Investigations into the Biosynthesis, Derivatization, and Purification of Jadomycins

by

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DEDICATION PAGE

To Science and Soccer

Para la Ciencia y el Fútbol

&

For everyone celebrating their un-birthday

Para todos aquellos que estén celebrando su no-cumpleaños

Para tod@s todo

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ABSTRACT

Nature produces many clinically used medicines in the form of natural products. These compounds can be isolated from a variety of sources, but bacteria have been shown to be the most prolific source. The strain *Streptomyces* has been thoroughly investigated for such natural products, with *Streptomyces venezuelae* ISP5230 showing the ability to produce the clinically used antibiotic chloramphenicol, as well as the jadomycins, a family of secondary metabolites. These secondary metabolites are produced through a biosynthetic pathway where the incorporation of the amino acid into the jadomycin structure is likely non-enzymatic. This allows for jadomycins to be readily derivatized, where upwards of twenty-five derivatives have been previously isolated. This work presents the amplification of the jadomycin library through the production of novel jadomycins, as well as their further diversification through the use of synthetic derivatization. The study of jadomycins is not only important chemically, but also biologically because they have been shown to possess anti-cancerous properties. For this reason, the two synthetic derivatives were assessed for biological activities and their results are discussed herein. Investigations were also carried out to develop a method to assess biological activity by nuclear magnetic resonance, and are presented. Finally, investigations into the jadomycin purification methodology were carried out using JadX, a potential regulatory protein in the jadomycin biosynthesis, and are discussed.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ε	molar absorptivity
λ_{max}	wavelength of maximal absorbance
Abs	absorbance
amu	atomic mass unit
APS	ammonium persulfate
ATCC	American type culture collection
ATMA	auto-tune and match
AU	arbitrary units
BBFO	broadband observe
BHI	brain heart infusion
bm	broad multiplet
br	broad
bs	broad singlet
CD_2Cl_2	deuterated dichloromethane
CD_3OD	deuterated methanol
CD_3OH	partially deuterated methanol
$CDCl_3$	deuterated chloroform
CFU	colony-forming unit
CH_3CN	acetonitrile
CH_3OH	methanol
Co	co-spot
COSY	correlation spectroscopy

CV	column volume
d	doublet
D_2O	deuterated water
DCC	*N*,*N*′-dicyclohexylcarbodiimide
DCM	dichloromethane
dd	doublet of doublets
ddH_2O	distilled, deionized water
dH_2O	distilled water
DMSO-d_6	deuterated dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
EPI	enhanced product ion
ESI	electrospray ionization
EtOAc	ethyl acetate
EtOH	ethanol
FPLC	fast protein liquid chromatography
H_2O	water
HCl	hydrochloric acid
His_6-tag	six-histidine tag
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry

HSQC	heteronuclear single-quantum correlation spectroscopy
hTopIIβ	human topoisomerase IIβ
I	observed signal intensity
I_{max}	maximal signal intensity
IPTG	isopropyl β-D-1-thiogalactopyranoside
ISP	international cooperative project for description and deposition of type cultures of *Streptomyces*
K_d	dissociation constant
KH_2PO_4	monopotassium phosphate
L	ligand concentration
LB	lysogeny broth
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LRMS	low-resolution mass spectrometry
m	multiplet
m/z	mass to charge
MeOH	methanol
Mj	major
Mn	minor
MOPS	3-(*N*-morpholino)propanesulfonic acid
MS/MS	tandem mass spectrometry
MSM	magnesium sulfate and MOPS containing production medium
MWCO	molecular weight cut-off
MYM	maltose, yeast extract, and malt extract containing growth medium

n-Bu_4NBr	tetra-*n*-butylammonium bromide
NaCl	sodium chloride
$NaH_2PO_4 \cdot H_2O$	sodium phosphate monobasic
NaOH	sodium hydroxide
NCI	national cancer institute
NCTC	national collection of type cultures
NH_4Cl	ammonium chloride
NMR	nuclear magnetic resonance
NMR-3	nuclear magnetic resonance research resource
NOESY	nuclear Overhauser effect spectroscopy
NRC-IMB	Canadian national research council institute for marine biosciences
OD	optical density
PBS	phosphate buffered saline
PKS	polyketide synthase
ppm	part per million
R_f	retention factor
ROESY	rotating-frame nuclear Overhauser effect
rpm	revolutions per minute
R_t	retention time
RT	room temperature
Rxn	reaction
s	singlet
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis

sec	seconds
SM	starting material
t	triplet
TAE	buffer solution containing Tris base, acetic acid, and EDTA
TEMED	tetramethylethylenediamine
TIC	total ion chromatogram
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
Tris	tris(hydroxymethyl)aminomethane
Tris-Cl	Tris-chloride
TSA	tripticase soy agar
TSB	tryptic soy broth
USA	United States of America
UV-Vis	ultra-violet-visible
WaterLOGSY	water-ligand observed via gradient spectroscopy

GLOSSARY

Biosynthetic pathway	The enzymatic reactions that lead to the production of a natural product
Gene cluster	A collection of genes that work toward a common goal, typically this involves the production of a natural product. These genes code for the enzymes used in the biosynthetic pathway.

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CHAPTER 1: INTRODUCTION

Naturally sourced medicines have been used for thousands of years.[1-4] They have had various applications, and can still be found in use today.[1,3-5] These medicines are generally prepared using whole plants, or large portions thereof.[1] However, modern science has strived to isolate the active ingredient from these natural sources.[3] The active ingredient comes in the form of chemical compounds known as natural products.

Natural products are produced by plants, fungi, and bacteria, and have long been responsible for many drugs and antibiotics.[4,6-10] Between 1981 and 2010, 26% of all newly approved drugs were natural products or their derivatives.[8] Of the cancer drugs approved within the same period, 34% were natural products or their derivatives, highlighting their importance within this drug category.[8] Because natural products are of such importance for medicine, their discovery and isolation has become paramount. In order to find novel natural products, researchers have begun investigating both bacterial and marine sources.[2,4,7,10] Both represent areas of research that remain relatively unexplored, have produced clinically relevant drugs, and present the greatest probability of discovering a potential new drug molecule.[2,4,7,10]

Natural products are not only important for medicine, but also present interesting chemistry. They often possess great structural diversity, with some containing structural features that have not been previously isolated or synthesized.[2,4,6,7,9-13] This structural diversity is important because it could impart different bioactivities to the isolated compounds if they are being used for medicinal research, or provide a novel synthetic target molecule, allowing for new synthetic techniques to be developed.[12,14-16] These new techniques are needed to synthesize natural products that contain synthetically

challenging scaffolds, such as macrocyclic ring systems or several stereocentres.[11,12,14,15] These structurally diverse, and often complicated, natural products are produced in nature through a variety of different biosynthetic pathways.[10,13- 15]

Biosynthesis is the stepwise process by which nature assembles compounds within living organisms.[10] This is done through the use of various specified enzymes within the organism, which systematically alter primary metabolites until a final natural product is produced.[10,13] Each of these biosynthetic pathways can produce a separate class of natural products including, polyketide synthases, non-ribosomal peptide synthases, and terpenes, among others.[10,13] The process of producing these natural products requires the organism to expend energy, which is done either because the natural product is a primary metabolite and necessary for the survival of the organism, or because it is a secondary metabolite and necessary for the defense of the organism from environmental stressors.[3,6,9,10,17] The natural products that will be presented herein are all secondary metabolites.

Secondary metabolites are non-essential for the survival of the organism, and only produced under stress conditions.[6,9,10,17] In nature, such conditions may include nutrient depletion or competition from an invasive organism.[3,6,10,17] When either of these situations are encountered, the secondary metabolite is produced and expelled from the organism into its immediate vicinity as a defense mechanism in order to ensure its survival.[3,6,10,17] These stress conditions can be replicated in a laboratory setting through nutrient depletion, heat or ethanol shock, or phage injection to induce production of a desired natural product. Such techniques can be used when chemical synthesis is unreasonable. Many natural product syntheses are still plagued by low yields over

numerous steps, and as such, the biosynthetic route is a more appealing alternative in some instances.[16] It is this latter route that is used here.

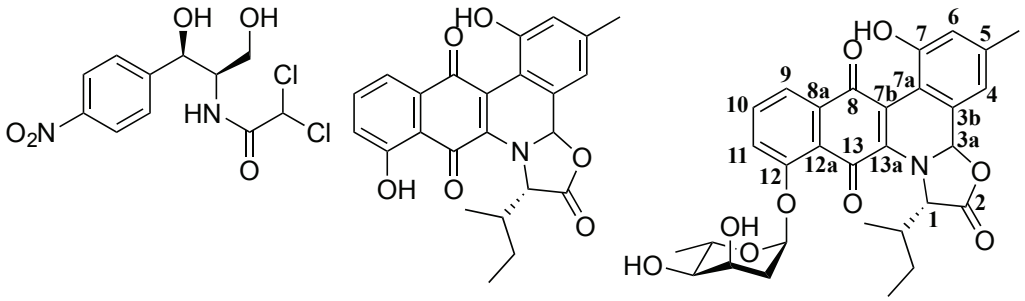
While many natural products have been discovered through their isolation from unique organisms, this is not the only method currently used to find these compounds. With advancements in genomic sequencing, genome mining has become a powerful tool for identifying organisms that could produce novel natural products.[4,9,13,18-22] An organism's genomic sequence is divided into gene clusters, which are collections of genes that work toward a common goal, such as the production of a natural product.[13,18,19,21,22] The organism's genomic sequence can then be analyzed using a program that identifies gene homology between these gene clusters and those of known natural product producers.[20-22] Using this approach, it is possible to determine what types of natural products an organism may be able to produce.[4,13,18-22] This provides the natural product chemist with the ability to selectively search for novel natural products. These may have been traditionally missed because these gene clusters were dormant, cryptic, or produced natural products that were missed because researchers screened them for a specific bioactivity.[18] Once a suitable organism has been selected that may produce a novel natural product, the natural product chemist may alter the growth conditions of the organism in an attempt to produce the natural product classes of interest. Genome mining may, therefore, be the future of natural product chemistry. This process may allow for the quick identification and isolation of novel natural product classes from both known and newly discovered organisms.

Once a novel natural product class has been discovered, it may be possible to biosynthetically alter its structure through the process of precursor-directed

biosynthesis.[23] This approach allows the natural product chemist to quickly generate novel natural products by altering the organism's growth conditions.[23] Precursor-directed biosynthesis relies on the organism's ability to uptake different reactants/primary metabolites and incorporate them into the natural product structure.[23] This has been previously shown to be an adequate route to obtaining novel natural products,[23] and is the approach that will be presented here.

1.1 Jadomycin Introduction

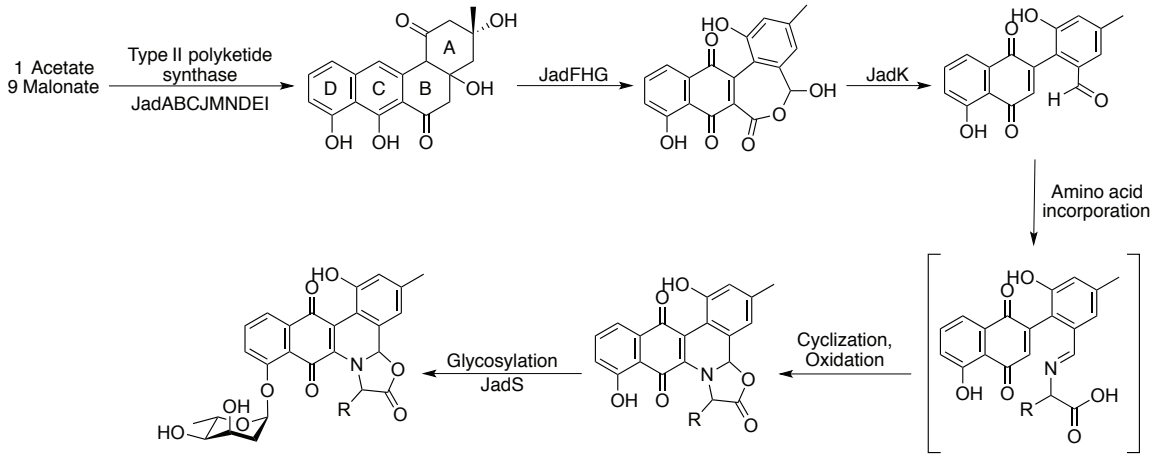
Although natural products are produced by a variety of different sources, bacteria have long been a prolific source of new drugs, with the genus *Streptomyces* responsible for 32% of all known bioactive metabolites.[24-26] One species, *Streptomyces venezuelae* ISP5230 (ATCC 10712), has been studied for its ability to produce a broad-spectrum antibiotic, chloramphenicol, which is widely used in the developing world (Figure 1).[27] While investigating chloramphenicol production conditions, the bacteria were inadvertently heat shocked, causing them to produce a family of secondary metabolites, known as the jadomycins.[28] This serendipitous discovery lead to the isolation and characterization of jadomycin A,[28] followed by the glycosylated jadomycin (jadomycin B)[29] (Figure 1). Since their initial discovery, upwards of twenty-five jadomycins have been isolated and characterized through the use of various laboratory stress conditions that include nutrient deprivation, ethanol shock, and heat shock.[30-32] The ability of the bacteria to produce a variety of jadomycins arises from a crucial step in the jadomycin's biosynthetic pathway.



Chloramphenicol Jadomycin A Jadomycin B

Figure 1. Structures of chloramphenicol, jadomycin A, and jadomycin B.

Jadomycins are produced *via* a unique type II polyketide synthase (PKS) biosynthetic pathway (Scheme 1).[33,34] Once the type II PKS enzymes have formed the jadomycin polyaromatic backbone, JadG causes the B-ring to open and form an oxepinone intermediate, *via* a Baeyer-Villiger oxidation (Scheme 1).[35-37] JadK then causes the seven-membered oxepinone ring to open and expose a reactive aldehyde intermediate (Scheme 1).[34,38] This newly formed intermediate can then react non-enzymatically with the sole amino acid in the production media to form an imine intermediate, which then spontaneously cyclizes to fully incorporate the amino acid within the jadomycin scaffold (Scheme 1).[35,38-42] Finally a glycosyl transferase, JadS, glycosylates the jadomycin aglycone with a rare 2,6-dideoxysugar, L-digitoxose, to yield the jadomycin of interest (Scheme 1).[34,35,43]



Scheme 1. Biosynthesis of jadomycins.

The non-enzymatic step of amino acid incorporation into the jadomycin structure is crucial for the production of novel jadomycins. Through the exploitation of this step, it has been possible to incorporate several different amino acids into the jadomycin structure, thereby yielding novel jadomycins.[30-32] This biosynthetic step has been deduced to be non-enzymatic due to a number of reasons. As of yet, no candidate enzymes have been identified to catalyze this step.[40] This was determined through the use of successive deletion mutants of the soil bacterium where various genes were systematically deleted in the hope of inhibiting jadomycin production. The genes necessary for amino acid incorporation were never identified. Additionally, amino acids placed within the production media as the sole nitrogen source for the bacteria become incorporated into the jadomycin structure without stereochemical alteration to the amino acid.[30-32] This was initially discovered using L-isoleucine in the bacterial production media, which produced jadomycin L-isoleucine (jadomycin B),[29] showing that the stereochemistry of the amino acid was preserved. The non-enzymatic nature of this step has also been deduced from the fact that jadomycins exhibit two diastereomers at the 3a position (Figure 1).[38,40,44] This implies that the amino acid does not necessarily add from one face of the jadomycin

backbone as would be expected in the case of an enzyme-catalyzed step as enzymes typically contain defined active sites where the components are added in a specific fashion. Synthetic studies have also suggested that the incorporation of the amino acid proceeds non-enzymatically, where spontaneous amino acid incorporation was observed during synthetic studies of the jadomycins.[45,46] Finally, as mentioned above, over twenty-five different amino acids, both proteinogenic and non-proteinogenic, have been successfully incorporated into the jadomycin structure.[30-32] This suggests a non-enzymatic addition because an enzymatic process would be expected to show greater specificity. The fact that so many amino acids have been successfully incorporated into the jadomycin structure demonstrates that this process is likely non-enzymatic.

The biosynthesis of jadomycins is still the preferred method of producing the natural products because their synthesis is challenging and low-yielding.[45,46] The first total synthesis of jadomycin was only accomplished in 2013.[46] Previously, the total synthesis was hampered by the addition of the L-digitoxose moiety.[45] Biosynthesis is also preferred because the bacteria have been shown to be capable of uptaking a variety of amino acids and incorporating them into the jadomycin structure. However, the bacteria do not uptake all amino acids presented to them, as will be discussed in Chapter 2.

Analytical techniques are important to determine whether the bacteria have successfully incorporated the amino acid into a jadomycin structure. These techniques include ultra-violet-visible (UV-Vis) spectroscopy,[30,32,38,47,48] high-performance liquid chromatography (HPLC),[42,47,48] and tandem mass spectrometry (LC-MS/MS).[30,31,38,42,47-50] UV-Vis spectroscopy is used to monitor both the cell growth, at 600 nm (OD_{600}),[30,32,38,47,48] and the production of coloured compounds, at 526 nm

(Abs_{526}),[32,38,47,48] where these coloured compounds are indicative of jadomycin production. HPLC is used to monitor the presence and purity of a potential jadomycin within the production media.[42,47,48] LC-MS/MS is an important technique for monitoring whether a jadomycin has been successfully produced. Jadomycin production is monitored using an enhanced product ion (EPI) scan, looking for the characteristic jadomycin fragmentation pattern.[31,42,47-50] The majority of jadomycins that have been previously isolated exhibit signals for the parent ion, the aglycone, and phenanthroviridin (Figure 2).[31,42,47-50] LC-MS/MS is, therefore, a useful tool to monitor both the production and isolation of jadomycins, and has even identified the presence of certain jadomycins that are not produced in isolatable quantities.

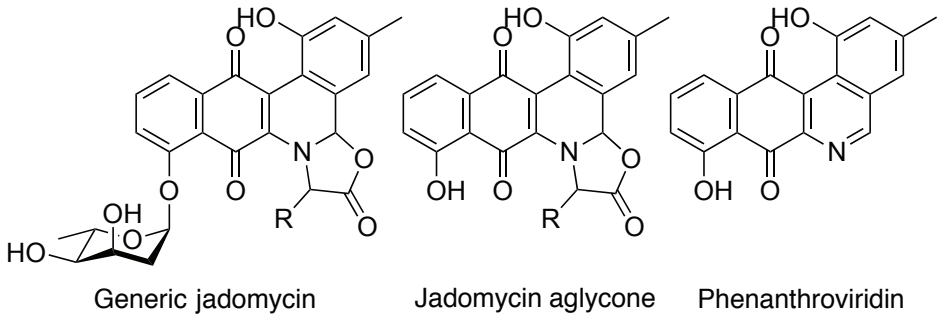


Figure 2. Structures of the typical fragmentation observed during tandem mass spectrometry analysis of jadomycins, where "R" represents the side chain of the amino acid being incorporated.

Natural product isolation is often hampered by a low abundance of the compound of interest. Jadomycins are no exception. In order to produce higher quantities of the jadomycin, a mutant strain of the soil bacterium is used, *Streptomyces venezuelae* ISP5230 VS1099.[47,48,51] This mutant has had the *jadW*$_2$ gene disrupted, a gene which is involved in the regulation of jadomycin production.[52] With this gene disrupted, the mutant strain of the bacteria is able to produce five to ten times higher concentrations of

the jadomycin of interest.[52] For this reason, *S. venezuelae* ISP5230 VS1099 was used throughout these studies.

Although the investigations presented here pertain to the production of jadomycins, these are not the only natural products that *S. venezuelae* ISP5230 may produce. Analyzing the genomic sequence of *S. venezuelae* ISP5230 reveals the presence of a number of potentially dormant or cryptic gene clusters, which were compared to known natural product producing organisms using the genome mining software antiSMASH 2.0.[21,22] The analysis showed that there were thirty-one gene clusters within the soil bacterium, of which only the chloramphenicol[53] and jadomycin[28] biosynthetic gene clusters have been thoroughly studied. The remaining gene clusters have the potential to produce novel natural products, whose classes are presented in Table 1. While investigations into jadomycins present the possibility of producing novel jadomycin analogues, investigations into these unexplored gene clusters could yield whole families of novel natural products. Therefore, the investigation of these gene clusters presents a potential future avenue of research for *Streptomyces venezuelae* ISP5230.

Table 1. Classes of natural products made by *Streptomyces venezuelae* ISP5230, as predicted by antiSMASH 2.0 software.

Class of Natural Product	Number of Gene Clusters That Produce the Natural Product
Type II Polyketide Synthase (PKS)	1
Type III PKS	2
Non-Ribosomal Peptide Synthase	3
Terpenes	4
Lantibiotics	1
Bacteriocins	3
Siderophores	3
Ectoines	1
Butyrolactones	2

Indoles	1
Melanins	2
Thiopeptides	1
Others (Not Specified)	4
Combinations of One or More	3

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CHAPTER 2: ISOLATION, SYNTHETIC DIVERSIFICATION, AND BIOLOGICAL EVALUATION OF JADOMYCIN 4-AMINO-L-PHENYLALANINE

Excerpts of this section were taken from Martinez-Farina, C. F.; Robertson, A. W.; Yin, H.; Monro, S.; McFarland, S. A.; Syvitski, R. T.; Jakeman, D. L. *Submitted to J. Nat. Prod.* **2014**. Manuscript ID: np-2014-009398[1]

2.1 Introduction

Due to the non-enzymatic biosynthetic incorporation of the amino acid into the jadomycin structure, it appears as though any amino acid can be incorporated into the jadomycin structure. However, this is not the case. Many proteinogenic and non-proteinogenic amino acids have been attempted, without success. These have included D-cysteine,[2] L-cysteine,[2] and L-proline.[3] This indicates that although the biosynthetic step of amino acid incorporation is non-enzymatic, its scope is not fully understood. Furthermore, the bacteria will not necessarily use amino acids that are structurally similar to those that have been previously isolated. It was hypothesized that analogues of L-phenylalanine may be successfully incorporated into the jadomycin structure because jadomycin L-phenylalanine had been successfully isolated[4] and 4-aminophenylalanine had been shown to be an intermediate in the *Streptomyces venezuelae* ISP5230 chloramphenicol biosynthesis.[5,6] To investigate this, the following phenylalanine analogues were investigated; 4-amino-D-phenylalanine (this work), 4-amino-L-phenylalanine (this work), 4-bromo-D-phenylalanine,[1] 4-bromo-L-phenylalanine,[1] 4-

chloro-D-phenylalanine,[1] 4-chloro-L-phenylalanine,[1] 4-cyano-D-phenylalanine (this work), 4-cyano-L-phenylalanine (this work), 2,4-dichloro-D-phenylalanine,[1] 3,4-dichloro-D-phenylalanine,[1] and 3,4-dichloro-L-phenylalanine[1] (Figure 3). Not all of the phenylalanine analogues attempted were successfully incorporated into the jadomycin structure. The attempted productions and isolations of jadomycin 4-amino-D-phenylalanine, jadomycin 4-amino-L-phenylalanine, jadomycin 4-cyano-D-phenylalanine, and jadomycin 4-cyano-L-phenylalanine will be discussed here.

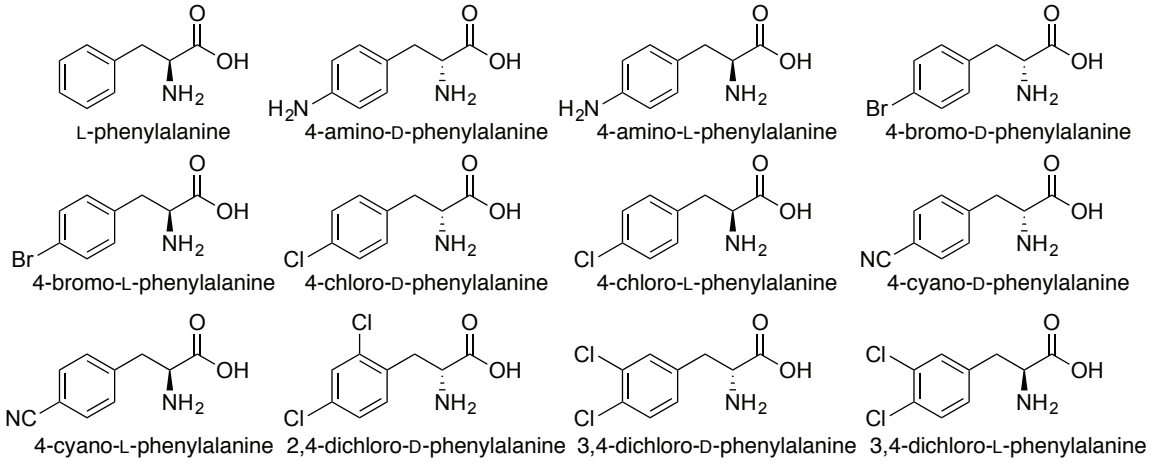


Figure 3. Structures of L-phenylalanine analogues that have been investigated as potential nitrogen sources for *Streptomyces venezuelae* ISP5230 incorporation into the jadomycin structure.

Natural product chemistry is not limited to the isolation and characterization of novel natural products. The natural product chemist must also be able to derivatize the natural products that are discovered.[7] The process of derivatization allows for an efficient method of expanding the natural product library since one novel natural product can be used to produce multiple novel derivatives.[1,7-9] Derivatization may be possible directly from the natural product isolated, if it possesses a chemical functionality that allows for

direct derivatization. If this is not the case, it may be possible to introduce these chemical functionalities through precursor-directed biosynthesis.[1,8,9]

Precursor-directed biosynthesis is a valuable tool for the derivatization of natural products through the insertion of chemical handles. These chemical handles can be used to alter the natural product through site-specific chemistry. This process of derivatization has been previously done using jadomycin *O*-propargyl-L-serine[8] and jadomycin L-ornithine (jadomycin Oct).[9] Both of these examples incorporated specific chemical handles, a terminal alkyne[8] and a primary amine,[9] respectively, which were then used to derivatize the parent compounds. Therefore, one can design novel jadomycins to undergo chemical derivatization through the use of amino acid analogues with potential chemical handles. It was this rationale that led to the investigation of the eleven phenylalanine analogues, where jadomycins containing amino- functionalities could be potentially derivatized through the use of succinimidyl esters. Their derivatization will be presented here, using (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester and phenoxyacetic acid *N*-hydroxysuccinimide ester (Figure 4). These succinimidyl esters were selected because past biological studies have suggested that jadomycin derivatives containing aromatic substituents exhibit the highest biological activity.[8]

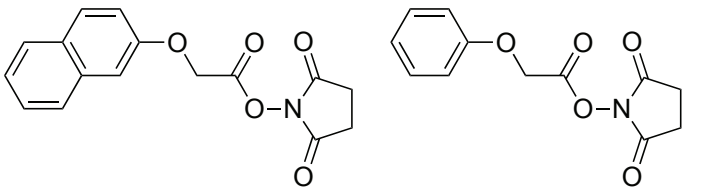


Figure 4. Succinimidyl esters used to derivatize jadomycin 4-amino-L-phenylalanine.

Since natural products, and their derivatives, are widely used in medicine, it is important to not only consider their chemical properties, but their biological properties as well.[10,11] These, much like a natural product's chemical properties, can be directly altered through the use of precursor-directed biosynthesis. Through the use of this technique it is possible to alter the lipophilicity or hydrophilicity of the natural product in question. These properties, for example, influence how easily a compound can cross cell membranes within an organism. These properties are important to consider when selecting possible amino acids to use for jadomycin productions because they have been shown to possess a variety of biological activity, such as cytotoxic activity against cancerous cell lines as tested at the National Cancer Institute (NCI) in the United States of America,[1,4,8,9,12] copper-mediated deoxyribonucleic acid (DNA) cleavage capability,[1,9,13,14] photo-dynamic inactivation of bacteria,[1,9] activity against yeast,[15] and activity against both gram positive and gram negative bacteria.[16] Novel jadomycins can, therefore, be tested for a variety of different biological activities. Through the biological evaluation of various novel jadomycins and their derivatives it may be possible to establish a structure activity relationship. For this reason, it is beneficial to produce a multitude of novel jadomycins and their derivatives. Currently, past biological studies suggest that jadomycin derivatives containing aromatic substituents exhibit the highest biological activity, which lead to the investigation of both succinimidyl esters presented above.[8] The biological activity of the jadomycin 4-amino-L-phenylalanine derivatives will be presented below.

2.2 Results & Discussion

2.2.1 Productions using 4-cyanophenylalanine enantiomers

While monitoring productions of jadomycin 4-cyanophenylalanine for cell growth (OD_{600}) and jadomycin production (Abs_{526}), it was found that while the cells were able to grow normally, as compared to past jadomycin work,[1,2,9] the jadomycin production was poor (Figure 5). Jadomycin production was also shown not to have worked based on HPLC and LC-MS/MS analysis and as such these conditions were not studied further.

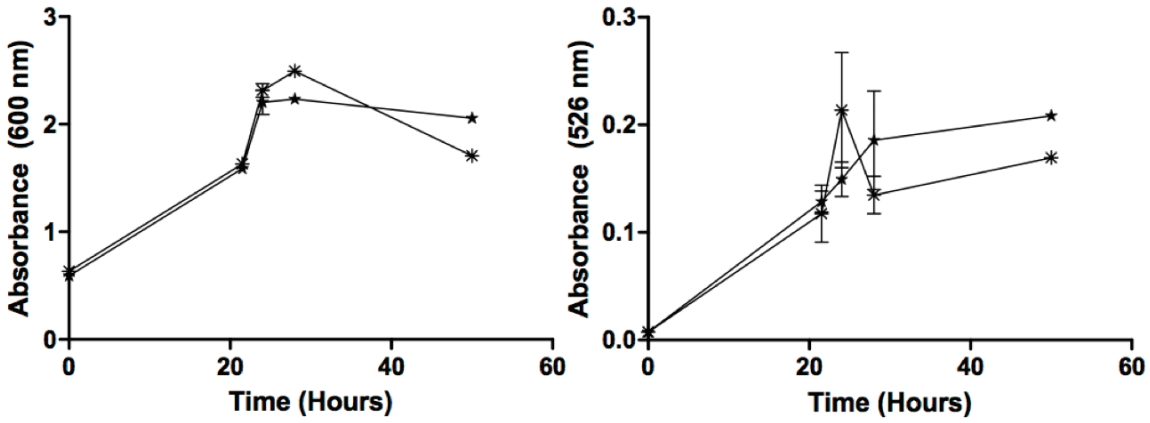


Figure 5. *Streptomyces venezuelae* ISP5230 fermentations in the presence of 4-cyanophenylalanine enantiomers. Monitoring cell growth at 600 nm and production of coloured compounds at 526 nm in the presence of 4-cyano-D-phenylalanine (❋) and 4-cyano-L-phenylalanine (★). Error bars correspond to standard deviation between triplicates.

2.2.2 Productions using 4-aminophenylalanine enantiomers

While monitoring productions of jadomycin 4-aminophenylalanine for cell growth (OD_{600}) and jadomycin production (Abs_{526}), it was found that the cells were able to grow normally, as compared to past jadomycin work,[1,2,9] and there appeared to be jadomycin production (Figure 6). Preliminary HPLC and LC-MS/MS results also suggested the presence of a jadomycin.

Figure 6 suggests that jadomycin production was more favourable using 4-amino-L-phenylalanine as opposed to the 4-amino-D-phenylalanine, based on higher Abs_{526}. As such, large-scale fermentations were carried out with 4-amino-L-phenylalanine.

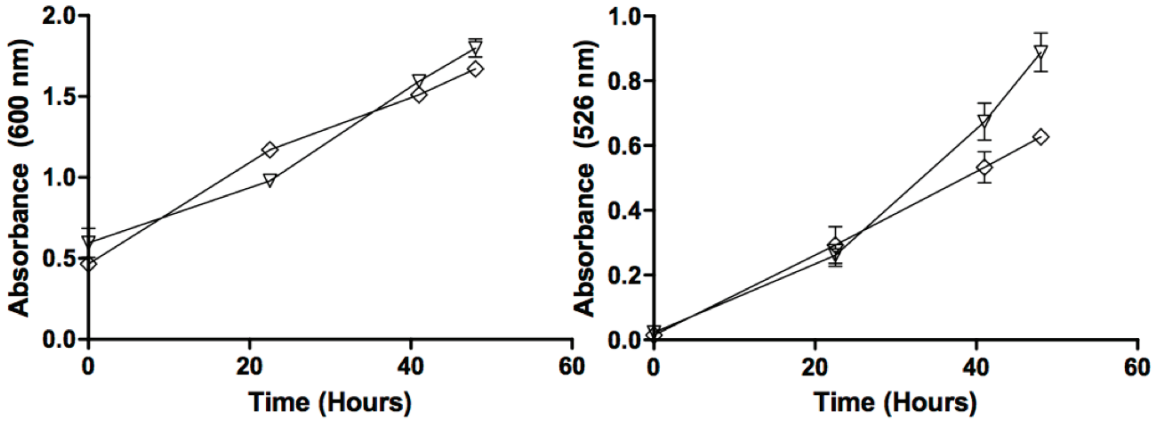


Figure 6. *Streptomyces venezuelae* ISP5230 fermentations in the presence of 4-aminophenylalanine enantiomers. Monitoring cell growth at 600 nm and production of coloured compounds at 526 nm in the presence of 4-amino-D-phenylalanine (◇) and 4-amino-L-phenylalanine (▽). Error bars correspond to standard deviation between triplicates.

Previous work within the laboratory had demonstrated that a water-ethyl acetate extraction was beneficial following the initial phenyl column for amino-containing jadomycins.[9] Following the extraction, however, jadomycin 4-amino-L-phenylalanine was found in both the aqueous and organic fractions. Although the extraction was unsuccessful in this regard, it was possible to remove some impurities that were insoluble in both fractions. Both the organic and aqueous fractions were pooled together and carried forward during purification, eventually yielding 12.6 mg of pure jadomycin 4-amino-L-phenylalanine, which was fully characterized (see section 2.4.7 & Appendix I).

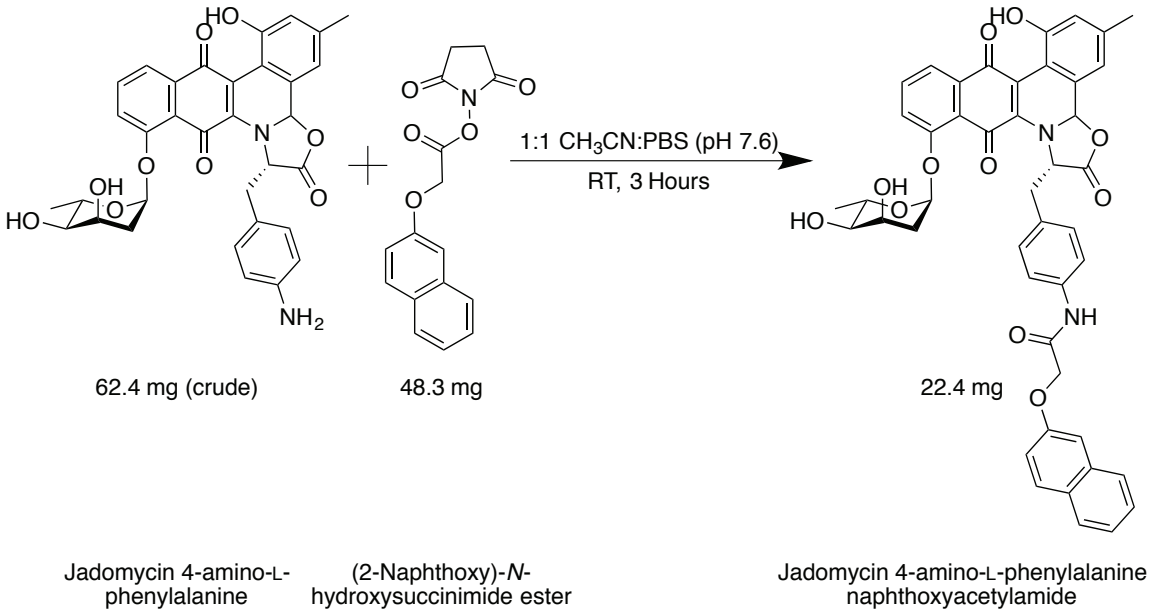
The structure of jadomycin 4-amino-L-phenylalanine was confirmed following NMR spectroscopy. The chemical shifts and connectivity observed are similar to recently

isolated jadomycins.[9] The connectivity of the jadomycin was established using correlation spectroscopy (COSY) NMR (Figure S2, see section 2.4.7 for numbering). The presence of the A ring was confirmed following COSY correlations between 4H and 6H. The D ring was confirmed following COSY correlations between 9H, 10H, and 11H. The connectivity about the sugar ring was confirmed following COSY correlations between 1″H, 2″H, 3″H, 4″H, 5″H, and 5″-CH_3. Finally, the amino acid aromatic ring connectivity was confirmed following COSY correlations between 3′H and 4′H. Heteronuclear multiple-bond correlation spectroscopy (HMBC) NMR (Figure S5 & Figure S6) was used to establish the incorporation of the amino acid into the jadomycin structure through correlations between 3aH and 1C as well as 1′C (see section 2.4.7 for numbering). HMBC NMR also correlated 9H to 8C, establishing the final connectivity to establish the correct jadomycin structure.

