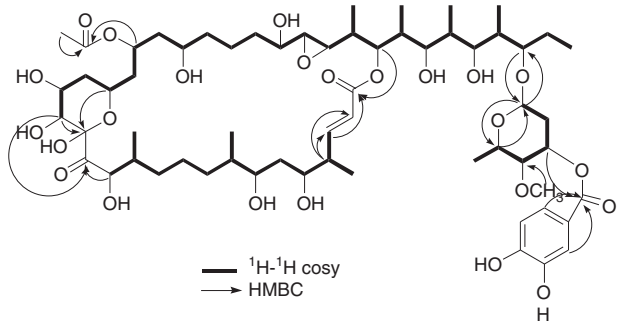

Figure 3 The key 2D NMR of compound **1**.

Kobayashi *et al.*[5] has reported that 1,3,5-trisubstituted acyclic compounds show a characteristic chemical shift that is dependent on the 1,3- and 3,5-relative configuration, but is independent of the functionalities present outside this structural motif. On this basis, the relative configuration of the C33−C35 position was the syn/syn-configuration, deduced by the significant upfield C-45 chemical shift (δ_c 5.09).[6,7] Supported by the universal NMR databases, the NOESY correlations between H-27 and H-28/H-29, H-28 and H-30, H-29 and H-43/H-31, H-30 and H-31/H-44, H-31 and H-33, H-33 and H-34/H-35, H-34 and H-32/H-46, and H-35 and H-33/H-37 yielded the relative configuration of C27–C37 (Figure 4). The NOESY correlation from H-16 to H-13 and H-16 to H-12 indicated that the relative configuration of the C12−C13 portion was syn. The coupling constant of H-16 (J=9.0 Hz) revealed that H-16 and H-17 both were in axial positions. Furthermore, H-19 also showed axial orientation as deduced by a NOESY correlation between H-17 and H-19. The relative configuration of the C4−C5 position was established by NOESY correlations of H-4 to H-2/H-3, and H-5 to H-2/H-3. The anomeric protons (δ 4.66 brd, J=8.5 Hz, H-1′) showed large coupling constants, representing the axial position of H-1′. NOESY correlations between H-1′ and H-3′ and between H-1′ and H-5′ together with the coupling constant of H-4′ (δ 3.04, dd, J=9.0, 9.0 Hz) revealed that the H-3′, H-4′ and H-5′ were all in axial positions. Finally, NOESY NMR correlations (Figure 4) were assigned the remaining relative configuration of **1**, which is shown in Figure 2.

Novonestmycin B (**2**) was purified as a white, amorphous powder with the molecular formula $C_{68}H_{110}O_{26}$, determined on the basis of NMR and HRESIMS data (*m/z* 1365.7174 $[M+Na]^+$, calcd for $C_{68}H_{110}O_{26}Na$, 1365.7178). Comparing the ^{13}C NMR data of **1** and **2** (Supplementary Figures S4 and S7), the obvious differences were the

Table 2 ^{1}H-NMR data of compounds 1 and 2 in CD_3OD 500 MHz (δ in p.p.m., J in Hz)

