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NOTE

Isolation and characterization of spliceostatin B, a new analogue of FR901464, from *Pseudomonas* sp. No. 2663

Xiangyang Liu[1,2], Sreya Biswas[1], Gong-Li Tang[3] and Yi-Qiang Cheng[1,2]

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FR901464 (Figure 1), a prototype pre-mRNA splicing inhibitor isolated from the culture broth of *Pseudomonas* sp. No. 2663, is a potent but unstable cytotoxic compound.[1–4] Spliceostatin A (Figure 1) is a methylated and more stable derivative of FR901464, shown to bind noncovalently to the splicing factor 3b subcomplex of the U2 small nuclear ribonucleoprotein particle of mammalian spliceosome, thus inhibiting pre-mRNA splicing and causing pre-mRNA leakage to the cytoplasm.[5,6] Recently we discovered thailanstatins A, B and C as three new and significantly more stable natural analogs of FR901464 from the culture broth of *Burkholderia thailandensis* MSMB43.[7] For evaluations of the stability, pre-mRNA splicing inhibitory activity and cytotoxicity of thailanstatins, we used FR901464 as a reference compound, which was freshly purified from the *Pseudomonas* sp. No. 2663 fermentation. During this purification optimization process, another analogous compound of FR901464, named spliceostatin B (1), was discovered. Herein we report the fermentation, isolation, chemical characterization and cytotoxicity of this new natural product.

The FR901464-producing strain *Pseudomonas* sp. No. 2663[2] was purchased from the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan. It was routinely activated from a glycerol stock on Luria-Bertani (LB) agar at 30 °C for 2 days as a starting plate. Several colonies from the plate were inoculated into a flask containing 250 ml of LB medium and incubated at 30 °C for 24 h on a rotary shaker (150 r.p.m.) to prepare a seed culture. For fermentation, the seed culture was inoculated at 2% (*v*/*v*) to each of the two fermentors (BioFlo IV, 20 l total volume, New Brunswick Scientific, Enfield, CT, USA); each contains 12 l of production medium consisting of soluble starch 1%, glycerin 1%, defatted soybean meal 1%, glucose 0.5%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.006% and antifoam-204 0.01% (pH 7.0), slightly modified from the literature.[2] The pH of bacterial culture was automatically maintained by the fermentor with 1 N HCl or 1 N NaOH, and the fermentation was proceeded at 25 °C for 48 h with an agitation of 200 r.p.m. and an air flow-rate of 4 l min^{-1}. The repeated fermentation with two fermentors led to an accumulation of 72 l of fermentation broth.

Fermentation broth was extracted three times with ethyl acetate (3:2, *v*/*v*), and the extracts were pooled and concentrated to dryness with a rotary evaporator at 35 °C. The resulting crude extract (25.2 g) was subjected to two steps of silica gel chromatography and one step of octadecyl-silica C_{18} chromatography on an YFLC AI-580 flash chromatography system (Yamazen, San Bruno, CA, USA), with elution monitored at UV 235 nm (see Supplementary Information: Scheme S1). In the first step, each 5 g of crude extract was suspended in 25 ml of ethyl acetate and mixed with 30 g of silica gel and packed into an injection column (3.0 × 12.5 cm, Catalog No. W830 silica gel, Yamazen), which was mounted atop a silica gel Universal Column (4.8 × 18.5 cm, 200 g silica gel, 40 µm, 60 Å). The column system was sequentially eluted by 1.1 l of each of the following solvents: hexane, hexane:ethyl acetate (3:1, *v*/*v*); hexane:ethyl acetate (1:1, *v*/*v*); ethyl acetate, ethyl acetate:acetone (1:1, *v*/*v*); and acetone, all at a flow rate of 50 ml min^{-1}. The ethyl acetate fraction containing 1 was concentrated to dryness at 35 °C. In the second step, the above resulting residue was suspended in 15 ml of acetone, mixed with 15 g of silica gel and packed into an injection column (2.0 × 6.5 cm, 14 g silica gel), which was mounted atop a silica gel Universal Column (2.6 × 12.0 cm, 40 g silica gel, 40 µm, 60 Å). The column system was sequentially eluted with chloroform, and 1, 2, 4 and 10% of acetone in chloroform, and finally acetone at 18 ml min^{-1}. In the third step, the 1% acetone fraction containing 1 was again concentrated and further fractionated on an octadecyl-silica C_{18} column system equipped with an injection column (2.0 × 6.5 cm, 14 g gel) and a