Investigations into the derivatization of jadomycin 4-amino-L-phenylalanine were carried out using crude samples following the initial phenyl column. This was done in order to maximize the yield of the reaction because there is inherent loss of the natural product during the purification process, mainly through breakdown.

2.2.3 Synthesis of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide

The reaction (Scheme 2) between jadomycin 4-amino-L-phenylalanine and (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester was allowed to proceed for three hours until completion, as determined by TLC (Figure S8), yielding jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide.



Scheme 2. Reaction between jadomycin 4-amino-L-phenylalanine and (2-naphthoxy)-*N*-hydroxysuccinimide ester to generate jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide.

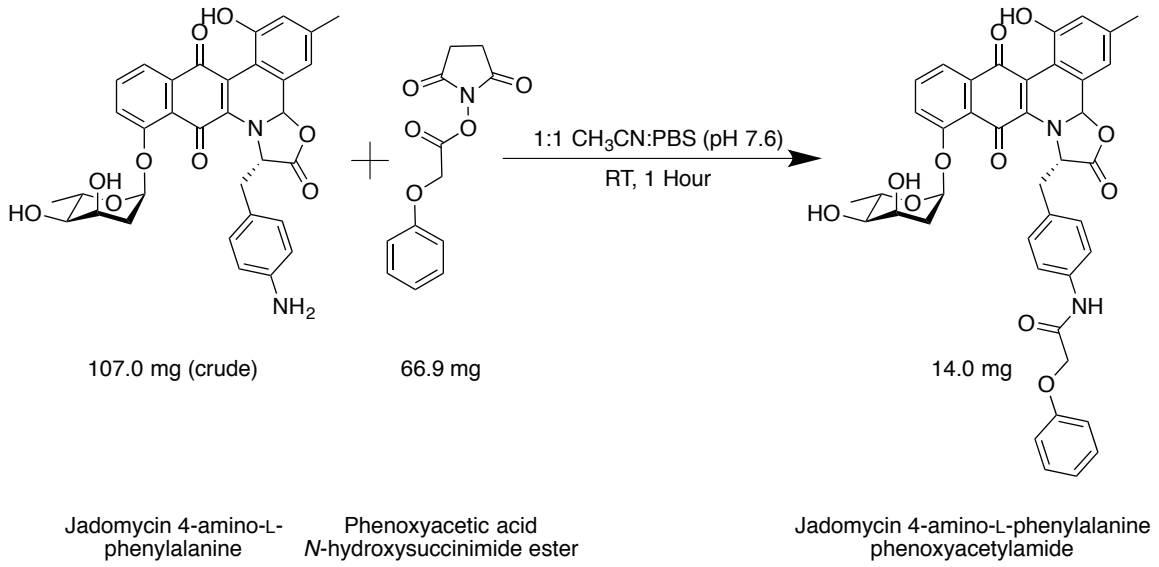
Following the reaction, an extraction using dichloromethane was successful in isolating the crude natural product in the organic phase. The organic phase was carried forward during purification, eventually yielding 12.6 mg of pure jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide, which was fully characterized (see section 2.4.9).

The structure of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide was confirmed following NMR spectroscopy. All of the same correlations that were found for jadomycin 4-amino-L-phenylalanine were present in the naphthoxy derivative. As well, the connectivity of the naphthoxy substituent had similar chemical shifts and connectivity to that of recently isolated jadomycins.[9] COSY correlations (Figure S11) between 11′H, 12′H, 13′H, and 14′H along with 16′H to 17′H and HMBC (Figure S14 & Figure S15) correlations between 7′H, 6′C, and 8′C (see section 2.4.9 for numbering) established the connectivity of the naphthoxy substituent. Finally, the connectivity of the sugar was

established through an HMBC correlation between 1″H and 12C (see section 2.4.9 for numbering).

2.2.4 Synthesis of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide

The reaction (Scheme 3) between jadomycin 4-amino-L-phenylalanine and phenoxyacetic acid *N*-hydroxysuccinimide ester was allowed to proceed for one hour until completion, as determined by TLC (Figure S16), yielding jadomycin 4-amino-L-phenylalanine phenoxyacetylamide.



Scheme 3. Reaction between jadomycin 4-amino-L-phenylalanine and phenoxyacetic acid *N*-hydroxysuccinimide ester to generate jadomycin 4-amino-L-phenylalanine phenoxyacetylamide.

Following the reaction, an extraction using dichloromethane was successful in isolating the crude natural product in the organic phase. The organic phase was carried forward during purification, eventually yielding 14.0 mg of pure jadomycin 4-amino-L-phenylalanine phenoxyacetylamide which was fully characterized (see section 2.4.10).

The structure of jadomycin 4-amino-L-phenylalanine phenoxyacetyl amide was confirmed following NMR spectroscopy. All of the same correlations that were found for jadomycin 4-amino-L-phenylalanine were present in the phenoxy derivative. As well, the connectivity of the phenoxy substituent had similar chemical shifts and connectivity to that of recently isolated jadomycins.[9] COSY correlations (Figure S19) between 9′H, 10′H, and 11′H and HMBC (Figure S22 & Figure S23) correlations between 7′H, 6′C, and 8′C (see section 2.4.10 for numbering) established the connectivity of the phenoxy substituent. Finally, the connectivity of the sugar was established through HMBC NMR through a correlation between 1″H and 12C (see section 2.4.10 for numbering).

2.2.5 Biological evaluation of jadomycin 4-amino-L-phenylalanine derivatives

National Cancer Institute (NCI) 60 DTP Human Tumor Cell Line Screen anticancer activity

All 60 DTP Human Tumor Cell Line Screen anticancer activity testing was conducted by the NCI (USA).

Although both jadomycin 4-amino-L-phenylalanine derivatives were submitted, only jadomycin 4-amino-L-phenylalanine phenoxyacetylamide was selected by the NCI for their anticancer activity screening. The results (Table S1) show modest cell-growth inhibition and cytotoxicity. The cell line that was affected the most by jadomycin 4-amino-L-phenylalanine phenoxyacetylamide was leukemia, which showed the highest cytotoxicity of all cell lines tested, with breast cancer exhibiting similar results (Table S1). However, jadomycin 4-amino-L-phenylalanine phenoxyacetylamide was deemed to not have enough activity to continue to the second round of testing.

Photodynamic DNA cleavage assay

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all photodynamic DNA cleavage assays.

The ability of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide to photodynamically cleave DNA was investigated. It appears as though jadomycin 4-amino-L-phenylalanine phenoxyacetylamide is capable of cleaving DNA after being exposed to light, as compared to DNA alone (Figure 7 & Figure 8). This is most evident at concentrations of 25 μM and higher (Figure 8). These results also appear to suggest that light is necessary for the cleavage of DNA, since there is a higher degree of cleavage when light is present than when it is absent (Figure 7 & Figure 8).

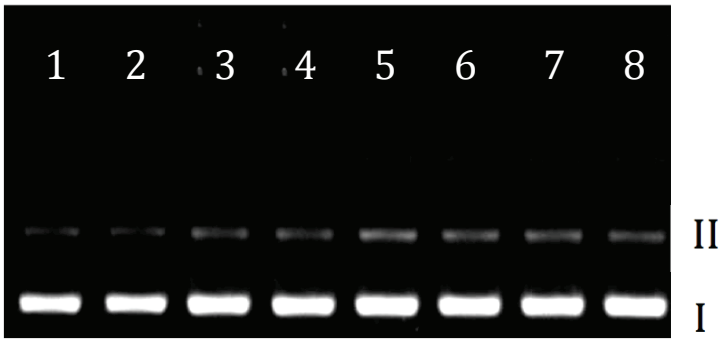


Figure 7. Photo-mediated DNA cleavage assay with jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Gel electrophoresis analysis of pUC19 DNA (20 μM bases) in a photocleavage assay, performed in 1% agarose gel cast with ethidium bromide, 1× TAE, 8 Vcm^{-1}, 30 min photoreactor with vis bulbs (14 Jcm^{-2}), in the presence of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Lane 1, DNA alone in the dark; lanes 2 – 7 different concentrations of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide: (2) 0 μM, hν; (3) 10 μM, hν; (4) 25 μM, hν; (5) 50 μM, hν; (6) 75 μM, hν; (7) 100 μM, hν; (8) 100 μM, in the dark. Form I (supercoiled) and form II (nicked) plasmid DNA are indicated.

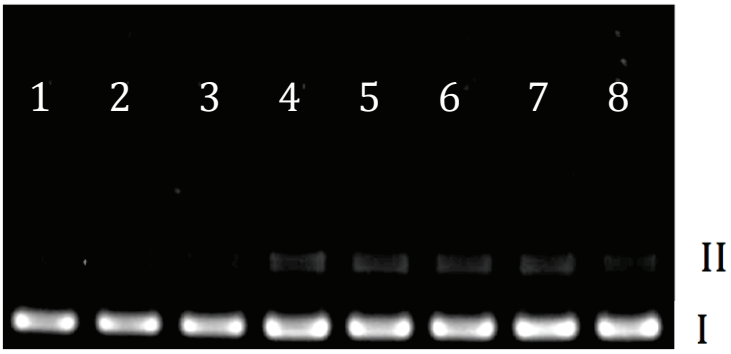


Figure 8. Photo-mediated DNA cleavage assay with jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Gel electrophoresis analysis of pUC19 DNA (20 μM bases) in a photocleavage assay, performed in 1% agarose gel stained with ethidium bromide following electrophoresis, 1× TAE, 8 Vcm^{-1}, 30 min photoreactor with vis bulbs (14 Jcm^{-2}), in the presence of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Lane 1, DNA alone in the dark; lanes 2 – 7 different concentrations of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide: (2) 0 μM, hν; (3) 10 μM, hν; (4) 25 μM, hν; (5) 50 μM, hν; (6) 75 μM, hν; (7) 100 μM, hν; (8) 100 μM, in the dark. Form I (supercoiled) and form II (nicked) plasmid DNA are indicated.

Photodynamic activation is a desirable property because a potential drug could be given throughout the body, but would only perform the desired effect where light is shone at the necessary intensity to observe a response. However, at all concentrations attempted there is not total cleavage of the DNA, where supercoiled DNA is shown to remain. These types of results have been previously seen for other jadomycin compounds.[9]

Copper-mediated DNA cleavage assays

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all copper-mediated DNA cleavage assays.

The abilities of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide and jadomycin 4-amino-L-phenylalanine phenoxyacetylamide to cleave DNA were investigated using a copper-mediated DNA cleavage assay. In both cases, it appears as

though the jadomycins were able to cleave DNA, to a greater extent, in the presence of cupric acetate (Figure 9 & Figure 10). This is evident when comparing the bands produced in the presence and absence of cupric acetate, as well as comparing these results to those obtained when DNA or cupric acetate were run by themselves as controls (Figure 9 & Figure 10). However, in both cases there is not total cleavage of the DNA, where there is still some remaining supercoiled DNA. These types of results have been previously seen for other jadomycin compounds.[9]

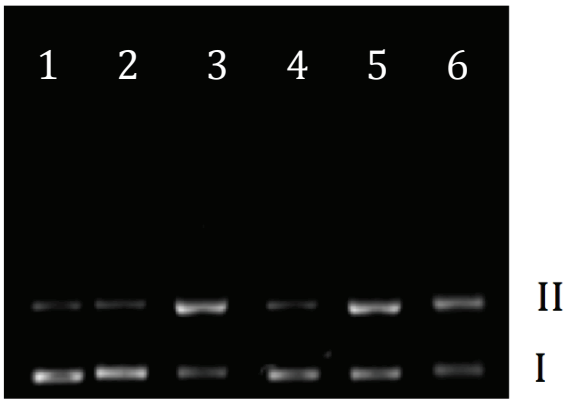


Figure 9. Copper-mediated DNA cleavage by jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide and jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Gel electrophoresis analysis of pUC19 DNA (20 μM bases) in a DNA cleavage assay, performed in 1% agarose gel containing 0.75 μgmL^{-1} ethidium bromide, 1× TAE, 8 Vcm^{-1}, overnight dark incubation, in the presence of two jadomycin compounds with/without cupric acetate. Lane 1, DNA alone; Lane 2, naphthoxy derivative (100 μM); Lane 3, naphthoxy derivative and Cu^{2+} (100 μM); Lane 4, phenoxy derivative (100 μM); Lane 5, phenoxy derivative and Cu^{2+} (100 μM); Lane 6, Cu^{2+} only (100 μM). Form I (supercoiled) and form II (nicked) plasmid DNA are indicated.

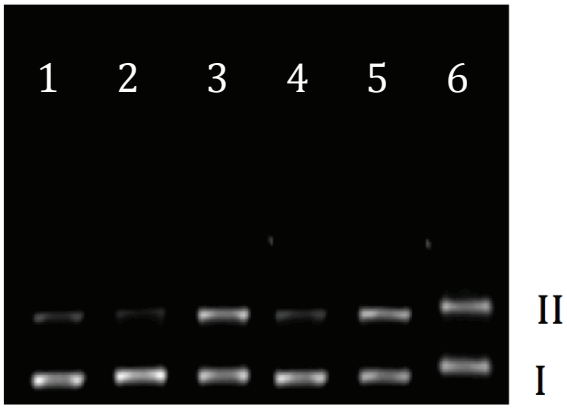


Figure 10. Copper-mediated DNA cleavage by jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide and jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. Gel electrophoresis analysis of pBR322 DNA (20 μM bases) in a DNA cleavage assay, performed in 1% agarose gel containing 0.75 μgmL^{-1} ethidium bromide, 1× TAE, 8 Vcm^{-1}, overnight dark incubation, in the presence of two jadomycin compounds with/without cupric acetate. Lane 1, DNA alone; Lane 2, naphthoxy derivative (100μM); Lane 3, naphthoxy derivative and Cu^{2+} (100 μM); Lane 4, phenoxy derivative (100μM); Lane 5, phenoxy derivative and Cu^{2+} (100 μM); Lane 6, Cu^{2+} only (100 μM). Form I (supercoiled) and form II (nicked) plasmid DNA are indicated.

Agar well diffusion assays

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all agar well diffusion assays.

Agar well diffusion assays were used to assess the antibacterial activity of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide and jadomycin 4-amino-L-phenylalanine phenoxyacetylamide. To test this, jadomycins were placed within the wells and *Streptococcus mutans* is grown on the plate. If there is antibacterial activity, there will be an area around the well where growth of *S. mutans* is inhibited. These experiments are conducted under dark and light conditions to determine whether there is light-activated antibacterial activity.

Under both dark and light conditions, no antibacterial activity was observed (Figure 11 & Table 2). The bacteria were able to grow to the border of the wells. Therefore, it appears as though both of these jadomycin derivatives do not exhibit antibacterial activity against *S. mutans*.

Table 2. *S. mutans* growth inhibition zones in the presence of jadomycins. Inhibition zones (mm) listed correspond to the diameter of the inhibition halo, including the 6 mm sample well.

Compound	Dark inhibition zone (mm)	Light inhibition zone (mm)
Naphthoxy derivative	6	6
Phenoxy derivative	6	6
6% EtOH	6	6

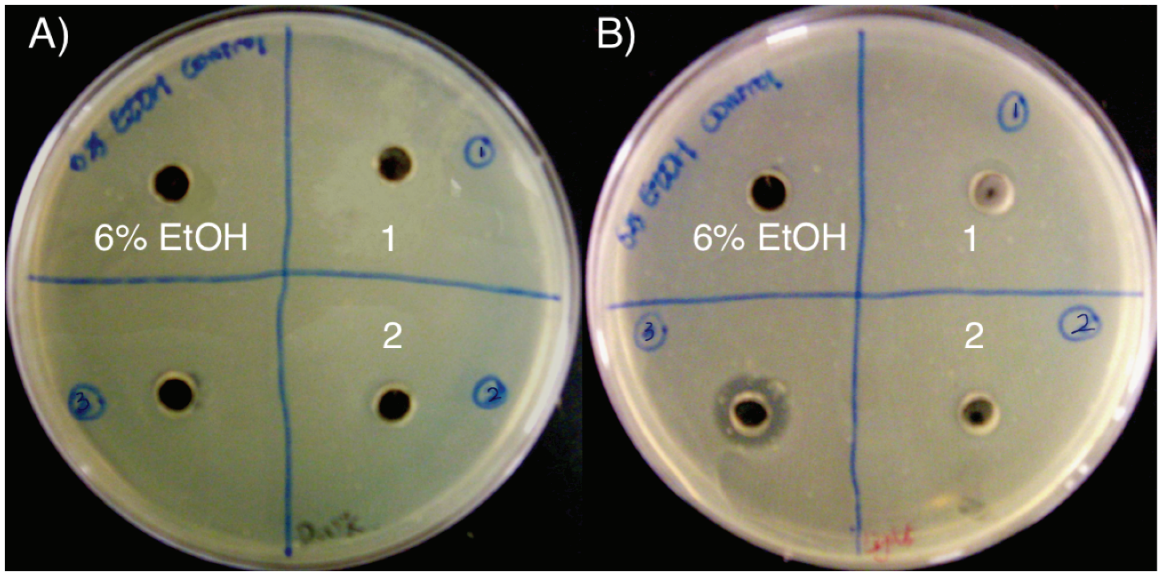


Figure 11. *S. mutans* growth inhibition in the presence of jadomycin 4-amino-L-phenylalanine derivatives. *S. mutans* growth plates showing agar well diffusion inhibition zones from (1) jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (300 μM), (2) jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (300 μM), and a 6% ethanol control under (A) dark and (B) light conditions.

2.3 Conclusions

The soil bacteria *Streptomyces venezuelae* ISP5230 VS1099 has been shown to successfully incorporate 4-aminophenylalanine enantiomers into the jadomycin structure,

leading to the successful production and isolation of a novel jadomycin, jadomycin 4-amino-L-phenylalanine. The fact that this phenylalanine analogue was incorporated into the jadomycin structure is significant because it has been shown that many other phenylalanine analogues have not been successfully used as sole nitrogen sources by the bacteria. This novel jadomycin has further significance because it is the first example of an oxazolone-ring containing jadomycin with a synthetically accessible amino functionality. This amino functionality allowed for this jadomycin to be successfully used as a chemical handle for further derivatization.

Two derivatives of jadomycin 4-amino-L-phenylalanine were successfully produced and isolated, effectively expanding the jadomycin library. The derivatization of this jadomycin using succinimidyl esters demonstrates that this is an effective means for derivatizing primary amine containing jadomycins. In the future, any jadomycins isolated with this moiety could easily be derivatized using this methodology, quickly generating novel jadomycin species. The two derivatives isolated were also subjected to biological evaluation.

Out of the two jadomycin 4-amino-L-phenylalanine derivatives prepared, the NCI accepted the phenoxy derivative, over the naphthoxy derivative, for testing against their 60 DTP human tumour cell line one-dose screen. The resulting evaluation did not show any appreciable cytotoxic activity (Table S1). Both derivatives were also tested for their DNA cleavage capability (Figure 7-9), as well as their ability to photodynamically inactivate bacteria (Figure 11). Neither derivative showed appreciable activity.

The lack of activity may potentially be explained through structural activity relationships. In the case of copper-induced DNA cleavage capability, both derivatives

presented here exhibited poor activity, in contrast to the naphthoxy and phenoxy derivatives of jadomycin Oct.[9] The lack of activity may, therefore, be potentially attributed to the presence of the 4-aminophenylalanine moiety. The same assessment is possible for the lack of activity in the photodynamic inactivation of bacteria.[9] However, both the jadomycin 4-amino-L-phenylalanine phenoxy derivative and the jadomycin Oct phenoxy derivative showed poor activity against the NCI 60 DTP human tumour cell line one-dose screen.[9] Although this suggests that the phenoxy moiety is responsible for the inactivity observed, many other factors may be at play. The ability to establish these structural activity relationships is of great interest and an important reason to explore the derivatization of natural products.

2.4 Experimental

All reagents were purchased from commercial sources and used without further purification. All solvents used for reactions and chromatographic methods were HPLC grade. Thin layer chromatography (TLC) plates were used to monitor jadomycin purification, reaction progress, and calculate R_f values. TLC plates were glass backed, normal phase silica (250 μm thickness) purchased from Silicyle®. All jadomycins prepared did not require chemical or ultraviolet (UV) visualization, since they are coloured compounds.

2.4.1 Purification methods

Flash chromatography

Flash chromatography was performed using a Biotage SP1™ unit (Biotage®) using pre-packed normal phase silica columns (25 g or 40 g) purchased from Silicyle® for all jadomycins. The solvent systems used for eluting the natural product from the column are specified in each respective section.

General methods for preparative thin layer chromatography

Preparative TLC was performed using 20 × 20 cm glass backed, normal phase silica TLC plates (Silicycle®, 1000 μm thickness). Preparative TLC was done by bringing up the natural product in minimal methanol in dichloromethane and spotting onto the plate. The plate was eluted in a specified solvent system. When the plate had been completely developed, it was removed from the solvent to allow air-drying, at which point it was placed into the same solvent system to allow for further elution of the compounds. This was repeated until sufficient separation had occurred to allow for the band of interest to be successfully removed. The band was removed by scraping the silica off of the plate, and the compound was then eluted off of the silica using the same solvent system as was used for development. The solvent was then removed *in vacuo*.

General methods for size exclusion chromatography

Size exclusion chromatography was performed using Sephadex™ LH-20 resin (GE Healthcare). The column was run using a specified solvent isocratically.

2.4.2 Compound characterization methods

All compounds were characterized using TLC, ultra-violet-visible (UV-Vis) spectroscopy, high-performance liquid chromatography (HPLC), liquid chromatography tandem mass spectrometry (LC-MS/MS), high-resolution mass spectrometry (HRMS), and 1D- and 2D- nuclear magnetic resonance (NMR) spectroscopy experiments.

General methods for ultra-violet-visible spectroscopy

All UV-Vis spectroscopy on novel jadomycin compounds was carried out on a SpectraMax Plus Microplate Reader (Molecular Devices), and analyzed using SoftMax® Pro Version 4.8 Software. Samples were dissolved in methanol, placed in a quartz cuvette (1 cm path length), and scanned over a 280 nm – 700 nm range using 1 nm intervals. Two separate dilutions were used (concentrations are listed with the appropriate characterization data) to calculate a series of extinction coefficients (ε) from several maximal absorbance wavelengths (λ_{max}).

High-performance liquid chromatography

HPLC of purified jadomycin and crude extracts were performed using a Hewlett Packard Series 1050 instrument equipped with an Agilent Zorbax 5 μm Rx-C18 column (150 cm × 4.6 mm). Jadomycins were monitored at an absorbance of 254 nm. HPLC was conducted using a linear gradient from 90:10 A:B to 40:60 A:B over 8.0 min followed by a plateau at 40:60 A:B from 8.0 to 10.0 min and finally a linear gradient from 40:60 A:B to 90:10 A:B over the remaining 5.0 min with a flow rate of 1.0 $mLmin^{-1}$. Buffer A is an aqueous buffer containing 12 mM *n*-Bu_4NBr, 10 mM KH_2PO_4, and 5% HPLC grade

acetonitrile (CH_3CN), at pH 4.0 and B is 100% HPLC grade CH_3CN. Samples were analyzed by injecting 20 µL aliquots.

Liquid chromatography tandem mass spectrometry

Low-resolution mass spectra were obtained using an Applied Biosystems hybrid triple quadrupole linear ion trap (2000 *Qtrap*) mass spectrometer equipped with an electrospray ionization (ESI) source. The software for analysis of mass spectra was Analyst version 1.4.1 (Applied Biosystems). The capillary voltage was set to 4500 kV with a declustering potential of 60 V and the curtain gas was set to 10 (arbitrary units). For sample analysis an Agilent 1100 HPLC system was connected to the mass spectrometer fitted with a Phenomenex Kinetex 2.6u (150 × 2.10 mm) C18 column. The buffer system used for the isocratic method was 30% 5 mM ammonium acetate pH 5.5 and 70 % acetonitrile with a flow rate of 120 $\mu L min^{-1}$. Samples were diluted to a concentration of approximately 500 µM in 100% HPLC grade methanol prior to injection of 5 µL. Enhanced product ion (EPI) scans, in positive mode, were used to detect jadomycin ions along with their characteristic fragments, aglycone and phenanthroviridin (Figure 2), to confirm identities using a collision energy of -60 V. Typical EPI scans were conducted using two steps; 130.0 amu to 320.0 amu (0.005 sec) and 300.0 amu to 900.0 amu (0.150 sec) to obtain better resolution.

High-resolution mass spectrometry

HRMS traces of all jadomycins were recorded on a Bruker Daltonics MicroTOF Focus Mass Spectrometer. Jadomycin 4-amino-L-phenylalanine used an ESI^+ source and both jadomycin 4-amino-L-phenylalanine derivatives required ESI^-

Nuclear magnetic resonance spectroscopy

NMR analyses of jadomycins were recorded using either a Bruker AV 500 MHz Spectrometer (^1H: 500 MHz, ^{13}C: 125 MHz) equipped with an auto-tune and match (ATMA) broadband observe (BBFO) SmartProbe located at the Nuclear Magnetic Resonance Research Resource (NMR-3) facility (Dalhousie University) or a Bruker AV-III 700 MHz Spectrometer (^1H: 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. The use of each is specified next to the appropriate spectra. Spectra were recorded in CD_3OD and $CDCl_3$. Chemical shifts are given in ppm and have been calibrated to residual solvent peaks (CD_3OD: 3.31 ppm; $CDCl_3$: 7.26 ppm). Structural characterization and signal assignments were accomplished using ^1H-NMR chemical shifts and multiplicities, and ^{13}C-NMR chemical shifts. In addition, ^1H-^1H correlated spectroscopy (COSY), ^1H-^{13}C heteronuclear single quantum coherence (HSQC), and ^1H-^{13}C heteronuclear multiple bond correlation (HMBC) NMR experiments were used in the NMR analyses.

2.4.3 Monitoring during jadomycin production methods

To monitor cell growth during jadomycin production 600 µL aliquots were removed from the production media and analyzed at 600 nm[1,3,4,9,17] on a SpectraMax Plus Microplate Reader (Molecular Devices). The cells were then removed from these aliquots *via* centrifugation (10,000 rpm, 4°C). The supernatant was then analyzed at 526 nm, using the same instrument, to determine jadomycin production.[1,4,9,17]

2.4.4 Media for jadomycin production methods

Preparation of MYM growth media

MYM growth media was prepared by addition of maltose (0.4%, w/v), yeast extract (0.4%, w/v), and malt extract (1%, w/v) to distilled, deionized water (ddH_2O). The pH was adjusted to 7.0 with sodium hydroxide (5 M) and hydrochloric acid (5 M) as necessary. The solution was then autoclaved.

Preparation of MSM production media

MSM production media was prepared by addition of magnesium sulphate (0.04%, w/v), MOPS (0.377%, w/v), salt solution (0.9%, v/v), trace mineral solution (0.45%, v/v), and 0.2% w/v ferrous sulphate solution (0.45%, v/v) to ddH_2O. The pH was adjusted to 7.5 by sodium hydroxide (5 M) and hydrochloric acid (5 M) as necessary. The solution was then autoclaved. The salt solution was made by addition of sodium chloride (1%, w/v) and calcium chloride (1%, w/v) to ddH_2O. The trace mineral solution was made by addition of zinc sulphate (0.088%, w/v), copper sulphate (0.0039%, w/v), manganese sulphate

(0.00061%, w/v), boric acid (0.00057%, w/v), and ammonium molybdate (0.00037%, w/v) to ddH_2O.

2.4.5 General jadomycin production methods

S. venezuelae ISP5230 VS1099 cultures were grown on MYM agar [maltose (0.4%, w/v), yeast extract (0.4%, w/v), malt extract (1%, w/v), agar (1.5%, w/v), apramycin (0.005% w/v)] incubated at 30°C for a period of two to four weeks. MYM growth media were inoculated with a loop of cells and incubated for a period of 16 – 24 hours with shaking (250 rpm) at 30°C. The cell growth period in the MYM media was established by observing cell growth under a microscope to determine whether cells were loosely packed and fibrous. The growth was terminated when cells reached this point. Once the initial growth period was complete, the bacteria were pelleted (8500 rpm, 4°C) and the supernatant decanted. The cell pellet from the 250 mL MYM cultures was re-suspended and washed with approximately 50 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, typically 15 mL for a cell pellet from a 250 mL culture.

The production media was prepared by addition of 30 – 60 mM of the corresponding amino acid to MSM solution (Table 3), then adjusting the pH to 7.5 using sodium hydroxide (5 M) and hydrochloric acid (5 M) as required, and autoclaving the solution. After sterilization, a separately filter sterilized 30% glucose solution (2%, v/v) and a separately autoclaved 9 mM phosphate solution (0.54%, v/v) were added to the MSM production media. The re-suspended *S. venezuelae* was then added to the production media to an absorbance of 0.6 at 600 nm (OD_{600}). The production media was

immediately ethanol shocked (3%, v/v) to induce jadomycin production. The media was incubated with shaking (250 rpm) at 30°C while being monitored *via* OD_{600}, Abs_{526}, and high-performance liquid chromatography (HPLC) for 48 hours. At 24 hours the pH was readjusted to 7.5 using sodium hydroxide (5 M) or hydrochloric acid (5 M) as required.

Table 3. Concentration of amino acid used in production of the corresponding jadomycin.

Amino acid to be incorporated	Concentration (mM)
4-cyano-D-phenylalanine	60
4-cyano-L-phenylalanine	60
4-amino-D-phenylalanine	30
4-amino-L-phenylalanine	30

Once production had been stopped, the bacteria were removed from the solution by filtration through Whatman #5 filters, followed by 0.45 μm and 0.22 μm Millipore filters. The production media was then passed through a phenyl column (Silicycle®) that had been preconditioned with ddH_2O. The production media was loaded onto the column that was subsequently washed with ddH_2O (6–8 L for a 2 L production) to remove all water-soluble material, and the crude natural product was eluted off of the column using 100% methanol (~250 mL for a 2 L production). The solvent was removed *in vacuo*, and the presence of the jadomycin of interest was examined using HPLC and LC-MS/MS analysis. Purification was performed as outlined in each respective section.

2.4.6 Productions using 4-cyanophenylalanine enantiomers

Productions using 4-cyanophenylalanine enantiomers were analytically assessed for jadomycin production following the typical 48-hour production period. Isolation was not carried forward.

2.4.7 Productions using 4-aminophenylalanine enantiomers

Productions using 4-aminophenylalanine enantiomers were analytically assessed for jadomycin production following the typical 48-hour production period.

Productions using 4-amino-L-phenylalanine were chosen to carry forward for characterization and isolation, as such large-scale fermentations were carried out.

The initial 70 g phenyl column (Silicycle®) yielded 205.9 mg of crude material. A water-ethyl acetate extraction was then attempted, but separation was not observed; therefore, both fractions were pooled together and carried forward as one. This yielded 93.1 mg of material, with the remaining mass being lost to impurities that were insoluble in both water and ethyl acetate. Purification was pursued using a 25 g silica flash chromatography column preconditioned with 20% CH_3OH in EtOAc. The material was eluted using a 20 $mLmin^{-1}$ flow rate collecting 9 mL fractions. Purification was accomplished using a gradient system with solvent A (20% CH_3OH in EtOAc) and solvent B (7:2:1 EtOAc:CH_3OH:H_2O). The column was run with an initial isocratic step of 100% solvent A (2 CV), followed by a linear increasing gradient of 0% to 100% solvent B (5 CV), with a final isocratic step of 100% solvent B (2 CV). Flash chromatography yielded 23.5 mg of crude material. Final purification was accomplished using a Sephadex™ LH-20 size exclusion column eluted with methanol. The solvent was

then removed *in vacuo* to yield the natural product in a 12.6 mg yield (6.3 mgL^{-1}) as a mixture of diastereomers (Mj:Mn 20:9) by ^{1}H-NMR spectroscopy. TLC R_f: 0.25 (5:5:1 CH_3CN:EtOAc:H_2O); HPLC R_t = 7.76 min; UV-Vis (3.0×10^{-4} and 3.8×10^{-5} M, MeOH): λ_{max} (ε) = 294 (24194), 539 (2057); LRMS (ESI^+): MS/MS (599) found 599 $[M+H]^+$, 469 [M+H-digitoxose]$^+$, 306 [M+H-digitoxose-$C_9H_{11}NO_2$]$^+$; HRMS (ESI^+) for $C_{33}H_{31}N_2O_9$ $[M+H]^+$: 599.2040 found, 599.2024 calculated; NMR spectra to follow (see Appendix I), see characterization table (Table 4 & 5) for numbering.

