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Investigations into the binding of jadomycin DS to human topoisomerase IIβ by WaterLOGSY NMR spectroscopy

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General Methods

All reagents were purchased from commercial sources and used without further purification. All solvents used for reactions and chromatographic methods were HPLC grade. Flash chromatography was performed on a Biotage SP1™ unit (Biotage®) using pre-packed 80 g normal phase silica columns from Silicyle®. Thin layer chromatography (TLC) plates used for monitoring reactions and calculating R_f values were glass backed, normal phase silica plates (250 μm thickness) purchased from Silicycle®. Compounds **3** and **4** did not require chemical or ultraviolet (UV) visualization, since they are coloured compounds. Compound **5** was prepared following literature protocol.[1] Preparative TLC plates used for purification were glass backed (20 cm × 20 cm) normal phase silica plates (1000 μm thickness) purchased from Silicycle®. Preparative TLC was conducted using a specified solvent system to develop the plate. The plate was then removed from the solvent, allowed to fully air-dry, then re-run in the same solvent system. This was repeated in order to achieve better separation. Once sufficient separation had been achieved the silica was scraped off of the plate, and the band of interest eluted using the same solvent system used for development. Size exclusion chromatography was carried out using Sephadex™ LH-20 resin (GE Healthcare). All compounds were characterized using high-performance liquid chromatography (HPLC, method outlined below), liquid chromatography tandem mass spectrometry (LC-MS/MS, method outlined below), high-resolution mass spectrometry (HRMS), and 1D- and 2D- nuclear magnetic resonance (NMR, method outlined below) spectroscopy experiments.

Jadomycin Production Methods

Preparation of MYM growth media

MYM growth media was prepared by addition of maltose (8 gL^{-1}), yeast extract (4 gL^{-1}), and malt extract (10 gL^{-1}) to one-litre of distilled, deionized water (ddH_2O). The pH was adjusted to 7.0 with NaOH (5 M) and HCl (5 M) as necessary.

Preparation of MSM production media

MSM production media was prepared by addition of $MgSO_4$ (0.8 g), MOPS (7.54 g), salt solution (18 mL), trace mineral solution (9 mL), and 0.2% w/v $FeSO_4 \cdot 7H_2O$ solution (9 mL) to two-litres ddH_2O. The pH was adjusted to 7.5 by NaOH (5 M) or HCl (5 M) as necessary. The salt solution was made by addition of NaCl (1%, w/v) and $CaCl_2$ (1%, w/v) to ddH_2O. The trace mineral solution was made by addition of $ZnSO_4 \cdot 7H_2O$ (0.088%, w/v), $CuSO_4 \cdot 5H_2O$ (0.0039%, w/v), $MnSO_4 \cdot 4H_2O$ (0.00061%, w/v), H_3BO_3 (0.00057%, w/v), and $(NH_4)Mo_7O_{24} \cdot 4H_2O$ (0.00037%, w/v) to ddH_2O.

General Jadomycin Production Methods

Streptomyces venezuelae ISP5230 VS1099 cultures were grown and stored on MYM agar [MYM growth media with 15 gL^{-1} agar and 50 μgmL^{-1} aparmycin] incubated at 30°C for a period of two to four weeks. MYM growth media were inoculated with a loop of cells

(1 cm × 1 cm) and incubated for a period of 16 – 24 hours with shaking (250 rpm) at 30°C. The growth was stopped when the cells were loosely packed and fibrous under a microscope. Once the initial growth period was complete, the bacteria were pelleted (8500 rpm, 4°C) and the supernatant decanted. The cell pellet from the 1 L MYM cultures was re-suspended and washed with approximately 100 mL of MSM solution. This process was repeated twice to ensure removal of all traces of MYM. The cells were then re-suspended in minimal MSM solution (~30 mL).

