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Isolation and characterization of jadomycin L from *Streptomyces venezuelae* ISP5230 for solid tumor efficacy studies*

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Abstract: Precursor-directed biosynthesis offers opportunities to modify natural products and obtain structurally complex metabolites without the need for chemical synthesis. However, such opportunities are limited owing to the inherent substrate specificity of biosynthetic enzymes. The jadomycins are a family of natural products produced by the soil microbe *Streptomyces venezuelae* ISP5230. Their biosynthesis contains one step that is potentially non-enzymatic, namely, the condensation of a biosynthetic aldehyde and an amino acid that leads to a uniquely substituted oxazolone ring. Variation of amino acids in the culture media enables the production of a wide array of substituted oxazolones. These analogs have been shown to have a variety of biological activities against cancer cell lines and also against Gram-positive bacteria. Herein, we report the first isolation and characterization of jadomycin L and jadomycin L aglycone from 8 L of bacterial culture for solid tumor efficacy studies.

Keywords: jadomycin; *Streptomyces venezuelae*; solid tumor efficacy; biosynthesis; precursor-directed.

INTRODUCTION

Soil bacteria have long been a source of structural diversity with a significant number of mankind's potent medicines being derived from these sources [1]. The genome sequencing of *Streptomyces* species led to the discovery that these bacteria have the potential to produce significantly more secondary metabolites than at first recognized [2,3]. *Streptomyces venezuelae* ISP5230 has long been known as a producer of chloramphenicol, a clinically used antibiotic [4]. In addition, under the correct nutritional environment and with either ethanol, heat, or phage shock, the organism also produces the jadomycins, first identified through the isolation of jadomycin aglycone and jadomycin B [5,6]. Two striking structural motifs were evident, the rare sugar L-digitoxose, and the unique oxazolone ring. Initial attempts to investigate the biosynthesis of the oxazolone ring and the role of the amino acid in the culture media were confounded by the difficulty in isolating the secondary metabolites, and the variability associated with their induction [7]. Subsequently, significant insight into the secondary metabolite gene cluster for jadomycin B biosynthesis was accomplished by Vining and coworkers [8–10]. Insight into the oxazolone ring formation was provided by studies from Rohr and coworkers who were able to isolate four new jadomycins [11] and through mass spectrometry (MS) studies where two fragmentations were ob-