Table 4. Jadomycin 4-amino-L-phenylalanine $3a_{Mj}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (*J*(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	3.34	obscured	53.9	unclear	3a, 13a
2			173.2		1' (b)
3a	5.84	s	91.8		1, 4, 5, 7a, 13a
3b					
4	6.76	s	120.9	6	3a, 5-CH_3, 7a
5			146.1		3a
5-CH_3	2.34	s	20.9		4, 6, 7a
6	6.84	s	120.6	4	5-CH_3
7			142.7		5-CH_3
7-OH					
7a			112.9		3a, 4, 6
7b					
8			186.4		9
8a			136.5		9, 10
9	7.83	d(7.5)	121.6	10	8, 8a, 11
10	7.75	t(9.3)	136.6	9, 11	8a, 12, 12a
11	7.52	d(8.4)	120.7	10	9
12			155.9		10
12a			121.0		10
13					
13a			132.0		3a
1' (a)	3.34	obscured	37.5	1' (b)	3a, 3'
1' (b)	3.08	dd(14.7, 9.2)	37.5	1' (a)	2, 3'
2'			134.2		4'
3'	7.33	d(8.2)	131.1	4'	1' (a), 1' (b), 3', 5'
4'	7.17	d(7.9)	125.2	3'	2', 4'
5'			146.2		3'
1"	5.94	s	95.1	2"	3", 5"
2"	2.14	d(7.3)	36.0	1", 3"	-
3"	3.89	d(2.1)	67.9	2", 4"	1"
3"OH					
4"	3.23	m	73.2	3", 5"	5"-CH_3
5"	3.84	m	57.4	4", 5"-CH_3	1", 5"-CH_3
5"-CH_3	1.14	d(6.0)	17.8	5"	4", 5"
MeOH	3.31		49.0		
Water	4.89				

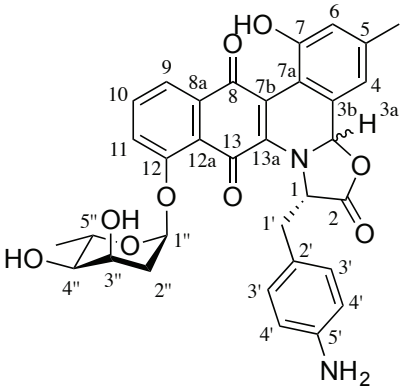
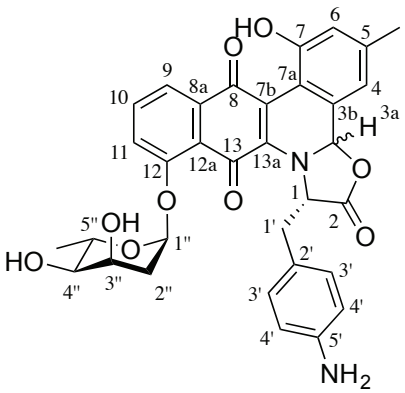


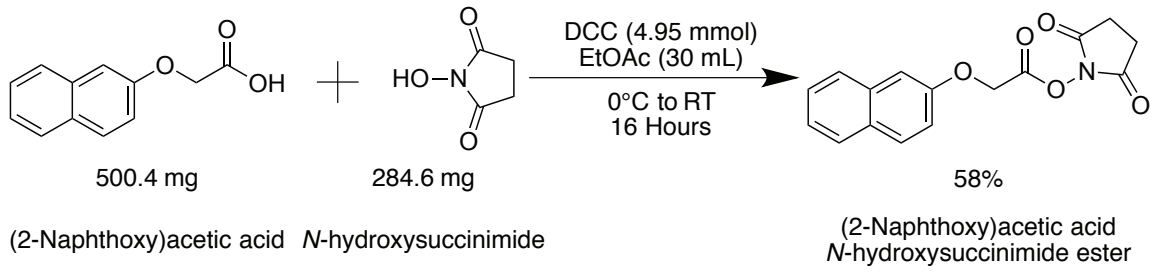
Table 5. Jadomycin 4-amino-L-phenylalanine $3a_{Mn}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (*J*(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	3.34	obscured	53.9	unclear	3a, 13a
2			173.2		1' (b)
3a	5.83	s	91.8		1, 4, 5, 7a, 13a
3b					
4	6.73	s	120.9	6	3a, 5-CH_3, 7a
5			146.1		3a
5-CH_3	2.33	s	20.9		4, 6, 7a
6	6.85	s	120.6	4	5-CH_3
7			142.7		5-CH_3
7-OH					
7a			112.9		3a, 4, 6
7b					
8			186.4		9
8a			136.5		9, 10
9	7.87	d(7.5)	121.6	10	8, 8a, 11
10	7.71	t(7.9)	136.6	9, 11	8a, 12, 12a
11	7.55	d(8.4)	120.7	10	9
12			155.9		10
12a			121.0		10
13					-
13a			132.0		3a
1' (a)	3.34	obscured	37.5	1' (b)	3a, 3'
1' (b)	3.08	dd(14.7, 9.2)	37.5	1' (a)	2, 3'
2'			134.2		4'
3'	7.35	obscured	131.1	4'	1' (a), 1' (b), 3', 5'
4'	7.17	d(7.9)	125.2	3'	2', 4'
5'			146.2		3'
1"	5.91	s	95.1	2"	3", 5"
2"	2.14	d(7.3)	36.0	1", 3"	-
3"	3.93	s	67.9	2", 4"	1"
3"OH					
4"	3.23	m	73.2	3", 5"	5"-CH_3
5"	3.80	m	66.3	4", 5"-CH_3	1", 5"-CH_3
5"-CH_3	1.17	d(6.1)	17.8	5"	4", 5"
MeOH	3.31		49.0		
Water	4.89				



2.4.8 Synthesis of (2-naphthoxy)acetic acid N-hydroxysuccinimide ester

(2-Naphthoxy)acetic acid (500.4 mg) and *N*-hydroxysuccinimide (284.6 mg) were dissolved in 15 mL of anhydrous ethyl acetate, with gentle stirring, in a 50 mL round bottom under nitrogen, and cooled to 0°C (Scheme 4). *N,N*′-dicyclohexylcarbodiimide (DCC; 1.0203 g) was dissolved in 15 mL of anhydrous ethyl acetate in a 50 mL round bottom under nitrogen. The DCC solution was then added drop-wise to the solution containing the acetic acid. Once all of the DCC solution had been added, the round bottom was removed from the ice bath and allowed to warm to room temperature overnight. Once the reaction is complete, the solid is removed *via* filtration through Whatman #5 filter paper. The solvent was then removed *in vacuo*. The solid was re-suspended in anhydrous ethyl ether (50 mL) and then washed with 75 mL anhydrous ethyl ether (3 x 25 mL). The solid is then dried and stored at 4°C under desiccation.



Scheme 4. Reaction between (2-naphthoxy)acetic acid and *N*-hydroxysuccinimide to generate (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester.

2.4.9 Synthesis of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide

Crude jadomycin 4-amino-L-phenylalanine (62.4 mg) was dissolved in 20 mL of 1:1 acetonitrile:phosphate buffered saline (PBS, pH 7.6) and added to (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester (48.3 mg) in a 25 mL round bottom flask drop-wise with gentle stirring. The flask was corked and protected from light. The reaction was

allowed to proceed for three hours until complete as determine by TLC (5:5:1 CH_3CN:EtOAc:H_2O, Figure S8).

Once the reaction was complete, the mixture was extracted with dichloromethane (DCM, 3 × 15 mL), with the organic layer being carried forward by removing the solvent *in vacuo*. The crude natural product was brought up in minimal methanol in DCM and run on a preparative TLC (5:5:1 CH_3CN:EtOAc:H_2O) yielding 26.7 mg of crude material. Final purification was accomplished using two consecutive Sephadex™ LH-20 size-exclusion columns eluting with 5:5:1 CH_3CN:EtOAc:H_2O. The solvent was removed *in vacuo* yielding the natural product derivative in a 22.4 mg yield as a mixture of diastereomers (Mj:Mn 25:21) by ^{1}H-NMR spectroscopy. TLC R_f: 0.45 (5:5:1 CH_3CN:EtOAc:H_2O); HPLC R_t = 10.34 min; UV-Vis (3.8×10^{-4} and 4.8×10^{-5} M, MeOH): λ_{max} (ε) = 299 (15916), 396 (2175), 546 (1635); LRMS (ESI^+): MS/MS (783) found 783 $[M+H]^+$, 653 $[M+H\text{-digitoxose}]^+$; HRMS ($ESI^-$) for $C_{46}H_{41}N_2O_{12}$ $[M+MeOH-H]^-$: 813.2678 found, 813.2665 calculated; NMR spectra to follow (see Appendix I), see characterization table (Table 6 & 7) for numbering.

Table 6. Jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide $3a_{Mj}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	4.58	t(3.6)	56.4	1' (a)	1', 2, 2', 13a
2			177.0		1, 1' (a), 1' (b)
3a	5.88	s	86.1		3b, 4, 7a, 13
3b			134.5		3a, 4
4	6.62	s	120.3	5-CH_3, 6	3a, 3b, 4, 5-CH_3, 7, 7a
5					
5-CH_3	2.30	s	21.0	4, 6	4, 6, 7a
6	6.84	s	120.4	5-CH_3, 4	5-CH_3, 6, 7, 7a
7			154.5		4, 6
7-OH					
7a			112.9		3a, 4, 5-CH_3, 6
7b					
8			186.2		9
8a			136.6		9, 10
9	7.88	m	122.0	10	8, 8a, 11
10	7.69	t(5.4)	136.3	9, 11	8a, 9, 11, 12
11	7.50	m	121.9	10	9, 12, 13
12			156.3		1", 10, 11
12a					
13			181.4		9
13a			170.4		1
1' (a)	3.16	m	38.3	1	1, 2, 2', 3'
1' (b)	3.04	d(5.1)	38.3		1, 2, 2', 3'
2'			136.4		1
3'	6.96	m	131.4	4'	1', 5'
4'	6.70	m	124.2	3'	
5'			145.3		3'
6'			170.2		
7' (linker)	4.64	d(3.5)	68.0		6', 8'
8'			156.9		
9'	7.13		108.6		
10'					
11'	7.63		130.6	12'	
12'	7.14		119.4	11', 13'	14'
13'	7.07		119.6	12',14'	
14'	7.52		128.6	13'	
15'					
16'	7.43		130.6	17'	8', 14'
17'	7.17		108.3	16'	
1"	5.72	s	96.3	2" axial	12, 3"
2"axial	2.01	m	35.9	1", 2" equa., 3"	1", 3", 4"
2"equa.	2.08	m	36.0	2" axial, 3"	1", 3", 4"
3"	3.88	m	67.5	2" axial, 2" equa., 4"	1", 5"
3"OH					
4"	3.14	m	73.6	3", 5"	5", 5"-CH_3
5"	3.73	m	66.2	4", 5"-CH_3	4", 5"-CH3
5"-CH_3	1.12	d(4.5)	17.9	5"	4", 5"
MeOH	3.31		49.0		
Water	4.93				

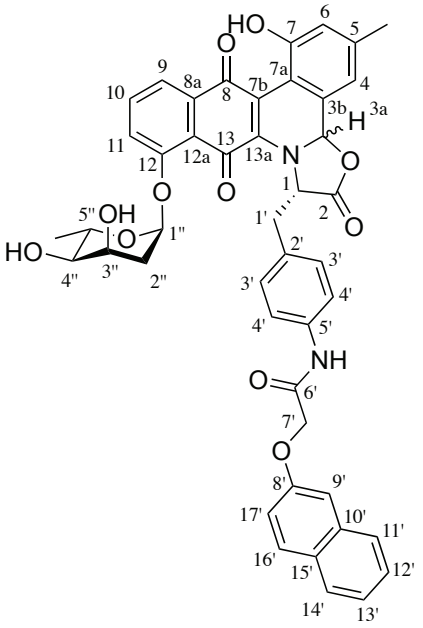
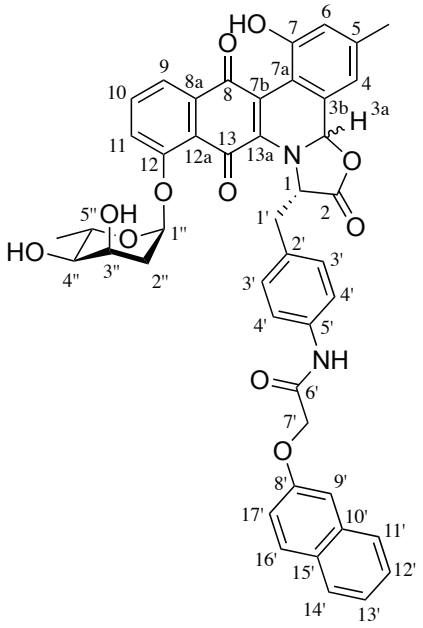


Table 7. Jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide $3a_{Mn}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	4.54	m	56.9	1' (b)	1', 2, 2', 13a
2			177.0		1, 1' (a), 1' (b)
3a	5.86	s	86.1		3b, 4, 7a, 13
3b			134.5		3a, 4
4	6.65	s	120.3	5-CH_3	3a, 3b, 4, 5-CH_3, 7, 7a
5					
5-CH_3	2.33	s	21.0	4, 6	4, 6, 7a
6	6.87	s	120.4	5-CH_3	5-CH_3, 6, 7, 7a
7			154.5		4, 6
7-OH					
7a			112.9		3a, 4, 5-CH_3, 6
7b					
8			186.2		9
8a			136.6		9, 10
9	7.88	m	122.0	10	8, 8a, 11
10	7.69	t(5.4)	136.3	9, 11	8a, 9, 11, 12
11	7.50	m	121.9	10	9, 12, 13
12			156.3		1", 10, 11
12a					
13			181.4		9
13a			170.4		1
1' (a)	3.16	m	38.2		1, 2, 2', 3'
1' (b)	3.02	d(5.3)	38.5	1	1, 2, 2', 3'
2'			136.4		1
3'	6.93	m	131.4	4'	1', 5'
4'	6.60	m	124.2	3'	
5'			145.3		3'
6'			170.2		
7' (linker)	4.68	d(5.6)	67.8		6', 8'
8'			156.9		
9'	7.13		108.6		
10'					
11'	7.66		127.8	12'	
12'	7.21		127.6	11', 13'	14'
13'	7.10		124.9	12', 14'	
14'	7.53		128.6	13'	
15'					
16'	7.43		130.6	17'	8', 14'
17'	7.17		108.3	16'	
1"	5.75	s	96.3	2" axial	12, 3"
2"axial	2.01	m	35.9	1", 2" equa., 3"	1", 3", 4"
2"equa.	2.08	m	36.0	2" axial, 3"	1", 3", 4"
3"	3.88	m	67.5	2" axial, 2" equa., 4"	1", 5"
3"OH					
4"	3.14	m	73.6	3", 5"	5", 5"-CH_3
5"	3.66	m	66.3	4", 5"-CH_3	4", 5"-CH3
5"-CH_3	1.19	d(4.5)	18.2	5"	4", 5"
MeOH	3.31		49.0		
Water	4.93				



2.4.10 Synthesis of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide

Crude jadomycin 4-amino-L-phenylalanine (107.0 mg) was dissolved in 20 mL of 1:1 acetonitrile:phosphate buffered saline (PBS, pH 7.6) and added to phenoxyacetic acid *N*-hydroxysuccinimide ester (66.9 mg, purchased from Sigma) in a 25 mL round bottom flask drop-wise with gentle stirring. The flask was corked and protected from light. The reaction was allowed to proceed for one hour until complete, as determined by TLC (5:5:1 CH_3CN:EtOAc:H_2O, Figure S16).

Once the reaction was complete, the mixture was extracted with dichloromethane (DCM, 4 × 20 mL), the organic layer was carried forward by removing the solvent *in vacuo*, yielding 88.6 mg of crude material. The crude material was then purified using a 40 g silica column preconditioned with 1:1 CH_3CN:EtOAc. The material was eluted using a 20 $mLmin^{-1}$ flow rate collecting 9 mL fractions. Purification was accomplished using a gradient system with solvent A (1:1 CH_3CN:EtOAc) and solvent B (5:5:1 CH_3CN:EtOAc:H_2O). The column was run with an initial isocratic step of 100% solvent A (1 CV), followed by a linear increasing gradient of 0% to 100% solvent B (10 CV), with a final isocratic step of 100% solvent B (10 CV). The solvent was removed *in vacuo*, yielding 22.4 mg of crude material, before being brought up in minimal methanol in DCM and run on a preparative TLC (5:5:1 CH_3CN:EtOAc:H_2O) yielding 17.8 mg of crude material. Final purification was accomplished using two consecutive Sephadex™ LH-20 size-exclusion columns eluting with 5:5:1 CH_3CN:EtOAc:H_2O. The solvent was removed *in vacuo* yielding the natural product derivative in a 14.0 mg yield as a mixture of diastereomers (Mj:Mn 2:1) by ^{1}H-NMR spectroscopy. TLC R_f: 0.70 (5:5:1 CH_3CN:EtOAc:H_2O); HPLC R_t = 9.87 min; UV-Vis (1.2 × 10^{-3} and 1.5 × 10^{-4} M,

MeOH): λ_{max} (ε) = 300 (4530), 392 (746), 548 (565); LRMS (ESI^+): MS/MS (733) found 733 $[M+H]^+$, 603 [M+H-digitoxose]$^+$; HRMS (ESI^-) for $C_{42}H_{39}N_2O_{12}$ $[M+MeOH-H]^-$: 763.2517 found, 763.2508 calculated; NMR spectra to follow (see Appendix I), see characterization table (Table 8 & 9) for numbering.

Table 8. Jadomycin 4-amino-L-phenylalanine phenoxyacetylamide $3a_{Mj}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J/(Hz))	δ ^{13}C (ppm)	COSY Correlation	HMBC Correlation
1	4.54	br, obscured	56.9	1' (a), 1' (b)	2, 3a, 1', 2'
2			177.4		1, 1'(a), 1'(b)
3a	5.74	s	92.8		1, 4, 7a, 7b, 13a
3b			130.6		6
4	6.69	s	120.4	5-CH_3, 6	3a, 5-CH_3, 6, 7a
5			143.0		5-CH_3
5-CH_3	2.33	s	21.1	4, 6	4, 5, 6
6	6.84	s	120.5	4, 5-CH_3	3b, 4, 5-CH_3, 7, 7a
7			155.9		6
7-OH					
7a			113.1		3a, 4, 6
7b			133.2		3a
8			186.5		9
8a			136.7		10, 11
9	7.92	m	121.9	10	8, 12a
10	7.75	m	136.4	9, 11	8a, 12, 12a
11	7.59	d(6.0)	122.4	10	12, 12a
12			156.5		10, 11, 1"
12a			120.8		9, 10, 11
13			181.2		
13a			144.9		3a
1' (a)	3.20	m	38.6	1	1, 2, 2', 3'
1' (b)	3.06	d(5.1)	38.6	1	1, 2, 2', 3'
2'			136.7		1, 1'(a), 1'(b), 4'
3'	7.09	d(6.0)	131.7	4'	1'(a), 1'(b), 5'
4'	6.93	d(5.9)	122.7	3'	2', 5'
5'			145.4		4'
6'			107.2		7'
7' (linker)	4.48	d(6.0)	66.6		6', 8'
8'			159.2		7', 9', 10'
9'	6.90	br, obscured	116.0	10'	8', 11'
10'	7.16	t(5.7)	131.5	9', 11'	8'
11'	6.86	br, obscured	120.9	10'	9'
1"	5.83	m	96.3	2" axial, 2" equa.	12, 3"
2" axial	2.07	m	36.1	1", 2" equa., 3"	
2" equa.	2.16	m	36.1	1", 2" axial, 3"	1", 3", 4"
3"	3.90	m	67.9	2" axial, 2" equa., 4"	1", 4"
3"OH					
4"	3.17	dd(7.1; 2.4)	73.9	3", 5"	5"
5"	3.76	m	66.4	4", 5"-CH_3	5"-CH_3
5"-CH_3	1.15	d(4.5)	18.1	5"	4", 5"
MeOH	3.31		49.0		
Water	4.89				

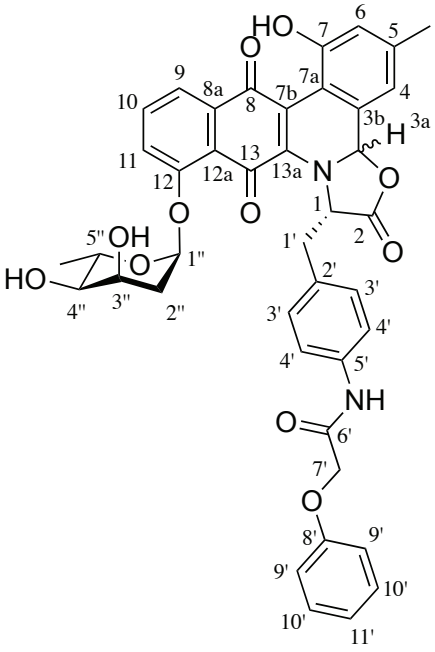
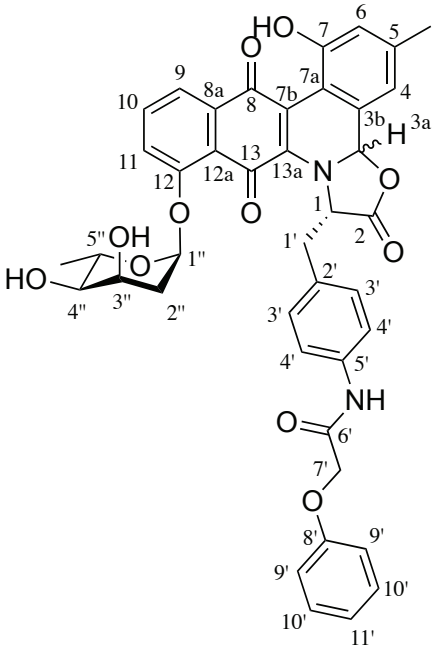


Table 9. Jadomycin 4-amino-L-phenylalanine phenoxyacetylamide $3a_{Mn}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J/(Hz))	δ ^{13}C (ppm)	COSY Correlation	HMBC Correlation
1	4.58	m	56.5	1' (a)	2, 3a, 1', 2'
2			177.2		1, 1'(a), 1'(b)
3a	5.73	s	92.7		1, 4, 7a, 7b, 13a
3b			130.6		6
4	6.67	s	120.2	5-CH_3, 6	3a, 5-CH_3, 6, 7a
5			142.9		5-CH_3
5-CH_3	2.32	s	21.1	4, 6	4, 5, 6
6	6.83	s	120.6	4, 5-CH_3	
7			155.7		6
7-OH					
7a			113.1		3a, 4, 6
7b			132.8		3a
8			186.5		9
8a			136.6		10, 11
9	7.92	m	122.0	10	8, 12a
10	7.75	m	136.4	9, 11	8a, 12, 12a
11	7.56	m	122.3	10	12, 12a
12			157.0		10, 11, 1"
12a			120.8		9, 10, 11
13			181.3		
13a			144.9		3a
1' (a)	3.22	m	38.2	1	1, 2, 2', 3'
1' (b)	3.04	d(5.1)	38.2		1, 2, 2', 3'
2'			136.8		1, 1'(a), 1'(b), 4'
3'	7.11	d(5.9)	131.6	4'	1'(a), 1'(b), 5'
4'	6.97	d(5.8)	122.6	3'	2', 5'
5'			145.4		4'
6'			170.3		7'
7' (linker)	4.54	br, obscured	66.3		6', 8'
8'			159.4		7', 9', 10'
9'	6.90	br, obscured	116.0	10'	8', 11'
10'	7.19	t(5.7)	131.5	9', 11'	8'
11'	6.86	br, obscured	121.0	10'	9'
1"	5.84	d(2.4)	97.0	2" axial, 2" equa.	12, 3"
2" axial	2.07	m	36.2	1", 2" equa., 3"	
2" equa.	2.23	m	36.2	1", 2" axial, 3"	1", 3", 4"
3"	3.93	m	67.8	2" axial, 2" equa., 4"	1", 4"
3"OH					
4"	3.14	dd(7.1; 2.3)	73.0	3", 5"	5"
5"	3.76	m	66.6	4", 5"-CH_3	5"-CH_3
5"-CH_3	1.19	d(4.4)	18.2	5"	4", 5"
MeOH	3.31		49.0		
Water	4.89				



2.4.11 Biological evaluation of jadomycin 4-amino-L-phenylalanine derivatives

National Cancer Institute (NCI) 60 DTP Human Tumor Cell Line Screen anticancer activity

All testing was conducted by the NCI (USA) following standardized procedures.

Photodynamic DNA cleavage assay

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all photodynamic DNA cleavage assays as described below.

Supercoiled plasmid (form I) was prepared by transforming NovaBlue cells (Novagen) and purifying them using QIAprep Spin miniprep kit (Qiagen), yielding ~30 μg of plasmid DNA per 20 mL culture. Jadomycin 4-amino-L-phenylalanine phenoxyacetylamide was dissolved in 99% ethanol and diluted using distilled water. The final assay contained <1% ethanol. The reaction mixture was prepared with transformed pUC19 plasmid (final concentration 130 ng, >95% form I) in 10 mM Tris-Cl (pH 8.5) diluted with Tris (pH 7.4, 5 mM) and sodium chloride (50 mM). The reaction mixture was diluted to 20 μL using ultrapure water. The tube (0.5 mL sterile microcentrifuge tube) was either kept at 37°C in the dark or irradiated with white light in a photoreactor (Luzchem LZC-4X, 7.72 $mWcm^{-2}$) for 30 minutes to yield an energy density of ~14 Jcm^{-2}. Once treatment had concluded, both samples were quenched by the addition of 4 μL gel loading buffer (0.025% bromophenol blue, 40% glycerol), loaded onto 1% agarose gels cast with 1× TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.2) containing ethidium bromide (0.75 μgmL^{-1}) and electrophoresed for 30 minutes at 80 Vcm^{-1} in 1× TAE. The

gel was visualized with UV-transillumination (UVP transilluminator) and processed using the Gel Doc-It Imaging System (UVP).

Copper-mediated DNA cleavage assays

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all copper-mediated DNA cleavage assays as described below.

Plasmid DNA cleavage assays were prepared as outlined above. They were performed in the presence or absence of 100 μM cupric acetate with 100 μM jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide or jadomycin 4-amino-L-phenylalanine phenoxyacetylamide in 20 μL reaction volumes. These reaction tubes were incubated at 37°C for 24 hours. The reaction was then quenched, the gel electrophoresed, and visualized as described above.

Bacterial culture

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all bacterial culture procedures aseptically as described below.

Streptococcus mutans (*S. mutans* Clarke, NCTC 10449) was proliferated in a culture tube containing 2 mL Brain Heart Infusion medium (BHI, Oxoid) and mixed with gentle stirring. The tube was loosely capped and incubated (37°C) for 24 hours. In order to check the purity of the growth, ten serial dilutions were made (10^{-1} to 10^{-8}) and 0.1 mL aliquots of each dilution were spread onto BHI agar plates (3.8% BHI), allowed to dry,

incubated (37°C) overnight, and assessed. The bacterial culture tube was then centrifuged (5000 rpm, 5 min), supernatant decanted, and re-suspended in 5 mL of BHI media. Frozen stock solutions (-80°C) were prepared from this media by mixing 500 µL aliquots of *S. mutans* into microfuge tubes with 500 µL of sterile 70% glycerol in water.

Plates for primary growth colonies were prepared by mixing 50 µL of frozen *S. mutans* stock solution (as prepared above) with 500 µL tryptic soy broth (TSB, Fluka 22092) in a sterile microfuge tube. 50 µL aliquots were applied to TSA plates (3% TSB in agar) in a quadrant streak method, allowed to dry, and incubated overnight. The purity of each plate was then assessed and 1-2 colonies were mixed well with 500 µL TSB in a microfuge tube. This solution was then used for secondary growth plates by transferring 50 µL of this mixture to a TSA plate by quadrant streaking, allowing it to dry, and incubating overnight. The purities of the secondary growth cultures were then verified and used for agar well diffusion assays.

Agar well diffusion assays

Huimin Yin and Susan Monro, of Dr. Sherri McFarland's lab at Acadia University, conducted all agar well diffusion assays as described below. All experiments using *S. mutans* were done from secondary growth colony plates less than one week old under aseptic conditions.

S. mutans inoculums were prepared by mixing secondary growth plate colonies with 5 mL sterile, distilled water in a 15 mL conical tube (VWR, Canada) to a turbidity matching a McFarland barium sulfate standard 3 (~9 x 10^8 $CFUmL^{-1}$). From this

inoculum, 500 µL were spread evenly on each TSA plate and allowed to dry. These plates were then divided into four quadrants each, into each of which a sterile, glass Pasteur pipette was used to make ~6 mm diameter holes. Jadomycins were then dissolved in ethanol to a concentration of 5 mM and then diluted to a final concentration of 300 µL (final ethanol concentration 6%). This solution was then added to the holes in the TSA plates in duplicate. A 6% ethanol in water control was also added to the holes in duplicate. The duplicates were separated for dark and light treatment. Plates subjected to the dark treatment were covered in foil and placed in a dark drawer. Plates subjected to light treatment were irradiated with white light in a photoreactor (Luzchem LZC-4X, 7.72 $mWcm^{-2}$) for sixty minutes to reach an energy density of ~28 Jcm^{-2}. The bacterial growth inhibition zones were then measured using Vernier calipers (Bel-Art, USA).

2.5 References

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CHAPTER 3: JADOMYCIN PRODUCTION

3.1 Introduction

Precursor-directed biosynthesis has been shown (Chapter 2) to be beneficial for the alteration of the jadomycin backbone through the incorporation of differing side-chains. These side-chains can be used to chemically derivatize the crude natural product, quickly expanding the jadomycin library. For this reason, the incorporation of 4-borono-DL-phenylalanine was investigated, and will be presented here. If successfully incorporated, jadomycin 4-borono-DL-phenylalanine could be synthetically derivatized through Suzuki coupling. This boronic acid containing amino acid was also chosen for investigation because boron is not commonly found within natural products.[1] There has also been evidence to the therapeutic potential possessed by boron-containing compounds.[2] Therapeutic potential is important because the jadomycins have been shown to possess a variety of biological activities.[3-11] If the incorporation of this amino acid were successful, it would be of greater importance because of the incorporation of the boron atom into a natural product. Although evidence has been provided to demonstrate precursor-directed biosynthesis' ability to alter the jadomycin structure through the modification of the amino acid side-chain, this method has also been shown to have a more dramatic effect.

Modification of the amino acid provided to the bacteria as their sole nitrogen source has not only affected the jadomycin amino acid side-chain, but also the structure of the oxazolone ring itself. Past jadomycin isolations have shown four structural motifs; the typical five-membered oxazolone ring with an amino acid side-chain (generic jadomycin),[3,12,13] the five-membered ring of jadomycin L-serine (jadomycin S) with a carboxylic acid side-chain,[14] the six-membered ring of jadomycin L-asparagine

(jadomycin LN),[3] and the eight-membered ring of jadomycin L-ornithine (jadomycin Oct)[7] (Figure 12). The three unusual structural motifs have all had various experiments to unequivocally elucidate their structures about the E-ring (Figure 12). The structure of jadomycin S was deduced using the chemical shift of 3aH following a recent total synthesis of the jadomycin (Figure 12).[14] The structure of jadomycin LN was deduced following a number of nuclear magnetic resonance (NMR) experiments, which included total correlation spectroscopy (TOCSY) NMR correlating the NH-proton to the 3a-proton, two-dimensional nuclear Overhauser effect spectroscopy (NOESY) NMR correlating the NH-proton to those at positions 3a and 4, heteronuclear single-quantum correlation (HSQC) spectroscopy NMR showing no carbon signal for the NH-proton, and the fact that the NH-proton signal integrated to one in the proton NMR instead of two if it were the free amino acid form.[3] The structure of jadomycin Oct was deduced following heteronuclear multiple-bond correlation (HMBC) spectroscopy NMR and rotating frame nuclear Overhauser effect spectroscopy (ROESY) NMR, which both correlated the proton at position 3a to that of position 3′.[7] The isolation of these jadomycin scaffolds provides further evidence that the amino acid incorporation is indeed non-enzymatic. It is interesting that the bacteria can incorporate a variety of amino acids into the jadomycin structure because, although the incorporation of the amino acid is non-enzymatic, there are many other enzymes involved in the production of the jadomycin (Scheme 1). The fact that these enzymes are still functional with a variety of amino acids present is interesting and shows a potentially wide substrate scope for this biosynthetic pathway. The production of various novel jadomycins is, therefore, important to probe the biosynthetic scope of the enzymes involved in the jadomycin production.

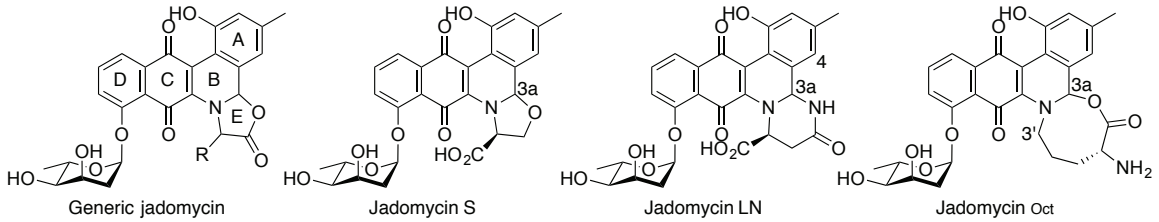
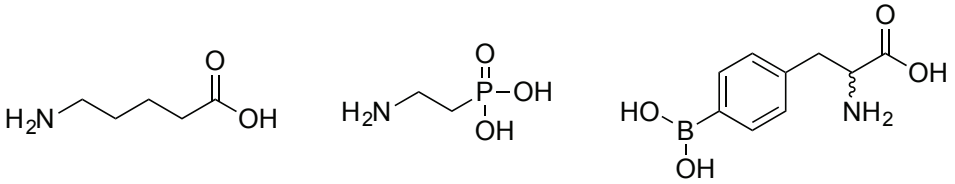


Figure 12. Structural motifs of jadomycins: generic jadomycin where "R" represents the amino acid side chain incorporated into the jadomycin structure, jadomycin LN, jadomycin S, and jadomycin Oct.

Jadomycin production has undergone many alterations since jadomycin B (Figure 1) was originally discovered.[15] These alterations have included changes to the production media, the length of the production period, and the point at which the cells are ethanol shocked.[15] The purification methods have also been altered. Because of these changes, it is thought that different production and purification techniques may lead to different jadomycin structures being isolated. It has been hypothesized that while the literature procedure produces the six-membered ring-containing jadomycin LN (Figure 12),[3] the current purification methodology, outlined in Chapter 2, may lead to the production of the five-membered oxazolone ring-containing jadomycin LN, similar to that of the generic jadomycin (Figure 12). This was hypothesized because previous purification techniques included the use of acids,[3] which are now avoided. This may cause the carboxylic acid to predominate and cause cyclization through the amide moiety forming the six-membered ring jadomycin (Figure 12).[3] To test this hypothesis, studies presented here were carried out to investigate how the purification methodology may affect the E-ring structural motif of jadomycin LN. Jadomycin LN productions were repeated using both current and literature methodologies, and the results are reported herein.

With the exploitation of the non-enzymatic amino acid incorporation being well documented for "traditional" amino acids, attempts were made to introduce "non-

traditional" amino acids. "Traditional" amino acids, those containing a carboxylic acid and a primary amine separated by a single carbon and have been successfully incorporated into the jadomycin structure in the past, as all examples presented thus far have shown.[3,12,13] However, attempts have not been made to incorporate straight chain, "non-traditional" amino acids. These "non-traditional" amino acids contain a carboxylic acid and a primary amine, but are separated by a straight alkyl chain. If these compounds were successfully incorporated into the jadomycin structure it would provide further evidence of a non-enzymatic process, because they are "non-traditional" amino acids. Investigations will be presented using 5-aminovaleric acid (Figure 13). If this "non-traditional" amino acid were successfully incorporated, it would alter the size of the jadomycin E-ring and allow for the possibility of creating a library of other similar jadomycins. As well, if successfully incorporated it may be possible to introduce other linear compounds that have similar properties to those of "non-traditional" amino acids.



5-aminovaleric acid 2-aminoethylphosphonic acid 4-borono-DL-phenylalanine

Figure 13. Structures of the amino acids investigated as potential nitrogen sources for *Streptomyces venezuelae* ISP5230 incorporation into the jadomycin structure.

If "non-traditional" amino acids are shown to successfully incorporate into the jadomycin structure, it may also be possible to incorporate phosphonic acids, specifically those that contain a primary amine because phosphonic acids are known to mimic carboxylic acids.[16] Phosphonic acids were first isolated from a natural source in 1959,[17] and many examples have been shown since. It may be possible to incorporate the first

naturally isolated phosphonic acid, 2-aminoethylphosphonic acid, into the jadomycin structure (Figure 13).[17] If this were the case, it would demonstrate that the bacteria could successfully incorporate many more compounds than originally thought into the jadomycin structure, greatly increasing the scope of the non-enzymatic amino acid incorporation step. The incorporation of this phosphonic acid would also be interesting because they have been shown to be stable toward phosphate cleaving enzymes and hydrolysis in free solution.[16,18] This could potentially make this compound more stable than other structurally similar jadomycins. It may also have interesting properties due to the fact that phosphonic acids are known to mimic both phosphates and carboxylic acids.[16] Here, 2-aminoethylphosphonic acid will be investigated as a possible nitrogen source for *Streptomyces venezuelae* ISP5230.

Beyond the production of jadomycins containing the "non-traditional" amino acids 5-aminovaleric acid and 2-aminoethylphosphonic acid, productions using the "traditional" amino acid 4-borono-DL-phenylalanine (Figure 13) were also attempted and are discussed below.

3.2 Results & Discussion

3.2.1 Productions using 4-borono-DL-phenylalanine

During the typical 48-hour production period, cell growth and production of jadomycin 4-borono-DL-phenylalanine were monitored at 600 nm and 526 nm, respectively (Figure 14). Figure 14 shows that the cells were able to grow in the presence of 4-borono-DL-phenylalanine and there appears to be production of something jadomycin-like. LC-

MS/MS analysis confirmed the presence of jadomycin 4-borono-DL-phenylalanine following the initial phenyl column (Figure 15).