Position	*1*	*2*	*Position*	*1*	*2*
2	5.91 (d, 16.0)	5.91 (d,16.0)	34	1.81, m	1.81, m
3	7.06 (dd, 7.5, 16.0)	7.07 (dd, 7.5, 16.0)	35	3.49 (d, 6.0)	3.49 (d, 6.0)
4	2.49, m	2.49, m	36	1.94, m	1.94, m
5	3.81, m	3.81, m	37	3.89, m	3.89, m
6	1.61, m	1.61, m	38	1.30, m	1.30, m
	1.44, m	1.44, m		1.50, m	1.50, m
7	3.84, m	3.84, m	39	0.97 (t, 7.0)	0.97 (t, 7.0)
8	1.55, m	1.55, m	40	1.11 (d, 7.0)	1.11 (d, 7.0)
9	1.43, m	1.43, m	41	0.89 (d, 7.0)	0.90 (d, 7.0)
	1.21, m	1.21, m			
10	1.49, m	1.49, m	42	0.81 (d, 7.0)	0.82 (d, 7.0)
	1.44, m	1.44, m			
11	1.55, m	1.55, m	43	1.07 (d, 6.0)	1.07 (d, 6.0)
	1.31, m	1.31, m			
12	2.26, m	2.26, m	44	0.86 (d, 7.0)	0.86 (d, 7.0)
13	4.58 (d, 3.5)	4.58 (d, 3.5)	45	0.91 (d, 7.0)	0.91 (d, 7.0)
16	3.54 (d, 9.0)	3.54 (d, 9.0)	46	0.76 (d, 7.0)	0.77 (d, 7.0)
17	3.84, m	3.84, m	1′	4.66 (brd, 8.5)	4.66 (brd, 8.5)
18	1.95, m	1.95, m	2′	2.24, m	2.24, m
	1.40, m	1.40, m		1.61, m	1.61, m
19	4.08, m	4.08, m	3′	5.04, m	5.04, m
20	1.78, m	1.78, m	4′	3.04 (dd, 9.0,9,0)	3.04 (dd, 9.0,9.0)
21	5.19, m	5.19, m	5′	3.40, m	3.40, m
22	1.58, m	1.58, m	6′	1.33 (d, 6.0)	1.33 (d, 6.0)
	1.45, m	1.45, m			
23	3.57, m	3.57, m	3″	7.46 (d, 2.0)	7.78 (d, 2.0)
24	1.59, m	1.59, m	6″	6.82 (d, 9.0)	6.90 (d, 9.0)
	1.46, m	1.46, m			
25	1.58, m	1.58, m	7″	7.46 (dd, 2.0,9.0)	7.66 (dd, 2.0,9.0)
	1.37, m	1.37, m			
26	1.42, m	1.42, m	1‴		5.14 (dd, 1.5,9.5)
	1.30, m	1.30, m			
27	3.42, m	3.42, m	2‴		2.03, m
					1.88, m
28	2.77 (dd, 1.5, 3.5)	2.77 (dd, 1.5, 3.5)	3‴		1.98, m
					1.50, m
29	2.73 (dd, 1.5, 8.5)	2.73 (dd, 1.5, 8.5)	4‴		3.54, br s
30	1.56, m	1.56, m	5‴		3.76 (q, 7.0)
31	5.33 (d, 10.5)	5.33 (d, 10.5)	6‴		1.26 (d, 7.0)
32	1.96, m	1.96, m	4′-OCH_3	3.51, s	3.51, s
33	3.38, m	3.38, m	21-O*C*H_3	2.07, s	2.06, s

additional six carbons (including one anomeric carbon, two oxygenated methine carbons, two methylene carbons and one methyl carbon). The ^{1}H–^{1}H COSY NMR spectrum of **1** indicated a structural correlation of H-1‴ to H-6‴. Furthermore, the HMBC correlation of H-1‴ to C-5‴ revealed the group was 2,3,6-trideoxyhexose moiety, which was linked to C-4″ by the HMBC correlation of H-1‴ to C-4″. The coupling constant of the anomeric proton H-1‴ (δ 5.14, dd, J = 1.5, 9.5 Hz) revealed an axial position of H-1‴. A NOESY correlation between H-1‴ and H-5‴ together with the coupling constant of H-5‴ (δ 3.76, q, J = 7.0 Hz) and H-4‴ (δ 3.54, br s) indicated that 4‴-OH and H-5‴ were both in axial positions. Thus, the structure of **2** was determined and is shown in Figure 2.

Assay of antimicrobial activity

The MICs of **1** and **2** against bacteria and fungi were determined by the agar dilution method (Table 4). Novonestmycins A and B showed weak activity against the Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus hemolytic*-β with the same MIC value of 25 μg ml^{-1}. The two compounds showed moderate activity against *Coniothyriopsis mangiferae*, *Botrytis cinerea*, *Fusarium graminearum* and *Alternaria solani*, with MIC values of 1.56, 12.5, 6.25 and 1.56 μg ml^{-1}, respectively. **1** and **2** also exhibited strong activity against the phytophathogenic fungi *Corynespora cassiicola*, *Rhizoctonia solani* and *Septoria nodorum*, with MIC values of 0.78, 0.39 and 0.78 μg ml^{-1}, respectively.

Cytotoxic activity

The newly compounds were evaluated for their *in vitro* cytotoxic effects against HepG2 (hepatocellular liver carcinoma), BGC-823 (gastric cancer), MCF-7 (breast adenocarcinoma) and Hela (cervical cancer) cell lines by the standard MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay using 5-FU (5-fluorouracil)

Table 3 ^{13}C-NMR data of compounds 1 and 2 in CD_3OD 125 MHz (δ in p.p.m.)