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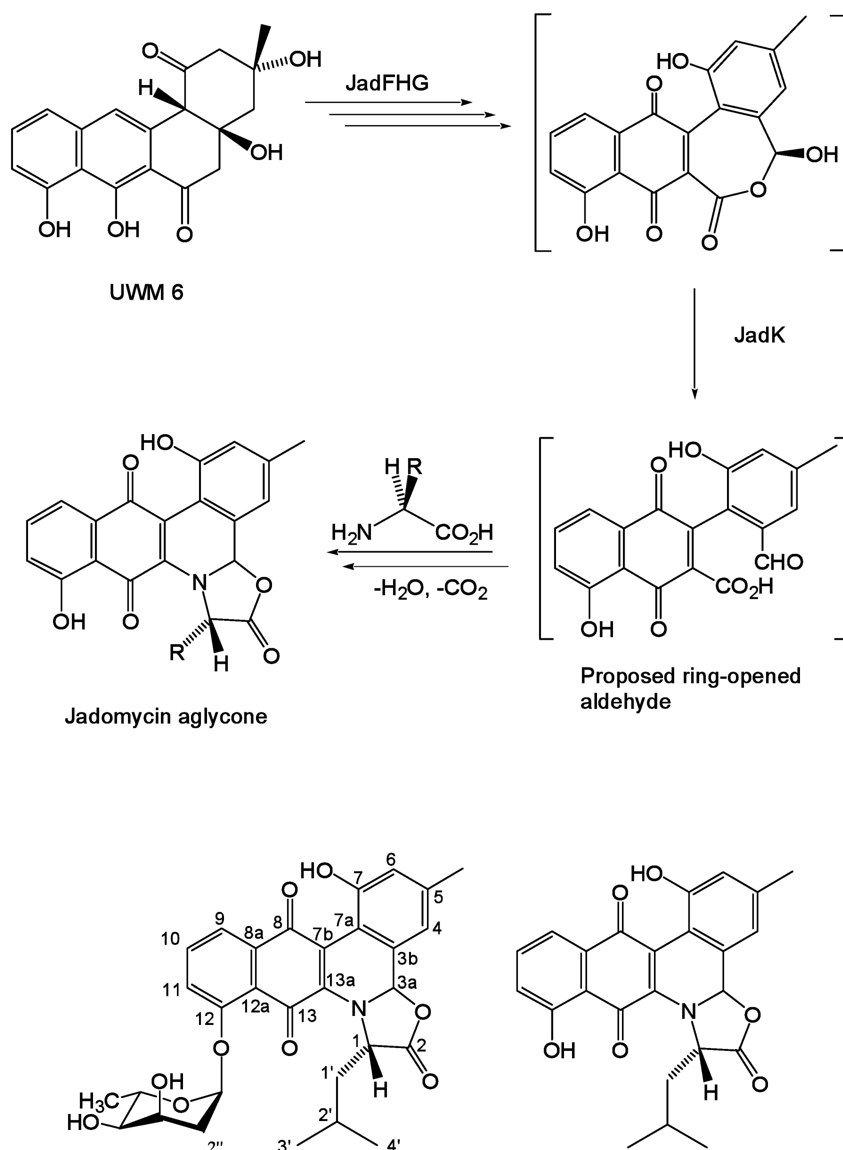
served in MS/MS experiments: the first corresponding to the cleavage of the glycosidic linkage and the second corresponding to the cleavage of the oxazolone ring [12,13]. Rohr and coworkers proposed a function for the early post-polyketide synthase (PKS) *jadFGH* and K genes involving the oxygenation of a known PKS intermediate, UWM 6, and formation, and subsequent hydrolysis, of a lactone-type Baeyer-Villiger product (Scheme 1) [14,15]. A similar cascade of post-PKS tailoring reactions has potentially been implicated in the gilvocarcin biosynthetic pathway [16]. We have exploited the reactivity of the ring-opened aldehyde intermediate with amino acids in the culture media, to isolate a series of new jadomycins. This was accomplished by culturing *S. venezuelae* ISP5230 on a minimal media with glucose as a carbon source and an amino acid as the sole nitrogen source. We have characterized



Scheme 1 The function of early post-PKS tailoring genes (*jadFHGK*) in the jadomycin biosynthetic cluster, R = various alkyl or aryl functionalities; (lower panel) Structures of jadomycin L (**1**) and jadomycin L aglycone (**2**).

jadomycins incorporating a wide array of proteinogenic and non-proteinogenic amino acids including α- and β-amino acids, and several non-proteinogenic amino acids with either *R*- or *S*-configuration at the chiral center [17]. We have also isolated derivatives with modified sugar substituents using blocked mutants in the L-digitoxose pathway [18]. In particular, isolation of products from the mutant strain blocked in the sugar C2-deoxygenase gene, *jadO*, demonstrated that the dideoxysugar biosynthetic enzymes are all capable of accepting substrates with the hydroxyl functionality maintained at C2, as a result of the isolation of a jadomycin analog with the presence of a sugar C2 hydroxyl substituent. Investigations into the stability of jadomycin B across a wide pH range enabled us to determine the existence of a ring-opened form of the jadomycins at basic pH, with a unique aldehyde functionality, clearly evident from the ^{1}H NMR [19]. The cytotoxicity of the jadomycins has been reported [17,20], and we have recently reported the effects of several jadomycins on Gram-positive pathogenic bacteria [21]. Herein, we report the first isolation of jadomycin L (**1**) and jadomycin L aglycone (**2**) from larger-scale *S. venezuelae* ISP5230 shaker cultures as a prelude to in vitro solid tumor efficacy studies in collaboration with the developmental therapeutics program at the National Cancer Institute, USA.