[1]Department of Biological Sciences, and Department of Chemistry and Biochemistry, University of Wisconsin–Milwaukee, Milwaukee, WI, USA; [2]UNT System College of Pharmacy, University of North Texas Health Science Center, Fort Worth, TX, USA and [3]State Key Laboratory of Bio-organic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai, China
Correspondence: Professor Dr Y-Q Cheng, Department of Biological Sciences, University of Wisconsin–Milwaukee, PO Box 413, Milwaukee, WI 53201, USA.
E-mail: ycheng@uwm.edu
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Universal Column (2.0 × 8.0 cm, 14 g gel, 50 μm, 120 Å). After loading the sample, the column system was first eluted with 5% acetonitrile for 3 min, then by a linear gradient of acetonitrile from 5 to 100% in 10 min, and finally by 100% acetonitrile for 14 min, with a flow rate of 20 ml min^{-1}. The fraction containing 1 was eluted at 15.7 min. In the final purification step, acetonitrile solution of 1 was purified with a Varian ProStar HPLC system (210 binary pump and 330 photodiode array detector, Varian, Palo Alto, CA, USA) equipped with an Agilent Prep-C18 column (21.2 × 250 mm, 10 μm, Agilent, Santa Clara, CA, USA) to give 28 mg of pure 1. As shown in Supplementary Figure S1, 1 was present in significant amount in the culture grown in fermentor but barely in that grown in shaking flask, suggesting that production of 1 is heavily culture condition-dependent.

The structure of 1 (Figure 1) was determined using a combination of UV, IR, HR-MS and NMR spectroscopic analyses (Supplementary Figures S2–S14) and was found to be a new compound after a SciFinder database search.

1 was obtained as a white powder and its physicochemical properties are summarized in Table 1. The IR spectra indicate the presence of hydroxyl (3355 cm^{-1}), carboxyl (2978 and 2938 cm^{-1}), carbonyl (1735 cm^{-1}), amide carbonyl (1667 cm^{-1}) and conjugated diene moieties (1635 cm^{-1}). HR ESI-MS revealed a quasi-molecular ion peak of *m/z* 504.2951 for $C_{28}H_{42}NO_7$ $[M+H]^+$ (calculated 504.2955) and suggested 503 as the MW and $C_{28}H_{41}NO_7$ as the molecular formula.

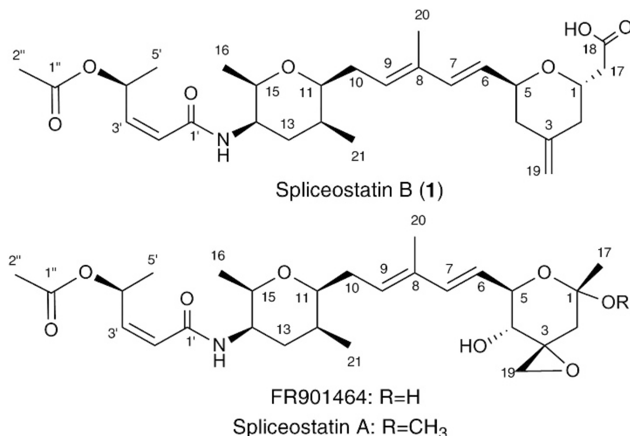

Figure 1 Structures of 1, FR901464 and spliceostatin A.

Table 1 Physicochemical properties of 1

Appearance	*White amorphous powder*
Elemental composition reported by HR ESI-MS $[M+H]^+$	$C_{28}H_{42}NO_7$
HR ESI-MS found	504.2951 $[M+H]^+$; 526.2767 $[M+Na]^+$; 542.2556 $[M+K]^+$
Calculated	504.2955 $[M+H]^+$
m.p. (°C)	77–83
UV $\lambda_{max}^{Acetonitrile}$ (nm; log ε)	236 (4.0)
IR (KBr) ν_{max} (cm^{-1})	3355, 2978, 2938, 1735, 1667, 1635, 1526, 1371, 1317, 1244, 1166, 1121, 1051
$[\alpha]_D^{25}$	+4.0 (CH_2Cl_2, c 0.1)
Solubility	DMSO, acetonitrile, acetone, water, $CHCl_3$, CH_2Cl_2

Abbreviation: DMSO, dimethyl sulfoxide.

The ^1H and ^{13}C NMR spectra in combination with ^1H–^{13}C HSQC NMR data (Table 2) of 1 exhibited signals of five methyl groups (CH_3-16, CH_3-20, CH_3-21, CH_3-5′ and CH_3-2″), six methylene groups (CH_2-2, CH_2-4, CH_2-10, CH_2-13, CH_2-17 and CH_2-19) and 12 methine group (CH-1, CH-5, CH-11, CH-12, CH-14, CH-15, CH-6, CH-7, CH-9, CH-2′, CH-3′ and CH-4′), as well as the two quaternary carbons (C-3 and C-8) and three carbonyl carbons (C-18, C-1′ and C-1″).

