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Culture conditions improving the production of jadomycin B

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Abstract The jadomycins are a unique family of benzoxazolophenanthridine antibiotics produced by *Streptomyces venezuelae* ISP5230 following heat or ethanol shock or phage infection. We have modified the culture conditions by altering the carbon source, buffer, inoculum size, and timing of ethanol shock, thereby reducing growing times and improving jadomycin B production. Our optimized conditions use glucose as the carbon source, MOPS as buffer, low concentrations of phosphate, a defined inoculum concentration and an immediate ethanol shock to induce jadomycin B production; results that contrast previous studies. The altered media will facilitate the isolation of related jadomycin B congeners.

Keywords Secondary metabolites · Jadomycin B · *Streptomyces venezuelae* ISP5230 · Biosynthesis · Ethanol shock

Introduction

Phage infection, heat shock and treatment with ethanol all induce *Streptomyces venezuelae* ISP5230 to produce a series of benzoxazolophenanthridine antibiotics when grown in nutrient-deprived media [1, 3]. Jadomycin aglycone was initially isolated from *S. venezuelae* ISP5230 cultures, and subsequently the glycosylated derivative jadomycin B was fully characterized (Fig. 1) [10]. The effects of varying culture conditions were initially investigated by Doull and co-workers [2], who reported the potential existence of structurally related jadomycins based on differences in the colour of cultures grown in media with various amino acids; they observed that one of the unique structural features of the jadomycins is an oxazolone ring incorporating an amino acid from the growth media. Rohr and colleagues [7] have recently characterized five of these postulated jadomycins and proposed a chemical insertion mechanism in which a polyketide-derived aldehyde intermediate couples with an amino acid to give an aldimine, which after cyclization forms a unique oxazolone ring. The amino acid side chain has an effect on the cytotoxic activities of jadomycins B, V, S, T and F [11]. We have determined that non-natural amino acids are also incorporated into the jadomycin oxazolone ring [4, 5] and recently described the effects of aqueous conditions upon the structure of the oxazolone ring [9]. At basic pH the antibiotic interconverts between two isomeric forms at C3 through a novel aldehyde intermediate, as shown in Fig. 2. To develop a defined media for growth studies and potentially aid our production and purification of jadomycins derived from diverse non-natural amino acids, we describe new culture conditions that reduce growth times and increase production of jadomycin B.

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Materials and methods

Cultures and growth conditions

Cultures of *S. venezuelae* VS1099, a mutant of strain ISP5230 disrupted in *jadW*$_2$ [10], were grown, from a frozen spore suspension, for ~3 weeks on MYM agar [maltose (0.4% w/v), yeast extract (0.4% w/v), malt extract (1% w/v) and agar 1.5% w/v] [2]. Surface growth and spores from the MYM agar were incubated at 250 rpm at 30 °C for 22 h in MYM broth to produce a vegetative inoculum. For preliminary production studies using galactose–isoleucine media, the methods of Doull et al. [2] and Rohr and colleagues [7] were used as described and repeated in triplicate. In experiments where

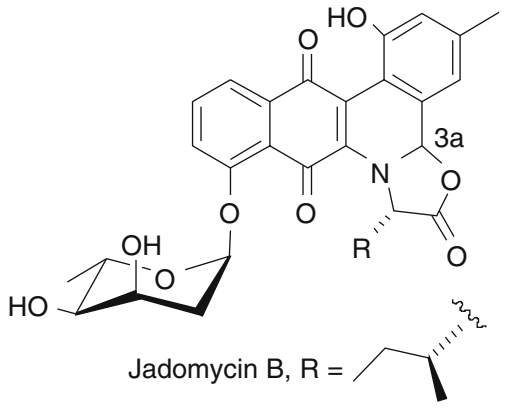

Fig. 1 Chemical structure of jadomycin B

vegetative inocula densities of 0.5–0.7 were used, it was necessary to use MYM volumes approximately one-half that of the final production culture to obtain sufficient cells (i.e. for 200 mL production culture, 100 mL vegetative inocula were required). The mycelium (100 mL) was pelleted by centrifugation at 4000×*g*; the pellet was washed twice with a buffered mineral salts solution (MSM; 40 mL), was resuspended in MSM (15 mL), and added to the production media to an initial OD_{600} of ~0.5–0.7. After inoculation, cultures were immediately shocked with absolute ethanol (3% v/v). All production cultures were grown as 25 mL aliquots in standard 125 mL Erlenmeyer flasks on a rotary shaker at 250 rpm at 30 °C.