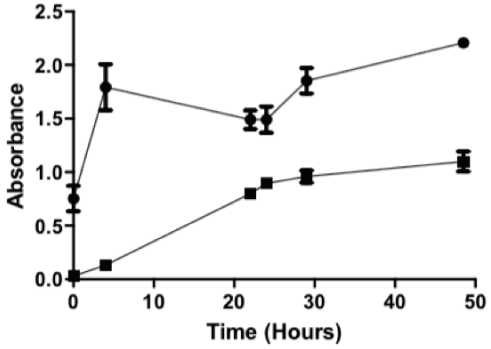


Figure 14. *Streptomyces venezuelae* ISP5230 fermentations in the presence of 4-borono-DL-phenylalanine. Monitoring cell growth at 600 nm (●) and production of coloured compounds at 526 nm (■). Error bars correspond to standard deviation between triplicates.

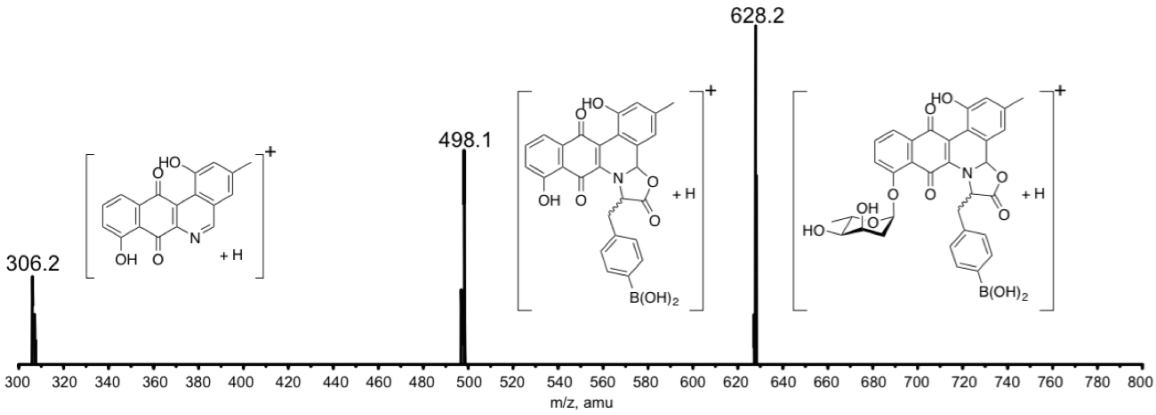


Figure 15. LC-MS/MS spectrum confirming the presence of jadomycin 4-borono-DL-phenylalanine, with the loss of L-digitoxose and the further fragmentation to phenanthroviridin clearly shown.

Once the presence of jadomycin 4-borono-DL-phenylalanine had been confirmed, the material was carried forward through the purification process. The purity of the sample was assessed following the first preparative TLC (Figure 16) which shows the presence of the jadomycin of interest, based on past laboratory work. The purity was again assessed following the second preparative TLC (Figure 17A) before final purification using Sephadex™ LH-20 yielded 4.6 mg of crude material (Figure 17B).

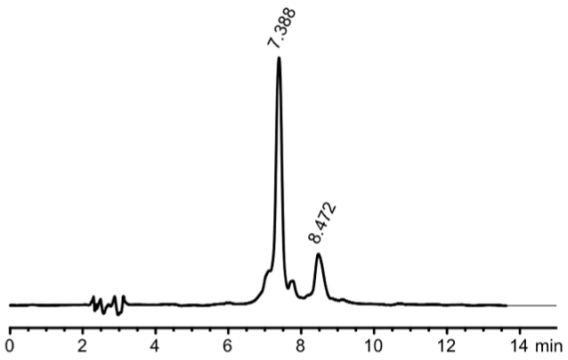


Figure 16. HPLC trace confirming the presence, and assessing the purity, of jadomycin 4-borono-DL-phenylalanine.

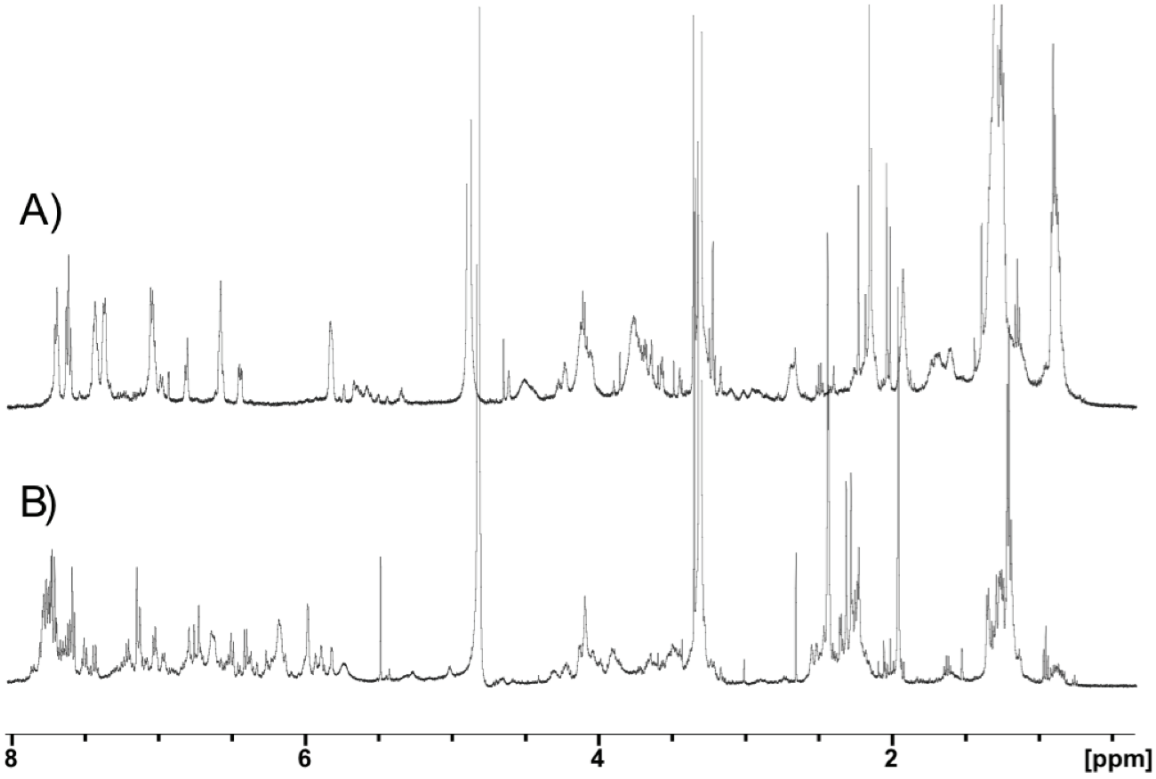


Figure 17. ^{1}H-NMR of jadomycin 4-borono-DL-phenylalanine following (A) preparative TLC (^{1}H: 700 MHz; in CD_3OD) and (B) Sephadex™ LH-20 size-exclusion column (^{1}H: 500 MHz; in CD_3OD).

As is clearly seen in Figure 17, breakdown of jadomycin 4-borono-DL-phenylalanine occurred following the Sephadex™ LH-20 size-exclusion column. This type of column does not generally cause jadomycins to breakdown. Breakdown may also have occurred due to the length of purification. Some jadomycins are known to readily

breakdown during the purification process. It may, therefore, be possible to isolate this material if a faster purification method is used and/or LH-20 is avoided.

Even with the breakdown observed, it was possible to tentatively characterize (section 3.4.1 & Appendix II) this compound from a COSY NMR experiment (Figure S26). These chemical shifts and connectivity were similar to those observed in Chapter 2. The connectivity of the A ring was determined through correlations between 4H and 6H. The D ring was determined through correlations between 9H, 10H, and 11H. The sugar ring was determined through correlations between 1″H, 2″H, 3″H, 4″H, 5″H, and 5″-CH_3. The amino acid aromatic ring was established through correlations between 3′H and 4′H. Finally, the E ring was determined through correlations between 1H and 1′H (see section 3.4.1 for numbering). The production and isolation should be repeated in order to afford more material to fully characterize the material and chemically derivatize the jadomycin.

3.2.2 Productions using L-asparagine

Since the original publication of the structure of jadomycin L-asparagine, jadomycin purification techniques have been altered within the Jakeman lab. This has led to the hypothesis that current methodology leads to the isolation of a five-membered ring species of jadomycin LN, while the literature procedure leads to the isolation of the six-membered ring species (Figure 18). In order to investigate this, a production of this jadomycin was carried out. Upon completion, LC-MS/MS analysis was done to identify that the jadomycin was present (Figure 19), and then the media was divided in equal

parts. Half of the material was purified using the current methodology, while the other half followed literature procedure.[3]

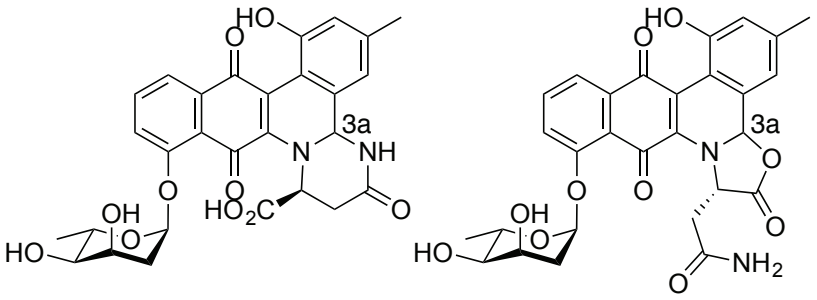


Figure 18. Structures of jadomycin LN following the incorporation of L-asparagine to form a six-membered ring or a five-membered ring.

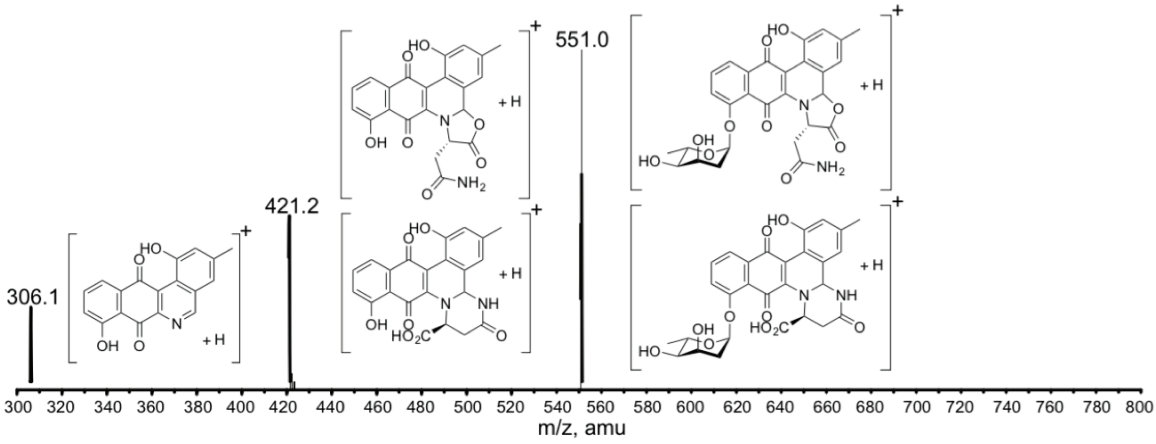


Figure 19. LC-MS/MS spectrum confirming the presence of jadomycin LN, with the loss of L-digitoxose and the further fragmentation to phenanthroviridin clearly shown.

Current purification methodology

Following the current purification methodology, outlined in Chapter 2, it was possible to isolate 12.2 mg (12.2 mgL^{-1}) of pure jadomycin LN, as shown by HPLC (Figure 20). This methodology should yield the five-membered ring jadomycin LN (Figure 18).

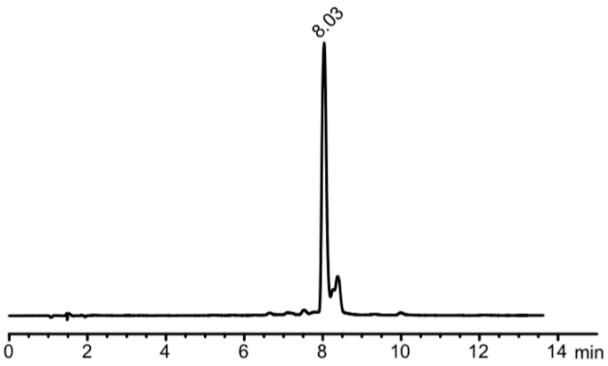


Figure 20. HPLC trace confirming the presence, and purity, of jadomycin LN from the current purification methodology.

Literature purification methodology[3]

Purification of jadomycin LN found that the natural product was in the 75% methanol in phosphate buffer fraction. Following the literature methodology it was possible to isolate 9.7 mg (9.7 mgL^{-1}) of pure jadomycin LN, as shown by HPLC (Figure 21). This methodology should yield the six-membered ring jadomycin LN (Figure 18).

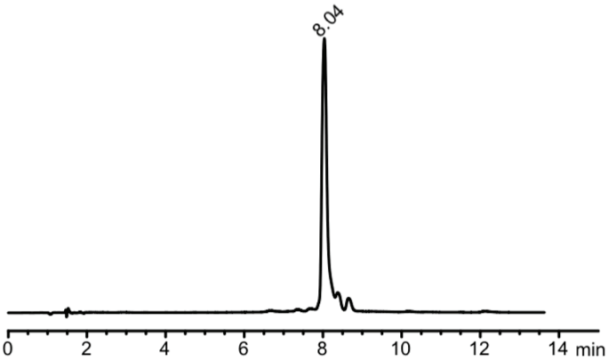


Figure 21. HPLC trace confirming the presence, and purity, of jadomycin LN from the literature purification methodology.

Comparison of current and literature jadomycin LN production methodology

Once the purification of jadomycin LN had been accomplished using both the current and literature methods, it was noted that neither purification method allowed for a substantial increased yield of the natural product. It was then necessary to perform experiments to

determine whether the five- or six-membered ring structures had been formed. As can be seen from the HPLC traces presented (Figure 20 and Figure 21), no difference in retention time can be observed. There is also no difference in the mass spectra, which is to be expected, as both would have the same molecular weight and fragmentation pattern. Therefore, NMR spectroscopy was used.

NMR spectra were recorded at various points throughout the purification process to both monitor the purification and to enable direct comparisons of the material. The first such NMR spectra were recorded of the 100% methanol wash from the initial phenyl column, using the current methodology, and of the 75% methanol in phosphate wash from the initial C18 column, using the literature methodology (Figure 22). The spectra indicate that the sample following the literature procedure is of higher purity at this stage. However, this may have been avoided if the current methodology involved washing the phenyl column with 50% methanol in water before eluting the crude natural product from the column. This would have removed some impurities prior to elution in 100% methanol. The other major difference arises from the fact that there are phenyl contaminants from the phenyl column used in the current methodology.

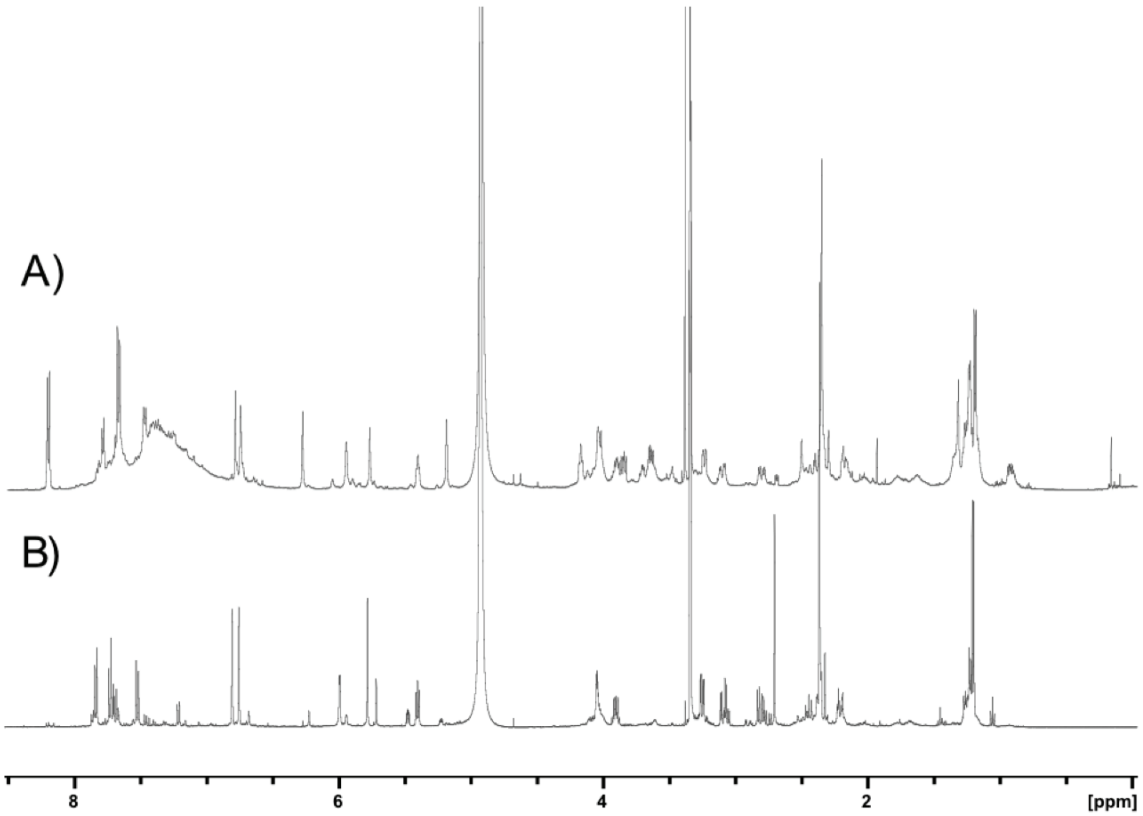


Figure 22. ^{1}H-NMR spectra of jadomycin LN following (A) the 100% methanol wash of the initial phenyl column from the current purification methodology and (B) the 75% methanol in phosphate wash of the initial C18 column from the literature purification methodology (^{1}H: 500 MHz; in CD_3OD).

Further NMR experiments were conducted after the preparative TLCs were completed following the current purification methodology and the flash chromatography C18 column was completed following the literature purification methodology (Figure 23). These two spectra indicate the material is of the same approximate purity and there appears to be no real difference between the spectra. The sole difference arises in the spectrum following the preparative TLCs where there is signal broadening around 1 ppm, which is typical if silica contaminant is present following preparative TLC.

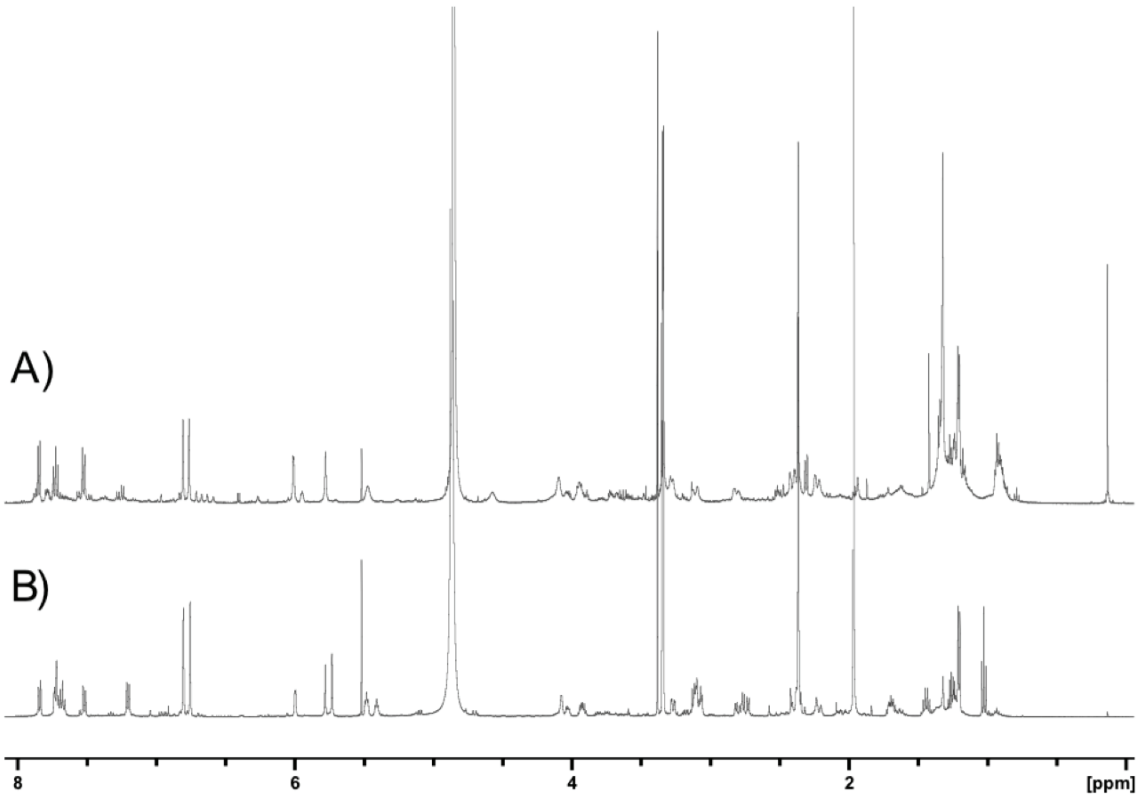


Figure 23. ^{1}H-NMR spectra of jadomycin LN following (A) preparative TLCs of the current purification methodology and (B) the flash chromatography C18 column of the literature methodology (^{1}H: 500 MHz; in CD_3OD).

NMR spectra were again recorded (Figure 24) following the current methodology's LH-20 column and the literature methodology's silica column. The sample prepared through the literature methodology appears slightly less pure than that following the current methodology (contaminants ~1 ppm), but are otherwise comparable (Figure 24). Since no differences are readily seen, it was believed that changing the solvent that the NMR sample was run in, may shed light on which species is present.

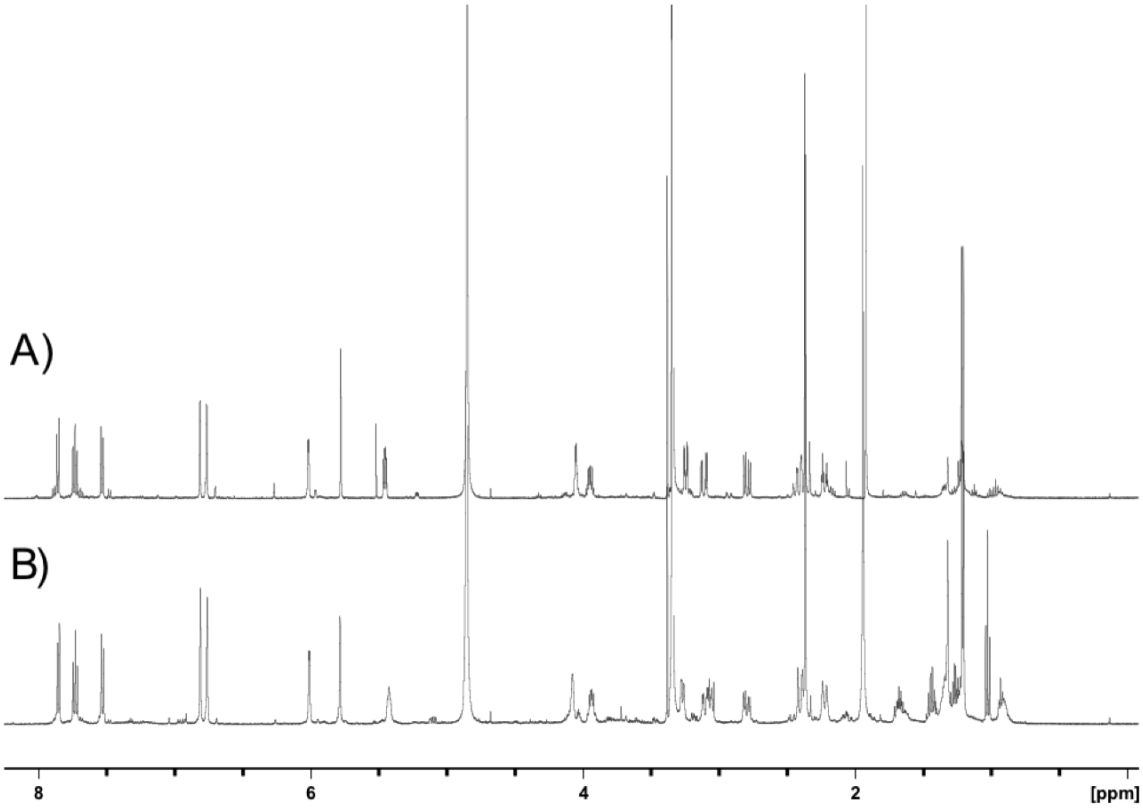


Figure 24. ^{1}H-NMR spectra of purified jadomycin LN following (A) the current purification methodology and (B) the literature methodology (^{1}H: 500 MHz; in CD_3OD).

The sample was then run in CD_3OH, rather than CD_3OD, to observe exchangeable protons (Figure 25). Specifically, CD_3OH should determine whether there is a carboxylic acid present and whether there are one or two protons on nitrogen, which would correspond to the six- or five-membered ring structures, respectively. Exchangeable protons were readily observed in the sample prepared following the literature methodology (~8.7 and ~11.1 ppm), while none were observed in the sample prepared following the current methodology. The lack of signals in the second sample does not effectively conclude whether the five- or six-membered ring jadomycin LN was isolated. This is because both structures have different exchangeable protons present. When these exchangeable protons are not visible, it is typical to acidify the solution in which NMR is

being recorded until they are visible. However, this cannot be done to the sample prepared using the current purification methodology. Both purification methodologies are quite similar, except for the fact that the literature methodology uses acetic acid. Therefore, if the sample prepared using the current methodology were to be acidified, it would not be possible to determine whether it is the addition of acid that leads to the production of the six-membered ring jadomycin LN. Since acidification of the solution was not feasible, 2D-NMR techniques were used to probe this further.

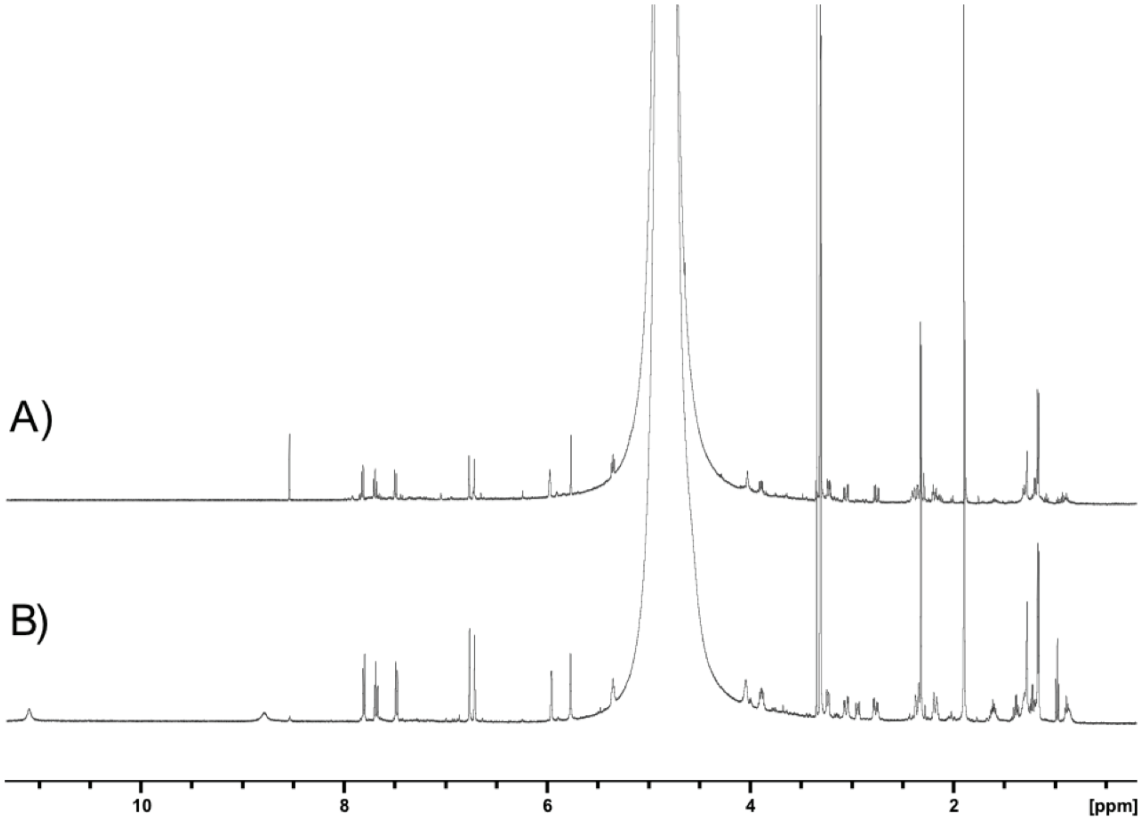


Figure 25. ^{1}H-NMR spectra of purified jadomycin LN following (A) the current purification methodology and (B) the literature methodology (^{1}H: 700 MHz; in CD_3OH).

Total correlated spectroscopy (TOCSY) NMR was performed to determine the connectivity of the jadomycin LN species. TOCSY NMR allows for correlations between all protons within any given spin system. This analysis is effective in jadomycin analysis

because it allows all protons within a ring system to be correlated. TOCSYs of different mixing times (60 ms and 120 ms) were recorded of both purification methods (Figure S27–S30). All four spectra show the same cross-peaks, and none have the 3a proton correlating to an exchangeable proton. In the literature, a 60 ms TOCSY is used to determine the connectivity between the 3a proton and the NH proton.[3] In my hands, it appears as though both methods, current and literature, produce structurally identical jadomycin LN samples. From the data, it appears as though both samples presented here are the five-membered ring jadomycin LN species. This is strongly corroborated through the use of the TOCSY experiments that demonstrate the connectivity associated with a five-membered ring jadomycin.

3.2.3 Investigations into a yellow-coloured compound from jadomycin DS purifications

While producing jadomycin DS for future studies (Chapters 4 & 5), an unknown yellow compound was isolated. This compound was isolated following the first preparative TLC of jadomycin DS, and investigations were undertaken to determine if it was a similar compound to that isolated during jadomycin lysine (jadomycin K) productions. Once purification had been completed, yielding 14.0 mg (7 mgL^{-1}) of pure material, the sample was analyzed by ^{1}H-NMR spectroscopy (Figure 26). It was discovered that the unknown yellow compound was L-digitoxosyl phenanthroviridin; the same compound previously isolated from jadomycin K productions (Figure 26A & Figure 27). Although the yield found was 14.0 mg, the true yield may be higher since some material may have been lost during the initial purification steps of jadomycin DS.

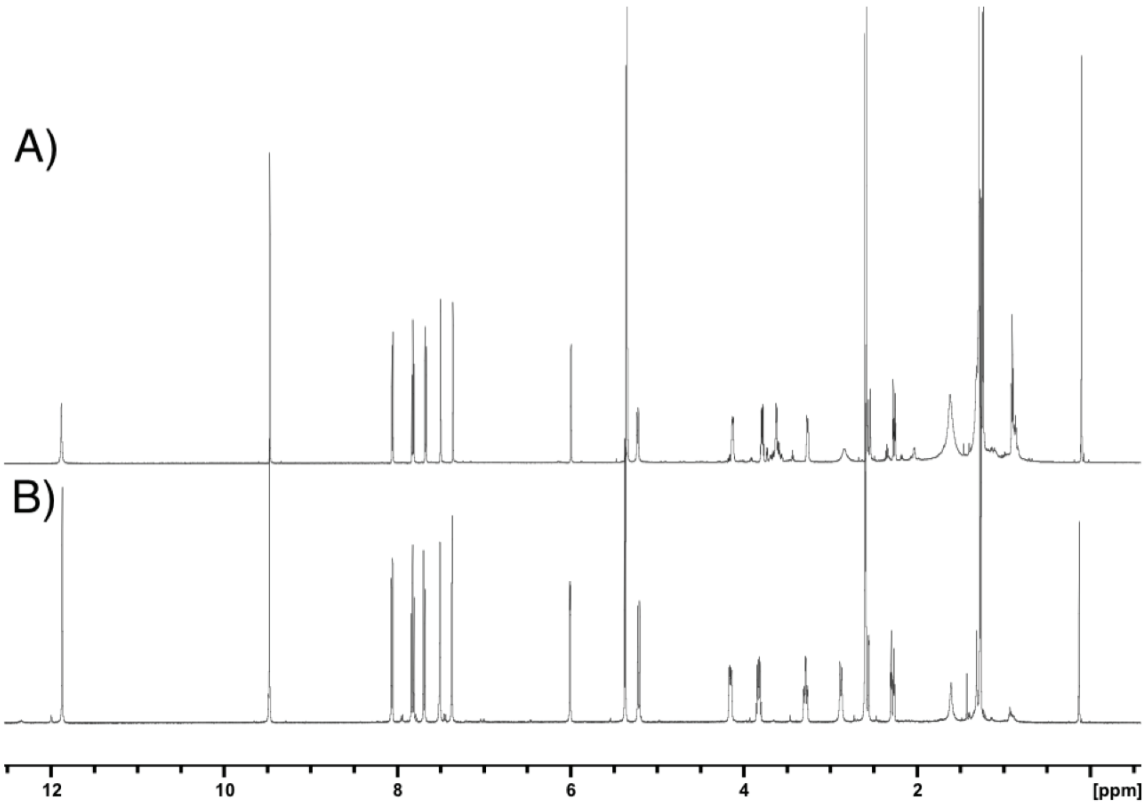


Figure 26. Comparison of L-digitoxosyl phenanthroviridin isolated from (A) a jadomycin K production and (B) a jadomycin DS production (^{1}H: 500 MHz; in CD_2Cl_2).

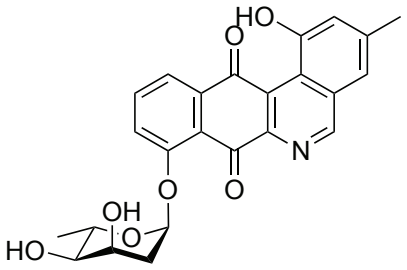


Figure 27. Structure of L-digitoxosyl phenanthroviridin.

The fact that L-digitoxosyl phenanthroviridin has been isolated in the productions of two separate jadomycins (jadomycin DS and jadomycin K) may signify that it is a natural product directly produced by *S. venezuelae* ISP5230 VS1099. In order to probe this further, a production of L-digitoxosyl phenanthroviridin was attempted and discussed below.

Production of L-digitoxosyl phenanthroviridin

In order to probe the ability of *S. venezuelae* ISP5230 VS1099 to produce L-digitoxosyl phenanthroviridin, the use of ammonium chloride as the sole nitrogen source for the bacteria, was explored. In theory, the bacteria should be able to use ammonium chloride, much as they use other amino acids, and incorporate it into the jadomycin backbone through the use of the imine intermediate (Scheme 1).

During the typical 48-hour production period, cell growth and production of L-digitoxosyl phenanthroviridin were monitored at 600 nm and 526 nm, respectively (Figure 28). Figure 28 shows that the cells were able to grow in the presence of ammonium chloride but were not able to produce anything jadomycin-like. However, this is expected since L-digitoxosyl phenanthroviridin is yellow in colour and not red/purple as jadomycins are, and it is this colour that is monitored at an absorbance of 526 nm. The productions were indeed a yellow-brown colour at the end of the production period.

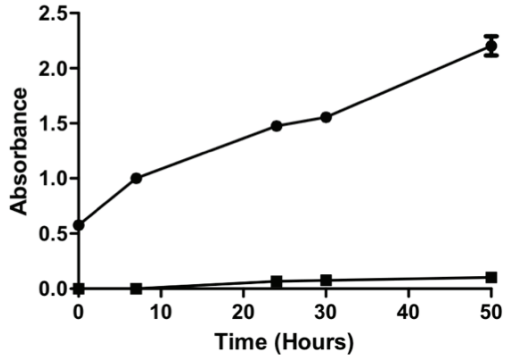


Figure 28. *Streptomyces venezuelae* ISP5230 fermentations in the presence of ammonium chloride. Monitoring cell growth at 600 nm (●) and production of coloured compounds at 526 nm (■). Error bars correspond to standard deviation between triplicates.