RESULTS AND DISCUSSION

S. venezuelae ISP5230 strain 1099 [10] was grown as described previously and added to an optimized minimal media containing L-leucine [22]. Immediately, ethanol was added to induce jadomycin biosynthesis. After 48 h, crude culture extracts were analyzed by electrospray ionization (ESI)-MS/MS to confirm the presence of jadomycin L. Under an enhanced product ion scan in positive mode, we observed the 550 $[M+H]^+$ ion, the fragmentation of the glycosidic linkage to the aglycone ion at 420 $[M+H-130]^+$ and cleavage of the oxazolone ring in the aglycone to the phenanthroviridin ion at 306 $[M+H-244]^+$ (Scheme 1). Capture of the crude secondary metabolite on reversed-phase media and elution with increasing methanol gave 720 mg crude secondary metabolite from 8 L culture. After three chromatographic steps greater than 10 mg L^{-1} jadomycin L was obtained (Table 1). Thin-layer chromatography (TLC) of the purified products is shown in Fig. 1. ^{1}H NMR spectra of jadomycin L and jadomycin L aglycone are shown in Fig. 2 and tabulated spectroscopic data in Tables 3–6.

Table 1 Quantity of jadomycin L at each purification step.

Purification stage method	Dry mass (mg)	Yield (mg/L)
Reversed-phase C18 capture	718	90
Normal-phase 7 × 20 cm	336	42
Reversed-phase 7 × 20 cm	259	32
Normal-phase 4 × 20 cm	95	12

Chemical shifts were generally in regions consistent with that of structurally related jadomycins that we have reported previously [17]. By integration and by 1D NOE NMR, the relative proportions of jadomycins in the 3a*S* form and in the 3a*R* form were determined. An NOE correlation between H-1and H-3a revealed the proton signals corresponding to the 3aS conformation, based on retention of the stereochemistry at H1. The 3a*S* form was the predominant form for both jadomycin L (5:3) and jadomycin L aglycone (5:4). Assignment of the remaining ^{1}H NMR peaks to either the 3a*R* or 3a*S* diastereomers was achieved using these integration ratios. The observed ratios between the 3a*S* and 3a*R* forms were consistent with those observed for the structurally related jadomycin B and jadomycin V, that contain L-isoleucine and L-valine, respectively [17]. Results from solid tumor efficacy studies will be reported in due course.

Fig. 1 TLC [MeOH/DCM (10 %)]: jadomycin L aglycone (**2**, left) and jadomycin L (**1**, right).