The MSM solution contained (per litre) $MgSO_4$ (0.4 g), MOPS (1.9 g), salt solution (9 mL of 1% w/v NaCl and 1% w/v $CaCl_2$), $FeSO_4 7H_2O$ (4.5 mL of 0.2% w/v) and trace mineral solution (4.5 mL). The trace mineral solution contained (per litre): $ZnSO_4 7H_2O$ (880 mg), $CuSO_4 5H_2O$ (39 mg), $MnSO_4 4H_2O$ (6.1 mg), H_3BO_3 (5.7 mg) and $(NH_4)_6Mo_7O_{24} 4H_2O$ (3.7 mg).

The glucose–MOPS (GM) media consisted of L-Ile (7.8 g L^{-1}) dissolved in MSM and adjusted to pH 7.5 (NaOH, 5 M). To avoid possible precipitation, glucose (30% w/v) and phosphate stock solutions (9 mM, 10.5 g K_2HPO_4:4.5 g KH_2PO_4) were autoclaved separately and added aseptically to the production media.

Phosphate and glucose experiments

The GM production media were identical to that described above except that various amounts of glucose (7, 17, 33, 66 and 166 mM) and phosphate (0.0, 0.001, 0.05, 0.1 and 1.0 mM) were added as required. Between 2 and 7 replicates were performed for the data presented in Fig. 5.

Carbon source experiments

Carbon source carryover during inoculation was minimized by stringent washing (four times) of the vegetative inocula. The GM production media were as described above except that sugar concentrations were based on molar equivalents of glucose and were as follows: maltotriose (11 mM); maltose and D-trehalose (16.5 mM); lactose, xylose, mannose, sucrose, glucose, and galactose (33 mM). Each experiment was performed in duplicate. Potassium phosphate solution was added aseptically to a final concentration of 0.05 mM.

MOPS experiments

The GM production media were as described above except that the final concentrations of MOPS were varied (10, 40, 80 and 100 mM), and glucose was added to a final concentration of either 33 or 66 mM. Each experiment was performed in duplicate. Potassium phosphate solution was added aseptically to a final concentration of 0.05 mM.

Potassium chloride experiments

The GM production media used were as described above except that different concentrations and sources of phosphate and potassium were used, namely: $NaH_2PO_4H_2O$ (9 mM) and KCl (10 mM); the final glucose concentration was 33 mM. Each experiment was performed in triplicate. Sample 1 contained only 0.08 mM potassium chloride; samples 2–6 all contained phosphate (0.05 mM) and varying concentrations of KCl (0, 0.08,

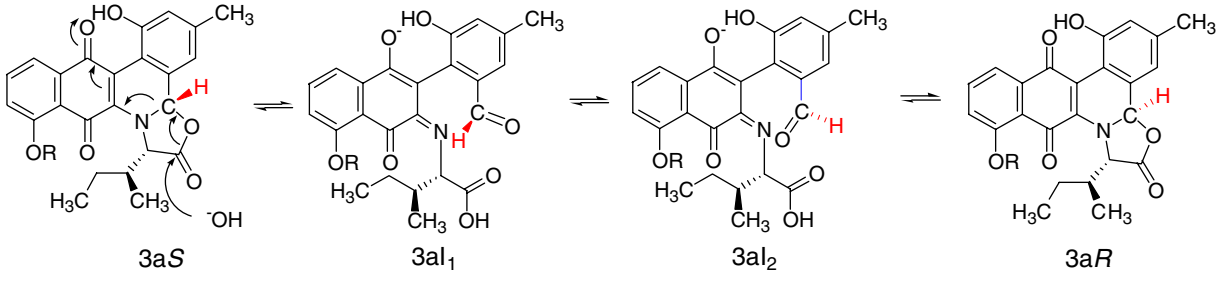

Fig. 2 A recently proposed mechanism to interconvert the C3 diastereomeric jadomycins [9]