Following the initial phenyl column a yellow compound was isolated, and TLC analysis suggested it was L-digitoxosyl phenanthroviridin. However, L-digitoxosyl phenanthroviridin was not present upon HPLC and LC-MS/MS analysis.

The fact that *S. venezuelae* ISP5230 VS1099 did not produce L-digitoxosyl phenanthroviridin in the presence of ammonium chloride may indicate that this compound is due to jadomycin-breakdown and isolated during jadomycin purification. Another explanation could be that ammonium chloride allows the bacteria to grow without providing a sufficient stress condition; as such the bacteria do not produce secondary metabolites. This has been corroborated by an experiment investigating the production of jadomycin DS, presented below. It may, therefore, be possible to produce L-digitoxosyl phenanthroviridin using an amino acid that has been shown not to incorporate into the jadomycin structure, but still allows for cell growth. However, it appears as though isolation of this natural product from jadomycin productions is readily accessible.

Investigations into the production of jadomycin DS in the presence of ammonium chloride

Two productions were conducted to further investigate the effect that ammonium chloride had on natural product production by *S. venezuelae*. To do this, productions of jadomycin DS were carried out in the presence or absence of equimolar ammonium chloride. During the typical 48-hour production period, the cell growth and production of coloured compounds were monitored (Figure 29). As can be seen, both methods allowed the bacteria to grow (Figure 29). However, the bacteria were able to grow to a higher

concentration in the presence of ammonium chloride than in the absence of it (Figure 29 & Table 10). This did not translate into higher production of coloured compounds, as determined by Abs_{526} (Figure 29 & Table 10).

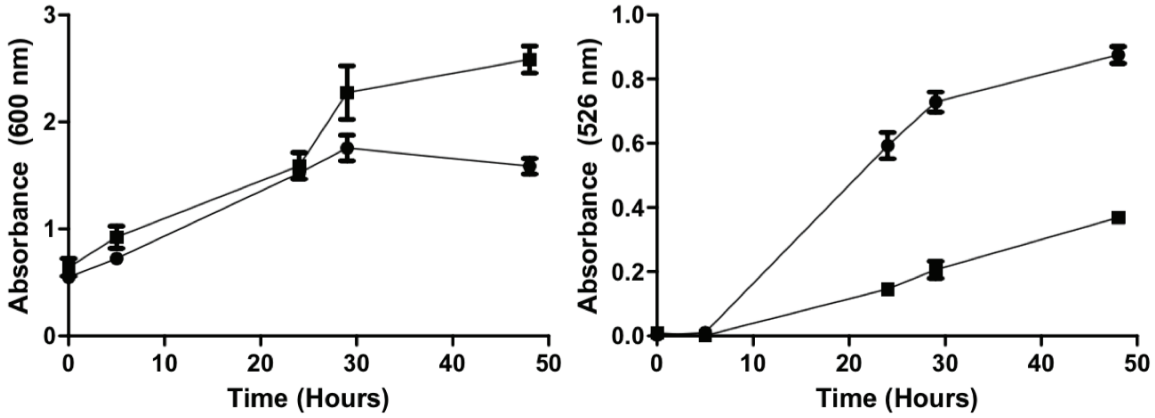


Figure 29. *Streptomyces venezuelae* ISP5230 fermentations in the presence of D-serine (●) and D-serine with equimolar ammonium chloride (■). Monitoring cell growth at 600 nm and production of coloured compounds at 526 nm. Error bars correspond to standard deviation between triplicates.

Table 10. Final absorbance readings for productions of jadomycin DS in the presence or absence of equimolar ammonium chloride.

Production Media	OD_{600} 48 hours (AU)	Abs_{526} 48 hours (AU)
D-serine	1.587	0.875
D-serine + NH_4Cl	2.583	0.368

Further evidence that jadomycin DS production was hampered by the presence of ammonium chloride was found following the conclusion of the 48-hour production period. Analysis of the production media directly following cell-removal showed that there was jadomycin DS produced in both productions attempted (R_t: ~9.3 min), but at a much lower concentration in the presence of ammonium chloride (Figure 30A). There were also a larger amount of impurities within the sample in the presence of ammonium chloride (Figure 30A). A higher concentration of jadomycin DS was also shown following the phenyl column (Figure 30B), where 7.8 mg of crude material were isolated

from the production using 60 mM D-serine and 2.4 mg of crude material were isolated from the production using 60 mM D-serine with 60 mM ammonium chloride. LC-MS/MS confirmed that both samples contained jadomycin DS (Figure 31).

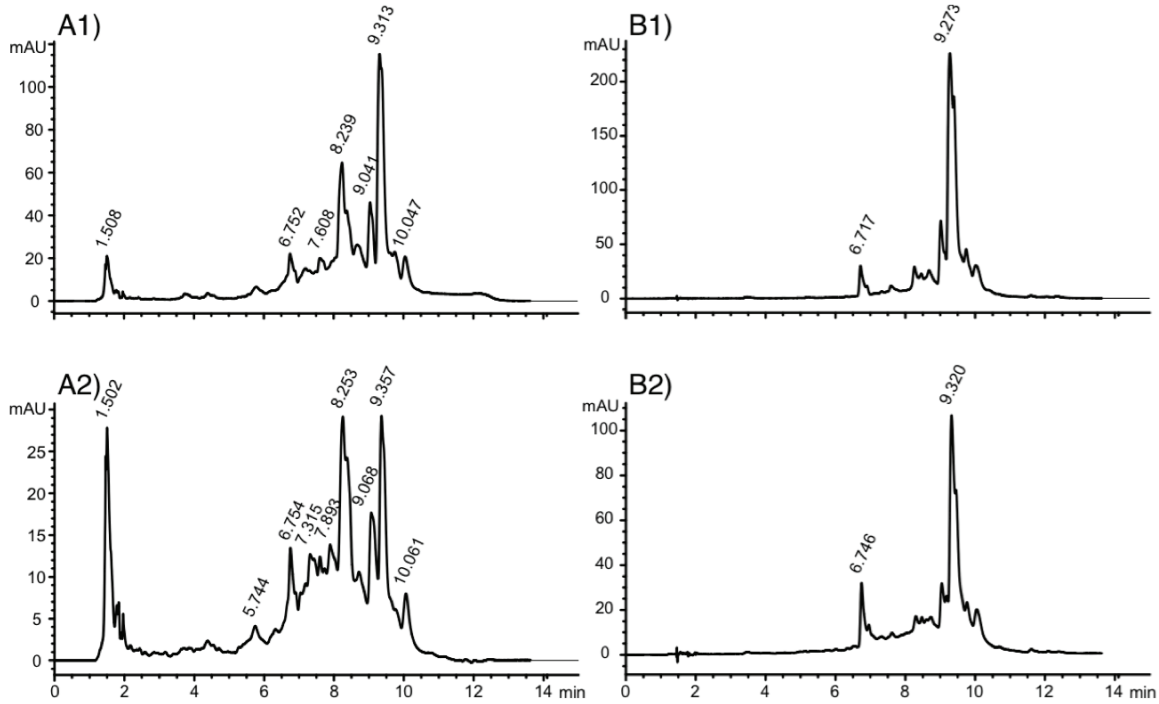


Figure 30. HPLC traces of (1) productions using D-serine and (2) productions using D-serine with equimolar ammonium chloride; after (A) the removal of cells from the production media and (B) the initial phenyl column.

From the evidence presented, it appears as though ammonium chloride does not present the cells with sufficient stress conditions to produce L-digitoxosyl phenanthroviridin nor jadomycins at normal amounts. In order for the bacteria to solely produce L-digitoxosyl phenanthroviridin, other culture conditions have to be investigated. These could include using amino acids that are known to not successfully produce jadomycins.

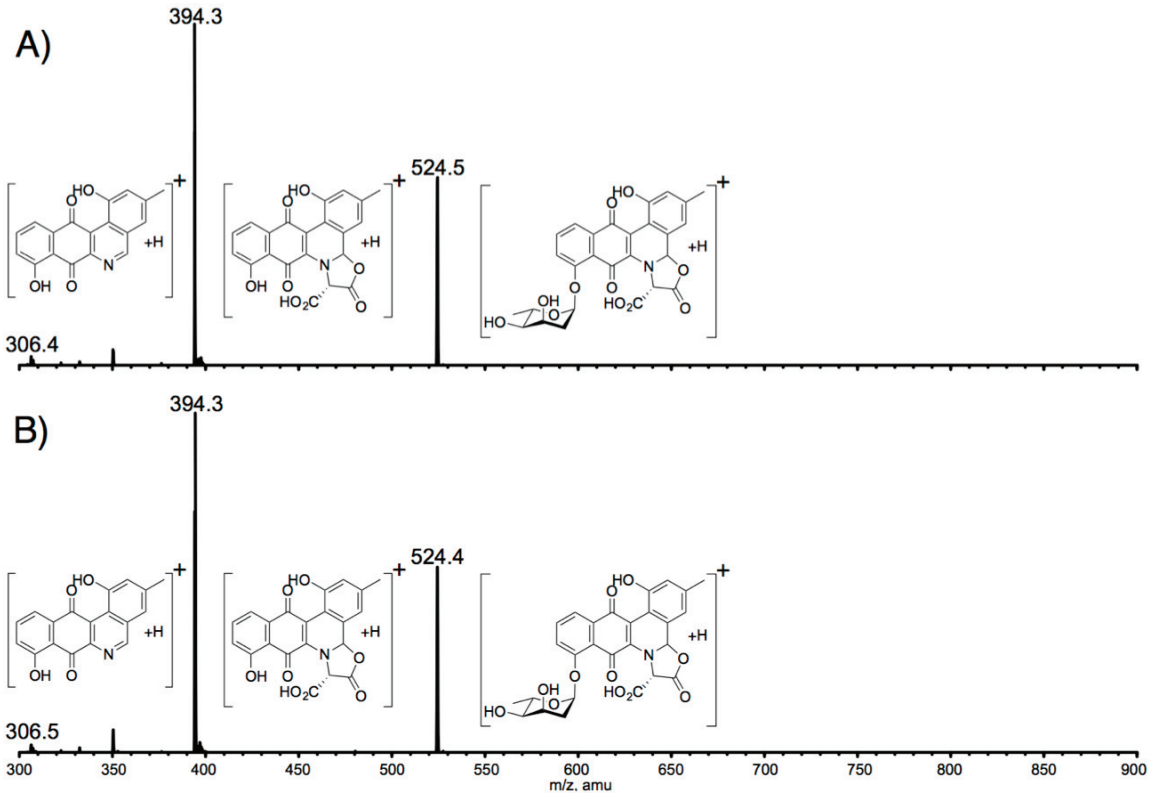


Figure 31. LC-MS/MS spectrum of (A) production with D-serine and (B) production with D-serine and equimolar ammonium chloride confirming the presence of jadomycin DS, with the loss of L-digitoxose and the further fragmentation to phenanthroviridin clearly shown.

3.2.4 Productions using 2-aminoethylphosphonic acid

2-Aminoethylphosphonic acid was investigated as a potential nitrogen source for *S. venezuelae* ISP5230 incorporation into the jadomycin structure. This was chosen as a "non-traditional" amino acid because phosphonic acids are known to mimic carboxylic acids. However, since phosphonic acids are not "traditional" amino acids, all productions were supplemented with additional nitrogen sources. The additional nitrogen sources investigated, L-proline[12] and ammonium chloride (current work), have been found to not produce jadomycins. The previous section also demonstrated that jadomycin DS could be produced in the presence of ammonium chloride. A production using D-serine as the sole nitrogen source was also conducted in parallel to the productions using 2-

aminoethylphosphonic acid to ensure cell viability. Data presented is to demonstrate cell viability only.

OD_{600} indicates that cell viability was not affected in the presence of the phosphonic acid (Figure 32). In fact, the cells grew to a higher extent in the presence of the phosphonic acid than the D-serine control (Figure 32). This did not translate into higher Abs_{526} readings (Figure 32). Both productions using phosphonic acids had poor Abs_{526} readings (Figure 32). Jadomycin 2-aminoethylphosphonic acid production was also monitored *via* the LC-MS/MS total ion chromatogram (TIC; Figure 32). Production of the natural product was only observed in productions supplemented with L-proline. This production demonstrates the presence of jadomycin 2-aminoethylphosphonic acid; however, the compound begins to breakdown in the second half of the production period (Figure 32). This would suggest that isolation might be difficult because of potential breakdown and decreased quantity of the natural product, but may benefit from a shorter production period

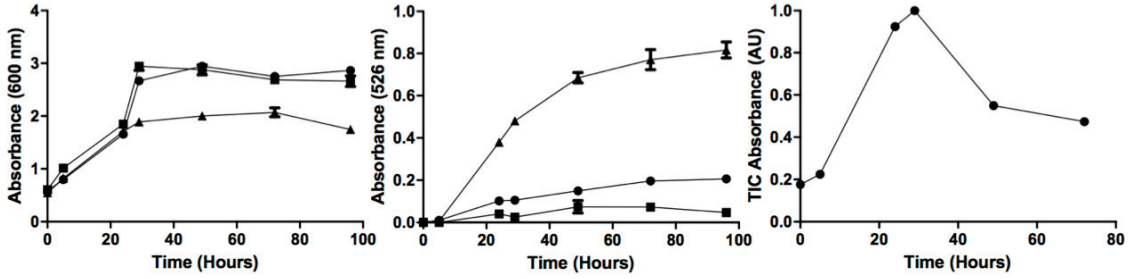


Figure 32. *Streptomyces venezuelae* ISP5230 fermentations in the presence of D-serine (▲), 2-aminoethylphosphonic acid with equimolar L-proline (●), and 2-aminoethylphosphonic acid with equimolar ammonium chloride (■). Monitoring cell growth at 600 nm, production of coloured compounds at 526 nm, and jadomycin 2-aminoethylphosphonic acid presence by LC-MS/MS TIC (normalized to the maximum reading). Error bars correspond to standard deviation between triplicates.

Once the phosphonic acid-containing productions were stopped, both were passed down the initial phenyl column. The production supplemented with L-proline was run as previously described. However, methanol was not sufficient to elute all coloured material off of the column. In an attempt to remove the remaining coloured material dichloromethane and 5:5:1 CH_3CN:EtOAc:H_2O were attempted as possible eluents. Dichloromethane did not remove the remaining material, and 5:5:1 CH_3CN:EtOAc:H_2O had minimal success. It was noted that the coloured material that had been eluted using this solvent system remained in the aqueous layer. To exploit this, the final solvent system used was 1:1 methanol:H_2O. This successfully eluted the remainder of the coloured material. All fractions were then concentrated separately for analysis. The production supplemented with ammonium chloride was also passed down an initial phenyl column. Although the TIC during the ninety-six hour production period did not show the presence of the natural product, the phenyl column was conducted to concentrate the potential jadomycin, if it had been produced. This material was run using the typical methodology, followed by 1:1 methanol:H_2O, which eluted the remaining coloured material. Again, the fractions were kept separate for analysis. LC-MS/MS analysis indicated that the natural product was present in the production supplemented with L-proline only (Figure 33). The production supplemented with ammonium chloride was, therefore, no longer investigated.

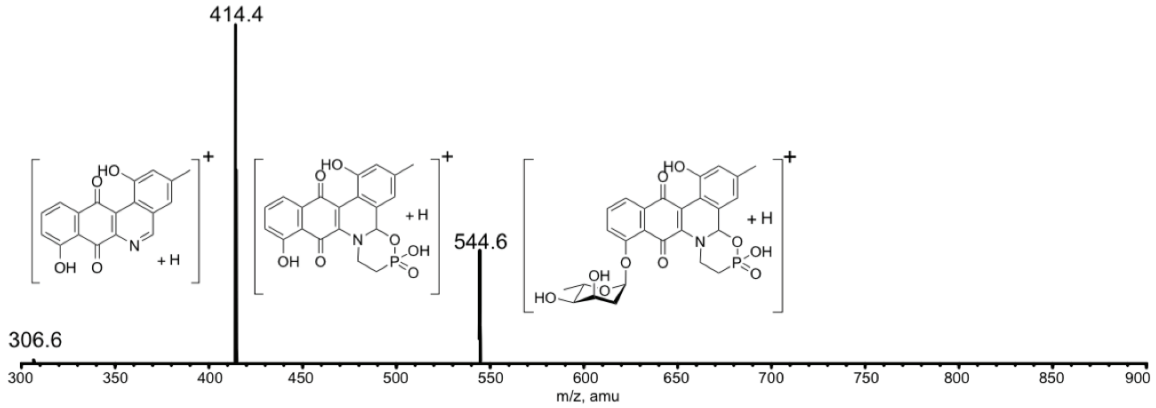


Figure 33. LC-MS/MS spectrum of production with 2-aminoethylphosphonic acid and equimolar L-proline confirming the presence of jadomycin 2-aminoethylphosphonic acid, with the loss of L-digitoxose and the further fragmentation to phenanthroviridin clearly shown.

LC-MS/MS analysis of the fractions following the initial phenyl column run on the production supplemented with L-proline indicated that the natural product of interest was likely in the 1:1 methanol:water fraction. HPLC analysis corroborated this (R_t ~8.2 min), with a potential jadomycin also being found in the coloured fraction that was eluted during the initial loading of the phenyl column (R_t ~7.6 min; Figure 34). The clear fraction that was eluted during the water wash was also analyzed, but found to contain mostly chloramphenicol (R_t ~6.8 min; Figure 34).

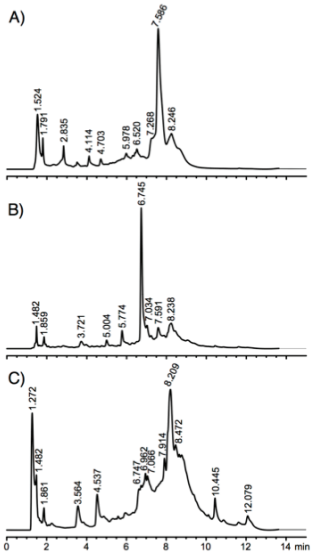


Figure 34. HPLC traces of fractions obtained following the initial phenyl column on production containing 2-aminoethylphosphonic acid and equimolar L-proline eluted during (A) the loading of the column (B) washing the column with ddH_2O and (C) elution with 1:1 methanol:water.

Finally, to determine whether a novel jadomycin had been produced, before the purification process was continued, NMR analysis was conducted. The coloured material that had been eluted while loading the production media contained a signal in the ^{31}P NMR; however, there were no characteristic jadomycin ^{1}H signals (Figure 35). Instead, the signals seem to arise from 2-aminoethylphosphonic acid and a potential sugar, as determined by NMR spectroscopy (Figure 35). The material eluted with 1:1 methanol:water did not have a ^{31}P-NMR signal, but appeared to contain aromatic ^{1}H-NMR signals, which could indicate a potential jadomycin (Figure 35). Since the jadomycin of interest should contain a phosphorous atom, it appears unlikely that this contains the jadomycin of interest. This sample exhibits poor purity, along with a small quantity of material; as such its investigation was discontinued. The spectra presented (Figure 35) show that the isolated material includes the starting material, 2-aminoethylphosphonic acid, as well as potential D-glucose that had been added to the production media.

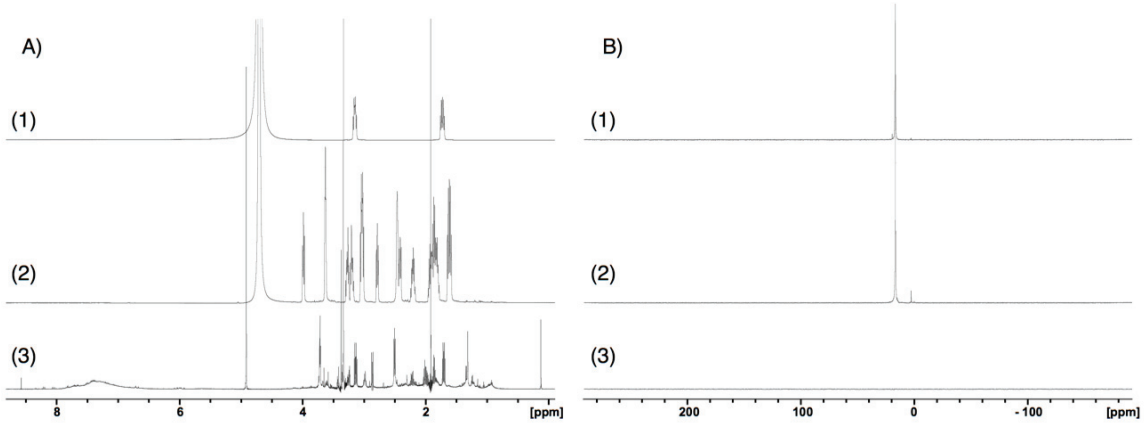


Figure 35. (A) ^{1}H-NMR and (B) ^{31}P-NMR of (1) 2-aminoethylphosphonic acid in D_2O, (2) loading flow-through in D_2O, and (3) 1:1 methanol:water elution in CD_3OD (^{1}H: 500 MHz, ^{31}P: 202 MHz).

3.2.5 Productions using 5-aminovaleric acid

In order to prepare the production media, a 6 M solution of aqueous 5-aminovaleric acid was prepared and filter sterilized separately from the autoclaved MSM production media. This was done to avoid potential self-cyclization of 5-aminovaleric acid in the autoclave. While monitoring for cell growth (OD_{600}) and jadomycin production (Abs_{526}), it was found that the cells were able to grow normally, as compared to past jadomycin work,[6,7] and there appeared to be jadomycin production (Figure 36). Preliminary HPLC and LC-MS/MS results also suggested the presence of a jadomycin.

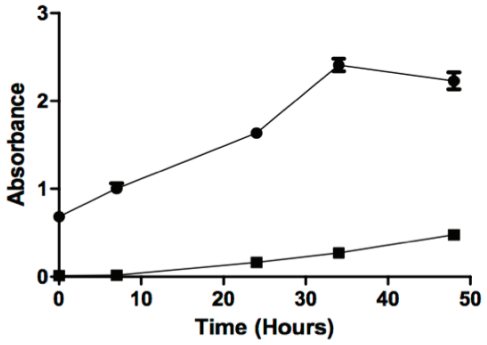


Figure 36. *Streptomyces venezuelae* ISP5230 fermentations in the presence of 5-aminovaleric acid. Monitoring cell growth at 600 nm (●) and production of coloured compounds at 526 nm (■). Error bars correspond to standard deviation between triplicates.

Following the purification methodology it was possible to isolate 4.5 mg (68.2 mgL^{-1}) of pure jadomycin 5-aminovaleric acid, which was fully characterized (see section 3.4.5).

Incorporation of 5-aminovaleric acid into the jadomycin structure can only occur through the formation of an eight-membered ring. This is an interesting compound

because there has only been one previously reported jadomycin with an eight-membered ring structure, jadomycin Oct. The eight-membered ring has been confirmed through key correlations in the HMBC NMR spectrum (3aH to 3′C; Figure S35 & Figure S36) and the nuclear Overhauser effect spectroscopy (NOESY) NMR spectrum (3aH to 3′H) (Figure S37 & Figure 37). The connectivity and chemical shifts observed are similar to those presented in Chapter 2 and of recently isolated eight-membered ring containing jadomycins.[7]

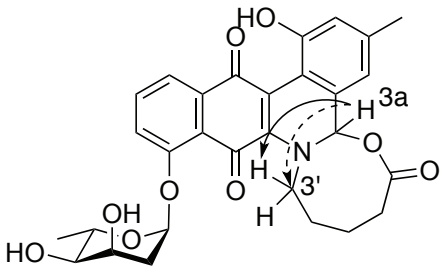


Figure 37. Structure of jadomycin 5-aminovaleric acid showing key structure elucidation correlations; plain arrow demonstrates NOESY NMR correlation and dashed arrow demonstrates HMBC NMR correlation.

3.3 Conclusions

The soil bacteria *Streptomyces venezuelae* ISP5230 VS1099 has been shown to exhibit substrate flexibility through its ability to successfully incorporate various amino acids, both traditional and "non-traditional", into the jadomycin structure. This provides further evidence that the incorporation of these amino acids likely occurs in a non-enzymatic fashion.

Exploitation of this non-enzymatic biosynthetic step resulted in the production and isolation of multiple novel jadomycins, effectively expanding the jadomycin library. The jadomycin that was successfully produced, isolated, and characterized was

jadomycin 5-aminovaleric acid. This jadomycin provides a significant advance for the jadomycin library because it provides a novel jadomycin scaffold.

Jadomycin 5-aminovaleric acid is the first non-substituted eight-membered ring jadomycin isolated. It is, therefore, also the first example of a straight-chain "non-traditional" amino acid to be isolated. The significance of this isolation is that the bacteria are capable of producing jadomycins utilizing linear amino acids. This could be exploited to produce jadomycins with differing ring sizes. Although some jadomycins with differing ring sizes have been isolated,[3,7] this would be the first example where the ring size was purposefully altered. Work is currently being undertaken to investigate whether this is possible using a variety of straight chain amino acids.

While 5-aminovaleric acid was successfully incorporated in isolatable quantities, the same was not true for the phosphonic acid containing jadomycin. Although the LC-MS/MS data (Figure 33) suggests that jadomycin 2-aminoethylphosphonic acid was successfully produced, it was not possible to isolate the compound of interest. This is likely because the material was not produced at sufficient concentrations. In order to remedy this fact, large-scale productions may be undertaken in order to isolate sufficient quantities for characterization. Another potential avenue for the production of jadomycin 2-aminoethylphosphonic acid could be the production of this novel natural product in the presence of L-proline or D-serine. This approach has been shown (current work) not to hinder the production of the jadomycin being investigated. It has also been shown that productions using novel amino acids in the presence of D-serine allow for the production of novel jadomycins, even when these amino acids do not seem to produce novel jadomycins when they are used in the absence of D-serine.[19] Future work should involve

investigating these potential avenues to produce a phosphonic acid containing jadomycin in appreciable yields. Although the material was not isolated, it is possible to determine that a phosphonic acid sufficiently mimics a carboxylic acid to become incorporated into a jadomycin. This presents the opportunity to investigate an entirely new class of phosphonic acid containing jadomycins.

The fact that jadomycin 2-aminoethylphosphonic acid was unsuccessfully incorporated into the jadomycin structure in isolatable quantities gives further evidence that the incorporation of novel amino acids is not as simple as exposing the bacteria to them.

Although the isolation of jadomycin 4-borono-DL-phenylalanine was not successful, it should be repeated. In the future, full characterization should be undertaken prior to the final LH-20 column to ensure there is no breakdown of material. Otherwise, the LH-20 purification procedure could be avoided altogether to prevent potential breakdown of the natural product. This should allow for the full characterization and isolation of this material.

Investigations into how the purification methodology affects the structure of jadomycin LN showed that, in my hands, both methodologies produced the five-membered ring isomer. No significant differences were observed between both samples, and the signals that were present all suggested the presence of the five-membered ring isomer.

Finally, L-digitoxosyl phenanthroviridin was successfully isolated from a production of jadomycin DS. The isolation of this compound from productions using D-serine and L-lysine, among others, suggests it is a breakdown product during the

jadomycin biosynthesis or their purification. Investigations into the production of L-digitoxosyl phenanthroviridin using ammonium chloride and production of jadomycin DS supplemented with ammonium chloride showed that the bacteria are not sufficiently stressed under these conditions, and as such will not produce L-digitoxosyl phenanthroviridin directly nor high concentrations of jadomycin DS.

Although many potential avenues towards the isolation of novel jadomycins have been presented, it is not the only potential natural product that may be isolated from these soil bacteria. Investigations into the remaining cryptic gene clusters could yield a multitude of novel natural product families. This, along with the continued study of jadomycins, will potentially yield multiple novel natural products in the near future.

3.4 Experimental

The production, purification, and characterization methods used were similar to those presented in Chapter 2. HRMS of jadomycin 5-aminovaleric acid used an ESI^+ source. NMR spectra were recorded in CD_2Cl_2, CD_3OD, CD_3OH, and D_2O. Chemical shifts are given in ppm and have been calibrated to residual solvent peaks (CD_2Cl_2: 5.32 ppm; CD_3OD: 3.31 ppm; CD_3OH: 3.31 ppm; D_2O: 4.79 ppm). Nuclear Overhauser effect spectroscopy (NOESY) NMR experiments were also used. Table 11 gives the concentrations of the amino acids used.

Table 11. Concentration of amino acid used in production of the corresponding jadomycin.

Amino acid to be incorporated	Concentration (mM)
4-borono-DL-phenylalanine	30
L-asparagine	60
2-aminoethylphosphonic acid + ammonium chloride	60 & 60

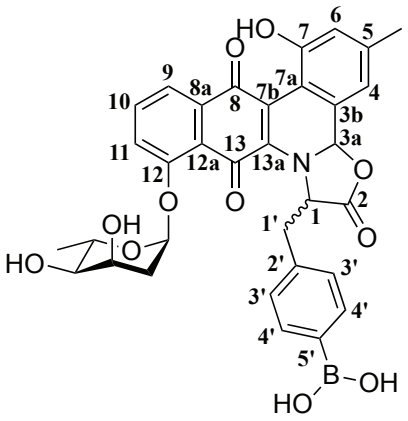
2-aminoethylphosphonic acid + L-proline	60 & 60
5-aminovaleric acid	60

3.4.1 Productions using 4-borono-DL-phenylalanine

The solvent was removed *in vacuo* following the initial phenyl column and purification was pursued using an 80 g silica flash chromatography column preconditioned with dichloromethane. The material was eluted using a 30 $mLmin^{-1}$ flow rate collecting 9 mL fractions. Purification was accomplished using a linear gradient system from 0% to 100% methanol in dichloromethane over 50 CV. The solvent was removed *in vacuo*, yielding 111.1 mg of crude material, before being brought up in minimal methanol in dichloromethane to be run on a preparative TLC (1:1 CH_3CN:EtOAc). The solvent was removed *in vacuo*, yielding 73.5 mg of crude material before running a second preparative TLC (5:5:1 CH_3CN:EtOAc:H_2O). This preparative TLC yielded 35.6 mg of crude material. Final purification was attempted using a Sephadex™ LH-20 size-exclusion column, eluting with 5:5:1 CH_3CN:EtOAc:H_2O, yielding 4.6 mg of crude material.

Table 12. Tentative characterization of jadomycin 4-borono-DL-phenylalanine.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J(Hz))	COSY Correlation
1	4.51	bs	1'
2			
3a	6.57	s	
3b			
4	6.44	m	6
5			
5-CH_3	2.15	s	
6	6.80	s	4
7			
7-OH			
7a			
7b			
8			
8a			
9	7.70	d(7.0)	10
10	7.61	t(10.5)	9, 11
11	7.44	d(7.0)	10
12			
12a			
13			
13a			
1'	3.77	m	1
2'			
3'	7.37	d(7.0)	4'
4'	7.05	d(7.0)	3'
1"	5.83	s	2"
2"	2.23	s	1", 3"
3"	2.66	m	2"
3"OH			
4"	3.22	s	5"
5"	4.08	m	4", 5"-CH_3
5"-CH_3	1.29	s	5"
MeOH	3.31		
Water	4.89		



3.4.2 Productions using L-asparagine

Following the 48-hour production period, the media was divided into two equal parts to be purified using different purification techniques.

Current purification methodology

The media (1 L) was passed down the phenyl column as previously described, yielding 56.2 mg of crude material. This material was then loaded onto an 80 g silica column preconditioned with DCM. The material was eluted using a 30 $mLmin^{-1}$ flow rate collecting 9 mL fractions. Purification was accomplished using a linear gradient system from 0% to 100% methanol in DCM over 50 CV. Crude material, as determined by TLC, was dried *in vacuo* before being brought up in minimal methanol in dichloromethane and run on a preparative TLC (10% methanol in dichloromethane) yielding 19.8 mg of crude material. A second preparative TLC was then run (5:5:1 CH_3CN:EtOAc:H_2O). Final purification was accomplished using a Sephadex™ LH-20 size-exclusion column, eluting with 100% methanol. Fractions containing jadomycin LN were pooled together, as determined by HPLC analysis. The solvent was then removed *in vacuo* to yield the natural product in a 12.2 mg yield (12.2 mgL^{-1}).

Literature purification methodology

Following the literature purification method,[3] 9.7 mg (9.7 mgL^{-1}) of pure natural product were isolated.

3.4.3 Investigations into a yellow-coloured compound from jadomycin DS purifications

After the initial preparative TLC of the jadomycin DS production, the yellow band was removed and purified independently. The material was brought up in minimal methanol in dichloromethane for a second preparative TLC using 1:1 CH_3CN:EtOAc, air-drying, and a second elution using 5:5:1 CH_3CN:EtOAc:H_2O. The solvent was then removed *in vacuo* yielding the compound of interest in a 14.0 mg yield (7.0 mgL^{-1}).

Production of L-digitoxosyl phenanthroviridin

Streptomyces venezuelae ISP5230 VS1099 were grown in the presence of ammonium chloride (60 mM) as the sole nitrogen source for the typical 48-hour production period.

Investigations into the production of jadomycin DS in the presence of ammonium chloride

Two productions of jadomycin DS were prepared; one containing 60 mM D-serine and the other containing 60 mM D-serine with 60 mM ammonium chloride. Following the initial phenyl column 7.8 mg of crude material were isolated from the production using 60 mM D-serine and 2.4 mg of crude material were isolated from the production using 60 mM D-serine with 60 mM ammonium chloride.

3.4.4 Productions using 2-aminoethylphosphonic acid

Productions using 2-aminoethylphosphonic acid (60 mM) were supplemented with L-proline (60 mM) or ammonium chloride (60 mM) as the sole nitrogen sources. Productions were allowed to proceed for 96 hours, instead of the typical 48 hours. Once

the production was stopped, the media was passed down a phenyl column. The phenyl column for productions using 2-aminoethylphosphonic acid and L-proline was eluted with ddH_2O (5 CV), followed by methanol (5 CV), dichloromethane (5 CV), 5:5:1 CH_3CN:EtOAc:H_2O (5 CV), and 1:1 methanol:H_2O (5 CV). The phenyl column for productions using 2-aminoethylphosphonic acid and ammonium chloride was eluted with ddH_2O (5 CV), followed by methanol (5 CV) and 1:1 methanol:H_2O (5 CV). All fractions were kept separate, and their solvent was removed *in vacuo* to determine whether a jadomycin had been produced.

3.4.5 Productions using 5-aminovaleric acid

A 6 M solution of aqueous 5-aminovaleric acid was prepared and filter sterilized before addition to the autoclaved MSM production media, to a final concentration of 60 mM. The solvent was removed *in vacuo* following the initial phenyl column yielding 16.4 mg of crude material. The crude natural product was brought up in minimal methanol in DCM for preparative TLC (15% methanol in DCM) yielding 15.2 mg of crude material. This was brought up in minimal methanol in DCM for a second preparative TLC. This preparative TLC was initially run in 1:1 CH_3CN:EtOAc, allowed to air dry, then run using 5:5:1 CH_3CN:EtOAc:H_2O. The solvent was removed *in vacuo*, yielding 5.2 mg of crude material. A final preparative TLC (15% methanol in DCM) was then used yielding the natural product in a 4.5 mg yield (68.2 mgL^{-1}) as a mixture of diastereomers (Mj:Mn 100:67) by ^{1}H-NMR. TLC R_f: 0.54 (10% methanol in DCM); HPLC R_t = 8.46 min; UV-Vis (3 x 10^{-4} and 3 x 10^{-5} M, MeOH): λ_{max} (ε) = 311 (10087), 522 (1167); LRMS (ESI^+): MS/MS (536) found 536 $[M+H]^+$, 406 [M+H-digitoxose]$^+$, 306 [M+H-digitoxose-

$C_5H_{10}O_2]^+$; HRMS (ESI^+) for $C_{29}H_{29}NO_9Na$ $[M+Na]^+$: 558.1753 found, 558.1735 calculated; NMR spectra to follow (see Appendix I), see characterization table for numbering.

