Two New 23-Membered Macrolactones from a Terrestrial Bacterium, *Streptomyces* sp. IMBJ01

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Two new unusual 23-membered macrolactones, named griseoviridins B (**1**) and C (**2**), along with the known analogue griseoviridin (**3**) were isolated from the culture of *Streptomyces* sp. IMBJ01, a terrestrial bacterium isolated from Wolong, Sichuan Province, China. The structures of **1** and **2** were elucidated on the basis of extensive spectroscopic data and their absolute configurations were determined by comparison of their CD spectra with those of compound **3**.

Introduction. – Polyketides (PKs) and nonribosomal peptides (NRPs) are two families of natural products with remarkable structural diversity and biological activities [1], while in different microorganisms key enzymes suitable for the biosynthesis of similar PKs and NRPs were known to be conservative in amino acid sequences, as well as encoding gene [2][3]. Recently, more and more results have indicated that the presence of PKs and NRPs genes was a good indicator for the selection of strains to find new compounds [4][5]. In our continuing search for new compounds from bacteria, a program combining genome screening and chemical analysis (HPLC/DAD (diode-array detection) and TLC) were used to screen our microorganism library. During the process, the terrestrial *Streptomycetes* IMBJ01 strain was selected for further research because it possesses both typical PKs and NRPs genes by PCR (polymerase chain reaction) screening and gene cloning, and showing interesting peaks with special UV absorptions by HPLC/DAD analysis. Investigation of both of this strain led to the discovery of two new unusual 23-membered macrolactones griseoviridins B[2]) (**1**) and C[2]) (**2**), along with the known analogue griseoviridin (=(1*S*,9*R*,11*S*,12*E*,14*E*,19*Z*,22*R*)-9,11-dihydroxy-22-methyl-6,23-dioxa-26-thia-2,17,27-triazatricyclo[17.5.2.1^{4,7}]heptacosa-4,7(27),12,14,19-pentaene-3,18,24-trione; **3**) (*Fig. 1*). In this article, we describe the isolation and structure elucidation of these two new griseoviridin analogues.

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[1]) These authors made equal contributions to this article.

[2]) Trivial atom numbering; for systematic names, see *Exper. Part.*

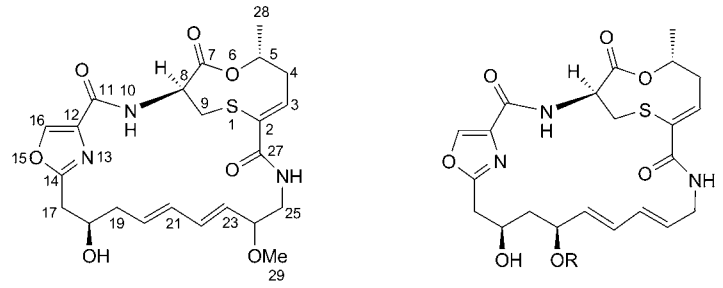

Fig. 1. *Compounds* **1**–**3** *from* Streptomyces *sp. IMBJ01*

Results and Discussion. – The crude extract was concentrated *in vacuo* and chromatographed by repeated column chromatography (silica gel, *Sephadex LH-20*) to yield, after semi-prep. HPLC, the two new compounds **1** and **2** and, after recrystallization, the known compound **3**.