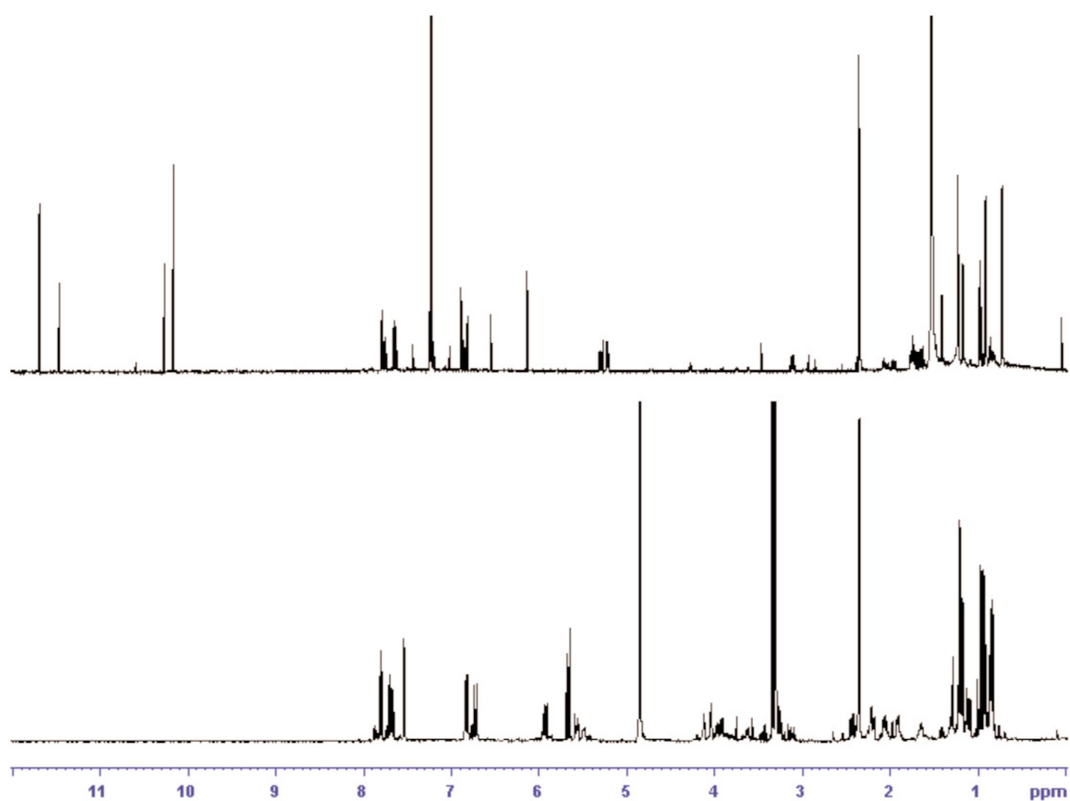


Fig. 2 ^{1}H NMR spectra of jadomycin L aglycone (**2**, in $CDCl_3$, top) and jadomycin L (**1**, in MeOD, bottom).

CONCLUSION

Methods to generate structural diversity of secondary metabolites are of increasing importance due to the potential of one variant to have optimum medicinal properties. However, the intrinsic substrate specificities associated with biosynthetic enzymes, and in particular the complex nature of PKSs, has meant that accessing structural variants has often been limited [23]. The jadomycin biosynthetic pathway contains a unique step, likely not enzymatically controlled, which has afforded a route to substituted variants of the oxazolone ring. These analogs have shown a spectrum of results in initial cell culture and antimicrobial evaluations. Further elaboration of this ring functionality is in progress in our laboratory.

EXPERIMENTAL

S. venezuelae ISP5230 strain VS1099 cultures were grown in maltose, yeast extract, and malt medium (16 × 250 mL) for 20 h at 30 °C. The cells were centrifuged at 4000 × g and resuspended in a glucose (33 mM), MOPS (40 mM, pH 7.5), and L-leucine (60 mM) minimal media (8 L) to an $o.d._{600}$ of 0.6 and immediately shocked with ethanol (7.5 mL per 250 mL culture) according to our previously reported method [22]. Cultures were incubated for 48 h until the A_{526} measured between 0.4 and 0.5. The cellular debris was removed from production media by suction filtration through no. 5 filter paper and then 0.22 μm MF filtration disks. The filtered media was passed through a reversed-phase capture C18 column (6 × 6 cm) which had been preconditioned with HPLC grade methanol. Water-soluble compounds and other metabolites were eluted using distilled water, followed by increasing amounts of methanol in water: 10, 20, 30, 40, and 60 %. The desired secondary metabolite eluted as a deep purple solution at 60 % methanol and the solvent removed in vacuo to yield 718 mg crude secondary metabolite from 8 L. TLC using normal-phase silica gel plates (10 % MeOH in dichloromethane, DCM, as eluant) confirmed the presence of jadomycin L (R_f = 0.4).

The crude material was loaded onto ISOLEUTE®HM-N sorbant using DCM and purified by automated normal-phase silica gel flash chromatography (7 × 20 cm) using a gradient of DCM to MeOH (30 %) at a flow rate of 45 mL min^{-1}. Jadomycin L aglycone (24 mg, R_f = 0.85) was essentially pure. Relevant jadomycin L fractions were combined, dried, applied to a reversed-phase C18 column (7 × 20 cm), and eluted using a gradient of water to acetonitrile (50 %) at a flow rate of 45 mL min^{-1}. Relevant fractions were combined and applied to a normal-phase column (4 × 22 cm) using a gradient of DCM to MeOH (30 %) and eluted at a rate of 60 mL min^{-1}.