The production media was prepared by addition of the corresponding amino acid to the MSM solution [30 mM 4-amino-L-phenylalanine, 60 mM D-serine, or 60 mM L-asparagine], then adjusting the pH to 7.5 using NaOH (5 M) and HCl (5 M) as required, and autoclaving the solution. After sterilization, a separately filter sterilized 30% glucose solution (20 mLL^{-1}) and a separately autoclaved 9 mM phosphate solution (5.4 mLL^{-1}) were added to the MSM production media. The re-suspended *S. venezuelae* was then added to the production media to an absorbance at 600 nm (Abs_{600}) of 0.6. The production media was immediately ethanol shocked (30 mLL^{-1}) to induce jadomycin production. The media was incubated with shaking (250 rpm) at 30°C while being monitored *via* Abs_{600}, Abs_{526}, and high-performance liquid chromatography (HPLC). At 24 hours the pH was readjusted to 7.5 using NaOH (5 M) or HCl (5 M) as required.

Once the production period had finished, the cells were removed *via* filtration using Whatman #5 filter paper, followed by 0.45 μm and 0.22 μm Millipore filters. The production media was then passed through an 80 g phenyl column (Silicycle®) that had been pre-conditioned using 100% methanol followed by distilled, deionized water. The material was loaded and washed with distilled, deionized water (6-8 L for a 2 L production) with the crude natural product being eluted off of the column using 100% methanol (~250 mL for a 2 L production). The solvent was then removed *in vacuo*. The presence of the natural product was checked by HPLC and LC-MS/MS. Purification was continued as outlined in the respective section.

Production using D-serine

Jadomycin DS (**3**) has been previously reported,[1] but was re-isolated for this study using the following conditions.

Following the phenyl column, the crude natural product was further purified using an 80 g silica column pre-conditioned with dichloromethane (DCM). The material was eluted using a 30 mL/min flow rate collecting 9 mL fractions. Purification was accomplished using a linear gradient system from 0% to 100% methanol in DCM over 50 CV. Fractions were analyzed by TLC, and those containing the compound of interest were combined and dried *in vacuo*. The crude material was brought up in minimal methanol in DCM and run on a preparative TLC (10% methanol in DCM) as previously described. Solvent was then removed *in vacuo*. A second preparative TLC was run (5:5:1 CH_3CN:EtOAc:H_2O) as previously described. Solvent was then removed *in vacuo*. Final purification was accomplished using a Chelex column, eluting with methanol. The solvent was then dried *in vacuo* yielding 101.4 mg of **3** (50.7 mgL^{-1}) as a mixture of diastereomers (Mj:Mn 100:75) by ^{1}H-NMR.

Human Topoisomerase IIβ Overexpression and Isolation Methods

The overexpression and isolation of hTopIIβ were conducted following literature procedure.[2] hTopIIβ was stored in phosphate buffered saline (PBS, pH 7.6) at -70°C in 50 μL aliquots.

β-Phosphoglucomutase Overexpression and Isolation Methods

The overexpression and isolation of β-Phosphoglucomutase were conducted following literature procedure. [3]

Bovine Serum Albumin

BSA was bought from Sigma Aldrich

Cps2L Overexpression and Isolation Methods

The overexpression and isolation of cps2L were conducted following literature procedure.[4]

DesR Overexpression and Isolation Methods

The overexpression and isolation of DesR were conducted following literature procedure.[5]

WaterLOGSY Methods [6, 7]

NMR analysis of jadomycin DS was recorded using a Bruker AV-III 700 MHz Spectrometer (^{1}H: 700 MHz, ^{13}C: 176 MHz) equipped with an ATMA 5 mm TCI cryoprobe located at the Canadian National Research Council Institute for Marine Biosciences (NRC-IMB) in Halifax, Nova Scotia. All spectra were recorded in a mixed solvent system (see below) and calibrated to residual solvent peaks (DOH: 4.71 ppm).