Position	1	2	Position	1	2
1	168.94, C	168.60, C	35	79.96, CH	79.63, CH
2	122.60, CH	122.45, CH	36	40.52, CH	40.19, CH
3	154.5, CH	154.42, CH	37	81.69, CH	81.27, CH
4	43.79, CH	43.47, CH	38	23.01, CH_2	22.68, CH_2
5	75.10, CH	74.77, CH	39	10.75, CH_3	10.42, CH_3
6	38.88,[a] CH_2	38.56,[a] CH_2	40	14.02, CH_3	13.69, CH_3
7	74.79, CH	74.46, CH	41	14.79, CH_3	14.44, CH_3
8	39.59, CH	39.26, CH	42	14.00, CH_3	13.66, CH_3
9	35.13, CH_2	34.80, CH_2	43	14.63, CH_3	14.13, CH_3
10	26.45, CH_2	26.24, CH_2	44	9.79, CH_3	9.46, CH_3
11	33.62, CH_2	33.29, CH_2	45	5.09, CH_3	4.75, CH_3
12	36.82, CH	36.48, CH	46	11.50, CH_3	11.16, CH_3
13	76.26, CH	75.93, CH	1′	98.49, CH	98.15, CH
14	208.50, C	208.17, C	2′	38.92,[a] CH_2	38.60,[a] CH_2
15	99.91, C	99.58, C	3′	75.44, CH	75.22, CH
16	75.54, CH	75.31, CH	4′	85.75, CH	85.46, CH
17	69.97, CH	69.93, CH	5′	71.28, CH	70.94, CH
18	40.96, CH_2	40.63, CH_2	6′	18.75, CH_3	18.41, CH_3
19	67.50, CH	67.17, CH	1″	167.79, C	167.11, C
20	42.11, CH_2	41.78, CH_2	2″	122.86, C	122.45, C
21	70.70, CH	70.37, CH	3″	117.79, CH	120.33, CH
22	44.08, CH_2	43.75, CH_2	4″	146.63, C	146.13, C
23	68.78, CH	68.45, CH	5″	152.29, C	154.20, C
24	38.63, CH_2	38.30, CH_2	6″	116.30, CH	116.99, CH
25	22.85, CH_2	22.53, CH_2	7″	124.08, CH	126.85, CH
26	35.82, CH_2	35.49, CH_2	1‴		102.86, CH
27	72.58, CH	72.23, CH	2‴		30.39, CH_2
28	64.02, CH	63.68, CH	3‴		25.22, CH_2
29	61.02, CH	60.68, CH	4‴		66.92, CH
30	40.88, CH	40.55, CH	5‴		75.31, CH
31	75.54, CH	75.58, CH	6‴		17.49, CH_3
32	38.83, CH	38.50, CH	4′-OCH_3	61.41CH_3	61.11, CH_3
33	78.66, CH	78.32, CH	21-O*C*CH_3	173.89 C	173.54, C
34	36.29, CH	35.97, CH	21-OC*CH_3*	21.60CH_3	21.26, CH_3

[a]Assignments within the same alphabets are interchangeable.

as a positive control. As shown in Table 5, the two compounds exhibited significant inhibitory activities against four different human tumor cell lines as well as one 5-FU-resistant human hepatocellular carcinoma cell line, with an IC_{50} of 0.15–0.48 and 0.24–1.34 μg ml^{-1}, respectively.

DISCUSSION

Novonestmycins A and B have closest related structure to notonesomycin A, a 32-membered macrolide antibiotic produced by notonesogenes 647-AV1.[8] Novonestmycin B and notonesomycin A have the same backbone and the difference between the two compounds is the substitution in the macrolide ring and benzene ring. Novonestmycins A and B showed strong inhibitory activity against some phytopathogenic fungi, but notonesomycin A displayed moderate antifungal activity. Another structurally related compound was liposidolide A, a 36-membered macrolide antibiotic produced by *S.* sp. RS-28, which also showed strongly antifungal activity against phytophathogenic fungi.[4] Brasilinolide A, another compound with similar structure, only showed moderate antifungal activity against *Aspergillus niger*.[9] These results suggested that 14-ketone groups may be important for the antifungal activity of this type of compound.

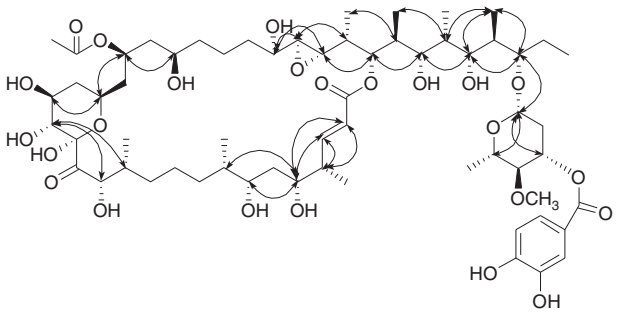

Figure 4 The key NOE correlations of compound **1**.