Table 13. Jadomycin 5-aminovaleric acid $3a_{Mj}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J/(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	2.28	obscured	38.2	1'	2
2			182.0		
3a	5.63	s	92.6		3', 4, 7a, 7b, 13, 13a
3b					
4	6.77	s	120.4	5-CH3, 6	3a, 5-CH3, 6, 7a, 7b
5			141.3		
5-CH_3	2.36	s	21.1	4, 6	4, 5, 6
6	6.82	s	120.6	4, 5-CH3	4, 5-CH3, 7, 7a
7			154.8		
7-OH					
7a			114.3		
7b			129.9		
8			183.0		
8a			136.8		
9	7.81	d(7.6)	121.3	10	8, 8a, 10, 11
10	7.72	obscured	136.7	9, 11	8a, 9, 11, 12
11	7.55	d(5.3)	120.6	10	9, 10, 12, 12a, 13
12			155.8		
12a			121.6		
13			185.8		
13a			154.0		
1'	1.56 - 1.65	bm	24.5	1, 2'	1, 2, 2', 3'
2'	1.86	obscured	32.2	1', 3'	1, 1', 3'
3'	4.00, 4.12	obscured	54.8	2', 3'	1', 2', 3a
1"	5.95	d(3.0)	96.5	2"	2", 3", 5", 12
2"	2.23	bm	36.4	1", 2", 3"	
2"	2.41	bm	36.4	1", 2", 3"	
3"	4.08	obscured	68.4	5"-CH3, 2", 4', 5'"	
3"OH					
4"	3.28	obscured	74.1	3", 5"	5", 5"-CH3
5"	3.93	bm	66.4	4", 3", 5"-CH3	
5"-CH_3	1.22	d(6.0)	18.2	3", 5"	4", 5"
MeOH	3.31		49.0		
Water	4.78				

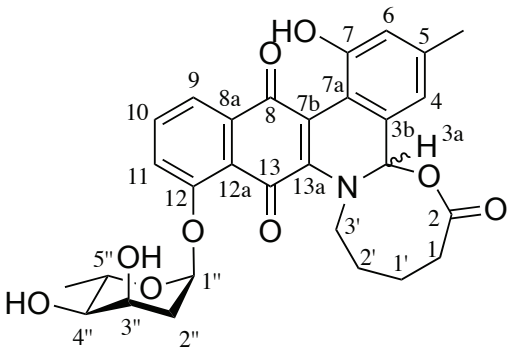
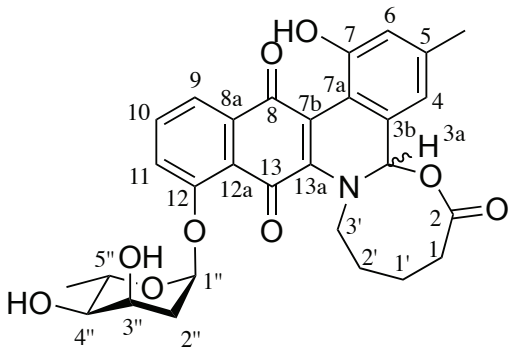


Table 14. Jadomycin 5-aminovaleric acid $3a_{Mn}$ diastereomer NMR data.

Position	δ ^{1}H (ppm)	Multiplicity + Coupling Constants (J/(Hz))	δ ^{13}C (ppm)	COSY	HMBC
1	2.28	obscured	38.2	1'	2
2			182.0		
3a	5.61	s	92.2		3', 4, 7a, 7b, 13, 13a
3b					
4	6.78	s	120.4	5-CH3, 6	3a, 5-CH3, 6, 7a, 7b
5			141.7		
5-CH_3	2.36	s	20.9	4, 6	4, 5, 6
6	6.82	s	120.6	4, 5-CH3	4, 5-CH3, 7, 7a
7			154.8		
7-OH					
7a			114.2		
7b			131.3		
8			184.0		
8a			136.8		
9	7.84	d(7.6)	121.6	10	8, 8a, 10, 11
10	7.72	obscured	136.7	9, 11	8a, 9, 11, 12
11	7.54	d(5.3)	121.3	10	9, 10, 12, 12a, 13
12			156.4		
12a			121.0		
13			184.2		
13a			151.3		
1'	1.56 - 1.65	bm	24.5	1, 2'	1, 2, 2', 3'
2'	1.65 - 1.74	obscured	32.0	1', 3'	1, 1', 3'
3'	3.80, 4.35	bm	55.6	2', 3'	1', 2', 3a
1"	5.93	d(3.0)	96.8	2"	2", 3", 5", 12
2"	2.23	bm	36.4	1", 2", 3"	
2"	2.41	bm	36.4	1", 2", 3"	
3"	4.08	obscured	68.1	5"-CH3, 2", 4', 5'"	
3"OH					
4"	3.28	obscured	74.1	3", 5"	5", 5"-CH3
5"	3.93	bm	66.5	4", 3", 5"-CH3	
5"-CH_3	1.22	d(6.0)	18.2	3", 5"	4", 5"
MeOH	3.31		49.0		
Water	4.78				



3.5 References

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CHAPTER 4: WATERLOGSY NMR EVALUATION OF JADOMYCIN DS

4.1 Introduction

Intelligent drug design involves producing candidate drugs that will interact with a specific target and elicit a desired response. In nature, these targets are often proteins but can be DNA or compounds within the cell membrane. Responses elicited from the binding of a ligand to a protein include olfaction in insects,[1] antibodies in humans,[2] or secondary metabolites impeding the advance of a competitive organism.[3-7] This last example will be examined further.

Organisms produce secondary metabolites as a form of self-defense from competitive organisms.[3-7] These secondary metabolites bind proteins found within the invasive organism, which elicits a response to either stop the invasion or kill the invasive organism.[8] For this reason, most secondary metabolites can be considered to have a potential protein target, and it is the job of the medicinal chemist to discover what that target may be.[8] Those that do not may bind DNA or another small molecule. This becomes more important when considering that many drugs are natural products, or derivatives thereof.[8,9] These natural products often exhibit highly selective biological activity and this selectivity is highly beneficial when converting a natural product into a candidate drug. Selectivity does not necessarily have to manifest itself through the production of a response once the protein has bound the ligand, it can also be shown by the ligand inhibiting the protein's function.[3] Therefore, the process of drug design is as much about producing a novel drug as identifying an adequate protein target.

The identification of adequate protein targets is critical for the treatment of various diseases, with great focus placed on cancer due to its increased prevalence in the

general population.[10] Cancer is a disease that begins within cells and causes them to function abnormally.[10] This is usually characterized through the rapid replication of these cells, and leads to malignant tumours.[10] Current cancer therapies, such as the inhibition of human topoisomerase IIβ, focus on the eradication of the tumour through destruction of the individual cancer cells.[10-12]

Human topoisomerase IIβ (hTopIIβ) is essential for DNA replication, and thus cell replication. During cell replication the two strands of the DNA double helix are separated to allow for binding of DNA polymerase and subsequent DNA replication.[13] The process of opening the double helix causes stress and strain to accumulate within the unopened portion of the DNA double helix.[13] hTopIIβ relieves this built up stress and strain by cleaving both strands of DNA and then ligating them again.[10,11,13] When hTopIIβ is inhibited DNA cannot successfully replicate, resulting in cell death. For this reason hTopIIβ is a valid drug target.

One form of current cancer therapy involves the introduction of hTopIIβ inhibitors directly into cancerous cells.[10-12] Two clinically used anti-cancer drugs that act through this mechanism are etoposide[11,12] and doxorubicin[10] (Figure 38). Since jadomycins are structurally similar to both of these compounds, it was thought they might also inhibit hTopIIβ. The lactone ring of etoposide and the anthraquinone moiety of doxorubicin are both present in jadomycin, and all exhibit fused ring structures. These similarities led to the investigation of jadomycin as both a ligand and inhibitor of hTopIIβ.

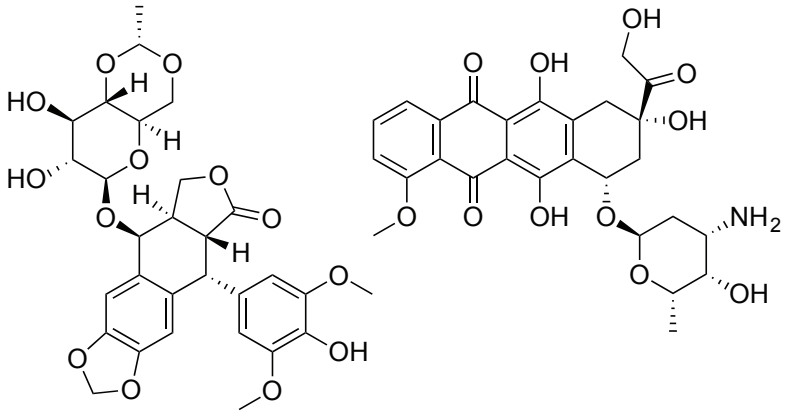

Figure 38. Structures of etoposide and doxorubicin.

Water ligand observed *via* gradient spectroscopy (WaterLOGSY) NMR was used to investigate the ability of hTopIIβ to bind jadomycin. WaterLOGSY NMR is used to qualitatively show whether a protein binds a specific ligand.[14,15] This is accomplished through the irradiation of bulk water within the NMR sample by the spectrometer. This magnetization is then transferred to the protein through protein-water interactions, and subsequently transferred from the protein to the bound ligand.[14,15] Once the ligand is released into free solution, a signal can be observed in the NMR spectrum.[14,15] In order to observe binding, the ligand must not bind the protein tightly and must be released back into free solution.[14,15] Since magnetization is transferred from the protein to the bound ligand, compounds that do not bind will not possess the magnetization of binding ligands. These non-binding ligands phase opposite to binding ligands.[14,15] For all WaterLOGSY NMR spectra presented, binders have been phased in the positive direction, while non-binders have been phased in the negative direction. Although it is important to qualitatively demonstrate that hTopIIβ binds jadomycins, it is of greater interest to quantitatively define this relationship.

Quantitation of how strongly hTopIIβ binds jadomycins will allow for the direct comparison between different jadomycin analogues as well as the known hTopIIβ inhibitors, etoposide and doxorubicin. Quantitation can be accomplished through the calculation of a dissociation constant (K_d) for the ligand-protein complex, where a smaller K_d indicates a stronger binding interaction. Since inhibition of hTopIIβ is desired, it would be beneficial if the jadomycin-protein complex had low K_d values. Quantitation of the jadomycin-protein complex was accomplished using WaterLOGSY NMR experiments.

Determination of the K_d by WaterLOGSY NMR relies on the relationship between the concentration of the ligand and the signal intensity observed.[16,17] This relationship is linear in the absence of protein and proton signals phase negatively.[16,17] However, in the presence of protein this relationship is no longer entirely linear.[16,17] To determine a K_d of the jadomycin–hTopIIβ complex a two-part experiment must be conducted.[16,17] NMR spectra were first recorded in the presence of protein with increasing concentrations of ligand.[16,17] The signal intensity of the ligand increases linearly with increasing concentration of ligand until it reaches a plateau.[16,17] The plateau indicates a point of protein saturation. If concentrations above the plateau are explored, the signal intensity will begin to decrease, as more ligands are unable to bind to the protein. The intensity of the ligand signal is then plotted as a function of ligand concentration, effectively producing a binding curve.[16,17] Although positive binding signals are clearly observed, they are slightly dampened in intensity. This is due to the ligands that are capable of binding the protein, but have not done so, and thus phase in the negative direction in the NMR spectrum.[16,17] To correct for this, the second part of the

two-part experiment must be performed. NMR spectra are taken for increasing concentrations of ligand in the absence of protein, which generates a negative linear standard curve correlating the signal intensity to the concentration of ligand.[16,17] To correct the intensities obtained in the first experiment, the standard curve is subtracted from the binding curve, which generates a third set of values.[16,17] These corrected signal intensities, when correlated to the concentration of the ligand, increase linearly until reaching a plateau.[16,17] The K_d value can then be obtained as the concentration midway to the plateau through regression analysis.[16,17]

Using WaterLOGSY NMR to determine the K_d of the jadomycin–hTopIIβ complex is beneficial for three reasons. Firstly, as previously mentioned, it is a relationship that can be quantified. Secondly, it allows for direct comparison between multiple jadomycin analogues. This is advantageous because it may allow for structural activity relationships to be established. Finally, because this method relies on the intensity of individual proton signals, it may be possible to determine which region of the molecule interacts with the protein more strongly. This could shed light on which part of the molecule is necessary for inhibition to occur and may allow for precursor-directed biosynthesis to produce compounds that show stronger inhibition of the protein.

Investigations into the determination of K_d values using jadomycin DS were carried out in the presence of hTopIIβ, and will be presented here.

4.2 Results and Discussion

The use of Chelex® was critical to the completion of these experiments. Chelex® is needed to remove any potential metal ions present within the experimental sample, and

aggregation of the ligand occurred when the sample was not passed through Chelex®. Ligand aggregates appear as binders even in the absence of the enzyme in question.

4.2.1 K_d determination with jadomycin DS and human topoisomerase IIβ

Binding of jadomycin DS to hTopIIβ was first investigated qualitatively, before quantitative K_d experiments were attempted. This showed that binding could be observed for this complex at adequate concentrations (Figure 39). Figure 39 demonstrates that the concentration used, 2 mM jadomycin DS, is appropriate to observe binding.

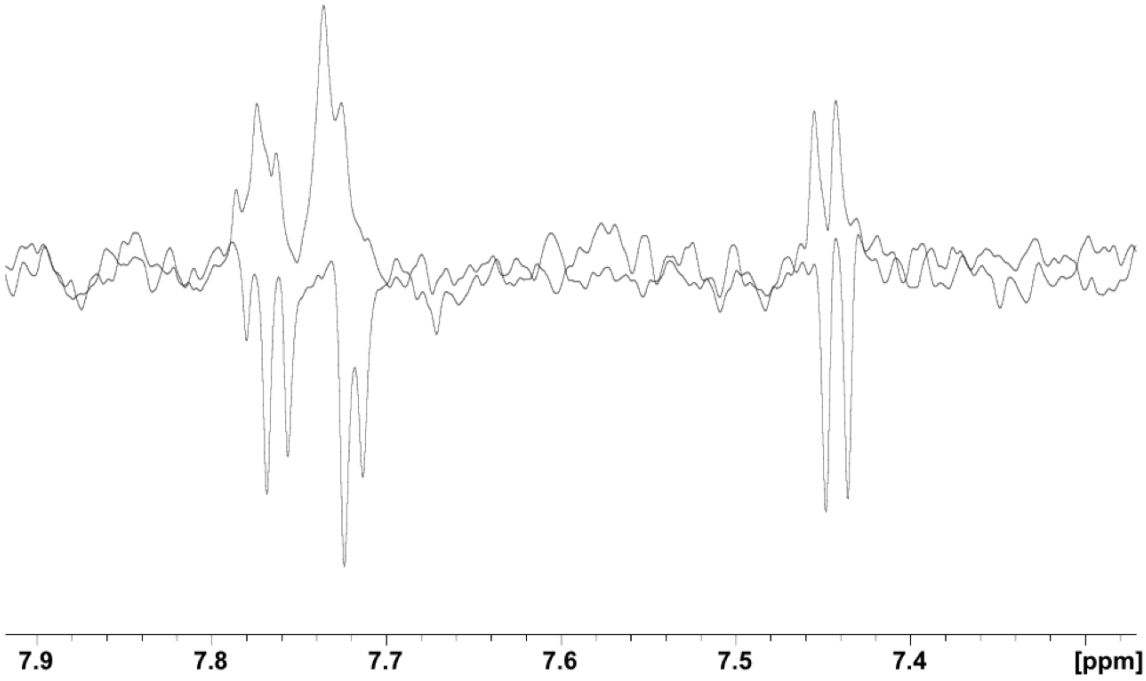

Figure 39. WaterLOGSY NMR of jadomycin DS (1 mM) in the presence (positive binding signals) and absence (negative non-binding signals) of 0.02 mM human topoisomerase IIβ. Signals highlighted correspond to protons 9H, 10H, and 11H.

Once binding had been established, experiments were run with varying concentrations of jadomycin in the presence or absence of hTopIIβ. Jadomycin DS concentrations begin above 1 mM because no binding was seen below 1 mM. The concentration range investigated provided sufficient data to obtain K_d values (Table 15)

from the curves generated (Figure 40). Higher concentrations were not investigated due to insolubility of the jadomycin. The data presented does not seem to suggest that any one portion of the jadomycin structure is responsible for its binding to hTopIIβ since the values obtained have a large range (3.83 mM – 12.74 mM). However, averaging all of the K_d values obtained gives a global K_d value for jadomycin DS binding to hTopIIβ of 9.78 mM.

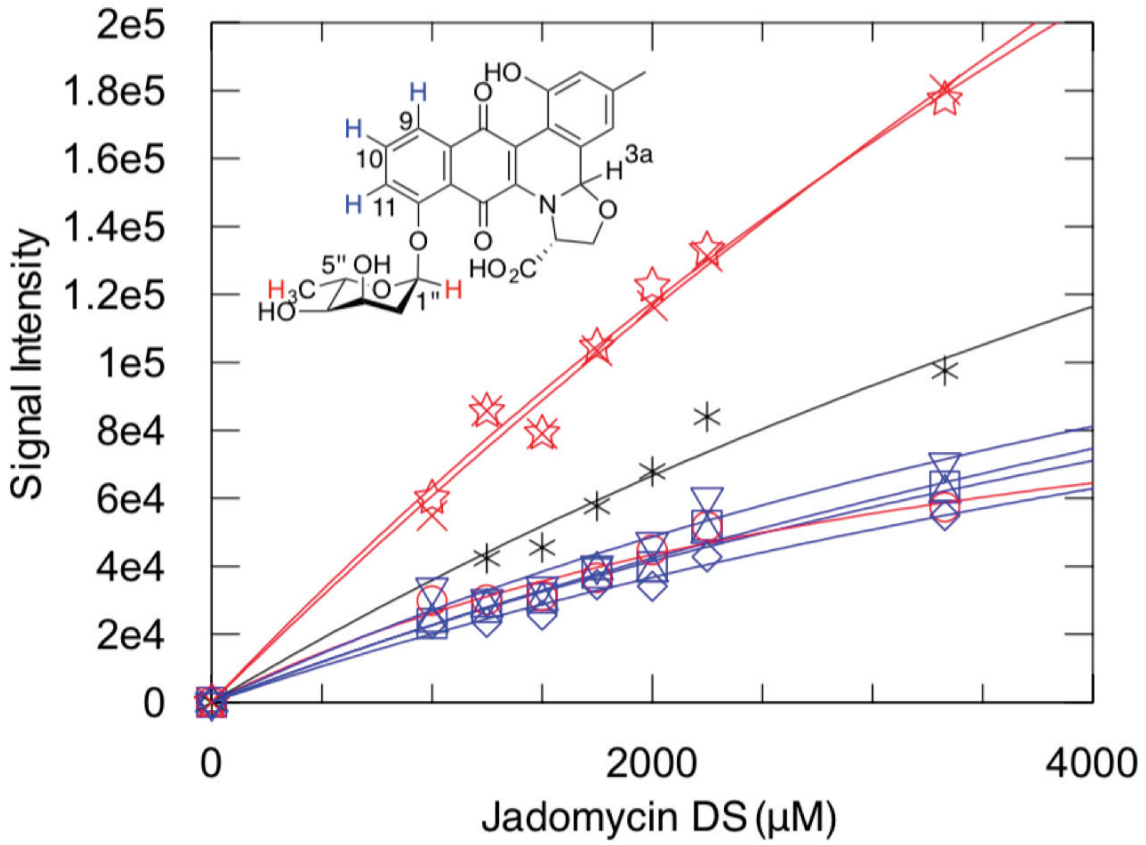

Figure 40. WaterLOGSY NMR peak intensity as a function of jadomycin DS concentration, in the presence of 0.02 μM human topoisomerase IIβ, for protons 3aH (✳), 9H (▽), 10H (♢), 11H left signal (□), 11H right signal (Δ), 1″H (○), 5″-CH_3 left signal (★), and 5″-CH_3 right signal (×).

Table 15. Dissociation constants obtained for each proton signal analyzed by WaterLOGSY NMR.

Proton	Chemical Shift δ (ppm)	K_d (mM)
3a	5.59	11.89
9	7.56	7.97
10	7.61	9.89
11	7.28	12.74
1″	5.71	3.82
5″-CH_3	1.24	12.35
Average		9.78

4.3 Conclusions

Through the use of WaterLOGSY NMR it was possible to determine that, as predicted, hTopIIβ is an adequate protein target to investigate jadomycin binding. The observed binding may be due to structural similarities previously highlighted between jadomycins and both etoposide and doxorubicin (Figure 38). This system is of interest because the jadomycins are known to possess anti-cancerous properties, as evaluated by the NCI, and this would allow further anti-cancer studies to be conducted in-house.

Using WaterLOGSY NMR it was possible to unequivocally show that jadomycin DS binds to hTopIIβ (Figure 39), which allowed the exploration of this system in a quantitative fashion. The K_d value obtained (9.78 mM) suggests that jadomycin DS is a weak binder of hTopIIβ. This is expected, as tight binders are not observed through WaterLOGSY NMR.[14,15] This is further corroborated by the fact that binding was not observed below a ligand concentration of 1 mM. When an excess of ligand is required to observe binding it may imply that binding is weak and non-specific. At such high concentrations it is assumed that the ligand is binding all over the enzyme. Additionally, determination of which portion of jadomycin DS interacts with hTopIIβ was not possible

because all of the values obtained showed a wide range. Although 1″H appears to have the lowest K_d value, one cannot say that the sugar moiety is responsible for the majority of the interactions because 5″-CH_3 has the second highest K_d value.

The K_d value obtained is much greater than that of etoposide binding to hTopIIβ (~5 μM).[12] Because of a higher K_d value, jadomycin DS does not appear to be able to replace etoposide as a clinically used hTopIIβ inhibitor. However, this may be an unfair comparison because the K_d of etoposide, as reported in the literature, was obtained using a nitrocellulose filter assay.[12] Although a K_d should be consistent regardless of the experiment used to calculate it, it would be beneficial to determine the K_d of hTopIIβ–etoposide using the presented WaterLOGSY method. If it is similar to that found with the nitrocellulose assay, it would further validate the WaterLOGSY experiment and allow for direct comparisons between assay methods in the future.

In an effort to obtain a K_d value for the hTopIIβ–etoposide that could be effectively compared to that obtained for jadomycin DS, WaterLOGSY NMR binding studies using etoposide in the presence and absence of hTopIIβ were attempted (data not shown). These experiments were found to be unsuccessful, with etoposide appearing as a non-binder both in the presence and absence of hTopIIβ. This can be attributed to the fact that etoposide is a tight-binder of hTopIIβ.[12] The process of K_d determination through WaterLOGSY NMR is limited to non-tight-binders.[14,15] WaterLOGSY NMR depends on the ligand exchanging quickly between the bound and unbound states in order to obtain signals in the WaterLOGSY spectra. Since this does not occur with tight-binders, the determination of a K_d cannot be done using the WaterLOGSY method presented here.

Therefore, direct comparison between etoposide and jadomycin DS was not possible using this methodology.

However, it may be possible to directly compare between jadomycin species, effectively establishing structural activity relationships in the future. This can be readily done through the investigation of other jadomycins that have high water solubility, such as jadomycin 4-amino-L-phenylalanine and jadomycin L-asparagine. Both of these jadomycins were successfully prepared in Chapters 2 and 3, and are actively being investigated in this regard. This is possible since it has been shown that WaterLOGSY NMR can be successfully used to quantitate the K_d value for a jadomycin–hTopIIβ complex.

4.4 Experimental

All jadomycin samples to be used for WaterLOGSY NMR binding studies were passed down a Chelex® column, eluted with methanol, and the solvent was then removed *in vacuo*.

All WaterLOGSY samples were composed of 80% PBS, 10% deuterated PBS, and 10% DMSO-d_6. PBS was prepared using 8.482 g NaCl and 6.9 g $NaH_2PO_4 \cdot H_2O$ in 1 L ddH_2O, and made to pH 7.6 with 1 M NaOH as necessary. Aliquots of this solution had the solvent removed *in vacuo* and were then brought up in D_2O to prepare deuterated PBS. Jadomycins were brought up in PBS (~5–10 mM) for use in WaterLOGSY experiments. The concentration of jadomycin varied between samples. Human topoisomerase IIβ was stored in PBS (3 mM). The enzyme was either present (0.02 mM)

or absent throughout the experiments, whether a positive curve or negative standard curve were being obtained, respectively.

In order to determine the K_d of each jadomycin-hTopIIβ binding complex two sets of experiments were run in all cases. The first has an increasing concentration of jadomycin in the absence of hTopIIβ. This generates negative signals in the WaterLOGSY spectrum and is used as the standard. The second has an increasing concentration of jadomycin in the presence of hTopIIβ. This generates the binding curve with positive signals in the WaterLOGSY spectrum. The spectra are then analyzed using ligand signals that are far from the water signal. In all cases, protons 3aH, 9H, 10H, 11H, 1″H, and 5″-CH_3 were chosen. Their respective peak intensities are recorded as a function of jadomycin concentration. The intensities of the negative standards are then subtracted from the positive binding signals to generate a third curve. This data can then be analyzed using GraFit regression analysis software to obtain a K_d, as given by the equation below where I is the observed signal intensity, I_{max} is the maximal signal intensity, and L is the ligand concentration.

$$I = \frac{-I_{max}}{1 + \left(\frac{L}{K_d}\right)} + I_{max}$$

Through the use of a range of ligand concentrations, it is possible for GraFit to estimate where the binding curve's plateau is found, generating I_{max}. The remaining parameters are defined through the specific ligand concentration at which the respective signal intensity is observed.

4.4.1 Nuclear magnetic resonance spectroscopy

WaterLOGSY NMR analyses of jadomycins were recorded using a 700 MHz Spectrometer (^{1}H: 700 MHz) located at the NRC-IMB. Spectra were recorded in 1:1:8 D_2O:DMSO-d_6:H_2O, with chemical shifts given in ppm and having been calibrated to the residual solvent peak; D_2O: 4.79 ppm.

4.4.2 K_d determination with jadomycin DS and human topoisomerase IIβ

The binding of jadomycin DS to hTopIIβ was investigated by varying the concentration of jadomycin DS in the presence or absence of hTopIIβ (Table 16).

Table 16. Preparation of jadomycin DS samples for WaterLOGSY NMR binding studies with human topoisomerase IIβ.

[Jadomycin DS] (mM)	[hTopIIβ] (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

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CHAPTER 5: JADX AS A JADOMYCIN PURIFICATION TECHNIQUE

5.1 Introduction

Natural product discovery heavily depends on the natural product chemist's ability to purify the desired compound from what is often a complex mixture of undesired compounds. There is a range of purification techniques that may be employed, from extractions to column chromatography.[1,2] However, these techniques do not always easily furnish novel natural products.

During the search for natural products, many difficulties can arise. A common complication is dereplication.[1,2] Dereplication is when a new, potential natural product source is being investigated, purification procedures are performed, and known natural products are isolated. Although it is interesting to discover new organisms that can produce known natural products, the aim of such studies is to discover novel natural products. Also, novel natural products may be missed altogether during the purification procedure because of bioassay-guided fractionation.[1,2] This technique analyzes the fractions collected from various chromatographic techniques against known medicinal targets.[1,2] Novel natural products may not be found because they do not show activity against the targets present in the bioassays used for screening.[1,2] Additionally, traditional purification techniques sometimes suffer from long turnaround times.[2] When trying to discover novel natural products, time is of the essence. This final issue is sometimes encountered during jadomycin purifications.

The current jadomycin purification methodology can sometimes involve numerous chromatographic techniques, leading to long turnaround times from the production of the jadomycin to its isolation and characterization. These long turnaround

times sometimes result in breakdown of the jadomycin of interest. Breakdown of the natural product can also occur while the compound is being dried *in vacuo* between chromatographic techniques. For this reason, the creation of a new jadomycin purification methodology was attempted.

For a new jadomycin purification technique to be effective it must minimize the number of chromatographic steps required, reduce the turnaround time, and successfully isolate small quantities of material. In an attempt to accomplish all three goals, jadomycin purification through protein binding was explored. If a protein could be immobilized on a solid support while simultaneously binding a jadomycin, it may be possible to use this methodology to purify novel jadomycins. The protein must bind tightly enough to adhere the jadomycin to the solid support, but weakly enough to allow the jadomycin to be eluted from the column upon addition of a second compound that binds the protein more tightly. This specific binding interaction would separate the jadomycin from all remaining impurities, which would be eluted off of the column immediately. In an attempt to develop such a purification technique, JadX was investigated as a protein that could potentially purify jadomycins through a binding interaction.

JadX is a protein found within the jadomycin biosynthetic pathway.[3] Its function is unknown, but it is believed to be involved in the regulation of jadomycin production and has been shown to bind jadomycin by WaterLOGSY.[3] Previous work in the Jakeman lab has led to recombinant expression of the *jadX* gene in *Escherichia coli*. JadX can be readily overexpressed and isolated from this organism.[3] Recombinantly expressed JadX has a C-terminal His_6-tag. This allows the protein to be immobilized onto a nickel (Ni^{2+}) affinity column.[3] Binding of JadX to jadomycins and the presence of a His_6-tag led to the

hypothesis that JadX could be utilized to develop an alternative jadomycin purification method. Ideally this methodology would allow for the isolation and characterization of jadomycins to be completed more efficiently than is presently possible. Investigations into the ability of JadX to be used as a novel jadomycin purification technique are presented here.

5.2 Results & Discussion

Various methods were attempted to establish whether jadomycin purification by a JadX-nickel column would be feasible.

5.2.1 Trial 1 – Pure jadomycin DS without JadX

Jadomycin DS was loaded onto a nickel column in the absence of JadX to determine whether it could directly bind to the nickel column. A pure sample was chosen to determine whether any jadomycin breakdown would occur from the nickel column alone.

Once jadomycin DS had been loaded onto the nickel column, it was washed using distilled, deionized water. The jadomycin was eluted off the column within one column volume, suggesting there are no interactions between the jadomycin and the nickel column. Therefore, if any purification is seen in future columns containing JadX it will be due to the protein binding the jadomycin, rather than an interaction with the nickel column directly. It was also noted that no jadomycin breakdown occurred while on the nickel column alone. HPLC analysis was carried out before and after the nickel column, and both traces are near identical (Figure 41). The entirety of the material was recovered

after this "purification" technique. Therefore, it appears as though the nickel column could be a potential jadomycin purification technique, with pre-bound JadX.

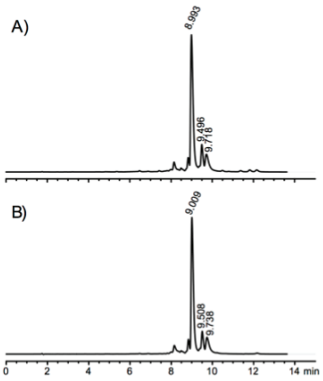

Figure 41. HPLC traces of jadomycin DS following Trial 1 (A) before the Ni^{2+} affinity column and (B) after the Ni^{2+} affinity column, in the absence of JadX.

5.2.2 Trial 2 – Pure jadomycin DS with JadX pre-bound to the nickel column

Jadomycin DS was loaded onto a nickel column previously loaded with JadX to determine whether pre-bound JadX could simultaneously bind jadomycin DS. A pure sample was chosen to determine whether any jadomycin breakdown would occur during this procedure.

Two molar equivalents of jadomycin DS, with respect to JadX, were loaded onto the column in case JadX could bind more than one molecule of jadomycin. However, not all of the material remained bound to the column, as assessed visually. The material that remained bound to the JadX-nickel column did so for more than one column volume of distilled, deionized water. Therefore, JadX was capable of binding jadomycin DS while pre-bound to the nickel column. Elution of jadomycin from the nickel column was then carried out using an increasing gradient of methanol in water. This solvent was chosen because methanol is an effective jadomycin eluent in other chromatographic methods. It

was hoped that methanol would cause the jadomycin to elute off of the column, while the JadX remained bound. This is preferred to eluting with imidazole, which would displace JadX, and would cause the JadX-jadomycin DS complex to be eluted together and require additional separation. The methanol in water solvent system was successful in eluting jadomycin DS (Figure 42), with the majority of the material being eluted in 10% methanol in water. The final elution system, 250 mM imidazole, was used to remove any remaining nickel-bound JadX. Fractions collected (10% methanol in water) contained a faint pink colour, typical of dilute jadomycins. However, upon removing the solvent *in vacuo* the solid was a yellow-brown colour. This is indicative of jadomycin breakdown. This may have been caused by the pH of the methanol in water eluent. Therefore, a new elution system should be investigated.

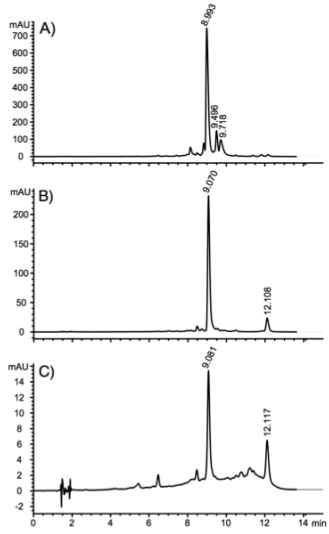

Figure 42. HPLC traces of jadomycin DS following Trial 2 (A) before the Ni^{2+} affinity column, and after the Ni^{2+} affinity column, with pre-bound JadX, eluted with (B) 10% MeOH in ddH_2O and (C) 20% MeOH in ddH_2O.

5.2.3 Trial 3 – Crude jadomycin DS without JadX

Jadomycin DS was loaded onto a nickel column in the absence of JadX to determine whether it could directly bind to the nickel column. A crude sample (following the initial

phenyl column) was chosen to determine whether any jadomycin purification would occur from the nickel column alone. The column was run as though JadX were present.

The solvent was changed from water to PBS buffer (pH 7.6) to avoid breakdown of the jadomycin while drying the sample following the column (as seen in trial 2). Once the sample had been loaded, yellow colour was eluted off of the column within one column volume of the initial PBS buffer wash. Continuation of the PBS buffer wash eventually eluted the jadomycin of interest (Figure 43) with higher purity than the loading material. The majority of the material was successfully eluted off of the column before the 10% methanol in PBS elution occurred. Therefore, it appears as though limited purification can be done using the nickel column alone when the sample is of low purity. This method allowed for the recovery of all of the material over the fractions eluted. However, it may be possible to obtain higher purity if the jadomycin can be more tightly bound, for a longer period of time.

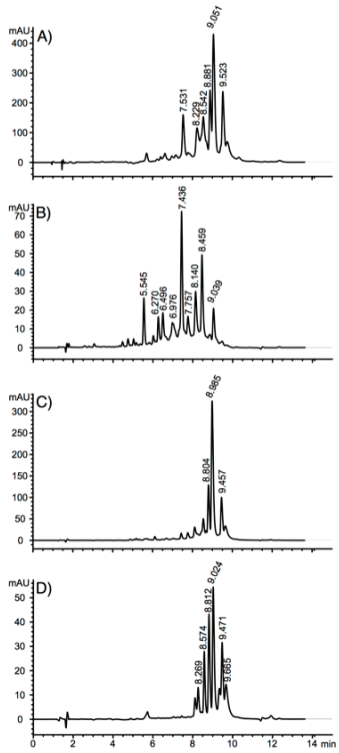

Figure 43. HPLC traces of jadomycin DS following Trial 3 (A) before the Ni^{2+} affinity column, and after the Ni^{2+} affinity column, in the absence of JadX, eluted with (B) 100% PBS buffer (<1 CV), (C) 100% PBS buffer (>1 CV), and (D) 10% MeOH in PBS buffer.

5.2.4 Trial 4 – Pure jadomycin DS with JadX pre-bound to the nickel column

Pure jadomycin DS was loaded onto a nickel column in the presence of JadX and purification was attempted using PBS buffer. The buffered system was chosen to prevent jadomycin breakdown upon removal of the solvent. A pure sample was chosen to determine whether any jadomycin breakdown would occur during this procedure.