Griseoviridin B[2]) (**1**), a colorless amorphous solid with a molecular formula $C_{23}H_{29}N_3O_7S$ based on the HR-ESI-MS peak at *m/z* 514.1328 ($[M + Na]^+$), indicated eleven degrees of unsaturation. IR Absorptions revealed the presence of amide and/or ester groups (1732 and 1674 cm^{-1}). The ^{13}C- and DEPT-NMR spectra (*Table*) displayed a Me (δ(C) 20.8), a MeO (δ(C) 56.3), four CH (three O-bearing at δ(C) 71.3, 67.6, and 78.3, and one N-bearing at δ(C) 50.5), and five CH_2 groups (δ(C) 37.9, 39.0, 34.6, 39.3, and 43.7), as well as nine sp^2 C-atoms and three C=O groups. Since eight of the eleven degrees of unsaturation were accounted for by the nine sp^2 C-atoms and three C=O groups, it was implied that **1** should contain three rings. Interpretation of the 2D-NMR data (*Fig. 2*) allowed the assignment of the constitution of compound **1**. Firstly, the methylated nine-membered thio-lactone moiety (fragment **a**) was constructed from COSY cross-peaks within two spin systems (H–C(3)/H–C(5)/Me(28); H–C(8)/CH_2(9)) and HMBC cross-peaks (H–C(4) and H–C(9)/C(2); H–C(8) and H–C(9)/C(7)), together with the chemical shift of C(2) (δ 130.6), C(5) (δ 71.3), C(7) (δ 170.9), and C(9) (δ 39.0) [6]. An NH was located at C(8) by the COSY cross-peak H–C(8)/NH(10) and confirmed by the chemical shift of H–C(8) (δ 4.48–4.53) and C(8) (δ 50.5). The fragment **b** from C(17) to N(26) was deduced from the COSY cross-peaks within the extended spin system H–C(17) to NH(26). The attachment of the MeO group at C(24) (δ 78.3) was determined by the chemical shifts and the HMBC cross-peak Me(29)/C(24). The (20*E*,22*E*) configuration of the two C=C bonds was deduced on the basis of the *trans* coupling constant ($J = 15.6$ and 15.1 Hz, resp.) between the olefinic H-atoms H–C(20)/H–C(21) and H–C(22)/H–C(23). The 2,4-disubstituted oxazole ring in fragment **c** was suggested by the chemical shift of C(12) (δ 134.8), C(14) (δ 159.4), and C(16) (δ 142.4), which was confirmed and further extended to C(11) by the HMBC cross-peaks H–C(16)/C(11), C(12), and C(14). Subsequently, fragments **a** and **b** were joined together *via* C(27) by the HMBC cross-peaks H–C(3)/C(27) and CH_2(25)/C(27), and fragments **b** and **c** were connected by the HMBC cross-peaks CH_2(17)/C(14). Finally, the gross structure of compound **1** was completed by the

Table. *^{1}H- and ^{13}C-NMR-Data* ((D_6)DMSO; 600 and 150 MHz, resp.) *of Compounds* **1** *and* **2**[1]). δ in ppm, J in Hz.

	1		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
C(2)		130.6 (*s*)		131.0 (*s*)
H–C(3)	7.33 (*dd*, *J* = 8.7, 8.2)	145.8 (*d*)	7.36 (*dd*, *J* = 8.7, 7.7)	145.2 (*d*)
CH_2(4)	2.79–2.85 (*m*)[a]),	37.9 (*t*)	2.88–2.91 (*m*)[a]),	38.0 (*t*)
	2.44 (*dd*, *J* = 12.3, 7.3)		2.43 (*dd*, *J* = 12.1, 7.6)	
H–C(5)	5.08–5.11 (*m*)	71.3 (*d*)	5.10–5.15 (*m*)	71.5 (*d*)
C(7)		170.9 (*s*)		171.4 (*s*)
H–C(8)	4.48–4.53 (*m*)	50.5 (*d*)	4.53–4.58 (*m*)	50.6 (*d*)
CH_2(9)	3.57–3.61 (*m*)[a]),	39.0 (*t*)	3.57–3.59 (*m*)[a]),	39.1 (*t*)
	2.60 (*dd*, *J* = 13.7, 10.9)		2.70 (*dd*, *J* = 15.4, 6.1)	
H–N(10)	7.22 (*d*, *J* = 7.8)		7.20 (*d*, *J* = 7.7)	
C(11)		162.2 (*s*)		163.1 (*s*)
C(12)		134.8 (*s*)		134.9 (*s*)
C(14)		159.4 (*s*)		159.3 (*s*)
H–C(16)	8.61 (*s*)	142.4 (*d*)	8.56 (*s*)	142.0 (*d*)
CH_2(17)	2.95 (*dd*, *J* = 13.74, 3.24),	34.6 (*t*)	2.87–2.88 (*m*)[a]),	36.0 (*t*)
	2.85–2.87 (*m*)[a])		2.81 (*dd*, *J* = 16.5, 9.9)	
H–C(18)	4.06–4.11 (*m*)	67.6 (*d*)	3.93–3.97 (*m*)	65.3 (*d*)
O*H*–C(18)	5.22 (*s*)		5.07 (*s*)	
CH_2(19)	2.20–2.24 (*m*),	39.3 (*t*)	1.62–1.66 (*m*),	42.5 (*t*)
	1.92–1.95 (*m*)		1.46–1.50 (*m*)	
H–C(20)	5.75–5.80 (*m*)	129.8 (*d*)	3.75 (*ddd*, *J* = 4.3, 3.3, 2.2)	79.7 (*d*)
H–C(21)	6.02 (*dd*, *J* = 15.6, 11.0)	134.0 (*d*)	5.32 (*dd*, *J* = 15.3, 9.9)	134 (*d*)
H–C(22)	6.34 (*dd*, *J* = 15.0, 10,5)	133.8 (*d*)	6.33 (*dd*, *J* = 15.3, 10.9)	132.8 (*d*)
H–C(23)	5.48 (*dd*, *J* = 15.1, 7.3)	129.4 (*d*)	5.98 (*dd*, *J* = 15.3, 10.9)	128.2 (*d*)
H–C(24)	3.93–3.95 (*m*)	78.3 (*d*)	5.83 (*ddd*, *J* = 15.3, 4.3, 3.3)	131.3 (*d*)
CH_2(25)	3.61–3.63, 3.13–3.16 (2*m*)	43.7 (*t*)	3.91–3.93, 3.80–3.83 (2*m*)	40.7 (*t*)
H–N(26)	7.80–7.82 (*m*)		8.33 (*dd*, *J* = 5.46, 5.52)	
C(27)		163.2 (*s*)		162.7 (*s*)
Me(28)	1.38 (*d*, *J* = 5.9)	20.8 (*d*)	1.38 (*d*, *J* = 5.4)	20.8 (*d*)
Me(29)	3.22 (*s*)	56.3 (*d*)	3.11 (*s*)	55.6 (*d*)

