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Jadomycins Derived from the Assimilation and Incorporation of Norvaline and Norleucine

Stephanie N. Dupuis,[†] Thomas Veinot,[‡] Susan M. A. Monro,[§] Susan E. Douglas,[⊥,∥] Raymond T. Syvitski,[†,⊥] Kerry B. Goralski,[‡,▽] Sherri A. McFarland,[§] and David L. Jakeman*[,†,‡]

[†]Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, Canada

[‡]College of Pharmacy, Dalhousie University, Halifax, Nova Scotia, Canada

[§]Department of Chemistry, Acadia University, Wolfville, Nova Scotia, Canada

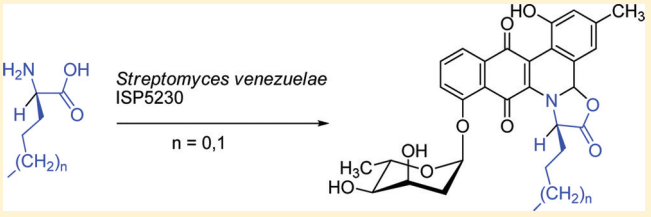
[⊥]Institute for Marine Biosciences, National Research Council of Canada, Halifax, Nova Scotia, Canada

[∥]Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada

[▽]Department of Pharmacology, Dalhousie University, Halifax, Nova Scotia, Canada

Supporting Information

ABSTRACT: *Streptomyces venezuelae* ISP5230 is recognized for the production of chloramphenicol and the jadomycin family of natural products. The jadomycins are angucycline natural products containing a unique oxazolone ring incorporating an amino acid present in the minimal culture media. Substitution of different amino acids results in products of varying biological activity. Analysis of cultures of *S. venezuelae* ISP5230 incubated with L- and D-norvaline and L- and D-norleucine indicated that only the D-configured amino acids were incorporated into the natural products. Subsequently, jadomycin DNV and jadomycin DNL were isolated and characterized (titers 4 and 9 mg L^{-1}, respectively). The compounds were evaluated in the National Cancer Institute cell line cancer growth inhibition and cytotoxicity screens, for antimicrobial activity against selected Gram-positive and Gram-negative bacteria, and as DNA-cleavage agents *in vitro*.



Polyketide synthases are multienzyme complexes responsible for the production of numerous bioactive natural products encompassing a broad scope of structural diversity. Many of the compounds produced by polyketide synthases have important roles as clinically approved drugs.[1] *Streptomyces venezuelae* ISP5230 (*Streptomyces venezuelae* ATCC 10712) is known to produce chloramphenicol and the jadomycin family of natural products. Analysis of the recently released genome sequence of the organism[2] using antiSMASH[3] resulted in the identification of 26 additional natural product biosynthetic gene clusters, including four additional polyketide synthase clusters. Another biosynthetic cluster was independently described as a novel lantibiotic biosynthetic cluster, although production of the natural product was not accomplished within *S. venezuelae* or within *S. lividans*, a heterologous host.[4] This illustrates a significant challenge associated with inducing anticipated cryptic biosynthetic gene clusters identified through genome sequencing.[5] Jadomycins A and B were serendipitously discovered to be induced through phage, heat, or ethanol shock[6,7] when *S. venezuelae* ISP5230 was cultivated in a minimal media.

The jadomycins are members of the angucycline family, which includes the landomycins,[8] urdamycins,[9,10] and kinamycins.[10] The polyaromatic backbone of angucyclines is generated through a type II polyketide synthase.[11] The structural diversity observed with products of polyketide synthases is in part due to the variety of mechanisms employed to cyclize the poly-β-ketone backbone.[12,13] In the case of the jadomycins, a unique structural feature is the oxazolone ring and the discovery that it is likely formed in a nonenzymatic fashion (Scheme 1).[14] The nonenzymatic nature of this step is supported by the total synthesis studies of jadomycin A,[15] where condensation of an aldehyde and a carboxyl-protected amino acid proceeded smoothly to form an aldimine that cyclized to furnish a hemiaminal. Upon deprotection of the carboxyl functionality, a second spontaneous cyclization occurred to form the oxazolone ring. The nonenzymatic incorporation of amino acids into the jadomycin oxazolone ring provides an opportunity to explore structure–activity relationships. As part of our structure–function studies on the jadomycins, a number of structurally related oxazolone analogues have been characterized and evaluated.[16−18] Different mechanisms of DNA cleavage have been identified for jadomycin B, jadomycin L, jadomycin G, and jadomycin *S*-Phe,[19] clearly exemplifying the importance of the substituents around the oxazolone ring in the *in vitro* DNA cleavage assays. Jadomycin L has been evaluated by the National Cancer Institute (NCI) in its 60-cell-line screening program,[20] and to provide further structure–activity studies, we embarked upon analyzing cultures of *S. venezuelae* ISP5230

