The Journal of Antibiotics (2018) 71:667–671
https://doi.org/10.1038/s41429-018-0052-0

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BRIEF COMMUNICATION

Two new spliceostatin analogs from the strain *Pseudomonas* sp. HS-NF-1408

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Received: 20 December 2017 / Revised: 3 March 2018 / Accepted: 7 March 2018 / Published online: 17 April 2018

Abstract

Two new spliceostatin derivatives, designed as spliceostatin H (**1**) and spliceostatin I (**2**), and one known compound FR901464 (**3**), were isolated from the strain *Pseudomonas* sp. HS-NF-1408. Their structures were determined by the comprehensive spectroscopic data, including 1D, 2D NMR, MS spectral analysis and comparison with data from the literature. Compound **1** exhibited potent cytotoxicity activity against A549 and HepG2 with IC_{50} values of 3.57 and 16.72 μg/ml, respectively.

The spliceostatin class of natural products, which has two highly functionalized tetrahydropyran rings linked by a diene chain, was reported to be potent cytotoxic agents via inhibition of the spliceosome, a key–protein complex in the biosynthesis of mature mRNA [1–3]. Due to the interesting architecture and biological activity, many natural and synthetic spliceostatin compounds have been described with the aim of discovering more potent drug leads [4–8]. In our ongoing effort to exploit novel bioactive compounds from microbial sources, the strain *Pseudomonas* sp. HS-NF-1408 obtained from a soil sample was selected for further study because of its cytotoxic activity against A549 and HepG2. As a result, two new members of the spliceostatins group, designed as spliceostatin H (**1**), spliceostatin I (**2**), and the known compound FR901464 (**3**) (Fig. 1), were isolated from the strain *Pseudomonas* sp. HS-NF-1408. In this paper, the details of fermentation, isolation, structure determination, and biological activity of two new derivatives are described.

The producing strain *Pseudomonas* sp. HS-NF-1408 was isolated from a soil sample collected from the Qingshan lake, located in Lin'an, Zhejiang province, China. The strain was identified as the genus *Pseudomonas* because its 16S rRNA sequence (accession no: MG386199 in the GenBank) showed a high similarity of 99% with that of the *Pseudomonas chlororaphis* strain XF10 (accession no: MF121986.1 in the GenBank) and it was deposited in the Pharmaceutical Research Culture Collection, Zhejiang Hisun Group Co. Ltd. with accession no: HS-NF-1408.

This strain was grown on an agar slant containing 30 g beef extract, 5 g peptone, and 15 g agar in 1.0 l of water, pH 7.0–7.2 and incubated for 6–7 days at 28 °C. The strain of stock culture was inoculated into 100 1.0 l Erlenmeyer flasks containing 36% volume of the seed medium at 30 °C for 24 h, shaken at 220 r.p.m. The seed medium consisted of polypeptone 1%, yeast extract 0.5%, and NaCl 0.5% in 1.0 l water, pH 7.0–7.2. All the media were sterilized at 121 °C for 20 min. Then, the entire culture was transferred into a 500 l fermentor containing 300 l of production medium consisting of defatted soybean meal 1%, corn steep liquor 0.5%, $(NH_4)_2SO_4$ 0.2%, soluble starch 1%, glycerin 1%, glucose 0.5%, $MgSO_4{\cdot}7H_2O$ 0.0006%, $CaCO_3$ 0.2%, adecanol LG-109 0.05%, and silicon KM-70 0.05% at pH 7.0–7.2. The fermentation was carried out at 28 °C for 6 days stirred at 220 r.p.m. with an aeration rate of 18,000 l of air per hour.

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Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0052-0) contains supplementary material, which is available to authorized users.