[a]) Overlapped.

linkage of C(11) and N(10) to generate an amide bond, according to the chemical shift of C(11) (δ 162.2), and to establish the molecular formula of griseoviridin B[2]) (**1**).

Griseoviridin C (**2**) was isolated as colorless amorphous powder. Its molecular formula was determined to be $C_{23}H_{29}N_3O_7S$ on the basis of HR-ESI-MS combined with ^{1}H- and ^{13}C-NMR data. The ^{1}H- and ^{13}C-NMR data of **2** (*Table*) were similar to those of griseoviridin (**3**), which suggested the presence of the same C-atom skeleton. The major difference was that OH–C(20) (δ 4.71) of **3** was replaced by a MeO group (δ 3.11 and 55.6 Me(29)) in **2**, and accordingly the chemical shift of C(20) in **2** was moved to lower field (δ 79.7) than in **3** (δ 69.7). The replacement was also confirmed by the HMBC cross-peak Me(29)O (δ 3.11)/C(20) (δ 79.7) (*Fig. 2*). The (21*E*,22*E*) config-

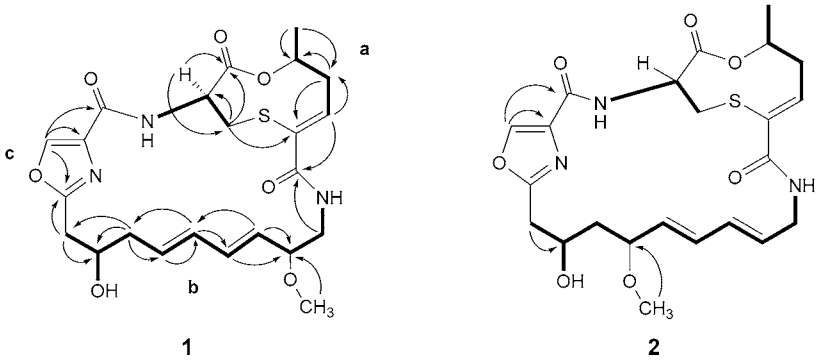

Fig. 2. *$^1H,^1H$-COSY* (—) *and key HMBC* (H → C) *features of compounds* **1** *and* **2**

urations of the C=C bonds were assigned on the basis of their coupling constants J(H–C(21), H–C(22)) (= 15.3 Hz) and J(H–C(23), H–C(24)) (= 15.3 Hz) which were the same as in **3**. Accordingly, compound **2** was named griseoviridin C[2]).