The key COSY and HMBC data were shown in Figure 2a. When the ^1H and ^{13}C NMR data were compared with those of FR901464,[3] the spectra showed overall similarities except for the absence of a methyl group and a hydroxyl group at the C1 and C4 positions, respectively. On the basis of molecular formula and ^{13}C chemical shifts, 1 has three more chemical shifts, where two (δ_C 38.7 p.p.m. and δ_C 174.7 p.p.m.) are attached to C1 atom, which was confirmed by the observation of correlations between 1-H and 17-H, and 1-H and 2-H (Table 2) in COSY spectrum. Another methylene group (δ_C 39.4 p.p.m. and δ_H 2.48 p.p.m., and δ_H 2.23 p.p.m.) indicated that this CH_2 is free of hydroxyl groups. Comparing with the methylene group (δ_C 48.0 p.p.m.) at C18 position in FR901464, the highly shifted methylene group (δ_C 111.4 p.p.m.) at C19 position indicated that it was a terminal methylene group with a double bond. According to the molecular formula and the already known NH group, there should be one hydroxyl group in the structure, which was not observable in the ^1H NMR spectrum. The presence of such a hydroxyl group in the terminal carboxyl moiety of 1 was confirmed by a positive bromocresol green visualization reaction (yellow spot on dark blue background) on TLC[8] (Supplementary Figure S15).

The relative configuration of 1 was determined to be the same as that of FR901464. The geometry of C2′ and C6 double bond was proposed as *cis* (*Z*) based on the vicinal coupling constants $J_{H2'–H3'} = 11.6$ Hz and $J_{H6–H7} = 15.8$ Hz (Table 2). The *trans* (*E*) configuration of the double bonds at C8–C9 was indicated by the chemical shift of C20 at 12.6 p.p.m. (<20 p.p.m.),[9] and the observations of the NOE correlations between 7-H and 9-H and between 6-H and 20-H (Table 2). Seven of the eight chiral carbons of 1 were assigned according to the related NOE correlations, which were divided into three parts. The configurations of the first part C1 ∼ C5 on the first tetrahydropyran ring were shown in Figure 2b. The cross peaks between 5-H and 17-H_2 in NOE spectrum suggested a 1, 3-diaxial relationship. In the relative stereostructure of the atoms C11 ∼ C15 in the second tetrahydropyran ring (Figure 2b), the observation of signals between 15-H and one of the 13-H_2, and between 15-H and 11-H pointed out the 1, 3-diaxial orientation. The observation of strong NOE correlations between 14-NH and 21-H_3 suggested a 1, 3-diaxial interaction between 14-NH and 12-CH_3.

The cytotoxicity of 1 was evaluated in three human cancer cell lines, HCT-116, MDA-MB-235 and H232A, with the MTT method.[10] 1 exhibited cytotoxic activity against these three cell lines with GI_{50} (the half-maximum growth inhibitory concentration) values of 1,152 ± 0.16 nM, 916.6 ± 1.20 nM and 893.6 ± 1.64 nM, respectively. FR901464 was reported to have GI_{50} values of 1.8 nM, 1.3 nM, 0.6 nM, 1.0 nM and 3.3 nM against MCF-7, A549, HCT-116, SW480 and P388 cell lines, respectively.[1] Apart from potentially differential cytotoxicities against different cell lines, 1 appeared to be a much weaker cytotoxic compound than FR901464.

Table 2 ^{13}C and ^{1}H NMR spectroscopic data for 1 ($CDCl_3$, 298 K)