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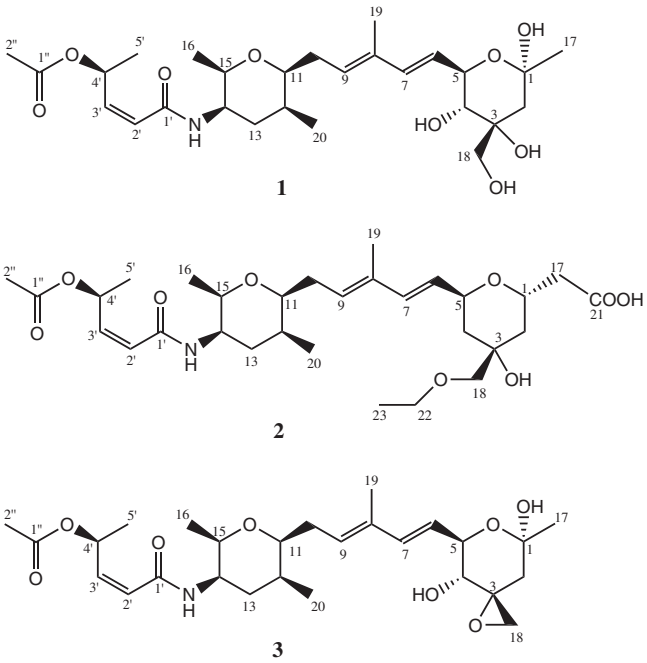

Fig. 1 Structures of compounds 1, 2 and 3

The final 300 l of broth from 500 l fermentor was filtered to separate mycelial cake and supernatant. The mycelial cake was extracted with MeOH (50 l) and the supernatant was subjected to a Diaion HP-20 resin (Mitsubushi Chemical, Tokyo, Japan) column eluting with 95% EtOH (50 l). The MeOH extract and the EtOH eluents were evaporated under reduced pressure to yield the crude extract. The crude extract was chromatographed on a silica gel (Qingdao Haiyang Chemical Group, Qingdao, China; 100–200 mesh) column and successively eluted with a stepwise gradient of $CHCl_3$/MeOH (100:0-50:50, v-v) to give five fractions (Fr.1-Fr.5) based on the TLC profiles. Fr.2 was subjected to another silica gel column eluted with n-hexane/acetone (95:5-50:50, v:v) to give three fractions (Fr.2-1 to Fr.2-3). Fr.2-1 was further isolated by preparative HPLC (Shimadzu LC-8A, Shimadzu-C18, 5 μm, $250 \times 20\ mm^2$ inner diameter; 20 ml/min; 220/254 nm; Shimadzu, Kyoto, Japan) eluting with a stepwise gradient MeOH/H_2O (50–100%, v/v, 40 min) to obtain five subfractions (Fr.2-1-1 to Fr.2-1-5) based on the retention times. Then, Fr.2-1-1 (t_R 10.6 min) was purified by semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5um, $9.4 \times 250\ mm^2$ inner diameter; 1.5 ml/min; 254 nm; Agilent, Palo Alto, CA, USA) eluting with CH_3CN:H_2O (50:50, v:v) to obtain spliceostatin H (**1**) (t_R 8.4 min, 30 mg). Fr.2-1-2 (t_R 12.1 min) was separated by semi-preparative HPLC eluting with CH_3CN:H_2O (40:60, v:v) to yield FR901464 (**3**) (t_R 12.1 min, 63 mg). Fr.2-1-4 (t_R 16.7 min) was isolated by semi-preparative HPLC eluting with CH_3CN:H_2O (50:50, v:v) to give spliceostatin I (**2**) (t_R 16.9 min,6.3 mg). ^1H and ^{13}C NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer (Bruker, Rheinstetten, Germany). The ESIMS and HRESIMS spectra were taken on a Q-TOF Micro LC–MS–MS mass spectrometer (Waters Co, Milford, MA, USA).