The absolute configurations at C(5), C(8), and C(18) in both **1** and **2** were deduced as (*R*), (*S*), and (*R*), respectively, the same as those in the known compound griseoviridin (**3**), based on their similar $[\alpha]_D$ values and CD spectra and together with the chemical shifts [7][8]. The absolute configuration at C(20) of **2** was assigned as (*S*) like that in **3** on the basis of a shared biosynthesis, while the configuration at C(24) of **1** remains undetermined. Compound **2** was suspected to be an artifact of **3**, produced during the process of isolation which involved MeOH. But no products were detected on stirring of **3** in MeOH (100% MeOH or MeOH containing 0.1% CF_3COOH) at room temperature with and without silica gel for 7 d.

Compounds **1**–**3** were tested for cytotoxic and antibiotic activities. None was active against the P388 and HL-60 cell lines or against *Enterobacter aerogenes*, *Staphylococcus aureus*, *Bacillus proteus*, *Micrococcus tetragenus*, *Bacillus subtilis*, and *Candida albicans* strains.

The streptogramin antibiotics are a family of natural products that have been isolated from soil *Streptomyces* [9], which are divided into two structurally different groups: 24-membered macrolactones (group A) such as the griseoviridins and madumycin [9][10], and cyclic hexadepsipetides (group B) such as etamycin [11]. Group A and group B exhibited synergetic activities against *Gram*-positive organisms when associated together. Further research led to the discovery of *Synercid* (*Aventis*), composed of two semisynthetic compounds belonging to streptogramin groups A and B, respectively, and approved by the FDA for the treatment of vancomycin-resistant bacteria in 2000 [12].

Griseoviridin (**3**), isolated from *Streptomyces griseus* in 1954 [9], was the first representative of the group A streptogramins, and is characterized by an unusual nine-membered thio-lactone encclosed in an 23-membered macrolactam. Attracted by the interesting structure, several synthetic studies of griseoviridin have been reported [13–16]. To the best of our knowledge, prior to our current study, griseoviridin (**3**) was the only known compound of a macrolactam type containing a nine-membered thio-lactone core.

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2; 200–300 mesh, 10–40 μm; *Qingdao Marine Chemical Inc.*, P. R. China), *Sephadex LH-20* (*GE Healthcare*, Sweden), and TLC: SiO_2 *GF254* (10–40 μm; *Qingdao Marine Chemical Inc.*, P. R. China). Optical rotations: *Jasco-P-1020* digital polarimeter. CD Spectra: *Jasco-J-810* spectropolarimeter; λ ([Θ]) in nm. UV Spectra: *Beckmen-DU-640* spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Nicolet-Nexus-470* spectrophotometer; KBr discs; $\tilde{\nu}$ in cm^{-1}. ^1H- and ^{13}C-NMR, DEPT, and 2D-NMR Spectra: *Jeol-Eclips-600* spectrometer; Me_4Si as internal standard, chemical shifts δ in ppm, and J in Hz. ESI-MS: *Micromass Q-TOF Ultima-Global-GAAo76* LC mass spectrometer.

Isolation and Identification of Streptomyces *sp. IMBJ01*. The isolate IMBJ01 was obtained from a mountain-soil sample collected at a hight above sea level of 3500 m in the Wolong nature conservation area, Sichuan Province, P. R. China, in 2004. Genomic DNA was isolated from cultured cells, and the nearly complete 16S rRNA gene was amplified by using the eubacterial primers 27f and 1492r [17][18]. 16S rRNA Gene sequence analysis was then conducted by means of the BLAST-network services provided by the NCBI (National Center for Biotechnology Information). The comparison of the 16S rDNA sequence of the strain IMBJ01 (GenBank acc. no. EU215800) with sequences in the NCBI nucleotide database revealed that the strain was affiliated with the genus *Streptomyces*.