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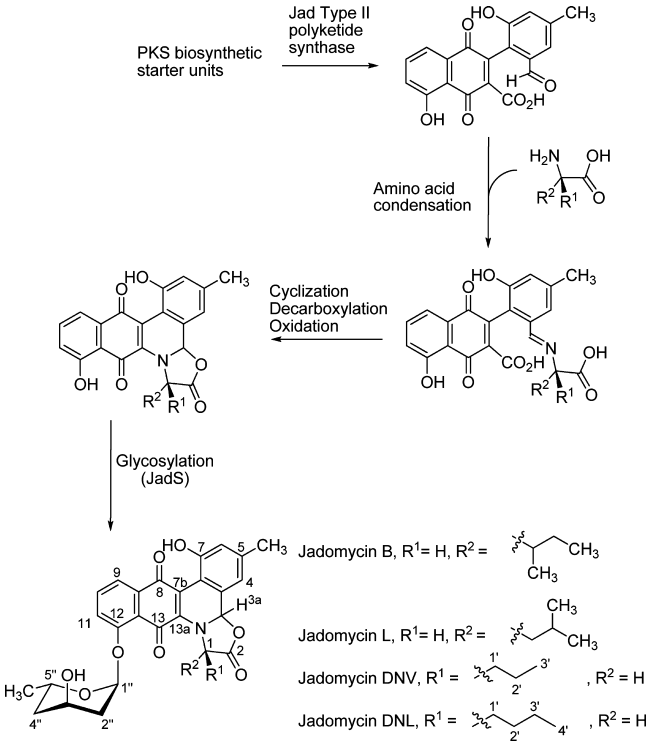
Scheme 1. Selected Jadomycin Biosynthetic Intermediates Outlining the Incorporation of an Amino Acid Present in the *Streptomyces venezuelae* ISP5230 Culture Medium



grown with the amino acids D-norvaline (DNV), L-norvaline, D-norleucine (DNL), and L-norleucine. Previously, we have shown that both enantiomers of valine, methionine, and phenylglycine were incorporated to form the anticipated jadomycins,[16] although the quantities of products isolated varied depending on the specific amino acid incorporated. Herein we report studies on the isolation of jadomycins DNV and DNL and aspects of their biological activity in cytotoxicity and antimicrobial studies.

RESULTS AND DISCUSSION

A 20 h liquid culture of *S. venezuelae* ISP5230 VS1099[21] grown in a standard maltose, yeast, and malt extract medium was used to inoculate 25 mL Erlenmeyer flasks, containing an optimized minimal medium for jadomycin production as described previously.[22] Cultures were monitored over a two-day period for increasing cell density by absorbance at 600 nm and for production of jadomycin by absorbance at 523 nm (Figure S1). The cultures of *S. venezuelae* ISP5230 grew comparably on all four amino acids, based on the doubling of the cell density over the 30 h time frame. These rates are consistent with the incubation of *S. venzuelae* ISP5230 on other amino acids as the sole nitrogen source.[16,22] We should clarify that the increase in absorbance at 600 nm is not a rigorous or unambiguous evaluation of cellular growth since we have not determined whether cellular materials are recycled or whether the amino acids are metabolized for primary growth. By contrast, there were significant differences with the absorbance values at 523 nm. Cultures grown on L-norvaline and L-norleucine failed to produce any significant colored materials consistent with an inability to produce jadomycins, whereas cultures grown on D-norvaline and D-norleucine were colored strongly, indicating the production of a jadomycin. This was further substantiated by LC-MS/MS, where the anticipated jadomycin analogues were observed in only the cultures containing the D-configured amino acids (Figure 1). The MS/MS fragmentation of the