Compound **1** was isolated as colorless oil with [α]-60 (*c* 0.02, EtOH) and UV (EtOH) λ_{max} nm (log ε): 236 (4.54). Its molecular formula was determined to be $C_{27}H_{43}NO_9$ by HRESIMS at *m/z* 548.2825 $[M + Na]^+$ (calcd as 548.2830 for $C_{27}H_{43}NO_9Na$) and NMR data (Table 1). The IR spectrum showed absorption bands for hydroxyl (3369 cm^{-1}) and carbonyl (1732 cm^{-1}) functionalities. The ^1H NMR spectrum of **1** (Table 1) displayed three doublet methyls at δ_H 1.04 ($J = 7.3$ Hz), 1.16 ($J = 6.4$ Hz) and 1.37 ($J = 6.5$ Hz), a singlet methyl at δ_H 1.39, an olefinic methyl at δ_H 1.81, an acetyl methyl at δ_H 2.04, seven methine proton signals from δ_H 3.30 to δ_H 4.39 and six downfield proton signals from δ_H 5.50 to δ_H 6.42. The ^{13}C NMR spectrum (Table 1) exhibited 27 resonances ascribed to six methyls at δ_C 12.7, 14.9, 18.0, 20.3, 21.1, 29.1, four methylenes (one oxygenated) at δ_C 33.1, 36.9, 41.7, 66.8, seven sp^3 methines (five oxygenated) at δ_C 30.6, 48.6, 70.0, 71.7, 71.8, 76.7, 82.2, one hemiketal carbon at δ_C 97.8, one oxygen-bearing quaternary sp^3 carbon at δ_C 74.8, five sp^2 methines at δ_C 123.4, 126.3, 129.8, 138.7, 144.8, one sp^2 quaternary carbon at δ_C 135.9 and two carbonyls at δ_C 167.5, 172.2. The ^1H and ^{13}C NMR data of **1** closely resembled those of **NP6**[9] except for the presence of a methylene carbon at δ_H 1.83, 1.90/δ_C 41.7 in **1** instead of the corresponding oxygenated methine (C-2) in **NP6**. The HMBC correlations (Fig. 2) from δ_H1.39 (H_3-17) and δ_H 3.39, 3.57 (H_2-18) to this carbon supported this assignment.

Table 1 ^{1}H and ^{13}C NMR data of compounds **1** (in CD_3OD), **2** and **3** (both in $CDCl_3$)

Position	δ_H (*ppm*, *J* in Hz)			δ_C (*ppm*)		
	1	2	3	1	2	3
1		4.42 m		97.8 (s)	70.2 (d)	96.4 (s)
2	1.83 d (14.2) 1.90 d (14.2)	1.85 dd (12.1, 5.9) 1.54 brd (12.1)	1.71 d (14.1) 2.37 d (14.1)	41.7 (t)	36.9 (t)	41.5 (t)
3				74.8 (s)	76.0 (s)	57.7 (s)
4	3.33 m	1.62 m	3.64 d (9.9)	71.7 (d)	40.2 (t)	67.8 (d)
5	4.36 dd (9.2,6.8)	4.53 m	4.35 m	71.8 (d)	68.9 (d)	73.5 (d)
6	5.69 dd (15.3, 6.8)	5.53 m	5.70 dd (15.7, 7.0)	126.3 (d)	128.5 (d)	123.8 (d)
7	6.40 d (15.3)	6.29 d (15.8)	6.43 d (15.7)	138.7 (d)	136.8 (q)	138.5 (d)
8				135.9 (s)	135.7 (s)	134.6 (s)
9	5.54 t (6.7)	5.51 m	5.52 t (6.8)	129.8 (d)	129.9 (d)	129.2 (d)
10	2.28 m 2.41 m	2.24 m 2.38 m	2.27 m 2.40 m	33.1 (t)	33.1 (t)	32.0 (t)
11	3.59 m	3.56 m	3.53 m	82.2 (d)	82.2 (d)	80.9 (d)
12	1.75 m	1.72 m	1.80 m	30.6 (d)	30.6 (d)	29.0 (d)
13	1.96 m	1.94 m	1.96 m	36.9 (t)	36.9 (t)	35.9 (t)
14	3.77 m	3.74 m	3.95 brd (7.8)	48.6 (d)	48.6 (d)	47.1 (d)
15	3.72 m	3.70 m	3.67 m	76.7 (d)	76.7 (d)	76.0 (d)
16	1.16 d (6.4)	1.14 d (16.4)	1.16 d (7.8)	18.0 (q)	18.1 (q)	17.8 (q)
17	1.39 s	3.10 dd (15.3,9.4) 2.63 dd (15.3, 5.1)	1.51 s	29.1 (q)	40.0 (t)	29.0 (q)
18	3.57 d (10.8) 3.39 d (10.8)	3.23 d (9.5) 3.26 d (9.5)	2.60 d (4.4) 3.13 d (4.4)	66.8 (t)	79.7(t)	47.8 (t)
19	1.81 brs	1.75 brs	1.80 brs	12.7 (q)	12.7 (q)	12.6 (q)
20	1.04 d (7.3)	1.02 d (7.3)	1.03 d (7.3)	14.9 (q)	14.9 (q)	15.0 (q)
21					175.8 (s)	
22		3.50 q (7.0)			68.0 (t)	
23		1.19 t (7.0)			15.4 (q)	
1′				167.5 (s)	167.5 (s)	164.9 (s)
2′	6.01 d (11.8)	5.98 d (11.8)	5.73 d (11.5)	123.4 (d)	123.4 (d)	122.5 (d)
3′	5.96 dd (11.8, 8.0)	5.95 dd (11.8, 7.3)	5.91 dd (11.5, 7.9)	144.8 (d)	144.8 (d)	143.6 (d)
4′	6.38 m	6.36 m	6.28 m	70.0 (d)	70.0 (d)	68.9 (d)
5′	1.37 d (6.5)	1.34 d (6.4)	1.40 d (6.5)	20.3 (q)	20.3 (q)	20.0 (q)
1″				172.2 (s)	172.2 (s)	170.4 (s)
2″	2.04 s	2.01 s	2.06 s	21.1 (q)	21.1 (q)	21.2 (q)