Detection of Polyketide Synthase Gene Fragments. The PCR approach for the detection of a type-I polyketide synthase (PKS I) and nonribosomal peptide synthetases (NRPS) was performed [19]. Comparison of PKS gene fragments (1399 nucleotides, GenBank acc. no. HQ625046) and NRPS gene fragments (717 nucleotides, GenBank acc. no. HQ625045) of the *Streptomyces* sp. IMBJ01 was accomplished with sequences in the NCBI nucleotide database available online using the blastx. One of the most closely related NRPS sequences was the putative NRPS gene of *Streptomyces griseus* subsp. griseus NBRC 13350 (91% similarity; GenBank acc. no. YP_001822165). The most closely related PKS sequences was the putative PKS type-I gene of *Streptomyces ambofaciens* ATCC 23877 (there is only 70% similarity; GenBank acc. no. CAJ88187).

Fermentation and Extraction of the Streptomyces *Strain*. A seed culture of IMBJ01 was inoculated into 500 ml *Erlenmeyer* flasks containing 100 ml of medium (glucose (20.0 g), yeast extract (10.0 g), soluble starch (10.0 g), beef extract (3.0 g), cornsteep liquor (3.0 g), KH_2PO_4 (0.5 g), $MgSO_4 \cdot 7\,H_2O$ (0.5 g), and $CaCO_3$ (2.0 g), dissolved in 1 l of seawater, pH 7.2). The flasks with a total of 30 l of liquid medium were incubated on a rotatory shaker at 165 rpm at 28°. After 7 d of cultivation, *Amberlite-XAD-7* resin (30 g/l) was added to adsorb extracellular secondary metabolites for 4 h before finishing cultivation. The resin and cell mass were then collected by filtration through cheesecloth and washed with deionized water to remove salts. The resin, cell mass, and cheesecloth were then extracted with 2 × 2 l of acetone, and the solvent was evaporated: 15.0 g of crude extract.

Purification. The crude extract (15.0 g) was fractionated by CC (SiO_2, step gradient 0 → 50% MeOH in $CHCl_3$). The 20% fraction was purified by CC (*Sephadex LH-20*, $CHCl_3$/MeOH 1:1) followed by recrystallization in MeOH: **3** (40 mg). The 30% fraction was subjected to reversed-phase HPLC (75% aq. MeOH): **1** (5 mg) and **2** (3 mg).

Griseoviridin B (=*(1S,9R,11E,13E,19Z,22R)-9-Hydroxy-15-methoxy-22-methyl-6,23-dioxa-26-thia-2,17,27-triazatricyclo[17.5.2.1^{4,7}]heptacosa-4,7(27),11,13,19-pentaene-3,18,24-trione*; **1**): Colorless solid. $[\alpha]_D^{24} = -190.5$ ($c = 0.2$, MeOH). CD (DMSO): 254 (+2.21), 282 (−0.95). UV(MeOH): 208.3 (3.9). ^1H- and ^{13}C-NMR: *Table*. ESI-MS: 514 ([M + Na]$^+$). HR-ESI-MS: 492.1812 ([M + H]$^+$, $C_{23}H_{30}N_3O_7S^+$; calc. 492.1804).

Griseoviridin C (=*(1S,9R,11S,12E,14E,19Z,22R)-9-Hydroxy-15-methoxy-22-methyl-6,23-dioxa-26-thia-2,17,27-triazatricyclo[17.5.2.1^{4,7}]heptacosa-4,7(27),12,14,19-pentaene-3,18,24-trione*; **2**). Colorless

solid. $[\alpha]_D^{24} = -192.6$ ($c = 0.2$, MeOH). CD (DMSO): 253 (+2.40), 288 (−2.46). UV (MeOH): 213.0 (4.0). ^{1}H- and ^{13}C-NMR: *Table.* ESI-MS: 514 ($[M + Na]^+$). HR-ESI-MS: 514.1636 ($[M + Na]^+$, $C_{23}H_{29}N_3NaO_7S^+$; calc. 514.1624).

Biological Assay. In the SRB (sulforhodamine B) assay [20], 200 µl of the cell suspensions were plated in 96-cell plates at a density of 2×10^5 cell/ml. Then, 2 µl of the test solns. (in MeOH) were added to each well, and the culture was further incubated for 24 h. The cells were fixed with 12% CCl_3COOH, and the cell layer was strained with 0.4% SRB. The absorbance of the SRB soln. was measured at 515 nm. Dose/response curves were generated, and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were caculated from the linear portion of log dose/response curves.

The antimicrobial activities were evaluated by an agar dilution method [21].

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