Jadomycin DS WaterLOGSY Experiments

Jadomycin DS samples for WaterLOGSY NMR experiments were prepared by varying the concentration of **3** in the presence or absence of constant human topoisomerase IIβ (Table S1). Both **3** and human topoisomerase IIβ were brought up in phosphate buffered saline (PBS, pH 7.6). PBS was used to prevent breakdown of the natural product during the experiment. Both deuterated and regular PBS must be used during the experiment to allow the spectrometer to lock on a signal and to allow transfer of magnetization from the bulk solvent to the enzyme, respectively. The samples also contain 10% DMSO-d_6 to prevent aggregation of the jadomycin DS. Aggregation is detrimental to the experiments because it leads to positive signal in the WaterLOGSY NMR spectrum, or binding signal, even when no enzyme is present for **3** to bind to (data not shown).

Table S1. Concentrations and volumes used during jadomycin DS WaterLOGSY NMR experiments.

[Jadomycin DS] (mM)	[Top IIβ] (mM)	Deuterated PBS (μL)	Regular PBS (μL)	DMSO-d_6 (μL)
0.50	0.02	60	480	60
0.75	0.02	60	480	60
1.00	0.02	60	480	60
1.25	0.02	60	480	60
1.50	0.02	60	480	60
1.75	0.02	60	480	60
2.00	0.02	60	480	60
2.25	0.02	60	480	60
3.33	0.02	60	480	60
1.33	0	60	480	60
2.00	0	60	480	60
2.66	0	60	480	60
3.33	0	60	480	60

The peaks corresponding to protons at positions 3a, 9, 10, 11, 1″, and 5″-CH_3 were chosen for analysis because they were far enough from the water signal to be reliably analyzed. The analysis of the WaterLOGSY spectra was done by determining the orientation of each proton signal in the spectrum, then specifically phasing each peak to obtain a reliable intensity. In order to correct for the fact that there will be some non-binding ligand in the presence of enzyme, leading to dampened positive signal, controls were run without enzyme (Table S1). This negative non-binding signal is then subtracted from the positive binding signal to give a corrected value. These values are then plotted as a function of jadomycin DS concentration in order to generate binding curves, which can be analyzed using regression analysis. This was done in order to obtain all dissociation constants presented (Figure S1–S7)

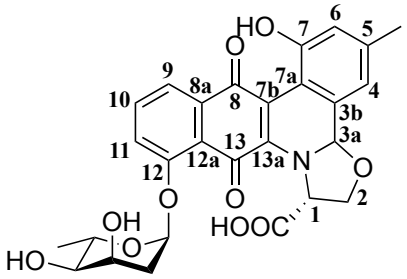

Figure S1. Fully labeled structure of jadomycin DS (**3**).

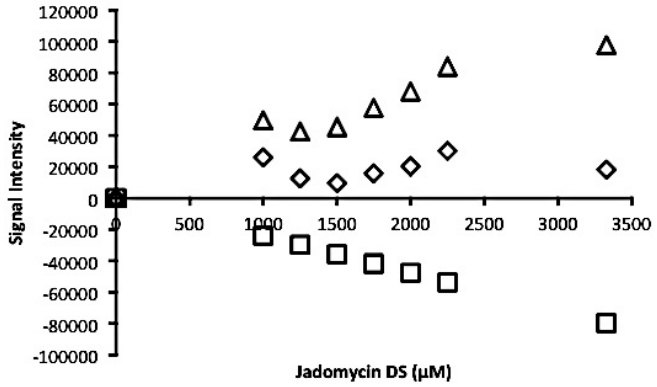

Figure S2. Compiled WaterLOGSY NMR K_D determination data compiled for 3aH showing the observed binding curve (♢), the negative standard curve (□), and the corrected values (Δ).