Thus, the gross structure of **1** was established to be a 2-dehydroxy derivative of **NP6**, as shown in Fig. 1. The ^{1}H–^{1}H COSY correlations (Fig. 2) of H-4/H-5/H-6/H-7, H-9/H_2-10/H-11/H-12/H_2-13/H-14/H-15/H_3-16, H-12/H_3-20, H-2′/H-3′/H-4′/H_3-5′ and the observed HMBC cross-peaks from H_3-17 to C-1, from H_2-18 to C-3 and C-4, from H_3-19 to C-7, C-8, and C-9, from H_3-2″ and H-4′ to C-1″ further confirmed the above structural assignment. In **1**, the coupling constants of H-7 (δ_H6.40, d, $J = 15.3$ Hz) and H-2′ (δ_H6.01, d, $J = 11.8$ Hz) unambiguously revealed the double bond geometry at C-6 and C-2′ to be *trans* and *cis*, respectively. In the NOESY spectrum, the NOE correlations (Fig. 2) between H-7 and H-9 indicated the double bond at C-8 was *trans*. In addition, the relative configuration for H-4 and H-5 was *anti* based on the *J* value between H-4/H-5 (9.2 Hz). The relative stereochemistry of **1** was assigned to be the same as that of FR901464 [8]. The NOE cross peaks from H_2-10 to H_3-20 and from H-4 to H_2-18 further supported the assignment. From this finding, the structure of **1** was established and named as spliceostatin H. All NMR spectra data of compound 1 are present in Supplementary file (Figure S1–S10).

Compound **2** was isolated as colorless oil with[α]-50 (*c* 0.02, EtOH) and UV (EtOH) λ_{max} nm (log ε): 235 (4.42). Its molecular formula was established as $C_{30}H_{47}NO_9$ by HRESIMS at *m/z* 566.3320 $[M + H]^+$ (calcd as 566.3324

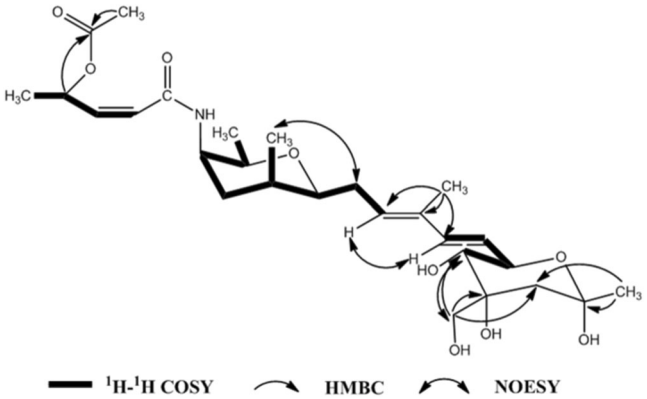

Fig. 2 Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of spliceostatin H (**2**)