One molar equivalent of jadomycin DS, with respect to JadX, was loaded onto the column since it had been demonstrated that two equivalents did not bind to the column entirely (Trial 2). This was shown to be adequate, because four column volumes of PBS buffer were required before any appreciable amount of jadomycin DS was eluted off of the column. Furthermore, from the HPLC analysis conducted after the column (Figure

44), it appears as though jadomycin DS is found within each solvent fraction collected. This may also be caused by the 1:1 molar ratio used in this experiment. The JadX may not strongly bind jadomycin DS, but rather slow it down throughout the column. This may lead to the material bleeding out over the entirety of the elution systems used. Although the material was eluted over multiple fractions, it was possible to recover all of the material. The fact that JadX does not tightly bind jadomycins has been investigated within the Jakeman lab, with binding studies indicating this to be true.[3]

Pure jadomycin DS was used to test whether sample degradation occurred during this purification procedure. HPLC analysis (Figure 44) demonstrates that the column introduces an impurity (HPLC R_t: ~12 min.). The presence of this impurity will have to be monitored when crude samples are attempted. If, for crude samples, purification of material with similar retention times to that of the jadomycin of interest were achieved, it would still be a feasible route towards jadomycin purification. Presumably, the newly introduced contaminant could be removed *via* preparative TLC or similar chromatographic techniques.

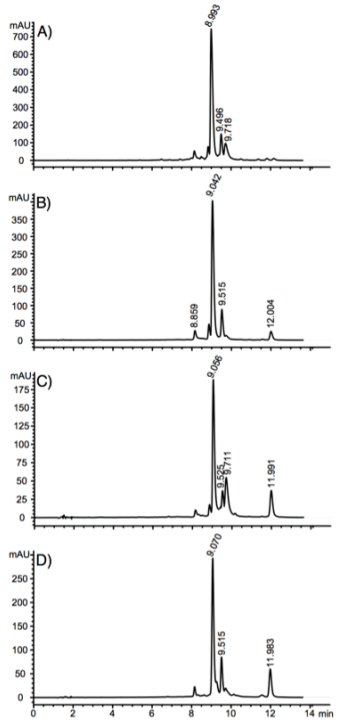

Figure 44. HPLC traces of jadomycin DS following Trial 4 (A) before the Ni^{2+} affinity column, and after the Ni^{2+} affinity column, with pre-bound JadX, eluted with (B) 100% PBS buffer, (C) 10% MeOH in PBS buffer, and (D) 50% MeOH in PBS buffer.

5.2.5 Trial 5 – Crude jadomycin DS with JadX pre-bound to the nickel column

Two equivalents of crude jadomycin DS were loaded onto a nickel column containing pre-bound JadX. Two equivalents were chosen assuming that fifty percent or more of the crude mixture would be contaminants. Therefore, the aim was to introduce approximately one equivalent of jadomycin DS, with the rest of the mass coming from impurities. The column was manually run using an increasing concentration of methanol in PBS buffer, after which all of the material was recovered over the various fractions eluted. However, the HPLC traces (Figure 45) suggest that methanol may not be necessary. As seen with Trial 4, jadomycin DS is found in various fractions and the cleanest fraction analyzed was after several column volumes of PBS buffer (Figure 45C). This provides further evidence that JadX does not tightly bind jadomycin DS, but rather slows it down throughout the

column. These results suggest that jadomycin purification may be achieved solely through PBS buffer elution.

The crude jadomycin sample already has the impurity (HPLC R_t: ~12 min.) noted in Trial 4 before the nickel column has been used. However, the peak becomes larger as purification progresses (Figure 45). This demonstrates that the column may be removing impurities with similar retention times to jadomycin DS, but causing breakdown of the compound during this procedure.

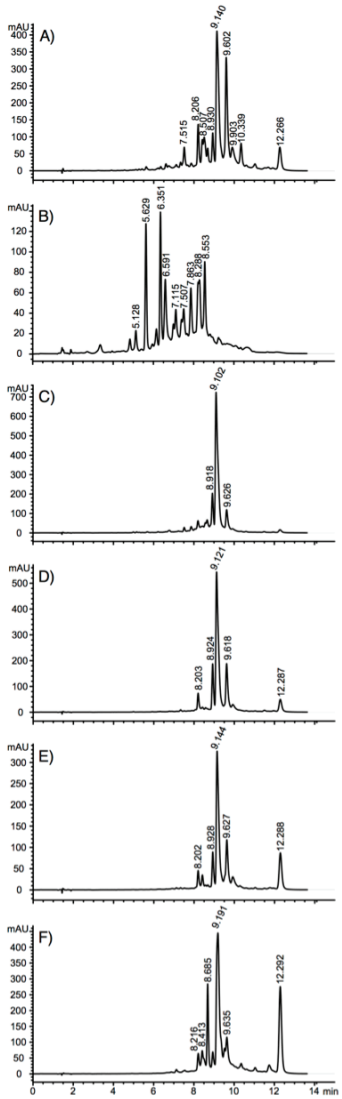

Figure 45. HPLC traces of jadomycin DS following Trial 5 (A) before the Ni^{2+} affinity column, and after the Ni^{2+} affinity column, with pre-bound JadX, eluted with (B) 100%

PBS buffer (<1 CV), (C) 100% PBS buffer (<10 CV), (D) 100% PBS buffer (≤20 CV), (E) 10% MeOH in PBS buffer, and (F) 50% MeOH in PBS buffer.

5.2.6 Trial 6 – Crude jadomycin B with JadX pre-bound to the nickel column

Crude jadomycin B was loaded onto a nickel column in the presence of JadX to determine whether this is a feasible route towards jadomycin purification. The procedure was conducted using fast protein liquid chromatography (FPLC) in an attempt to standardize the procedure.

Two equivalents of crude jadomycin B were loaded onto a nickel column containing pre-bound JadX. Again, two equivalents were chosen assuming that fifty percent or more of the crude mixture would be contaminants. Jadomycin B was chosen to expand the scope of the investigations into the novel purification technique. Previous lab work (data not shown) indicated that jadomycin B would be eluted in 50% methanol in PBS buffer. Therefore, the gradient was designed to have a plateau at this concentration. However, an instrument malfunction occurred just as this plateau was reached. The procedure was monitored using Abs_{256}, Abs_{280}, and Abs_{526} (Figure 46), which indicated that jadomycin B was still present when the instrument malfunctioned. Visual inspection also indicated that there was bound jadomycin B remaining. Therefore, manual elution was done to elute the remaining material.

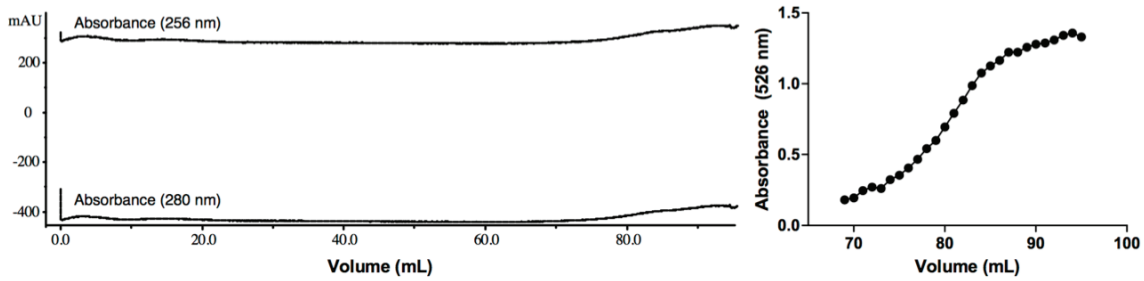

Figure 46. Absorbencies (256 nm, 280 nm, and 526 nm) obtained while eluting jadomycin B from the Ni^{2+} affinity column, with pre-bound JadX.

When manual elution was continued a new elution method was attempted. Residual methanol was removed using PBS buffer, before attempting to elute jadomycin B using a solution of phenol. Phenol was used in an attempt to outcompete JadX binding to jadomycin B. It was thought that phenol might be able to displace jadomycin B, through tighter binding with JadX. However, this was not the case and no jadomycin B was eluted in the presence of phenol. As such, PBS buffer was used to remove any remaining phenol solution, before jadomycin B was eluted using 50% methanol in PBS buffer. This removed the remaining jadomycin B quickly, suggesting that the FPLC method was adequate, had the instrumentation not failed. All of the material was recovered. HPLC analysis of the fractions did not yield any insight as to whether jadomycin B had been purified any further using this procedure (Figure 47). This was later explained when purification of jadomycin B was carried out showing that HPLC analysis is unreliable in determining the purity of jadomycin B where a ^{1}H-NMR spectrum shows a pure jadomycin B sample while the HPLC trace suggests that it is still impure (Figure 48).

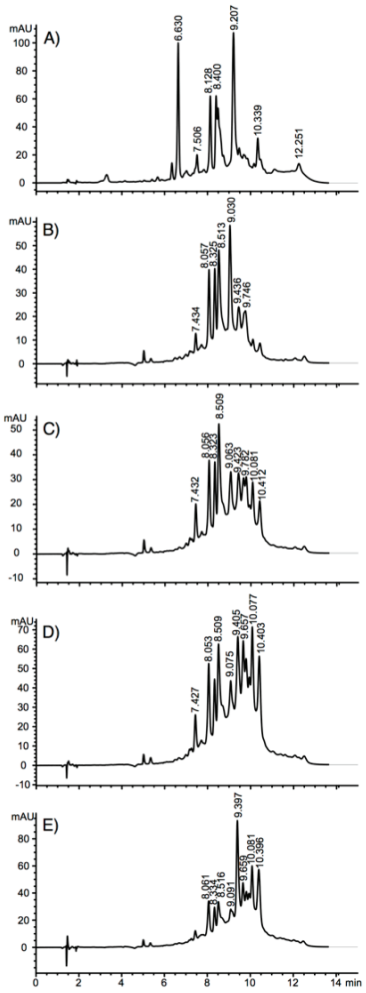

Figure 47. HPLC traces of jadomycin B following Trial 6 (A) before the Ni^{2+} affinity column, and after the Ni^{2+} affinity column, with pre-bound JadX, FPLC fractions (B) F8, (C) F14, (D) G2, and (E) G5.

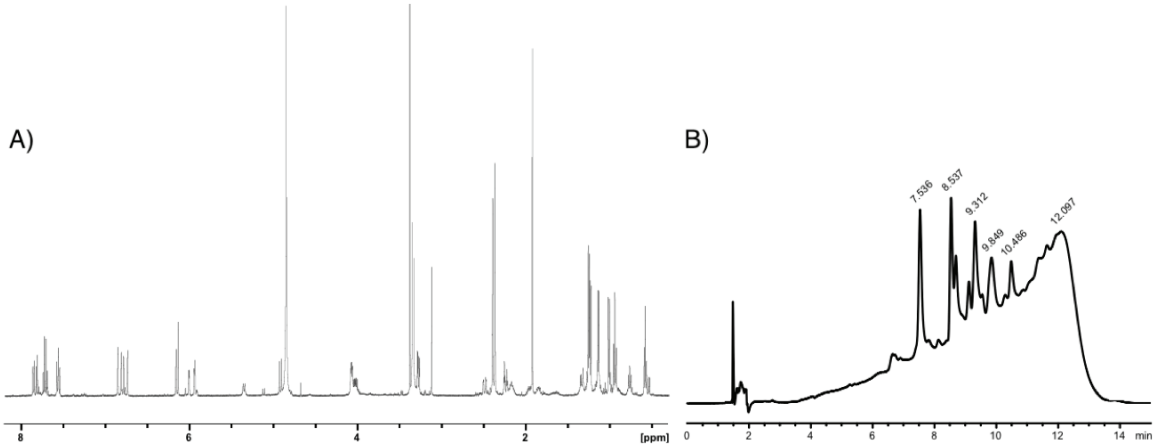

Figure 48. (A) ^{1}H-NMR spectrum (^{1}H: 500 MHz; in CD_3OD) and (B) HPLC trace of jadomycin B following the Sephadex™ LH-20 size-exclusion column, collected on the same sample on the same day.

5.3 Conclusions

Although current jadomycin purification methodology is adequate, it could be improved upon in order to generate higher quantities of isolated material in less time. This would be beneficial in order to prevent the inherent breakdown that occurs over various chromatographic purification steps. In an effort to provide such a methodology, the ability of JadX to bind jadomycins while being bound to a nickel affinity column was investigated.

These investigations showed that it was possible to bind jadomycins to JadX that had been immobilized on a solid surface. This was determined by the fact that the jadomycin remained bound to the JadX-nickel affinity column following various washes, while it took less volume to elute it off of the nickel affinity column in the absence of JadX. The fact that JadX successfully bound jadomycins was expected as this has been previously shown through NMR binding experiments.[3]

Once it had been determined that the JadX-nickel affinity column would successfully bind jadomycins, purification of various jadomycin samples was attempted. Purification was attempted on both pure and impure samples. Both samples, once passed down the JadX-nickel affinity column, appeared to demonstrate higher purity in the HPLC traces at retention times similar to the natural product of interest. However, there was an impurity that was consistently added into the samples. This impurity should be easily removed through other chromatographic techniques, and this possibility should be investigated further in the future. Furthermore, it was found (Trial 5) that it might be possible to solely purify jadomycin DS using PBS buffer. This finding could be beneficial because it may be possible to reuse the JadX-affinity column when neither

methanol nor imidazole is used to elute the column. Methanol affects the protein by causing it to precipitate and can denature the protein, while imidazole buffer causes the protein to be eluted off of the solid support. The ability to reuse the column would be beneficial since columns could be run in quicker succession if JadX was not eluted off of the column and would not require JadX to be freshly isolated every time a novel jadomycin was investigated. The practicality of reusing a JadX-nickel affinity column should be explored in the future.

The preliminary results presented here demonstrate that it may be possible to purify crude jadomycin samples using a JadX-nickel affinity column. As mentioned, this would be beneficial because it would reduce the time and chromatographic steps necessary to purify novel jadomycins. For this reason, further experiments should be conducted to determine whether this purification methodology is feasible. Future studies should focus on exploring the ability of this purification methodology on various jadomycin samples.

5.4 Experimental

5.4.1 Preparation of LB growth media

LB growth media was prepared by addition of tryptone (1.0%, w/v), sodium chloride (1.0%, w/v), and yeast extract (0.5%, w/v) to distilled water (dH_2O). The pH was adjusted to 7.5 with sodium hydroxide (5 M) and hydrochloric acid (5 M) as necessary.

5.4.2 JadX production methods

Escherichia coli BL21DE3 pET-28a_*jadX* cells are stored at -70°C as a glycerol suspension, of which 25 µL is used to inoculate 25 mL of LB growth media, with kanamycin (50 μgmL^{-1}). The solution was incubated overnight with shaking (250 rpm) at 37°C. The following morning, 10 mL from the growth media was removed and placed into 1 L of fresh LB media, with kanamycin (50 μgmL^{-1}). The solution was incubated with shaking (250 rpm) at 37°C, and monitored *via* OD_{600}. Once the OD_{600} was between 0.6 and 0.8 (~3 hours), a 500 µL sample was removed, and JadX overexpression was induced with IPTG (1 mM). The cells from the 500 µL sample were pelleted using centrifugation (13,000× g; 3 minutes) and the supernatant discarded. The pellet was stored at -70°C. The LB growth media was grown overnight with shaking (250 rpm) at 17°C. The following day a second sample with an equivalent number of cells, as determined by OD_{600}, as the 500 µL sample from the previous day was removed. The cells were pelleted as previously described. LB growth media cells were then pelleted *via* centrifugation (3500 rpm; 4°C) and the supernatant discarded.

5.4.3 JadX purification methods

E. coli BL21DE3 pET-28a_*jadX* cells were re-suspended in 16 mL of 25 mM imidazole buffer [8.766 g sodium chloride, 1.211 g Tris base, and 0.851 g imidazole into 500 mL ddH_2O; pH 8] with 1 mL 10% Triton X-100, 3 mL glycerol, 10 mg lysozyme, and 1 μgmL^{-1} DNAse solution. This solution was stirred for one hour on ice and then the cells were lysed by sonication, pelleted (13,000× g; 10 minutes), and discarded. JadX was purified using a HisTrap™ HP nickel affinity column (5 mL) pre-conditioned using 25

mM imidazole buffer. The cell lysate was loaded onto the column, which was run using 20% 250 mM imidazole buffer in 25 mM imidazole buffer (15 CV). JadX was eluted with 100% 250 mM imidazole buffer (5 CV). JadX was then concentrated using 10 000 MWCO Amicon® Ultra centrifugal filters (50 mL; 4°C; 3750 rpm). The protein was removed from the filter using 2.5 mL PBS buffer [8.482 g NaCl and 6.9 g $NaH_2PO_4 \cdot H_2O$ in 1 L ddH_2O (pH 7.6)]. Residual imidazole was removed using a PD-10 Desalting Column preconditioned with PBS buffer. The column was run under gravity, eluting the protein in 3.5 mL PBS buffer. JadX was then quantified using UV-Vis (ε = 22460 M^{-1} cm^{-1}; 280 nm).

5.4.4 SDS-PAGE gel methods

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gels were prepared using a resolving and stacking gel. The resolving gel was made using 1.24 mL sterile ddH_2O, 1.35 mL resolving buffer [1.5 M Tris HCl, pH 8.8], 2.75 mL acrylamide-bisacrylamide solution [29 g acrylamide, 1 g *N*,*N*′-methylenebisacrylamide, 100 mL dH_2O], 27.5 µL 20% SDS solution, 110 µL 10% ammonium persulfate (APS) [1 g APS, 10 mL dH_2O], and 6 µL tetramethylethylenediamine (TEMED). This was allowed to polymerize, protected by a layer of isopropanol. Once the gel had polymerized, the isopropanol was removed and replaced by the stacking gel. The stacking gel was made using 1.166 mL sterile ddH_2O, 0.333 mL acrylamide solution [30 g acrylamide, 0.8 g bis-acrylamide, 100 mL dH_2O], 0.5 mL stacking buffer [1.5 M Tris HCl, pH 6.8], 10 µL 20% SDS solution, 60 µL 10% APS, and 6 µL TEMED. The stacking gel was allowed to polymerize above the resolving gel with a comb inserted to create sample-wells. All gels

were run using 1× SDS-PAGE running buffer [1.0 g SDS, 3.0 g Tris base, 14.41 g glycine, 1 L ddH_2O].

Samples for SDS-PAGE analysis were prepared by the addition of 20 μL ddH_2O and using 5 μL of this solution with 5 μL of 2× loading dye solution [10 mL stacking buffer, 6 mL 20% SDS solution, 30 mL glycerol, 15 mL β-mercaptoethanol, 1.8 mg bromophenol blue, and 50 mL dH_2O] and 1 μL 20% SDS. These solutions were then incubated in a thermocycler at 95°C for 10 minutes. The solutions were then added to the SDS-PAGE gel to be run. All samples were run alongside a broad range (10-230 kDa) prestained protein ladder. Once a gel had finished running, it was stained with AcquaStain while shaking for approximately 15 minutes.

5.4.5 Trial 1 – Pure jadomycin DS without JadX

Pure jadomycin DS (2.9 mg in ddH_2O) was loaded onto a nickel column (5 mL) preconditioned with distilled, deionized water. The column was then manually washed with distilled, deionized water until all visible colours had been removed (<1 CV). The solvent was then removed *in vacuo*.

5.4.6 Trial 2 – Pure jadomycin DS with JadX pre-bound to the nickel column

JadX (~120 mg) was bound to a nickel column (5 mL) preconditioned with distilled, deionized water. Jadomycin DS (5.9 mg in ddH_2O; ~2 equivalents) was then loaded onto this JadX-nickel column. The column was then manually washed using ddH_2O (10 CV), followed by 10% methanol in ddH_2O (6 CV), 20% methanol in ddH_2O (6 CV), 30% methanol in ddH_2O (6 CV), 50% methanol in ddH_2O (6 CV), 70% methanol in ddH_2O (6

CV), 100% methanol (6 CV), and 100% 250 mM imidazole buffer (6 CV). All fractions within an elution plateau were combined and their solvent was removed *in vacuo*.

5.4.7 Trial 3 – Crude jadomycin DS without JadX

Crude jadomycin DS (10.0 mg in PBS buffer, pH 7.6) was loaded onto a nickel column (5 mL) preconditioned with PBS buffer. The column was then manually washed using PBS buffer (20 CV), followed by 10% methanol in PBS buffer (12 CV), 50% methanol in PBS buffer (8 CV), 100% methanol (8 CV), and 100% 250 mM imidazole buffer (8 CV). All fractions within an elution plateau were combined and their solvent was removed *in vacuo*. Residual salts were removed by solubilizing the material in methanol and filtering through a Kimwipe®.

5.4.8 Trial 4 – Pure jadomycin DS with JadX pre-bound to the nickel column

JadX (~150 mg) was bound to a nickel column (5 mL) preconditioned with PBS buffer. Jadomycin DS (4.6 mg in ddH_2O; ~1 equivalent) was then loaded onto this JadX-nickel column. The column was then manually washed using PBS buffer (20 CV), followed by 10% methanol in PBS (12 CV), 50% methanol in PBS (8 CV), 100% methanol (8 CV), and 100% 250 mM imidazole buffer (8 CV). All fractions within an elution plateau were combined and their solvent was removed *in vacuo*. Residual salts were removed by solubilizing the material in methanol and filtering through a Kimwipe®.

5.4.9 Trial 5 – Crude jadomycin DS with JadX pre-bound to the nickel column

JadX (~120 mg) was bound to a nickel column (5 mL) preconditioned with PBS buffer. Crude jadomycin DS (5.0 mg in PBS buffer; ~2 equivalents) was then loaded onto this JadX-nickel column. The column was then manually washed using PBS buffer (20 CV), followed by 10% methanol in PBS buffer (12 CV), 50% methanol in PBS buffer (8 CV), 100% methanol (8 CV), and 100% 250 mM imidazole buffer (8 CV). All fractions within an elution plateau were combined and their solvent was removed *in vacuo*. Residual salts were removed by solubilizing the material in methanol and filtering through a Kimwipe®.

5.4.10 Trial 6 – Crude jadomycin B with JadX pre-bound to the nickel column

JadX (~120 mg) was bound to a nickel column (5 mL) preconditioned with PBS buffer. Crude jadomycin B (5.0 mg in PBS buffer; ~2 equivalents) was then loaded onto this JadX-nickel column. The column was run using fast protein liquid chromatography (FPLC) and washed using PBS buffer (5 CV), followed by a linear gradient of 0% to 50% methanol in PBS (14 CV). The instrument then malfunctioned, therefore, manual elution was continued using 100% PBS buffer (1 CV), followed by 1 M phenol (2 CV), 100% PBS buffer (2 CV), and 50% methanol in PBS (3 CV). FPLC fractions were automatically monitored *via* Abs_{256} and Abs_{280}, and manually *via* Abs_{526} where potential jadomycin had been eluted. The solvent was removed *in vacuo*. Residual salts were removed by solubilizing the material in methanol and filtering through a Kimwipe®.

5.5 References

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3. Robertson, A. W.; Martinez-Farina, C. F.; McCormick, N. E.; Syvitski, R. T.; Jakeman, D. L. NMR-binding studies towards the functional characterization of JadX, a multi-natural product binding protein. *In progress*

CHAPTER 6: GENERAL CONCLUSION

Through the use of various amino acids, it has been shown that *Streptomyces venezuelae* ISP5230 VS1099 is capable of incorporating many structurally diverse amino acids into the jadomycin structure. This can be explained by the fact that the incorporation of the amino acid is likely non-enzymatic.[1-6] The exploitation of this non-enzymatic step allowed for the production of novel jadomycins using 4-amino-L-phenylalanine, 4-borono-DL-phenylalanine, and 5-aminovaleric acid, as well as the reproduction of jadomycin L-asparagine. Each of the novel jadomycins is an important addition to the jadomycin library.

Although jadomycin 4-borono-DL-phenylalanine broke down during the purification process, sufficient analytical analysis was conducted to unequivocally show its successful production. This jadomycin should be easily isolated following another production with the amino acid with the avoidance of an LH-20 column and shorter purification period. Once this occurs, it will be a boron-containing natural product, which are rare in nature.[7] It will also be possible to chemically derivatize this compound. The importance of chemical derivatization demonstrates the significance of the incorporation of 4-amino-L-phenylalanine into the jadomycin structure. Through the incorporation of an amino functionality, it has been shown that chemical derivatization was possible through the use of succinimidyl esters, where two derivatives were successfully prepared, jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide and jadomycin 4-amino-L-phenylalalnine phenoxyacetylamide. Finally, the incorporation of 5-aminovaleric acid is a significant addition to the jadomycin library because it demonstrates the ability of the bacteria to incorporate linear “non-traditional” amino acids into the jadomycin structure

and the formation of alternate E-ring ring sizes. This finding suggests that other "non-traditional" amino acids can be incorporated into the jadomycin structure in order to alter the size of the E-ring further. Such investigations are currently underway. Once completed, all of these novel jadomycins should be assessed for biological activity. Although new amino acids were successfully incorporated into the jadomycin structures, not all amino acids attempted generated jadomycins.

Investigations into the incorporation of 4-cyanophenylalanine enantiomers and 2-aminoethylphosphonic acid into the jadomycin structure did not yield jadomycins in appreciable yields. This demonstrates that although the amino acid incorporation is non-enzymatic, it does not necessarily mean that all amino acids will be successfully incorporated.[1-6] Since jadomycin 2-aminoethylphosphonic acid was analytically observed, it might be beneficial to investigate this amino acid further. Future work could include the production of this jadomycin from a mixture of amino acids, since this was shown to not affect the production of jadomycin DS in Chapter 3. This technique may also be used to investigate whether productions using 4-cyanophenylalanine enantiomers are successful under these conditions. If the production of these jadomycins can be improved through their production using two amino acids, their biological activity should be investigated in addition to those of the novel jadomycins mentioned above.

The evaluation of the jadomycins' biological activity is important because past jadomycins have been shown to possess various forms of biological activity.[5,6,8-14] Examples were presented for both jadomycin 4-amino-L-phenylalanine derivatives produced, where neither exhibited remarkable biological activity. However, it has also been shown that biological evaluation of these jadomycins may be possible within the

Jakeman laboratory. Such evaluation can be accomplished through binding studies using human topoisomerase IIβ.

The binding of hTopIIβ to jadomycin DS was successfully observed using WaterLOGSY NMR. This technique was also useful to quantitate this relationship, establishing a global K_d of 9.78 mM for this complex. Using this approach, it may be possible to establish dissociation constants for numerous water-soluble jadomycins with respect to hTopIIβ. This allows for the biological evaluation of jadomycins within the Jakeman laboratory. Jadomycin-protein binding was also explored as a novel method of jadomycin purification.

Since jadomycin purification can be lengthy, often requiring multiple chromatographic techniques, a novel purification method was investigated using JadX to bind jadomycins while immobilized on column resin. The results presented show that this method is feasible, but should be explored further using other water-soluble jadomycins. If the use of a JadX-nickel affinity column is found to be successful, it may be possible to quickly purify the small quantities of material necessary for the characterization of novel jadomycins. The ability of isolating and characterizing jadomycins more efficiently would allow for the exploration of various amino acids in a timely manner.

Through these and past investigations it has been shown that *Streptomyces venezuelae* ISP5230 readily produces a diverse family of natural products, known as the jadomycins.[8,9,15] However, genome mining has shown that the bacteria appear to be capable of producing a number of other distinct natural products.[16,17] The exploration of these potential natural products, in conjunction with further jadomycin research, has the

potential of uncovering numerous novel natural products of both chemical and biological importance. The search for these unknown natural products should guide future research.

6.1 References

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APPENDIX I: NMR, ETC FOR CHAPTER 2

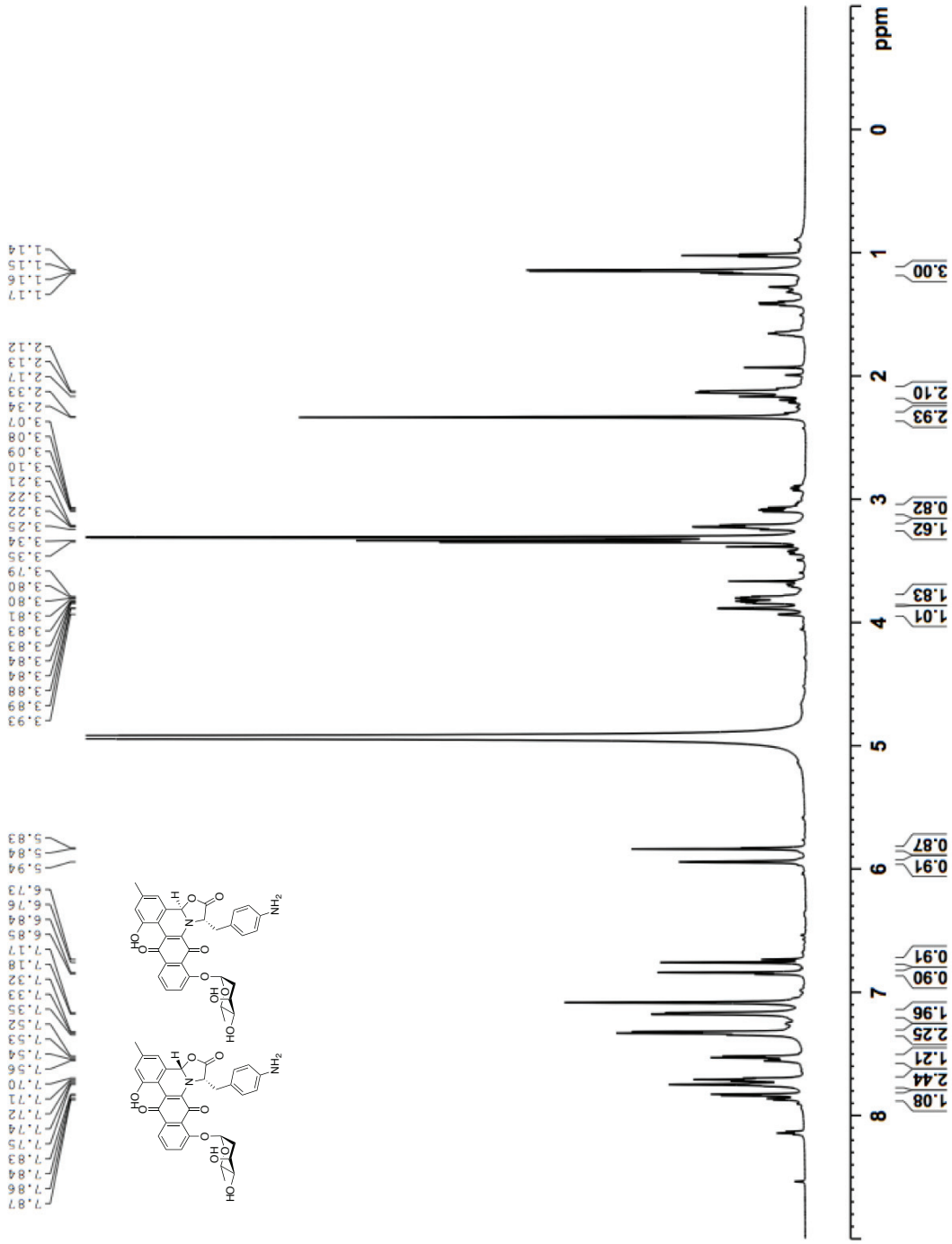


Figure S1. ^{1}H-NMR spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD (^{1}H: 700 MHz).

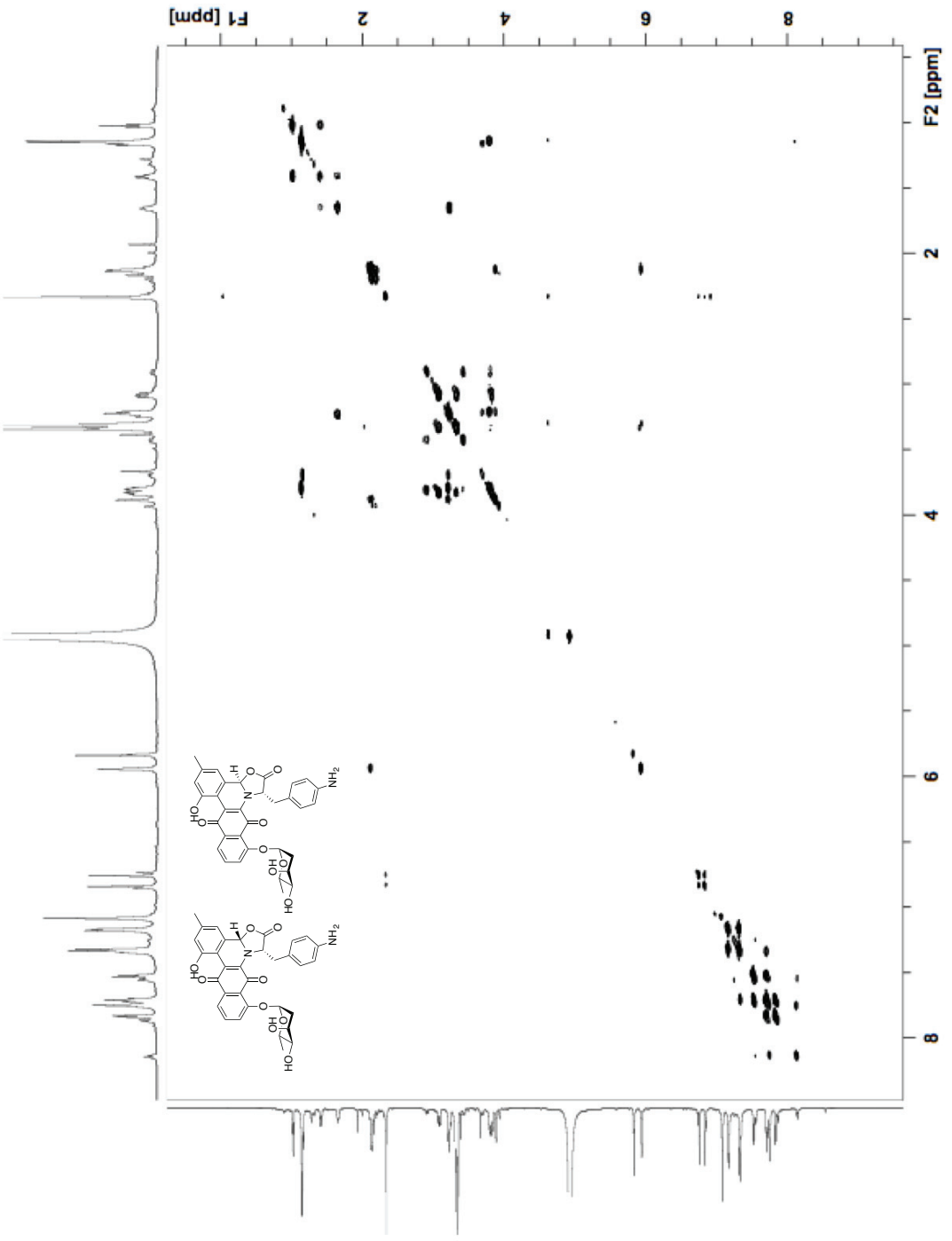


Figure S2. ^{1}H-^{1}H COSY spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD.