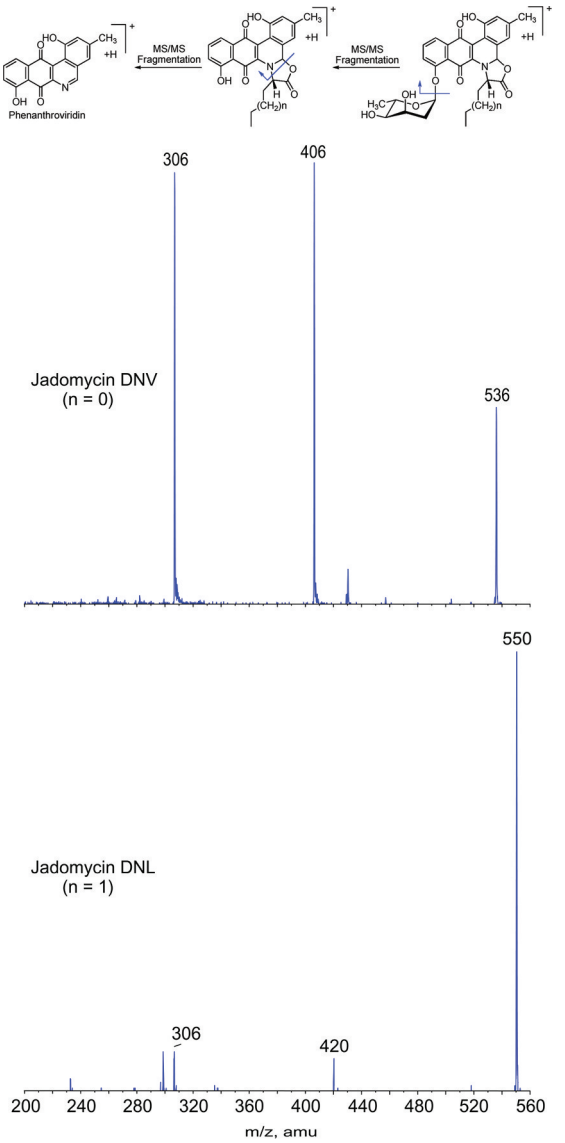


Figure 1. MS/MS fragmentation of jadomycins DNV and DNL. The first fragmentation corresponds to the formation of the aglycone and the second fragmentation to the formation of the phenanthroviridin.

jadomycins is highly self-consistent: the first fragmentation corresponds to the cleavage of the glycosidic linkage and loss of the L-digitoxose moiety, and the second fragmentation step corresponds to the cleavage of the oxazolone ring and formation of phenanthroviridin. The reasons that these two nonproteinogenic L-amino acids were not incorporated remains unclear. It could be due to a lack of uptake of the amino acids by *S. venezuelae* ISP5230 or an indication that the formation of the aldimine intermediate (Scheme 1) may be regulated biosynthetically.

Cultures of *S. venezuelae* ISP5230 VS1099 incubated on D-norvaline and D-norleucine were scaled up to 2 L volume and monitored spectrophotometrically. The products were captured using a reversed-phased C_{18} column, and the eluted material (56 and 84 mg, respectively) was purified via column

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Table 1. Summary of GI_{50}, TGI_{50}, and LC_{50} Values for Jadomycins DNV and DNL in the NCI 60 Cancer Cell Line Screen[a]

		GI_{50} (μM)		TGI (μM)		LC_{50} (μM)	
compound	concentration range (nM–μM)	median (range)	n	median (range)	n	median (range)	n
jadomycin DNV	3.2–32	4.9 (0.6–13.5)	59	10 (1.1–31)	57	19 (2.4–31.3)	41
jadomycin DNL	3.2–32	4.2 (0.5–9.6)	59	8.7 (0.9–26.3)	56	18 (1.8–31.7)	44

[a] GI_{50}, concentration required to inhibit cell growth by 50%; TGI, concentration required to inhibit growth by 100%; LC_{50}, concentration required to cause lethality in 50% of the cells. All values are expressed as median (range), where n = the number of cancer cell lines out of a maximum of 59 in which GI_{50}, TGI_{50}, and LC_{50} were quantifiable below the maximal jadomycin concentration of 32 μM.

chromatography. The products were characterized using UV–vis, IR, LRMS, HRMS, and NMR. The LC-MS/MS was consistent with that observed on the small cultures, and signals in the ^{1}H NMR spectra were assigned using a combination of COSY, 1D TOCSY, and 1D NOE experiments, whereas carbon resonances were assigned using HSQC and HMBC experiments. Jadomycins DNV and DNL were purified as diastereomeric mixtures, with a 3a*S*:3a*R* ratio of 1.67:1 and 1.65:1, respectively. These diastereomeric ratios observed are consistent with those reported for other aliphatic jadomycin derivatives including jadomycin B (with an L-leucine side chain) and jadomycin V (with an L-valine side chain).[16]