0.16, 0.32 and 0.64 mM); sample 7 (used as a control) was the same as the glucose sample used in the carbon source experiments.

L-Ile experiments

The GM production media were as described above except that L-Ile was used at different concentrations (15, 30, 45, 60, 75 mM). The final glucose concentration was 33 mM. Experiments were performed in triplicate. Phosphate from a sterile stock solution of sodium dihydrogen phosphate was added to a final concentration of 0.05 mM.

Analyses

Periodically during the growth of cultures, 1 mL aliquots were removed aseptically and cell densities were recorded on a SpectraMax-plus 384 spectrophotometer at 600 nm using a cuvette with a 1 cm path length. The samples were then centrifuged (10,000 rpm) in 1.5 mL microfuge tubes and the culture supernatant absorbances were measured at 526 nm. Values estimated for the production of jadomycin B by this method correlated well with those obtained by previously reported methods in which the cultures were extracted with ethyl acetate, or the metabolite was eluted from a 1 mL solid phase extraction cartridge [2]; this also allowed measurements with small culture volumes to be taken at multiple time-points. Samples were prepared for thin layer chromatographic (TLC) analysis by passing centrifuged 1 mL culture volumes through RP-18 columns (4 mL, Argonaut), washing the column with water (4 mL), eluting with MeOH (2.5 mL), concentrating to 20 µl. This was spotted on normal phase silica gel plates (Silicycle Inc.). Plates were run in DCM:MeOH:H_2O, (95.6:4.0:0.2) jadomycin B, $R_f = 0.5$.

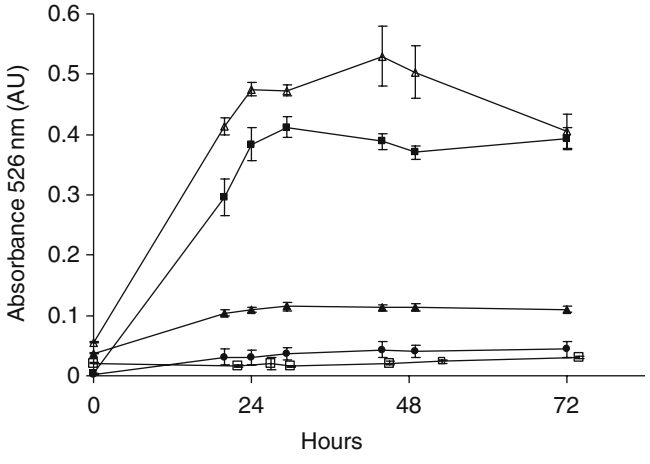

Fig. 3 Effects of different culture conditions on jadomycin B production. (*Filled triangle*) Doull et al. [2]; (*open square*) Doull et al. [2] with no media carryover; (*open triangle*) Doull et al. [3] plus 10% malt extract; (*filled circle*) Rohr and colleagues [7]; (*filled square*) GM media

Results and discussion

Preliminary growth of *S. venezuelae* ISP5230 in galactose–isoleucine production medium [2] and corresponding media with L-Ile replaced by other amino acids (to obtain jadomycins with modified oxazolone ring substituents) gave differing rates of bacterial growth, as well as marked variation in the quantities of the analogues produced; results that impeded isolation and characterization. We therefore investigated culture conditions affecting the production of jadomycin B by *S. venezuelae* ISP5230, aiming to use superior conditions to produce other congeners.