The structures of the purified jadomycin L and jadomycin L aglycone were confirmed by mass spectrometry and NMR spectroscopy. Mass spectra were recorded using ESI on a 2000Q trap linear ion trap instrument. Samples were scanned in positive mode over a range of 300–700 *m/z* and then in MS/MS mode (Table 2). NMR spectra were recorded using a Bruker AV 500 instrument at 500 MHz. Spectra were recorded in MeOD (jadomycin L) or $CDCl_3$ (jadomycin L aglycone). Chemical shift values (δ in ppm) were calibrated to residual solvent peak (MeOH at 3.31 ppm in MeOD, $CHCl_3$ at 7.24 ppm in $CDCl_3$). Peak assignment was achieved using chemical shifts and peak multiplicities from the proton spectra as well as through the use of ^1H-^1H COSY, 1D TOCSY, and 1D NOE experiments (Tables 3–6). Assignment of the ^{13}C spectra was achieved through heteronuclear multiple-quantum coherence (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments. We were unable to assign all ^{13}C resonances, despite varying the J value (5–10 Hz) for the HMBC experiments due to the concentration of the sample.

Table 2 Yields and analytical mass spectrometric data for each purified compound.

Purified compound	Dry mass (mg)	Yield (mg/L)	Molecular weight (g/mol)	ESI-MS peaks observed (*m/z*)	ESI-MS peak assignments
Jadomycin L (**1**)	95	12	549	572	$[M+Na]^+$
				550	$[M+H]^+$
				420	$[M+H\text{-digitoxose}]^+$
				306	$[M+H\text{-digitoxose-}C_6H_{10}O_2]^+$
Jadomycin L aglycone (**2**)	24	3	419	420	$[M+H]^+$
				306	$[M+H\text{-}C_6H_{10}O_2]^+$

Table 3 Jadomycin L 3a*R* NMR data.

Position	δ ^{1}H (ppm)	Multiplicity [J(Hz)]	δ ^{13}C (ppm)	COSY	TOCSY
1	5.58	m	62.85	1'	
2	–	–		–	
3a	5.69	s	84.49	–	
3b	–	–		–	
4	6.75	d (1.2)	119.04	5-CH_3	
5	–	–	139.55	–	
5-CH_3	2.36	d (7.3)	19.62	4, 6	
6	6.82	q (0.7)	119.10	5-CH_3	
7	–	–	155.65	–	
7a	–	–		–	
7b	–	–		–	
8	–	–	183.04	–	
8a	–	–	137.44	–	
9	7.82	dd (8.6, 1.0)	119.71	10	
10	7.70	t (8.6)	134.85	9, 11	
11	7.54	dd (8.6, 1.0)	119.38	10	
12	–	–	155.13	–	
12a	–	–		–	
13	–	–		–	
13a	–	–		–	
1'	1.92	m	38.26	1, 2'	
2'	1.22	m	24.47	1', 3', 4'	
3'	0.86	d (6.6)	22.21	2'	4', 2', 1', 1
4'	0.84	d (6.6)	20.02	2'	3', 2', 1', 1
1"	5.94	d (3.1)	94.66	2"ax, 2"eq	
2"ax	2.19	m	34.3	2"eq, 1", 3"	
2"eq	2.46	m	34.41	2"ax, 3", 1"	
3"	4.13	m	67.08	2"ax, 2"eq, 4"	
4"	3.34	dd (9.9, 3.3)	72.62	5", 3"	
5"	3.97	m	64.88	5"-CH_3, 4"	
5"-CH_3	1.18	d (6.2)	16.76	5"	5", 4", 3"
water	4.86	s	–	–	
MeOH	3.35	s	48.18	–	
MeOD	3.31	p	47.85	–	

Table 4 Jadomycin L 3a*S* NMR data.