Cytotoxicity Assays. Jadomycins DNV and DNL were evaluated by the NCI against six leukemia, nine non-small-cell lung carcinoma, six colon, six central nervous system, nine melanoma, seven ovarian, eight renal, two prostate, and six breast cancer cell lines at five doses spanning a 5 $\log_{10}$ concentration range. At each dose the percent growth and mean optical density were determined according to the protocol described on the NCI Web site.[23] The optical densities were plotted versus jadomycin concentration to obtain GI_{50} (the concentration at which growth inhibition is half its maximal value), TGI (the concentration of test drug that causes total growth inhibition), and LC_{50} (the concentration at which half the cells are killed). The compiled median GI_{50}, TGI, and LC_{50} values for all 59 cell lines are summarized here for each compound (Table 1). Both compounds inhibited the growth of the majority of the 59 cancer cells that were tested. The median GI_{50} and TGI values were in the low micromolar range, indicating similar growth-inhibition potency for the two jadomycins. Within the concentration ranges tested, these same jadomycins demonstrated lethality in either 41 or 44 of the 59 cancer cells, as measured by the LC_{50} values. The GI_{50}, TGI, and LC_{50} of jadomycins DNV and DNL grouped according to cancer cell type are shown in Figure S2, highlighting their general efficacy with respect to growth inhibition and lethality across the different classes of cancer cells. An exception is the leukemia cell lines, all of which were resistant to jadomycin DNV- and DNL-mediated lethality.

Antimicrobial Assays. Jadomycins have been shown to have antibacterial activity against Gram-positive organisms.[24] We screened the compounds against five strains of Gram-positive bacteria (*Staphylococcus aureus* C622, *Staphylococcus aureus* MRSA C623, *Staphylococcus epidermis* C960, *Bacillus subtilis* C971, and *Enterococcus faecalis* C625) and three strains of Gram-negative bacteria (*Salmonella enterica typhimurium* C587, *Escherichia coli* C498, and *Pseudomonas aeruginosa* H187) using Clinical and Laboratory Standard Institutes methods. The activity was measured as the minimum inhibitory concentration (MIC), the lowest concentration of compound that inhibited bacterial growth. Neither of the compounds showed activity against the Gram-negative strains. Both compounds were active against *S. epidermidis* C960 (MIC_{50} 4 μg mL^{-1}) and a clinical MRSA strain (MIC_{50} 32 μg mL^{-1}). It is evident from the data that the presence of the additional methylene unit that distinguishes jadomycins DNV and DNL does not alter the antimicrobial activity of the two jadomycins.

Copper-Mediated DNA Cleavage. Jadomycins DNV and DNL produced no detectable relaxation of supercoiled (form I) plasmid DNA alone (Figure S3). However, in the presence of equimolar $Cu(OAc)_2$, both jadomycins yielded concentration-dependent single-strand (nicked) breaks (form II), as shown in lanes 2–6. The EC_{50} values for jadomycins DNV and DNL were determined to be 86 and 144 μM, respectively.

EXPERIMENTAL SECTION

General Experimental Procedures. All reagents used were purchased from commercial sources and used without further purification. All solvents used were HPLC grade. Glass-backed normal-phase silica gel plates were used throughout (SiliCycle). Preparative TLC plates were 20 × 20 cm and 1000 μm in thickness (SiliCycle). Flash chromatography was performed on a Biotage SP1 unit using prepackaged columns (Biotage, SiliCycle). UV–vis spectra were recorded using a Varian Cary 50 Bio UV–visible spectrophotometer. NMR spectra were recorded using a Bruker Avance 500 instrument (^{1}H at 500 MHz, ^{13}C at 125 MHz) with broadband observe probe at the Nuclear Magnetic Resonance Research Resource (NMR-3), Dalhousie University. Spectra were recorded in $CDCl_3$, MeOD, or D_2O. 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 $CDCl_3$, H_2O at 4.71 ppm in D_2O). Peak assignment was achieved using chemical shifts and peak multiplicities from the proton spectra as well as through the use of ^{1}H–^{1}H COSY and, where noted, 1D TOCSY and 1D NOE experiments. Assignment of the ^{13}C spectra was achieved through HSQC and, where noted, HMBC experiments. Not all ^{13}C resonances could be assigned, despite varying the J value for the HMBC experiments. Low-resolution mass spectra were recorded using electrospray ionization (ESI) on a 2000Qtrap linear ion trap instrument (Applied Biosystems). Samples were scanned in positive mode over a range of 300–700 m/z (Q1) and then in MS/MS mode. High-resolution mass spectra were recorded using ESI on a Bruker Daltonics MicroTOF instrument in positive mode from 50 to 1500 m/z. Samples were run in HPLC grade methanol and blanked against samples of 100% HPLC grade methanol.