The conditions described by Doull et al. resulted in the production of jadomycin B (Fig. 3), but the consistency of production in different experiments was poor, even though replicates within an experiment were consistent. We also tried growth conditions (Rohr and colleagues [7]) that differed from that of Doull et al. only in the volume of starter culture and the volume and timing of ethanol shock, but surprisingly failed to obtain significant jadomycin B production (Fig. 3). The larger volume of starter culture used in the Doull growth media [2] caused us to investigate the role of MYM media components, particularly malt extract due to its sugar content, on production. The addition of malt extract (0.4% w/v) to the galactose–isoleucine media caused a significant improvement in jadomycin B production (Fig. 3). The influence of the malt extract on carryover was confirmed by washing, in a buffered salt solution, (MSM) the vegetative mycelium transferred to the galactose-isoleucine production media. This severely limited jadomycin B production (Fig. 3). TLC analysis of these samples is shown in Fig. 4, demonstrating that the significant secondary metabolite is jadomycin B. Other advantages observed from adding malt extract were improved culture growth rates, although reproducibility between experiments remained poor. We attributed this to interactions between the phosphate buffer and other components forming insoluble precipitates, such as calcium phosphate, in the galactose–isoleucine media during autoclaving. To reduce the precipitation problem and to better control the culture pH, we replaced the phosphate buffering agent in the medium with morpholinopropanesulfonic acid (MOPS). This also enabled us to investigate the effects of phosphate on jadomycin B biosynthesis, as this nutrient is known to regulate secondary metabolite production [6, 8]. In addition, the timing of ethanol shock is critical for the production of jadomycin B [2] and the possibility that a specific concentration of cells could be required for optimum jadomycin B production led us to determine the bacterial cell density when cultures were treated with ethanol. The cultures of *S. venezuelae* ISP5230 in the galactose–isoleucine–malt (GalIM) medium grew

with sufficiently uniform dispersion for turbidity (OD_{600}) to be used to measure growth. Transferring washed vegetative inoculum from overnight MYM cultures into the GalIM medium gave an initial culture OD_{600} value of approximately 0.2. When ethanol was added after 6.5 h, the OD_{600} had increased to 0.5–0.6. The concentration of cells at shock time in our experiments using the conditions of Rohr and colleagues was significantly less OD_{600} (0.1–0.2) and this may explain why we were unable to observe jadomycin B production using their culture conditions. To further facilitate the production of jadomycin B, we investigated the effect of shocking the vegetative mycelium immediately after its transfer into the production medium. Thus, a 24 h vegetative inoculum was centrifuged, washed and the cells resuspended in GalIM production media, to a final turbidity of OD_{600} ~0.6, and shocked immediately. After 24 h incubation, jadomycin B had been produced in titres comparable to those obtained previously by waiting to shock until 6.5 h after inoculation, thereby reducing culturing times required to produce jadomycin B.

We attempted to define a culture medium for antibiotic production based on glucose (as the most significant carbon source component of malt extract) in contrast to galactose (with potential cost savings), with a limiting concentration of phosphate to potentially aid production. To avoid carryover, the *S. venezuelae* ISP5230 vegetative mycelium was washed by centrifuging and re-suspending cells in MSM solution. Production media containing differing phosphate and glucose concentrations and a fixed concentration of L-Ile were inoculated with washed mycelium to a final turbidity of OD_{600} ~0.6, and shocked immediately with ethanol. Figure 5 shows the results of varying phosphate and glucose concentrations on jadomycin B production. The lowest glucose concentration tested (7 mM) did not support significant production. However, production occured at all other glucose concentrations. The effect of phosphate was much more pronounced. With no phosphate the jadomycin B titres were very low. At phosphate concentrations of 0.01 and 1 mM jadomcyin B titres were higher, but with phosphate concentrations of 0.05 and 0.1 mM the values were comparable to levels of jadomycin B produced in preliminary experiments in the GalIM media.

