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Novel and expanded jadomycins incorporating non-proteogenic amino acids

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Abstract—Jadomycin B is a secondary metabolite produced, in response to stress, by *Streptomyces venezuelae* ISP5230 grown in nutrient-deprived media. We present definitive electrospray ionization mass spectrometry data identifying a series of novel jadomycins with non-proteogenic amino acids incorporated into the oxazolone ring of the secondary metabolite, and strengthening evidence for the existence of an aldimine intermediate in the biosynthetic pathway. We also demonstrate that the size of the oxazolone ring can be expanded by incorporating β-amino acids.

The structural diversity of natural products is a source of both chemical inspiration and medicinal potential. The jadomycins (Scheme 1A) are a series of glycosylated secondary metabolites containing an oxazolone ring fused onto an angucycline polyketide skeleton.[1] Sequencing the gene cluster responsible for jadomycin B production has rationalized many important steps in the biosynthetic pathway and identified several unique genes with unknown functionality.[2,3] However, to date, no candidate genes have been identified that appear to catalyze oxazolone ring synthesis. Therefore, our studies have led us to investigate the scope of the unusual transformation required to form the oxazolone ring during the biosynthesis of the glycosylated natural product. Recently, formation of this ring has been postulated to proceed chemically through condensation of a polyketide-derived intermediate with a reactive aldehyde functionality and an amino acid.[4] The resulting aldimine intermediate may be subsequently cyclized and transformed into the glycosylated jadomycin derivative (Scheme 1A).

Our recent results support this chemical mechanism by identifying jadomycin derivatives formed not only from naturally occurring L-amino acids, but also from D-Val, D-Ile and two synthetic amino acids, threonine-*O*-methyl ether and threonine-*O*-methoxymethylene ether.[5] The derived jadomycins were identified through the analysis of electrospray ionization mass spectrometry (ESI-MS) enhanced product ion (EPI) scan data of culture extracts. The ESI-MS/MS data clearly showed an ion corresponding to the jadomycin derivative (**1**), which was first fragmented by MS/MS to the deglycosylated jadomycin congener (**2**), and subsequently, by cleaving the oxazolone ring, to form the phenanthroviridin (**3**) (Scheme 1B). Herein, we provide evidence for the formation of other novel jadomycin derivatives based on non-proteogenic amino acids and the first report of jadomycin derivatives containing an oxazinanone (6-membered) ring.

Cultures containing DL-4-fluorophenylalanine, 2-aminoisobutyric acid, (*R*)- and (*S*)-phenylglycine, β-alanine, DL-3-aminoisobutyric acid and isoleucine (as control) were grown in shake flasks as described previously.[1,6] After 24 h, the characteristic orange colour corresponding with jadomycin B production developed in the isoleucine cultures; however, an additional 24 h growth was required for significant colour development with the other nitrogen sources, potentially indicating slower uptake processes. Thin layer chromatographic analysis (data not shown) of the reversed-phase column eluates indicated the presence of a major coloured component in each sample. The extracts were then analyzed by ESI-MS EPI (Fig. 1 and Table 1).

The molecular ion corresponding to the postulated parent molecule was identified in each case, and an EPI scan confirmed the breakdown of the proposed