Position	δ_C^{a} *(p.p.m.)*	*HSQC*	δ_H^{b} *(mult, J Hz)*	*COSY*	*HMBC*	*NOE*
1	69.0	CH	4.35 (qui, 5.7)	2, 17	18	2, 4, 6, 17
2	39.1	CH_2	2.11 (q, 6.6)		3, 4, 17, 19	1, 19
3	140.6	C				
4	39.4	CH_2	2.48 (overlap), 2.23 (overlap)		3, 5, 6, 19	5, 19
5	73.2	CH	4.42 (q, 5.2)	4, 6	1, 3, 6, 7	4, 6, 7, 17
6	125.8	CH	5.62 (dd, 15.8, 6.1)	5, 7	4, 5, 8	1, 4, 5, 7, 20
7	137.1	CH	6.26 (d, 15.84)	6	5, 8, 9, 20	5, 6, 9, 10, 20
8	134.8	C				
9	128.5	CH	5.44 (t, 6.9)	10	7, 10, 11, 21	7, 10, 11, 12, 20
10	31.8	CH_2	2.39 (overlap); 2.24 (overlap)		8, 9, 11, 12	9, 11
11	81.2	CH	3.57 (qt, 7.1, 5.0)	10	15, 21	9, 10, 12, 13
12	28.9	CH	1.79 (overlap)	21		10, 11, 13, 21
13	35.8	CH_2	1.99 (s); 1.94 (overlap)		11, 14, 15	11, 14, 15, 21
14	47.0	CH	3.95 (d, 7.1)	13, NH		NH, 13, 16
15	76.4	CH	3.72 (qd, 6.3, 2.0)	16	16	13, 16, 11, 14
16	17.7	CH_3	1.18 (d, 6.4)	15	14, 15	NH, 14, 15
17	38.7	CH_2	2.71 (dd, 15.1, 8.1); 2.50 (overlap)		1, 2, 18	1, 5
18	174.7	C=O				
19	111.4	CH_2	4.85 (d, 10.4)		2	2, 4
20	12.6	CH_3	1.74 (s)		7, 8, 9	6, 10
21	14.8	CH_3	1.02 (d, 7.3)	12	11, 12, 13	NH, 10, 12, 13
1′	165.1	C=O				
2′	122.5	CH	5.80 (d, 11.6)	3′	1′, 3′	NH
3′	143.8	CH	5.91 (dd, 11.5, 5.8)	2′, 4′	1′, 2′	5′
4′	69.1	CH	6.31 (qui, 6.62)	3′, 5′	1″	2′, 3′, 5′, 21
5′	20.0	CH_3	1.40 (d, 6.5)	4′	3′	3′, 4′
1″	170.4	C=O				
2″	21.3	CH_3	2.06 (s)	9, 11	1″	
		NH^c	6.46 (d, 8.80)	14	14, 15, 1′	2′, 14, 16, 21
		OH^d				

^{a}The ^{13}C NMR was measured at 125 MHz.
^{b}The ^{1}H NMR was measured at 500 MHz.
^{c}The 15N-HSQC was measured at 50 MHz.
^{d}The hydroxyl group was not observed in NMR spectra.

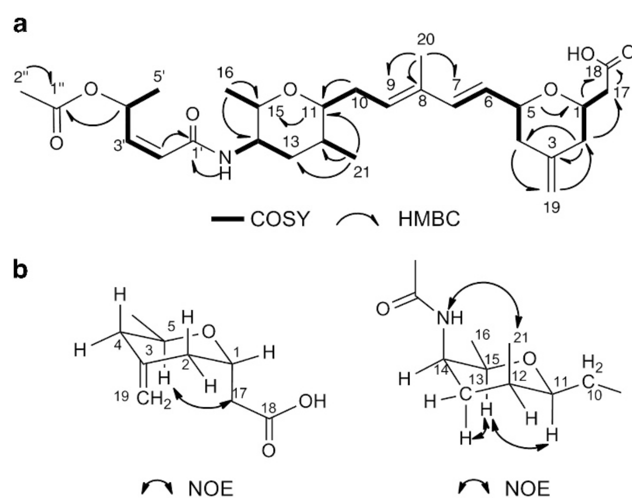

Figure 2 Key COSY and HMBC correlations of 1 (**a**), and key NOE correlations of 1 fragments (**b**).

1 differs structurally from FR901464 at four points (Figure 1): substitution of an epoxide group at C3 position with a terminal methylene moiety, presence of a carboxyl moiety at C17 position, and absence of two hydroxyl groups at C1 and C4 positions, respectively. It was reported that a loss of C4 hydrogen bond donor decreases the cytotoxicity of meayamycin B (a synthetic FR901464 analog) about fourfold.[11] The importance of the C3 epoxide moiety for bioactivity has been documented in two independent studies. First, substitution of the C3 epoxide moiety in FR901464 by a terminal methylene group decreases the cytotoxicity about fivefold.[5] Second, a non-epoxide analog of meayamycin that still contains the oxygen atom at the C3 position completely lost the activity, with IC_{50} values changed from 0.02 nM to >10 000 nM.[11] Although there is no concrete evidence about the influence of the carboxyl moiety at C17 position on the bioactivity of 1, our studies on thailanstatins suggested that this moiety is critical to compound stability under weak alkaline conditions.[7] Collectively, the absence of an epoxide moiety at C3 position and a hydroxyl group at C4 position of 1 most likely contributes to its weak cytotoxicity, which, in turn, supports the importance of those functionalities in FR901464 class of natural or synthetic compounds.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)