To see whether further improvements could be made, we examined the effects of glucose, maltotriose, maltose, galactose, xylose, mannose, sucrose, lactose and trehalose on jadomycin B production. We chose concentrations equivalent to 33 mM glucose for monosaccharides and for di- and trisaccharides we based the concentrations on moles of glucose equivalents. For example, with maltotriose which contains three glucosyl functionalities per molecule of maltotriose, we used a concentration of 11 mM. Phosphate levels were at a sub-millimolar level (0.05 mM). The effects on jadomycin B production over 72 h are shown in Fig. 6, and confirm that glucose is the most appropriate carbon source for jadomycin B production in *S. venezuelae* ISP5230. Further, *S. venezuelae* ISP5230 is capable of producing jadomycin B when grown on mannose, maltotriose and maltose at higher levels than when the previously used sugar (galactose) is the sole carbon source. The TLC analysis of culture extracts after 72 h confirmed that jadomycin B was the major secondary metabolite produced.

The effect of MOPS buffer at much higher levels (100 mM) than we used in the preceding experiments is reported to maintain culture pH, but reduce jadomycin B titres [2]. We therefore assessed the effects of MOPS buffer at concentrations between 10 and 100 mM, and at

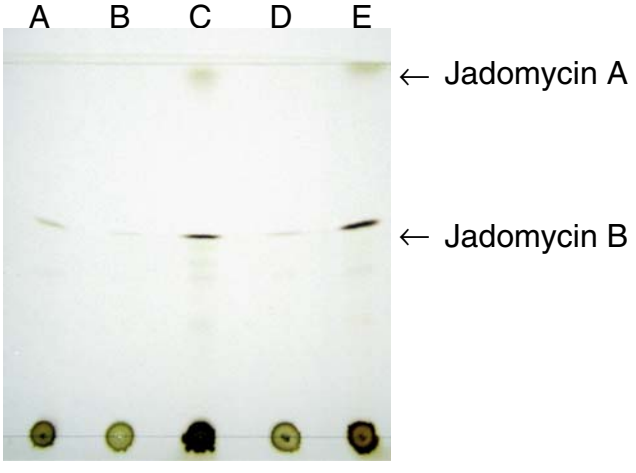

Fig. 4 TLC analysis of culture extracts from differing conditions after 40 h showing the production of jadomycin B. (*A*) Doull et al. [2]; (*B*) Doull et al. [2] with no media carryover; (*C*) Doull et al. [3] plus 10% malt extract; (*D*) Rohr and colleagues [7]; (*E*) GM media

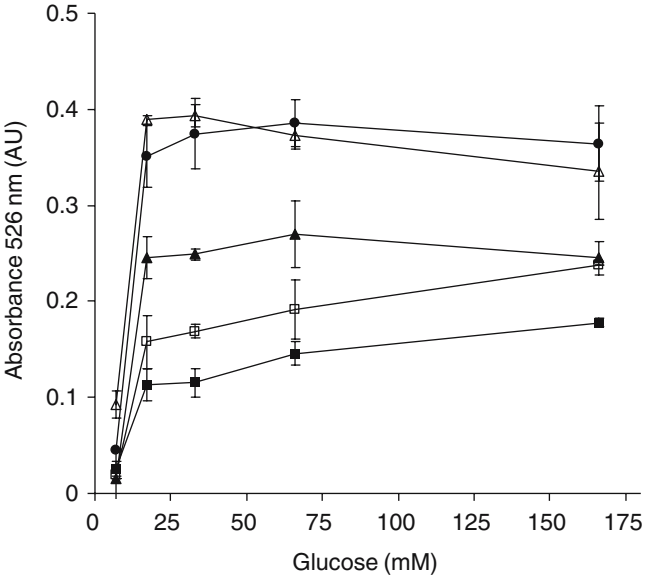

Fig. 5 Effects of glucose and phosphate on jadomycin B production after 24 h. (*Filled triangle*) 1.0 mM; (*filled circle*) 0.1 mM; (*open triangle*) 0.05 mM; (*open square*) 0.01 mM; (*filled square*) 0.0 mM phosphate