Position	δ ^{1}H (ppm)	Multiplicity [J(Hz)]	δ ^{13}C (ppm)	COSY	TOCSY
1	5.49	m	60.7	1'	
2	–	–		–	
3a	5.66	s	88.43	–	
3b	–	–		–	
4	6.71	d (1.2)	118.63	5-CH_3	
5	–	–	139.55	–	
5-CH_3	2.36	d (7.3)	19.62	4, 6	
6	6.84	q (0.7)	118.81	5-CH_3	
7	–	–	155.65	–	
7a	–	–		–	
7b	–	–		–	
8	–	–	183.04	–	
8a	–	–	137.44	–	
9	7.80	dd (8.6, 1.0)	119.71	10	
10	7.68	t (8.6)	134.85	9, 11	
11	7.54	dd (8.6, 1.0)	119.38	10	
12	–	–	155.13	–	
12a	–	–		–	
13	–	–		–	
13a	–	–		–	
1'	2.07	m	41.7	1, 2'	
2'	1.66	m	24.94	1', 3', 4'	
3'	0.97	d (6.6)	21.25	2'	4', 2', 1', 1
4'	0.93	d (6.6)	22.51	2'	3', 2', 1', 1
1"	5.91	d (3.1)	95.34	2"ax, 2"eq	
2"ax	2.22	m	34.3	2"eq, 1", 3"	
2"eq	2.43	m	34.41	2"ax, 3", 1"	
3"	4.05	m	67.08	2"ax, 2"eq, 4"	
4"	3.26	dd (9.9, 3.3)	72.62	5", 3"	
5"	3.93	m	64.88	5"-CH_3, 4"	
5"-CH_3	1.21	d (6.2)	16.76	5"	5", 4", 3"
water	4.86	s	–	–	
MeOH	3.35	s	48.18	–	
MeOD	3.31	p	47.85	–	

Table 5 Jadomycin L aglycone 3a*R* NMR data.

Position	δ ^{1}H (ppm)	Multiplicity [J(Hz)]	δ ^{13}C (ppm)	COSY	TOCSY
1	5.31	dd (10.0, 3.5)	57.4	1'	1', 2', 3', 4'
2	–	–		–	
3a	6.56	s	86.7	–	
3b	–	–	111.87	–	
4	6.86	m	114.02	5-CH_3	
5	–	–	143.31	–	
5-CH_3	2.35	d (6.2)	21.25	–	
6	6.90	m	121.11	5-CH_3	
7	–	–		–	
7-OH	10.28	s	–	–	
7a	–	–		–	

(*continues on next page*)

Table 5 (*Continued*).

Position	δ ^{1}H (ppm)	Multiplicity [J(Hz)]	δ ^{13}C (ppm)	COSY	TOCSY
7b	–	–		–	
8	–	–		–	
8a	–	–		–	
9	7.76	dd (7.5, 1.1)		10	
10	7.64	t (7.5)	137.68	9, 11	
11	7.21	dd (7.5, 1.1)	123.95	10	
12	–	–		–	
12-OH	11.47	s	–	–	
12a	–	–		–	
13	–	–		–	
13a	–	–		–	
1'	1.97	m	37.93	1, 2'	
2'	2.06	m		1', 3', 4'	
3'	1.17	d (6.6)	21.25	2'	4', 2', 1', 1
4'	0.98	d (6.6)	23.53	2'	3', 2', 1', 1
$CDCl_3$	7.24	s	77.2	–	

Table 6 Jadomycin L aglycone 3a*S* NMR data.

Position	δ ^{1}H (ppm)	Multiplicity [J(Hz)]	δ ^{13}C (ppm)	COSY	TOCSY
1	5.23	dd (10.0, 3.5)	57.4	1'	1', 2', 3', 4'
2	–	–		–	
3a	6.14	s		87.1	–
3b	–	–	111.87	–	
4	6.82	m	114.02	5-CH_3	
5	–	–	143.31	–	
5-CH_3	2.35	d (6.2)	21.25	–	
6	6.9	m	121.11	5-CH_3	
7	–	–		–	
7-OH	10.17	s	–	–	
7a	–	–		–	
7b	–	–		–	
8	–	–		–	
8a	–	–		–	
9	7.80	dd (7.5, 1.1)		10	
10	7.65	t x2 (7.5)	137.68	9, 11	
11	7.24	dd (7.5, 1.1)	123.95	10	
12	–	–		–	
12-OH	11.69	s	–	–	
12a	–	–		–	
13	–	–		–	
13a	–	–		–	
1'	1.75	m	42.90	1, 2'	
2'	1.66	m		1', 3', 4'	
3'	0.92	d (6.6)	21.25	2'	4', 2', 1', 1
4'	0.73	d (6.6)	23.53	2'	3', 2', 1', 1
$CDCl_3$	7.24	s	77.2	–	

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