Culturing *Streptomyces venezuelae* ISP5230 for Jadomycin Production. *Streptomyces venezuelae* ISP5230 VS1099[21] colonies were grown on MYM-agar plates [maltose (0.4% w/v), yeast extract (0.4% w/v), malt extract (1% w/v), and agar (1.5% w/v)] for two to four weeks. Single colonies were used to inoculate MYM media [4 × 250 mL in four 1 L flasks; maltose (0.4% w/v), yeast extract (0.4% w/v), malt extract (1% w/v); pH 7.0], which was then shaken at 250 rpm at 30 °C for 20 h. The resulting broth was centrifuged at 3750 rpm for 15 min, and the pellet was washed with MSM medium. MSM medium consisted of the following, per liter: $MgSO_4$ (0.4 g), MOPS (1.9 g), salt solution (9 mL of 1% w/v NaCl and 1% w/v $CaCl_2$), $FeSO_4{\cdot}7H_2O$ (4.5 mL of 0.2% w/v), and trace mineral solution (4.5 mL). The trace mineral solution contained the following, per liter: $ZnSO_4{\cdot}7H_2O$ (880 mg), $CuSO_4{\cdot}5H_2O$ (39 mg), $MnSO_4{\cdot}4H_2O$ (6.1 mg), H_3BO_3 (5.7 mg), and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (3.7 mg). Culture media were prepared by dissolving the selected amino acid in 2 L of MSM

media to a final concentration of 60 mM, adjusting the pH to 7.5 with 5 M NaOH, and autoclaving the solution (8 × 250 mL in eight 1 L flasks). Subsequently, glucose (to a final concentration of 33 mM) and phosphate (to a final concentration of 50 μM) were added aseptically, and the *S. venezuelae* pellet slurry was added until the OD_{600} reached 0.6. The culture medium was immediately shocked using 100% ethanol (3% v/v in the medium) and shaken at 30 °C for 48 h until the A_{526} measured between 0.5 and 1.0. Aliquots were taken and read as described previously.[20] The cellular debris was removed from production media by suction filtration through No. 5 filter paper, then 0.45 and 0.22 μm MF filtration disks. The filtered media was passed through a reversed-phase capture C_{18} column (6 × 6 cm; Biotage) that had been preconditioned with HPLC-grade methanol. Water-soluble compounds and media components were eluted using distilled water (until the flow-through was colorless, 10 L), followed by increasing amounts of methanol in water: 10%, 20%, 30%, and 40% (approximately 250 mL each). The desired secondary metabolite was eluted as a deep purple solution at 60% methanol. Solvent was removed *in vacuo* to yield crude secondary metabolite. Thin-layer chromatography using normal-phase silica gel plates (10:90 MeOH/DCM as eluant) provided evidence for the presence of a jadomycin derivative due to the deep burgundy color.

Jadomycin DNV. Cultures were grown using D-norvaline MSM medium (2 L) as described above to yield crude jadomycin DNV (56 mg). This material was loaded onto minimal ISOLEUTEHM-N sorbant using CH_2Cl_2 and purified by normal-phase silica gel flash chromatography (22 cm × 4.0 cm) using a gradient of CH_2Cl_2 to MeOH (50%) at a flow rate of 45 mL min^{-1}. Relevant jadomycin fractions were combined, dried (25 mg), applied to a reversed-phase C_{18} column (15 cm × 3.0 cm), and eluted using a gradient of water to acetonitrile (50%) at a flow rate of 25 mL min^{-1}. Relevant fractions were combined, dried (13 mg), and further purified by preparative TLC (5:95 MeOH/CH_2Cl_2), affording the pure secondary metabolite as a deep purple powder (8.5 mg, 4.3 mg/L), as a mixture of diastereomers (3a*S*/3a*R* = 55:33). R_f = 0.42 (10:90 MeOH/CH_2Cl_2). UV−vis (1.87×10^{-5} M, MeOH): λ_{max} (ε) = 291 (15 812), 315 (16 562), 492 (2680), 529 (2037), 605 (1340), 754 (429). For NMR ($CDCl_3$) chemical shift tables see Tables S1 and S2. LRMS (ESI^+): Q1 found 538 *m*/*z* $[M + Na]^+$, 536 *m*/*z* $[M + H]^+$; MS/MS (536) found 406 $[M + H - \text{digitoxose}]^+$, 306 $[M + H - C_6H_{10}O_2]^+$. HRMS (ESI^+) for $C_{29}H_{29}N_1O_9Na$ $[M + Na]^+$: calcd 558.1735; found 558.1706.