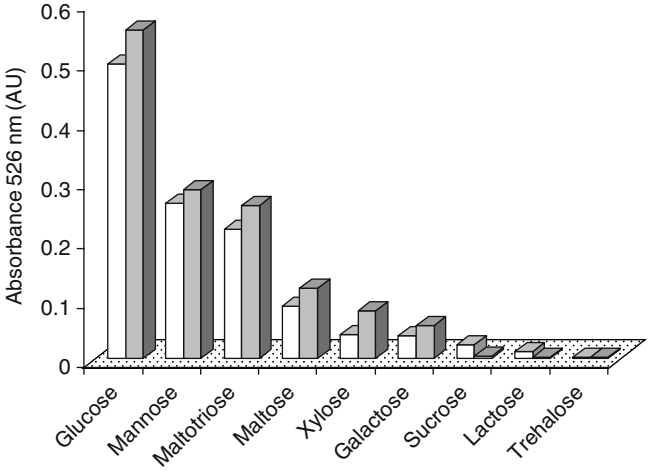

Fig. 6 Effects of carbon source on jadomycin B production. *Clear bars* 24 h; *shaded bars* 72 h

two different glucose concentrations (33 and 66 mM), over 68 h (Fig. 7). Jadomycin B titres were highest at the lower concentrations of MOPS (10 mM). However, at the two lowest MOPS concentrations, the cultures were less effectively buffered; the pH values dropped one pH unit over the first 23 h, but remained approximately constant for the next 48 h. A doubling of the glucose concentration in the GM media had no significant effect on jadomycin B titres over the duration of the experiment.

We next investigated the role of potassium ions in the GM production media. Our standard conditions for the three previous experiments used potassium phosphate as phosphate source. To vary the potassium ion content of the medium, we replaced potassium phosphate with sodium phosphate at the same concentration (50 μM). Potassium chloride was added to vary potassium ion levels. The results presented in Fig. 8 show that increasing concentrations of potassium chloride from 80 to 640 μM decreased production of jadomycin B two-fold in comparison to cultures with no additional potassium. It appears that potassium ions are detrimental to jadomycin B production in the *S. venezuelae* ISP5230 GM media we have developed.

Our long-term interest in generating jadomycin B analogues derived from non-proteogenic amino acids, from commercial or synthetic sources, caused us to assess the influence of amino acid concentration present in GM production media on the production of jadomycin B, so as to minimize waste of the amino acid. Increasing concentrations of L-Ile from 15 to 75 mM in the medium resulted in higher jadomycin B titres (Fig. 9), although at concentrations above 45 mM titres did not change significantly. Interestingly, increasing the L-Ile concentration did not significantly alter the rate of jadomycin B production over the 2 days studied. Thus, it appears that a 45 mM concentration of L-Ile is sufficient for efficient conversion to jadomycin B; however, it is anticipated that this will need to be re-evaluated for each amino acid used, since the ability of *S. venezuelae* ISP5230 to metabolize specific amino acids may have a significant impact on intracellular amino acid concentration, or the rate of biosynthesis of secondary metabolites.

Production of jadomycin B using our optimized culture conditions are shown in Figs. 3 and 4 where we use glucose as carbon source, MOPS (10 mM), sodium phosphate (50 μM), a defined vegetative inoculum, and an immediate shock on transfer to the GM production media.

In summary, we have developed a robust and chemically defined medium for producing jadomycin B that yields higher quantities of the natural product in comparison to previously reported media, in a more rapid and reproducible way. The defined nature of the medium will enable us to assess the effects of additional nutrients on jadomycin B production. We have currently determined