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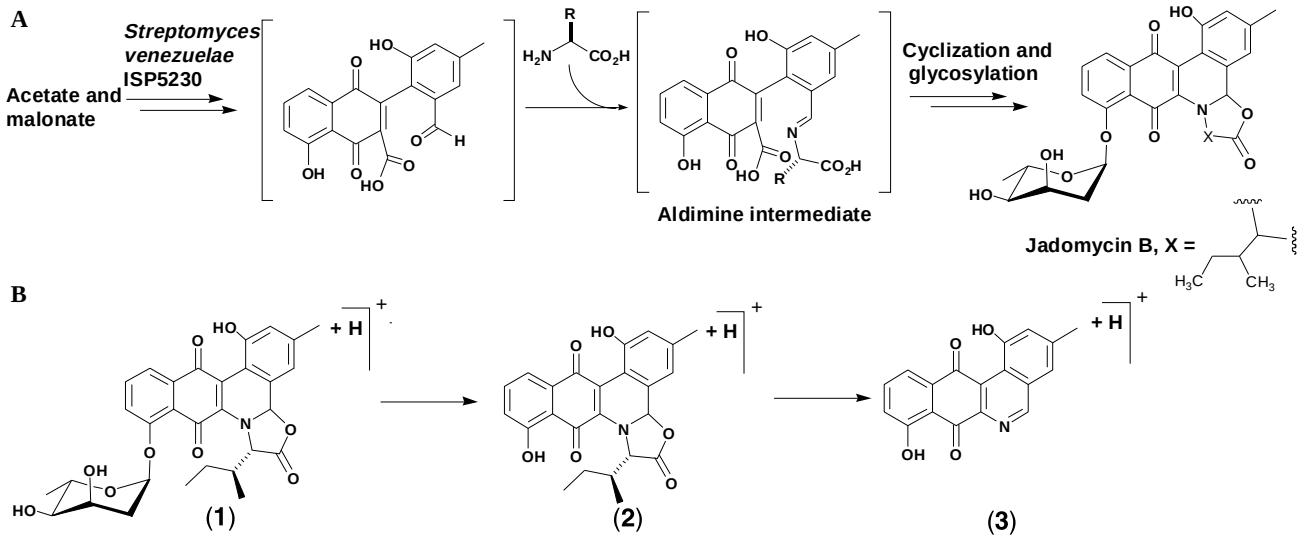


Scheme 1. (A) Schematic representation of key intermediates in the biosynthesis of jadomycin B derivatives: formation of a reactive aldehyde; condensation with an amino acid; cyclization and glycosylation. (B) Fragmentation pathway of jadomycin B based on its ESI-MS enhanced product ion spectrum. Under these MS/MS conditions the jadomycin B ion (**1**) fragments first into its aglycone ion (**2**) and subsequently into the phenanthroviridin ion (**3**) (*m*/*z* 306.1).[5]