Interestingly, **1** and **2** exhibited better inhibitory activities against four human tumor cell lines compared with 5-FU. Importantly, **1** and **2** both were active against 5-FU-resistant BEL-7402/5-FU cell line (0.27 and 0.42 μg ml^{-1}, respectively); as compared with the positive control, 5-FU was shown to be inactive (100 μg ml^{-1}). Compound **1** showed stronger cytotoxicity than **2** against all test cancer cell lines, indicating that the sugar moiety linked to the protocatechuic acid part may decrease cytotoxicity.

METHODS

General experimental procedures

Optical rotations were determined on a Perkin-Elmer model 341 polarimeter (Perkin-Elmer Inc., San Jose, CA, USA). IR spectra were obtained on a Perkin-Elmer Spectrum 577 (Perkin-Elmer Inc.) spectrophotometer. NMR spectra, including HSQC, HMBC and COSY, were recorded on a Bruker AVANCE-500 instrument with tetramethylsilane as an internal standard (Bruker BioSpin group, Karlsruhe, Germany). ESI-MS and HR-ESI-MS data were obtained on Waters LC-MS (Waters Corp., Boston, MA, USA) and Thermo Q-Tof Micro mass spectrometers (Thermo Scientific, Bremen, Germany), respectively. Preparative HPLC was performed a Waters Autopurification system coupled with 2998 DAD detector (Waters Corp.), using Sunfire Prep C_{18} OBD (5 μm, 19 × 250 mm, Wexford, Ireland) column.

Taxonomy of the strain HBERC-20821

The strain HBERC-20821 was isolated on ISP-2 and humic acid–vitamin agar, from a soil sample collected at Wawushan Hill, Sichuan province, PR China, in 2010. The International Streptomyces Project (ISP) media recommended by Shirling and Goebel[10] and the media recommended by Waksman[11] were used to investigate the morphological and physiological characteristics. Cultures were routinely observed after incubation for 2–3 weeks at 28 °C. The utilization of carbon sources was tested by determining the growth on Pridham and Gottlieb's medium containing 1% carbon source at 28 °C. Morphological properties were observed with a scanning electron microscope (model HITACHI SU8010, Tokyo, Japan). Extraction of genomic DNA, PCR amplification and sequencing of 16S rRNA were carried out using the universal methods.[12] It was identified as *S. phytohabitans* and deposited at the China Center for Type Culture Collection (CCTCC) Wuhan University, PR China, with the code number CCTCC M2013379. Identification of the strain was carried out by sequence analysis of 16S rRNA gene using the DDBJ-BLAST search.

Fermentation

The strain HBERC-20821 was inoculated in 100 ml of the seed medium (1.0% mannitol, 2.5% soybean flour, 0.5% yeast extract, 0.4% NaCl, 0.3% $CaCO_3$ in tapwater, pH 7.0) in a 500-ml Erlenmeyer flask with baffle from the glycerol stock, and cultured at 28 °C, for 96 h on a rotary shaker (150 r.p.m.). Ten ml of the seed culture was inoculated in 10 × 500 ml flasks each containing 100 ml of the seed medium, and cultivated for 24 h in the same conditions. Tank fermentation was carried out with a 50-l stainless steel fermentor (Shanghai Baoxing Bioengineering Equipment Co., Ltd, Shanghai, China) in a 30-l medium (1.0% mannitol, 2.5% soybean flour, 0.5% yeast extract, 0.4% NaCl,

Table 4 Antimicrobial activity of the compounds 1 and 2

	MIC ($\mu g\,ml^{-1}$)[b]			*MIC* ($\mu g\,ml^{-1}$) [b]	
Test organism [a]	*1*	*2*	*Test organism*	*1*	*2*
Coniothyriopsis mangiferae	1.56	1.56	*Phytophthora infestans*	>100	>100
Corynespora cassiicola	0.78	0.78	*Pseudomonas solanacearum*	>100	>100
Botrytis cinerea ACCC30091	12.5	12.5	*P. aeruginosa* ATCC 9027	>100	>100
Rhizoctonia solani ACCC30283	0.39	0.39	*Streptococcus hemolytic-β* CMCC 32120	25	25
Alternaria solani	1.56	1.56	*Staphylococcus aureus* ATCC 25923	25	25
Septoria nodorum	0.78	0.78	*Escherichia coli* ATCC 25922	>100	>100
Fusarium graminearum ACCC30068	6.25	6.25	*Salmonella typhimurium* H9812	>100	>100
F. oxysporum ACCC30024	>100	>100	*Erwinia carotovora* ACCC12104	25	25

[a]For fungi: 1×10^4 spores per ml, For bacteria 1×10^6 CFU per ml.
[b]Inhibited growth rate>50%. The MIC value of positive control was not displayed (penicillin and streptomycin for bacteria; cycloheximide for fungi).