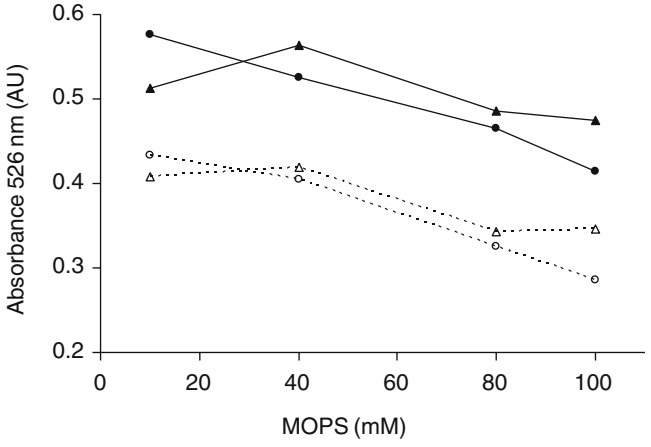

Fig. 7 Effects of MOPS buffer and glucose concentration on jadomycin B production. (*Open circle*) 23 h, 33 mM; (*filled circle*) 68 h, 33 mM; (*open triangle*) 23 h, 66 mM; (*filled triangle*) 68 h, 66 mM

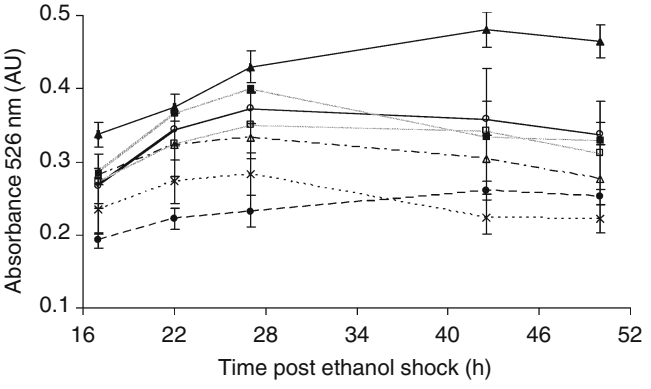

Fig. 8 Effects of KCl on jadomycin B production. (*Filled triangle*) 0 μM KCl, 50 μM NaH_2PO_4; (*filled square*) 80 μM K^+, 50 μM PO_4^{3-} (standard); (*open circle*) 80 μM KCl, 50 μM NaH_2PO_4; (*open triangle*) 160 μM KCl, 50 μM NaH_2PO_4; (*open square*) 320 μM KCl, 50 μM NaH_2PO_4; (*times*) 640 μM KCl, 50 μM NaH_2PO_4; (*filled circle*) 80 μM KCl, 0 μM NaH_2PO_4

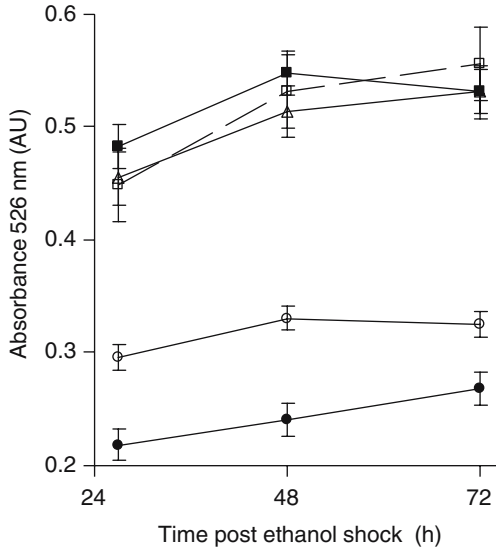

Fig. 9 Effects of L-Ile concentration on the production of jadomcyin B. (*Filled circle*) 15 mM; (*open circle*) 30 mM; (*filled square*) 45 mM; (*open triangle*) 60 mM; (*open square*) 75 mM

that glucose is the preferred carbon source and that jadomycin B biosynthesis is optimal with sub-millimolar levels of phosphate using MOPS buffer (10 mM) to maintain pH. We have also increased the inoculum size to a defined level in the GM production medium, making it feasible to administer ethanol shock immediately. These changes in the growth conditions for *S. venezuelae* ISP5230 have enabled us to isolate novel jadomycins by growing the organism on different amino acids. The structures and bioactivity of these analogues will be reported in due course.

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