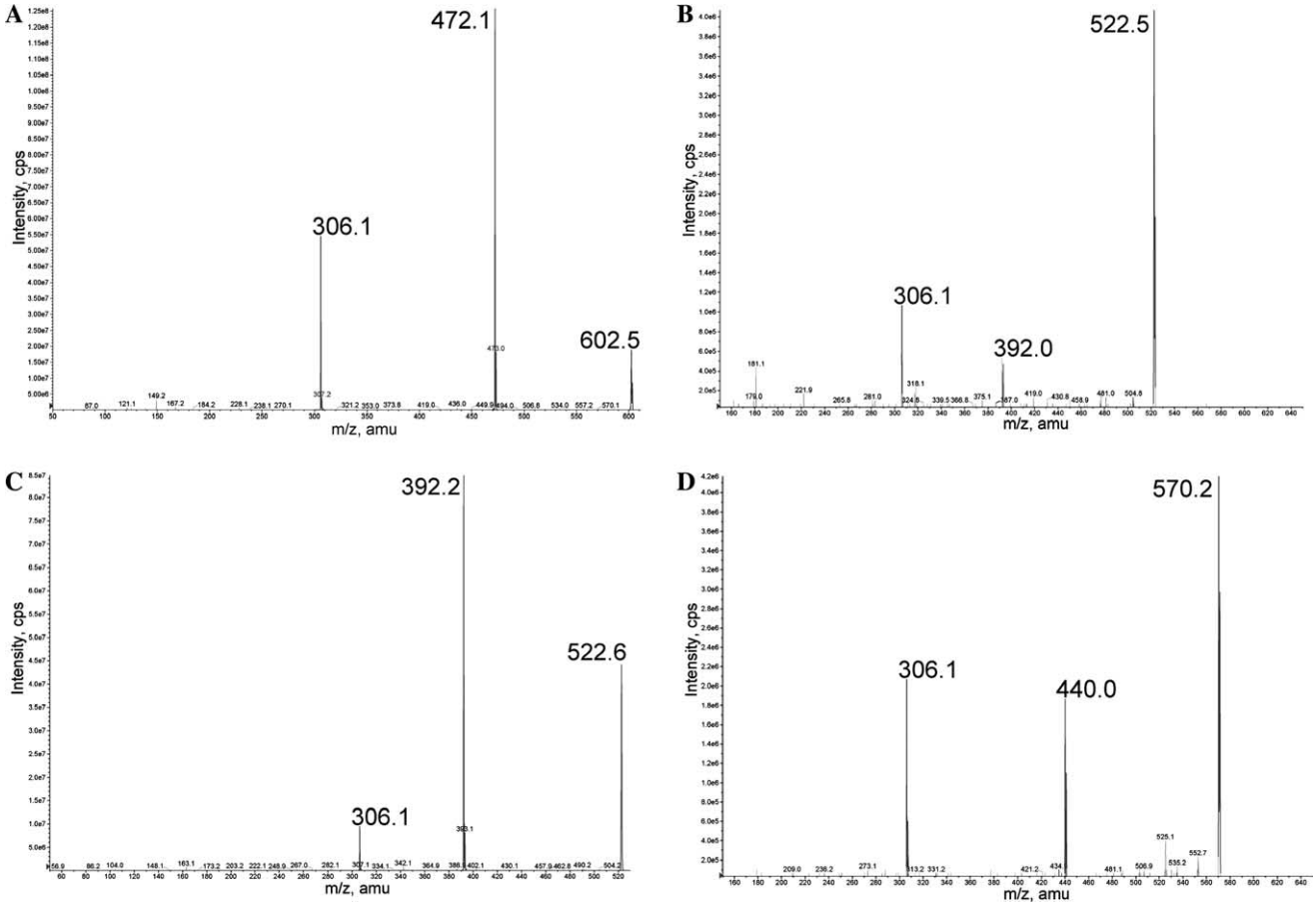
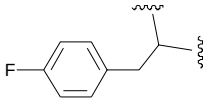
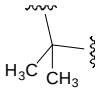
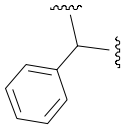
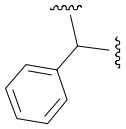
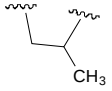
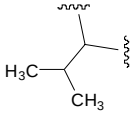
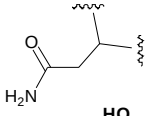
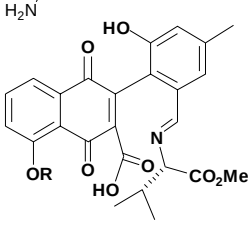


Figure 1. Representative ESI-MS EPI scan data for the MS/MS breakdown of jadomycin derivatives formed from incorporating (A), DL-4-fluorophenylalanine; (B), 2-aminoisobutyric acid; (C), DL-3-aminoisobutyric acid and (D), (*R*)-phenylglycine.

parent ion directly within the mass spectrometer to the aglycone ion, and subsequently to the phenanthroviridin ion, providing unequivocal evidence for the structures of the parent jadomycins. All jadomycin derivatives showed this fragmentation pattern, including those produced when VS1099 was grown on β-alanine or DL-3-aminoisobutyric acid. The proposed β-alanine and DL-3-aminoisobutyric acid derived prod-

Table 1. ESI-MS EPI data observed for culture extracts of *Streptomyces venezuelae* ISP5230 grown on specific nitrogen sources

Nitrogen source used in growth	EPI-MS/MS data of novel jadomycins		
	Parent ion	Aglycone ion	Proposed structure X =
DL-4-Fluorophenylalanine	602.5	472.1	
2-Aminoisobutyric acid	522.5	392.0	
(*R*)-Phenylglycine	570.0	440.0	
(*S*)-Phenylglycine	570.0	440.0	
β-Alanine	508.2	378.1	
DL-3-Aminoisobutyric acid	522.6	392.2	
D-Val and L-Asn[a]	536.4	406.1	
	551.4	421.1	
L-Valine methyl ester	596.4		
	466.3		(**4**) R = L-digitoxosyl, (**5**) R = H

[a] When both amino acids were fed simultaneously peaks observed from incorporation of D-Val and L-Asn were of similar intensity.

ucts are the first examples of jadomycin derivatives containing a six-membered oxazinanone ring. This parallel to jadomycins containing five-membered rings, provides strong spectrometric evidence for jadomycin derivatives containing six-membered rings.