Table 5 Cytotoxic activity of the compounds 1 and 2 against human tumor cells

	In vitro cytotoxicity IC_{50}[a] ($\mu g\,ml^{-1}$)				
Compd. No.	*HepG2*	*BGC-823*	*MCF-7*	*Hela*	*BEL-7402/5-FU*
1	0.15±0.05	0.23±0.01	0.48±0.13	0.21±0.09	0.27±0.04
2	0.24±0.01	0.50±0.01	1.34±0.08	0.42±0.10	0.42±0.04
5-FU[b]	10.09±1.48	10.06±0.85	13.94±2.62	10.36±0.54	>100.00

Abbreviations: BEL-7402/5-FU, 5-fluorouracil-resistant human hepatocellular carcinoma cell line; BGC-823, human gastric cancer cell line; Hela, human cervical cancer cell line; HepG2, human hepatocellular liver carcinoma cell line; MCF-7, human breast adenocarcinoma cell line. [a]IC_{50}—compound concentration required to inhibit tumor cell proliferation by 50%.
[b]5-Fluorouracil, known chemotherapy, for comparison purpose.

0.3% $CaCO_3$, 0.1% polyether antifoam). The fermentation was incubated at 28 °C for 120 h with aeration at 30 l min^{-1} and agitation at 300 r.p.m.

Extraction and isolation

The culture broth (25 l) was absorbed with 1-l HP-20 resin (Mitsubishi Chemical, Tokyo, Japan) for 3 h, then the resin was columned and washed with water (4 l) and 90% ethanol (3 l), respectively. The filtered ethanol eluent was concentrated *in vacuo* to give 42-g crude extracts. The extracts (20 g) were dissolved with methnol (20 ml) and then passed though a 0.22-μm microporous membrane. The filter was purified by preparative HPLC (solvent: CH_3CN/H_2O; column: Sunfire C_{18}, 5 μm, 19×250 mm; the flow rate was 27 ml min^{-1}; elution gradient: 0–2 min, 5% CH_3CN; 2–27 min, 5–100% CH_3CN; 27–32 min, 100% CH_3CN; 32–37 min, 100-5% CH_3CN; 37–40 min, 5% CH_3CN; the injected volume was 1000 μl; Diode Array Detector (DAD) detection). Compound **1** (15.3 mg) and compound **2** (20.5 mg) were eluted with retention times of 22.4 and 23.7 min, respectively.

Assay of antimicrobial activity

The *in vitro* antimicrobial activities of compounds **1** and **2** were determined on nutrient agar on a 96-well plate with a twofold serial dilution method.[13] After 72 h of incubation (for a bacterial strain it was 24 h), the optical density of the medium was measured at 490 nm (OD_{490}) with a microplate reader (Thermo Scientific, Bremen, Germany). The cultural conditions were as follows: Fungi PDA medium (Potato 20%, Dextrose 2%, Agar 2%), 28 °C, 72 h; Bacteria LB medium (Yeast extract 0.5%, Tryptone 1%, NaCl 1%), 37 °C, 24 h.

Assay of cytotoxic activity

The cytotoxicity of the compounds was evaluated in tumor cell lines with the MTT assay. Briefly, the cells were seeded on a 96-well plate. After 24 h, the medium was replaced with fresh medium (2% fetal calf serum) and twofold serial dilution of the compounds was added to the cells. After 72 h of exposure, the medium was removed and MTT was added at a concentration of 5 mg ml^{-1} and incubated for 4 h at 37 °C. Dimethylsulfoxide (50 μl per well) was added to dissolve the MTT formazan, and the optical density of the cells was measured at 570 nm (OD_{570}) with a microplate reader. All data were analyzed with SPSS software, and the 50% inhibitory concentrations (IC_{50}) of each compound for the different cell lines were determined. Assays were performed in triplicate on three independent experiments.

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