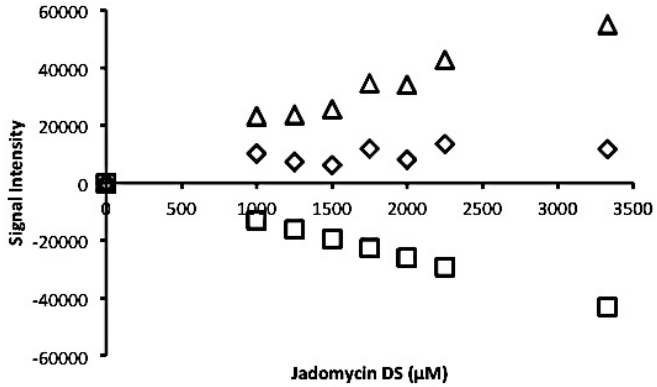

Figure S3. Compiled WaterLOGSY NMR K_D determination data compiled for 9H showing the observed binding curve (♢), the negative standard curve (□), and the corrected values (Δ).

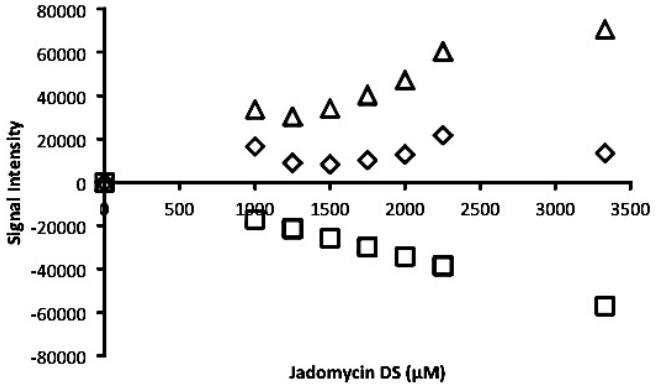

Figure S4. Compiled WaterLOGSY NMR K_D determination data compiled for 10H showing the observed binding curve (◊), the negative standard curve (□), and the corrected values (Δ).

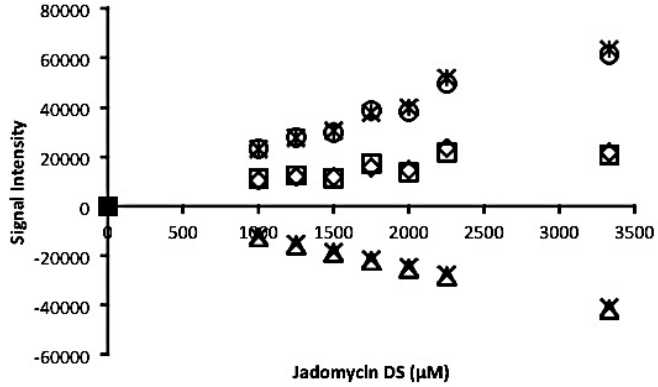

Figure S5. Compiled WaterLOGSY NMR K_D determination data compiled for 11H showing the observed binding curve for the left signal (◊) and right signal (□), the negative standard curve for the left signal (Δ) and the right signal (×), and the corrected values for the left signal (∗) and the right signal (O).

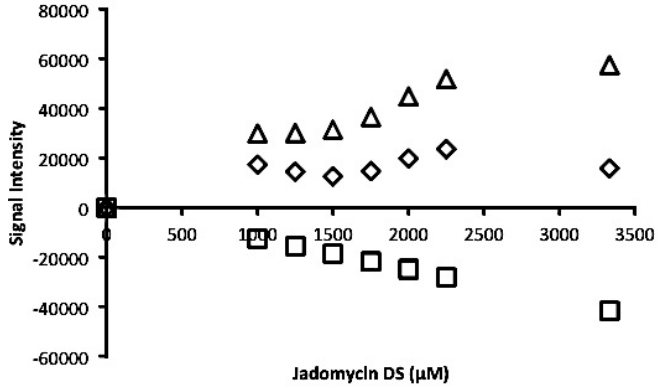

Figure S6. Compiled WaterLOGSY NMR K_D determination data compiled for 1″H showing the observed binding curve (◊), the negative standard curve (□), and the corrected values (Δ).