Jadomycin DNL. Cultures were grown using D-norleucine MSM medium (2 L) as described above to yield crude jadomycin DNL (84 mg). The crude material was loaded onto minimal ISOLEUTEHM-N sorbant using CH_2Cl_2 and purified by normal-phase silica gel flash chromatography (22 cm × 4.0 cm) using a gradient of CH_2Cl_2 to MeOH (50%) at a flow rate of 45 mL min^{-1}. Relevant jadomycin fractions were combined, dried (47 mg), applied to a reversed-phase C_{18} column (15 cm × 3.0 cm), and eluted using a gradient of water to acetonitrile (50%) at a flow rate of 25 mL min^{-1}. Relevant fractions were combined, dried (29 mg), and further purified by preparative TLC (5:95 MeOH/CH_2Cl_2), affording the pure secondary metabolite as a deep purple powder (17 mg, 8.5 mg/L), as a mixture of diastereomers (3a*S*/3a*R* = 56:34). R_f = 0.45 (10:90 MeOH/CH_2Cl_2). UV−vis (1.82×10^{-5} M, MeOH): λ_{max} (ε) = 291 (18 425), 315 (20 570), 492 (3025), 529 (2475), 669 (715), 754 (275). For NMR ($CDCl_3$) chemical shifts see Tables S3 and S4. LRMS (ESI^+): Q1 found 572 *m*/*z* $[M + Na]^+$, 550 *m*/*z* $[M + H]^+$; MS/MS (550) found 420 $[M + H - \text{digitoxose}]^+$, 306 $[M + H - C_6H_{10}O_2]^+$. HRMS (ESI^+) for $C_{30}H_{31}N_1O_9Na$ $[M + Na]^+$: calcd 572.1891; found 572.1884.

Copper-Mediated DNA Cleavage Assays. Supercoiled plasmid (form I) was prepared by transformation of NovaBlue cells (Novagen) followed by purification using the QIAprep Spin miniprep kit (Qiagen) to yield approximately 30 μg of plasmid DNA per 20 mL culture. Jadomycins DNV and DNL were dissolved initially in 95% EtOH, and subsequent dilutions were made with water (distilled, deionized Milli-Q), where the final assay tubes contained <1% EtOH. Reaction mixtures (20 μL total volume) were prepared in 0.5 mL sterile microfuge tubes. Transformed pUC19 plasmid (final concentration 130 ng, or 20 μM bases, >95% form I) was delivered to the assay tubes as a solution in 10 mM Tris-Cl (pH 8.5) and diluted with Tris (pH 7.4, final concentration 5 mM) and NaCl (final concentration 50 mM). Solutions of the jadomycins, premixed 1:1 with $Cu(OAc)_2$ where appropriate, were added to give the final concentrations, and the reaction mixtures were diluted to a final volume of 20 μL with ultrapure water. Reaction mixtures were incubated at 37 °C for 2 h. All samples were quenched by the addition of gel loading buffer (4 μL), loaded onto 1% agarose gels containing ethidium bromide (0.75 μg mL^{-1}), and electrophoresed for 30 min at 8 V cm^{-1} in 1 × TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2). The bands were visualized with UV transillumination (UVP transilluminator) using the Gel-Doc-It Imaging system (UVP) or GNU Image Manipulation Program (GIMP). The EC_{50} values were fitted using Kaleidagraph.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Three figures (bacterial growth, cytotoxicity data, and DNA cleavage) and four tables of assigned NMR data and ^1H, COSY, HMBC, and HSQC spectra for both jadomycins. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: + 1 902 494 7159. E-mail: david.jakeman@dal.ca.

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