If a chemical insertion process, rather than an enzymatic one, is involved in jadomycin biosynthesis, then cultures grown in media containing mixtures of amino acids might be expected to produce comparable quantities of each jadomycin derivative. Analysis of the mass spectrometric data from an extract of *Streptomyces venezuelae* cultures grown with equimolar D-valine and L-asparagine present in the medium clearly indicates the presence of both jadomycin derivatives in the methanol extracts, as shown in Table 1, corroborating this mechanistic hypothesis.

To assess whether the aldimine intermediate proposed as a prerequisite to oxazolone ring formation during jadomycin biosynthesis could be observed (Scheme 1A), we have conducted experiments using L-valine methyl ester as the sole nitrogen source. Due to the presence of the methyl ester functionality, the proposed aldimine intermediate should be unable to cyclize and could potentially be isolated or detected. Mass spectrometric analysis of the partially purified culture extracts from growth on L-valine methyl ester clearly identified two relevant peaks, the first corresponding to the anticipated molecular ion for the aldimine intermediate, formed by condensation of L-valine methyl ester with a polyketide-derived aldehyde, and the second corresponding to the glycosylated aldimine intermediate. Neither of these peaks broke down under increasingly vigorous

MS/MS conditions, indicating that neither parent ion contained an oxazolone ring. These two peaks provide direct evidence for the formation of the aldimine intermediate and also indicate that the glycosyltransferase, JadS, does not require an oxazolone ring in its aglycone substrate.

The incorporation of DL-4-fluorophenylalanine, 2-aminoisobutyric acid, and both (*R*)- and (*S*)-phenylglycine into jadomycin derivatives provides evidence for a non-enzymatic route to the oxazolone ring. A similar non-enzymatic process has been proposed during betaxanthin biosynthesis.[7] In the case of the jadomycins, this non-enzymatic route is now substantiated by direct mass spectrometric observation of the aldimine intermediate using cultures grown on L-valine methyl ester. Identification of predictable products, arising from incorporation of β-alanine and DL-3-aminoisobutyric acid, demonstrates an unexpected reactivity of the jadomycins' biosynthetic machinery and discovery of a new class of jadomycins with an oxazinanone ring. Further investigations are in progress to structurally characterize and assess the bioactivity of these novel metabolites.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.08.047.

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6. Cultures of *Streptomyces venezuelae* ISP5230 strain VS1099 were grown as described previously,[1] except that we have developed improved protocols that stabilize the culture pH and reduce growth times. Full details will be presented elsewhere, but briefly: in the production media morpholinepropanesulfonic acid (10 mM) replaced the phosphate as a buffer, and cultures were inoculated with sufficient VS1099 mycelium to give an initial OD_{600} of 0.6. Vegetative inoculum for the production media was grown for 22 h in maltose, yeast extract and malt (MYM) medium and concentrated by centrifugation at 4000*g*. Absolute ethanol (3%) was added to production media immediately after inoculation to stimulate jadomycin biosynthesis. Cultures (25 mL) were grown in Erlenmeyer flasks (125 mL) at 30 °C on a rotary shaker at 230 rpm for up to 48 h. Cultures were harvested by filtration, and the clarified broth (25 mL) was passed through a 2 g ISOELUTE Flash C18 column; the column was washed with water and adsorbed coloured components were eluted with MeOH. Samples were standardized to $OD_{313} = 0.01$ in 50% methanol (0.5% formic acid) for ESI-MS analysis. The ESI-MS/MS (EPI) analysis was performed on an Applied Biosystems-MDS SCIEX triple quadrupole linear ion trap with an ambient source temperature. For the enhanced product ion scans nitrogen was used as collision gas. The sample was infused by syringe pump into the turbospray ion source at a flow rate of 10 μl/min. The mass spectrometer settings were: needle voltage 5.5 kV; nitrogen was used as the curtain and nebulizer gas (GS1); the curtain gas and GS1 was set to 20. EMS scan: mass range *m*/*z* 100–700; scan speed 4000 Da/s; trap fill time 20 ms. EPI scan: mass range Q3 *m*/*z* 150–600; scan speed 4000 Da/s; trap fill time 50 ms; Q1 resolution 'low' setting.
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