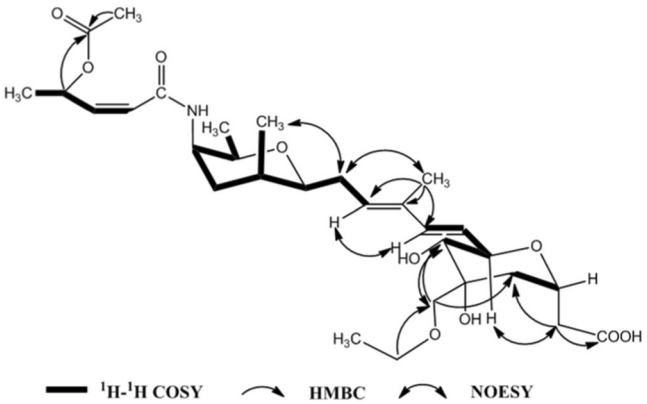

Fig. 3 Key ^{1}H-^{1}H COSY, HMBC, and NOESY correlations of spliceostatin I (**2**)

Table 2 Cytotoxic activity of compounds **1**, **2** and **3** against selected human tumor cell lines

	IC_{50} (μg/ml)			
Compound	1	2	3	doxorubicin
HepG2	16.72	101.93	0.50	0.58
A549	3.57	25.81	0.09	0.12

for$C_{30}H_{48}NO_9$) and NMR data (Table 1), requiring 8° of unsaturation. The IR absorption bands at 3431 cm^{-1}, 1724 cm^{-1} were characteristics of hydroxyl and carbonyl groups. The ^{1}H NMR data (Table 1) of **2** displayed three doublet methyls (δ_H 1.02, 1.14, 1.34), one triplet methyl (δ_H 1.19), an olefinic methyl (δ_H 1.75) and an acetyl methyl (δ_H 2.01). The ^{13}C NMR data (Table 1) showed resonances for 30 carbons, which included six methyls, three double bonds, seven sp^3 methylenes (including two oxygenated at δ_C 68.0 and 79.7), seven sp^3 methines (five oxygenated), three carbonyls and one oxygen-bearing quaternary carbon (δ_C 76.0). Comparision of the NMR spectroscopic data and analyses of the 2D NMR spectra revealed the gross structure of **2** to be closely related to **NP7** [9] except for the absence of an acetyl group and the presence of one ethyl group (δ_H/δ_C 1.19/15.4, and 3.50/68.0) in **2**. The ethyl group was connected with the C-18 methylene via an oxygen atom, as evident from the methylene protons (δ_H 3.50) of the ethyl moiety exhibiting HMBC correlations (Fig. 3) to C-18 (δ_C 79.7). The large coupling constant of H-7 (δ_H 6.29, $J = 15.8$ Hz) and the NOE correlation (Fig. 3) between H_3-19 and H_2-10 indicated that the geometry of the two double bond at C-6 and C-8 were all *trans*. The coupling constant of H-2′ (δ_H 5.98, d, $J = 11.8$ Hz) revealed the double bond at C-2′ was *cis*. The cross peaks between H-5 and H_2-17 in NOESY spectrum suggested a 1, 3-diaxial relationship. On the basis of biogenetic considerations, the stereochemistry of other chiral centers was assigned as that of **1** according to the NOE correlations from H_2-18 to H-4, from H_3-20 to H_2-10 and the concurrence with **1**. Therefore, the structure of **2** was established and named as spliceostatin I. The *O*-ethyl group suggested that this compound may be an artifact. So, it was analyzed by HPLC together with the methanol extract of the fermentation broth. The absence of **2** in the extract suggested the ethoxy group was elaborated in the course of the extraction or purification process. All NMR spectra data of compound **2** are present in Supplementary file (Figure S11–S20).

Compound **3** was identified as FR901464 by comparing the NMR spectral data with those reported in literature [8].

The cytotoxicity of compounds **1**, **2** and **3** were assayed for growth-inhibition activity in vitro against two human tumor cell lines, human hepatocellular liver carcinoma cells

HepG2 and human lung tumor cells A549 according to the CCK8 colorimetric method as reported in our previous papers [10, 11] using doxorubicin as positive control (Table 2). As a result, compound **1** exhibited cytotoxic activity against the two tumor cell lines and compound **2** only exhibited potent cytotoxic activity against A549 cell lines.

Acknowledgements This work was supported in part by grants from the National Natural Youth Science Foundation of China (No. 31701858), Youth innovation talent program for general undergraduate colleges of Heilongjiang Province (UNPYSCT-2017017), the Heilongjiang Postdoctoral Fund (LBH-Z17015) and the 'Young Talents' Project of Northeast Agricultural University (17QC14).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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