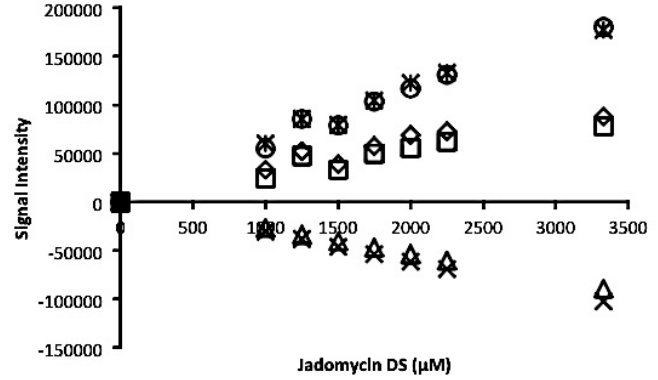

Figure S7. Compiled WaterLOGSY NMR K_D determination data compiled for 5″-CH_3 showing the observed binding curve for the left signal (◊) and right signal (□), the

negative standard curve for the left signal (Δ) and the right signal (×), and the corrected values for the left signal (∗) and the right signal (O).

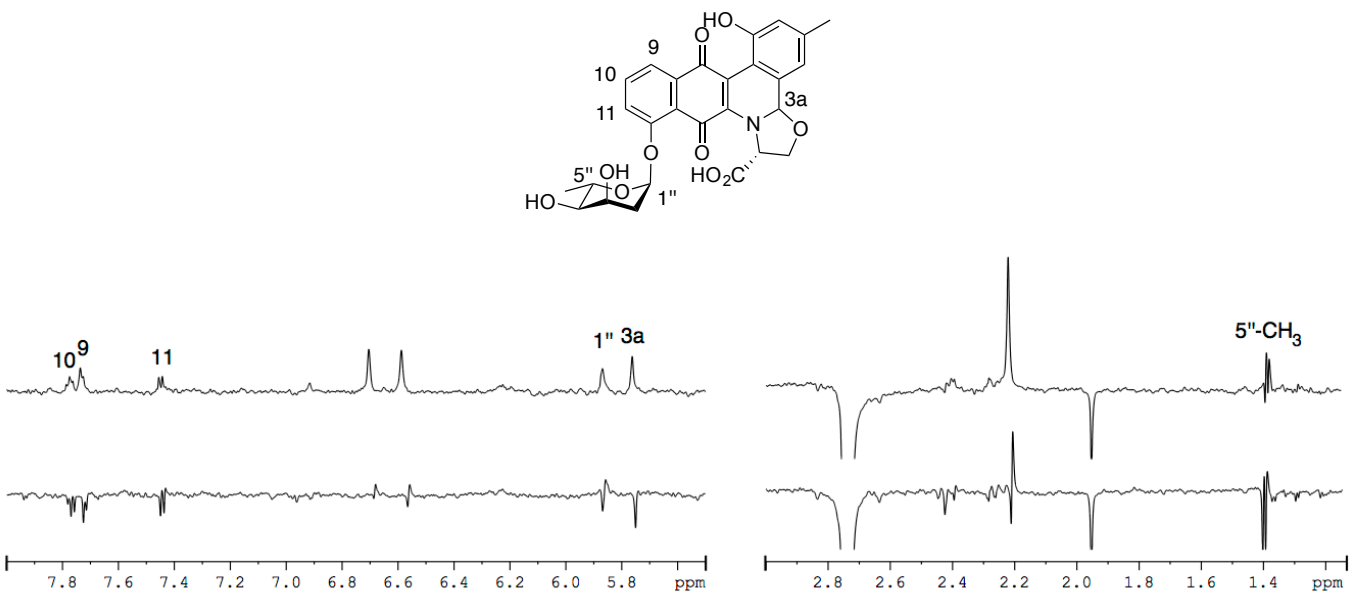

Figure S8. WaterLOGSY spectra of jadomycin DS (2 mM) in the presence of hTopIIβ (0.02 mM, top) and in its absence (bottom), highlighting protons used for K_D determination (^{1}H: 700 MHz).

References

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