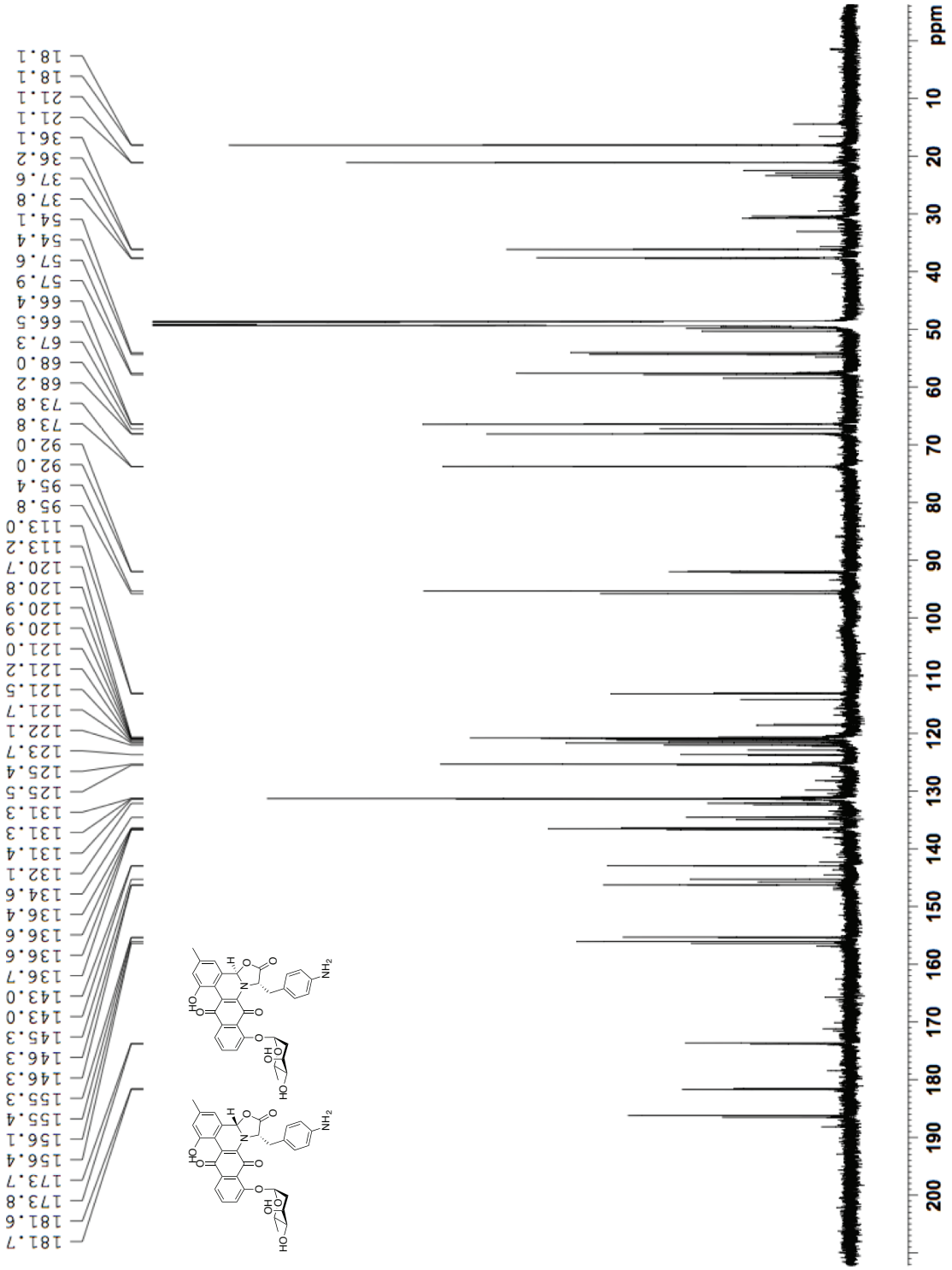


Figure S3. ^{13}C-NMR spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD (^{13}C: 176 MHz).

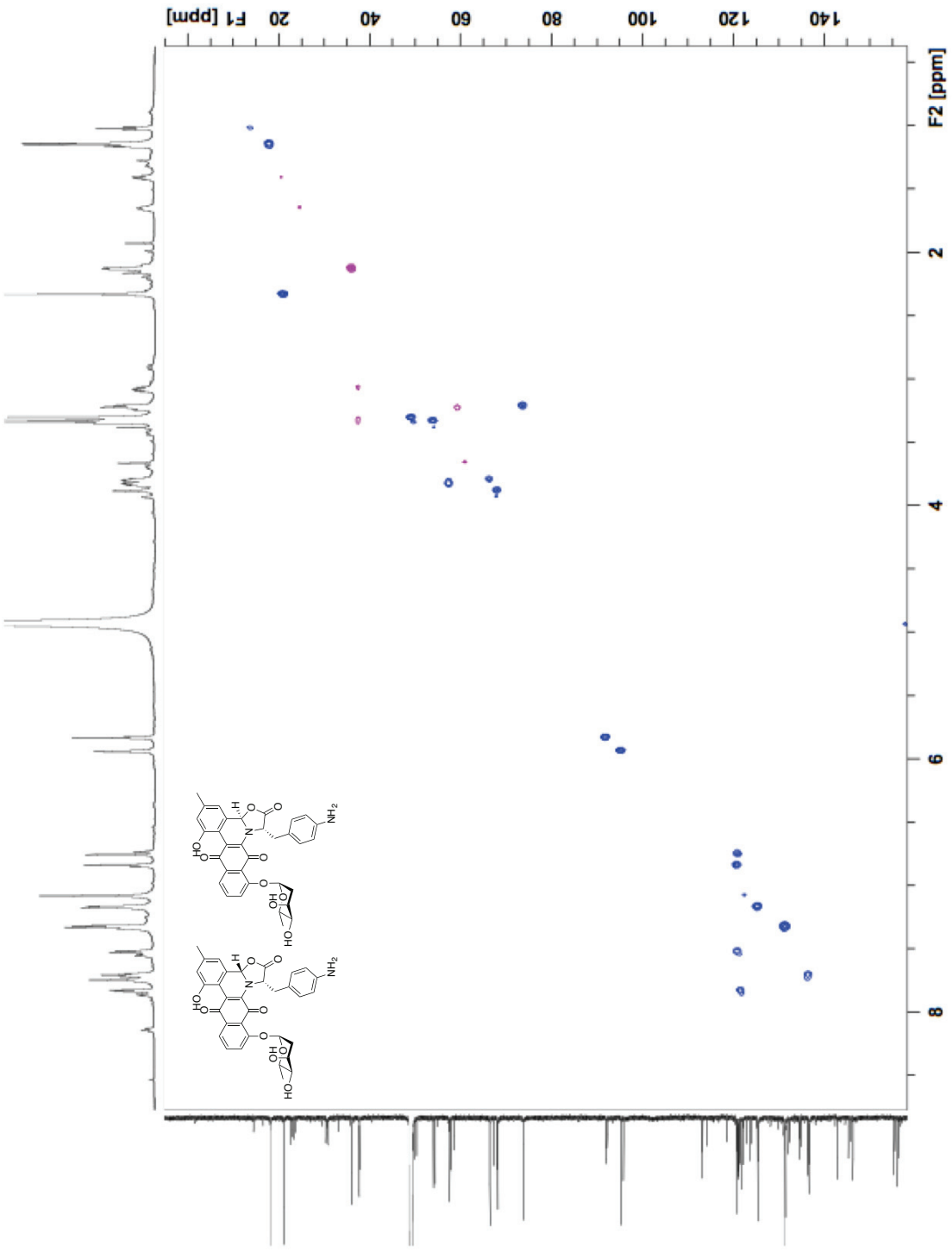


Figure S4. Edited-HSQC (^{1}H-^{13}C) spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD.

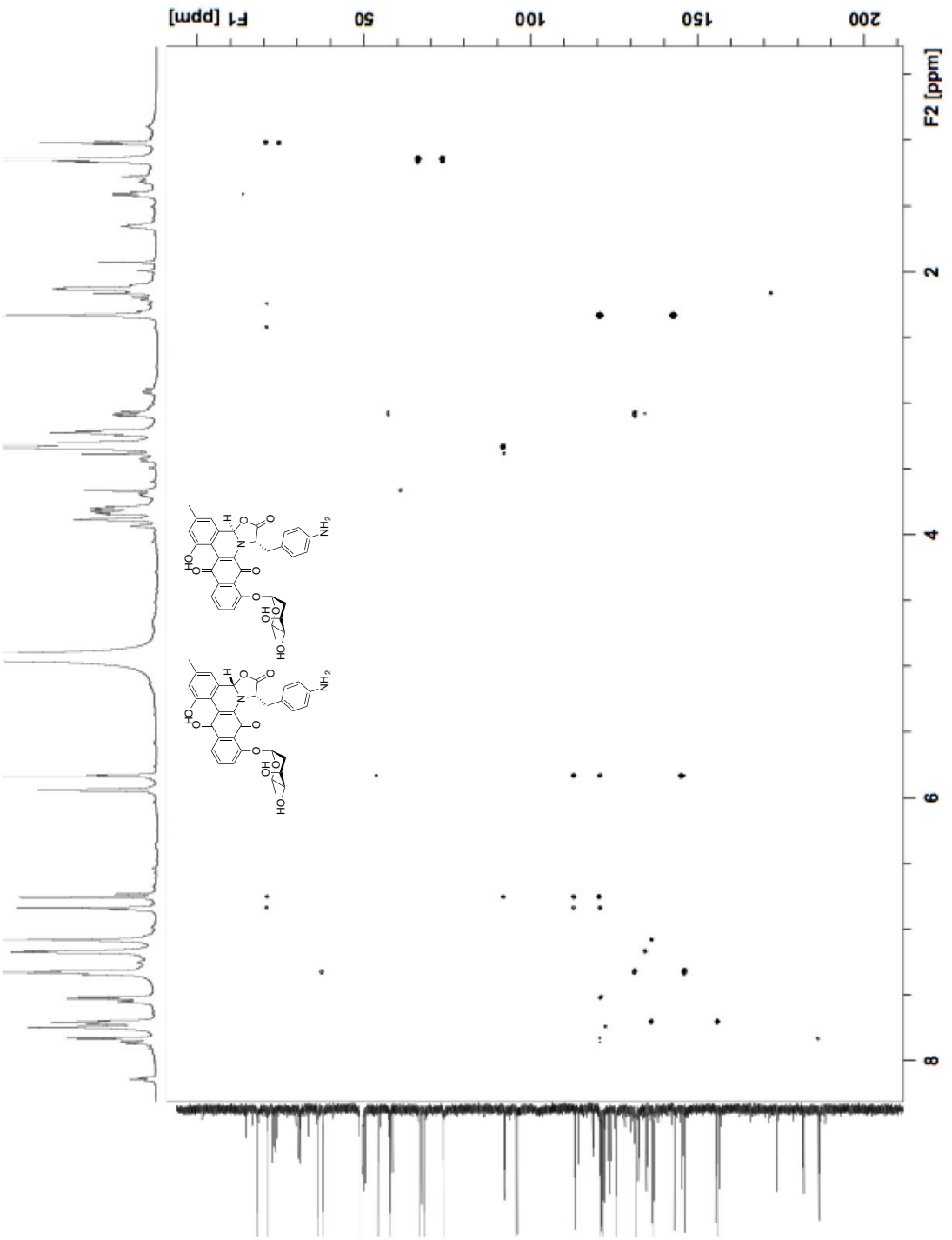


Figure S5. ^{1}H-^{13}C HMBC (6.5 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD.

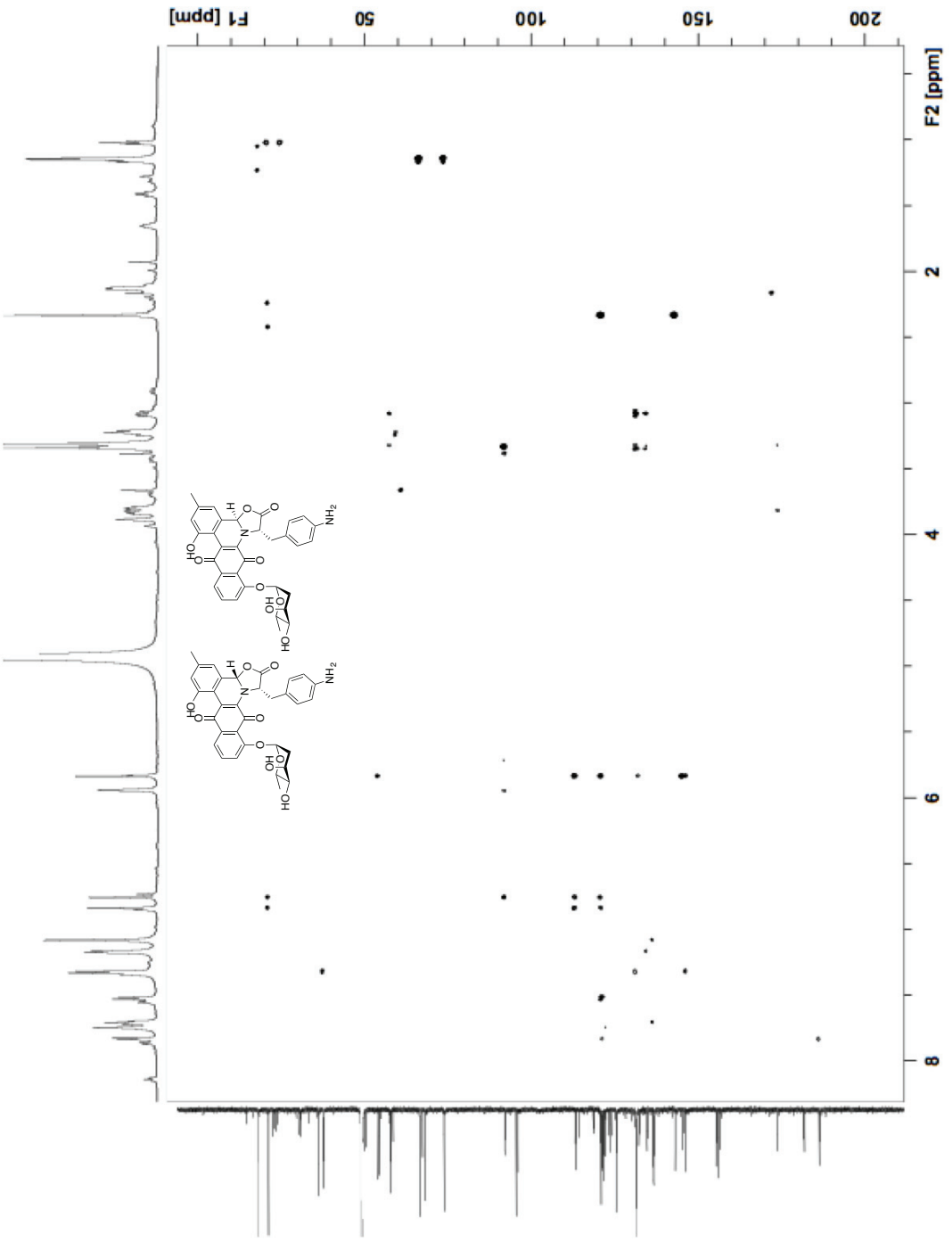


Figure S6. ^{1}H-^{13}C HMBC (25 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine (diastereomeric mixture) in CD_3OD.

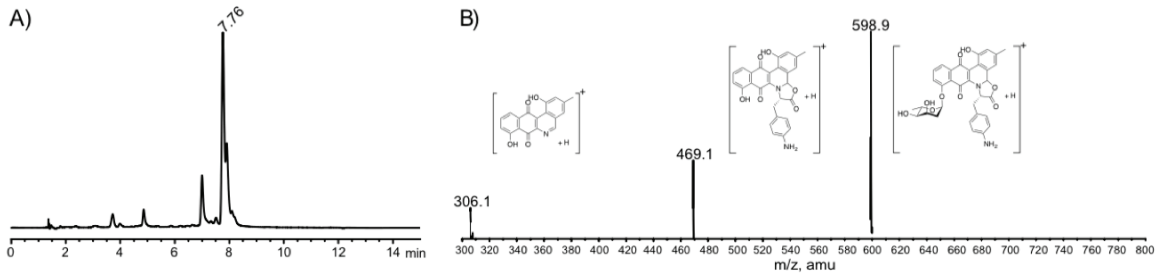


Figure S7. (A) HPLC trace and (B) mass spectrum of jadomycin 4-amino-L-phenylalanine. The mass spectrum shows the parent compound, the loss of the L-digitoxose, and phenanthroviridin.

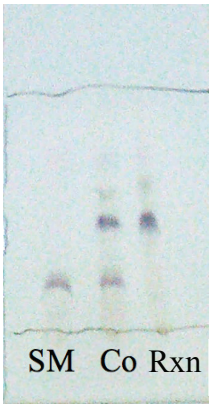


Figure S8. Normal phase silica TLC (5:5:1 CH_3CN:EtOAc:H_2O) comparing starting material (SM), reaction mixture between jadomycin 4-amino-L-phenylalanine and (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester after three hours (Rxn), and a co-spot of both (Co).

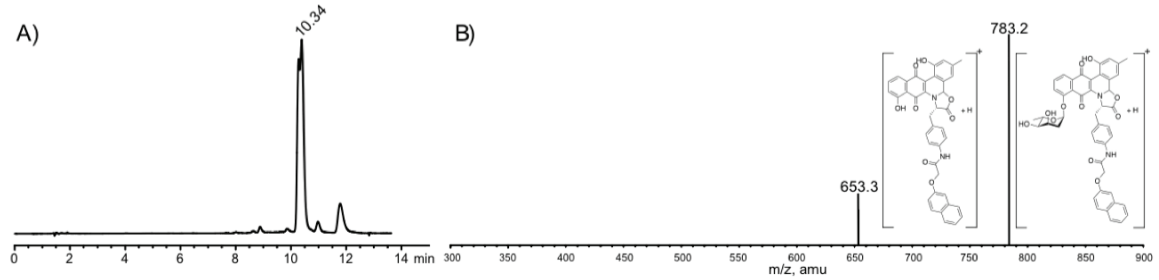


Figure S9. (A) HPLC trace and (B) mass spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetyl amide. The mass spectrum shows the parent compound and the loss of the L-digitoxose moiety.

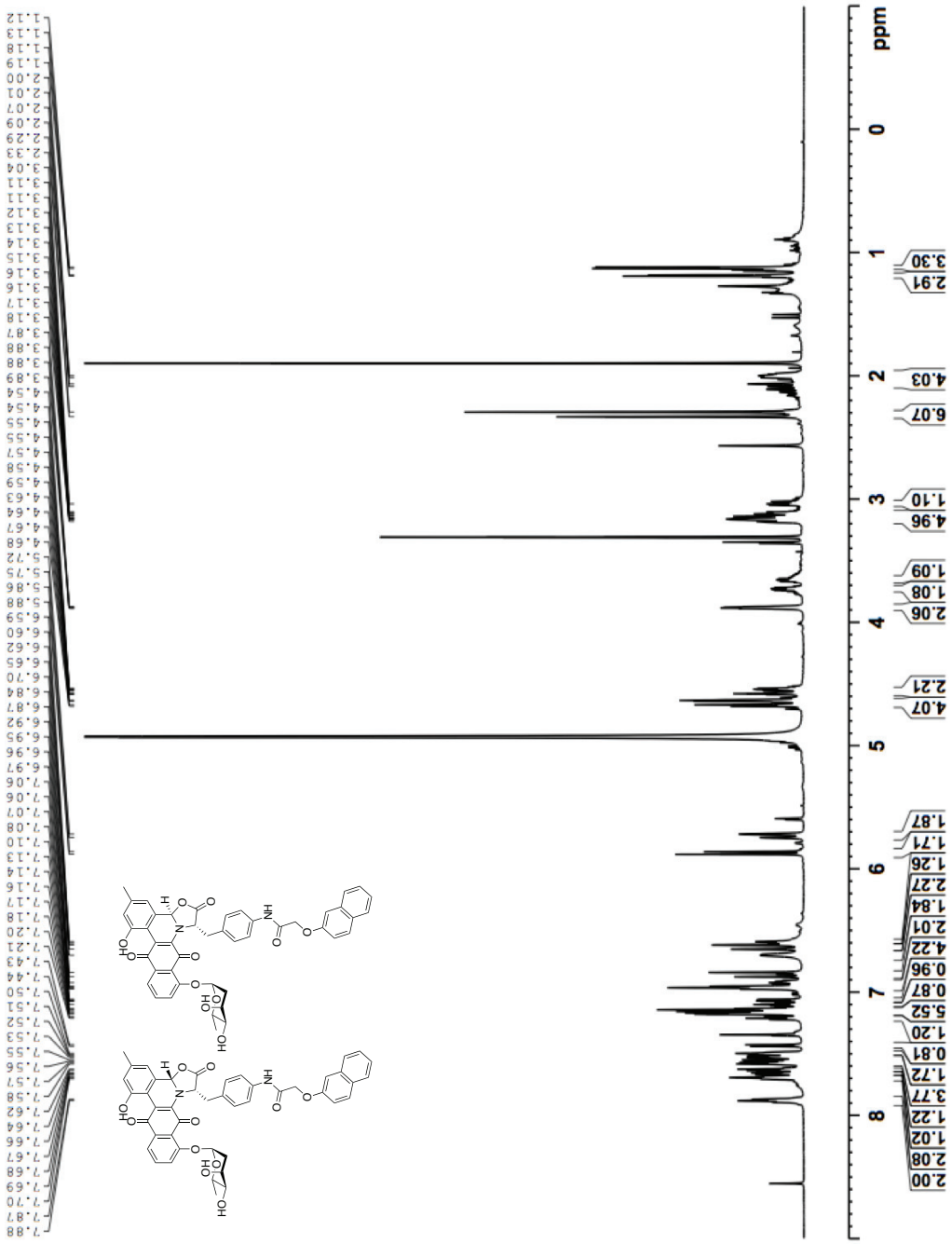


Figure S10. ^{1}H-NMR spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD (^{1}H: 700 MHz).

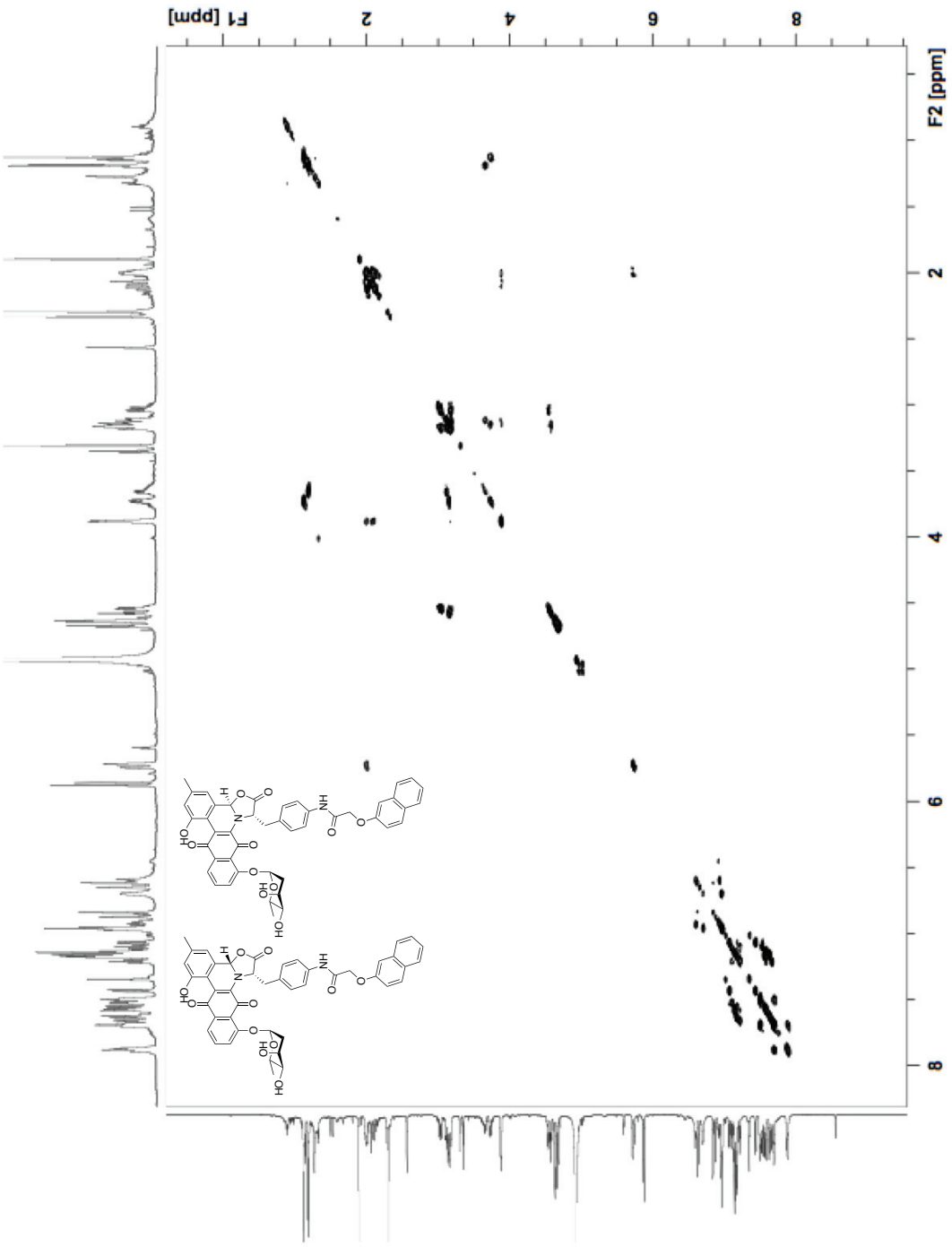


Figure S11. ^{1}H-^{1}H COSY spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD.

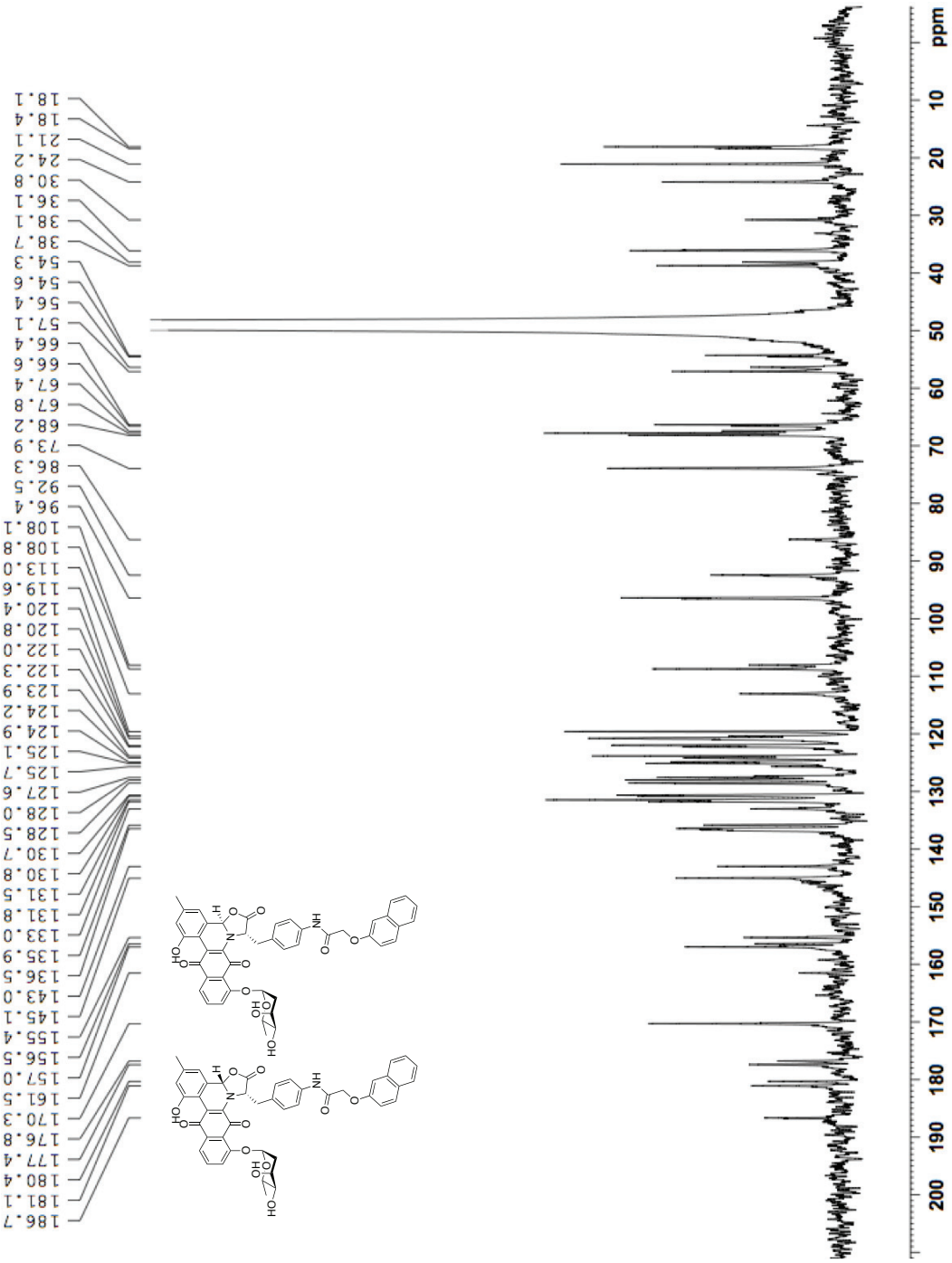


Figure S12. ^{13}C-NMR spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD (^{13}C: 176 MHz).

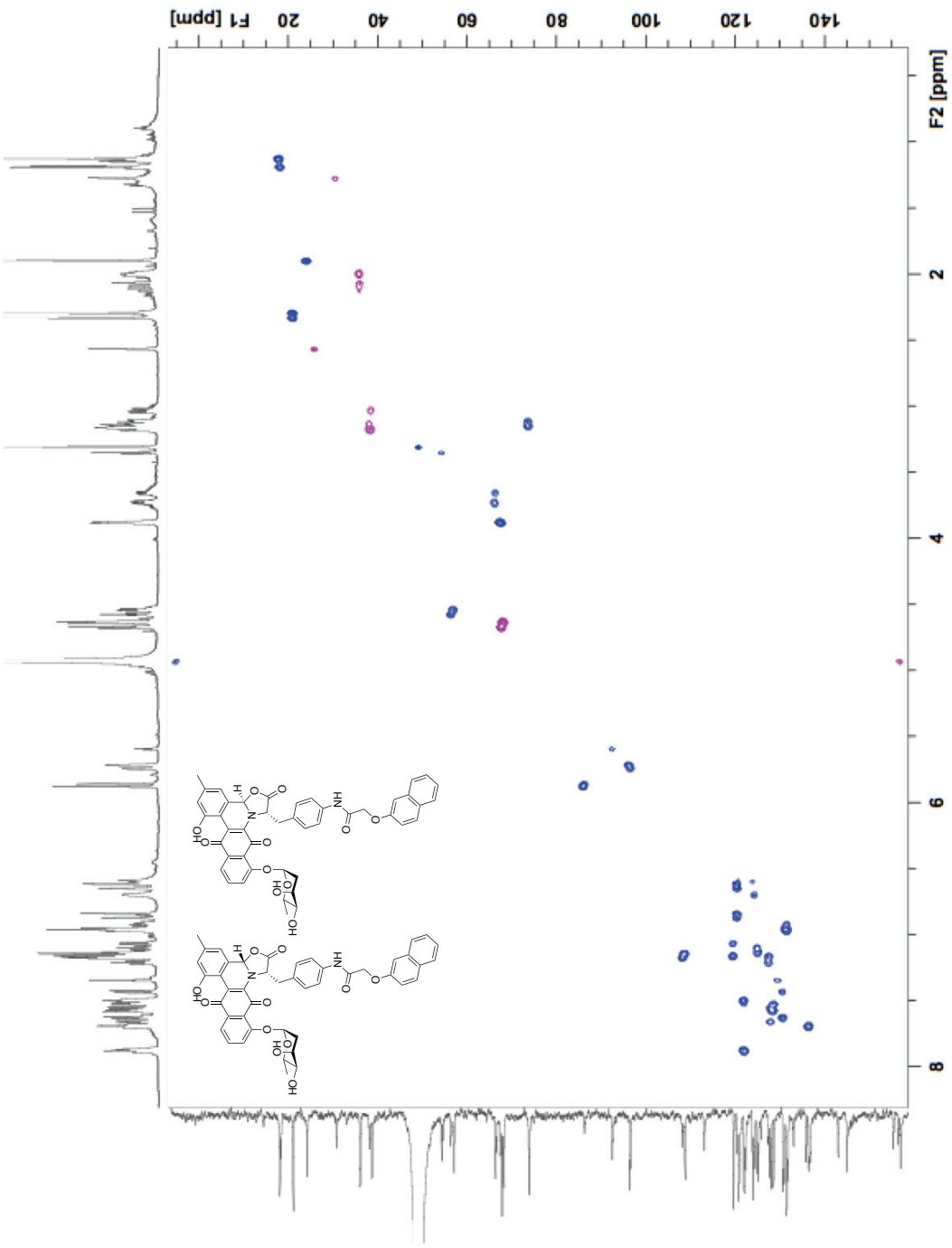


Figure S13. Edited-HSQC (^{1}H-^{13}C) spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD.

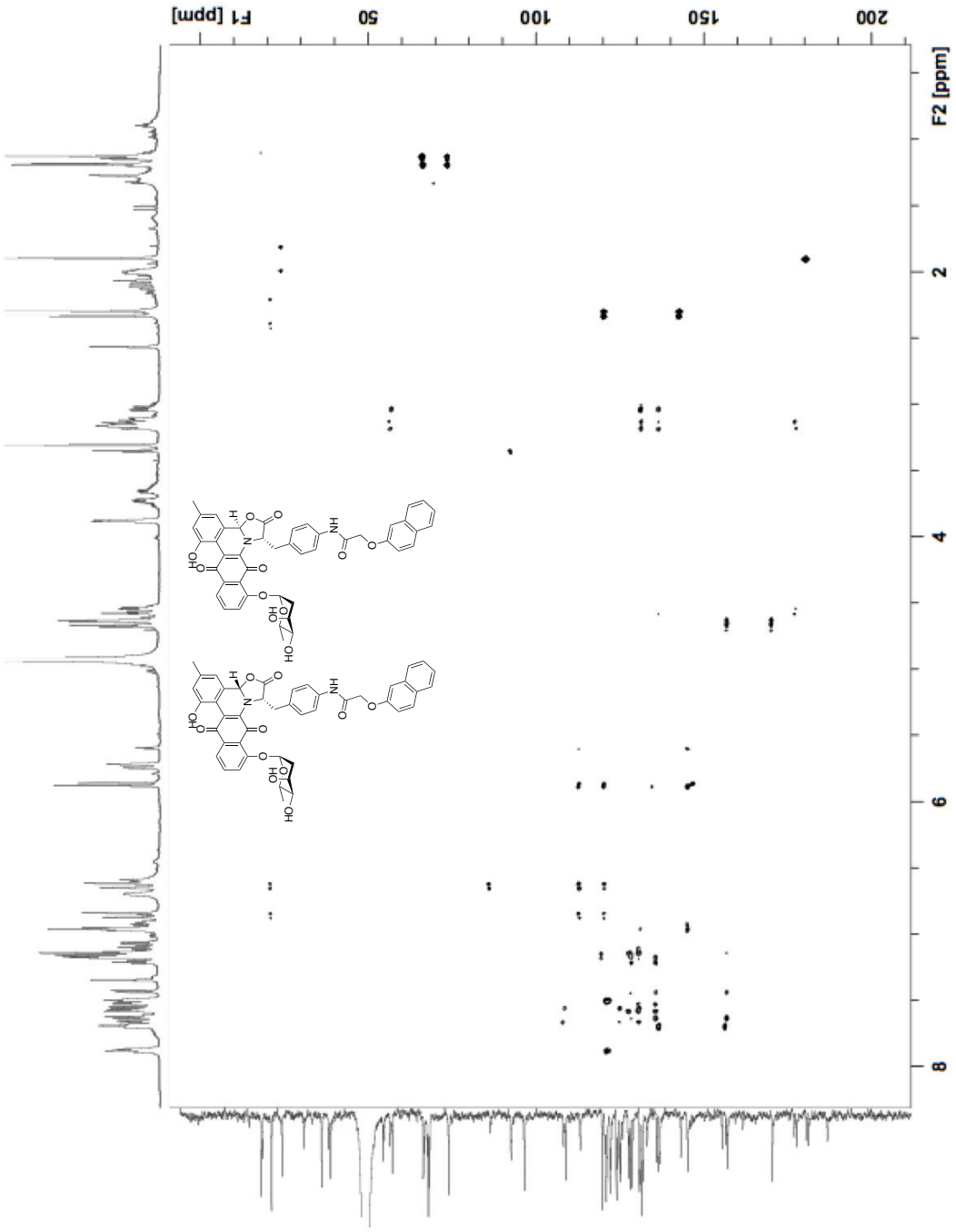


Figure S14. ^{1}H-^{13}C HMBC (6.5 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD.

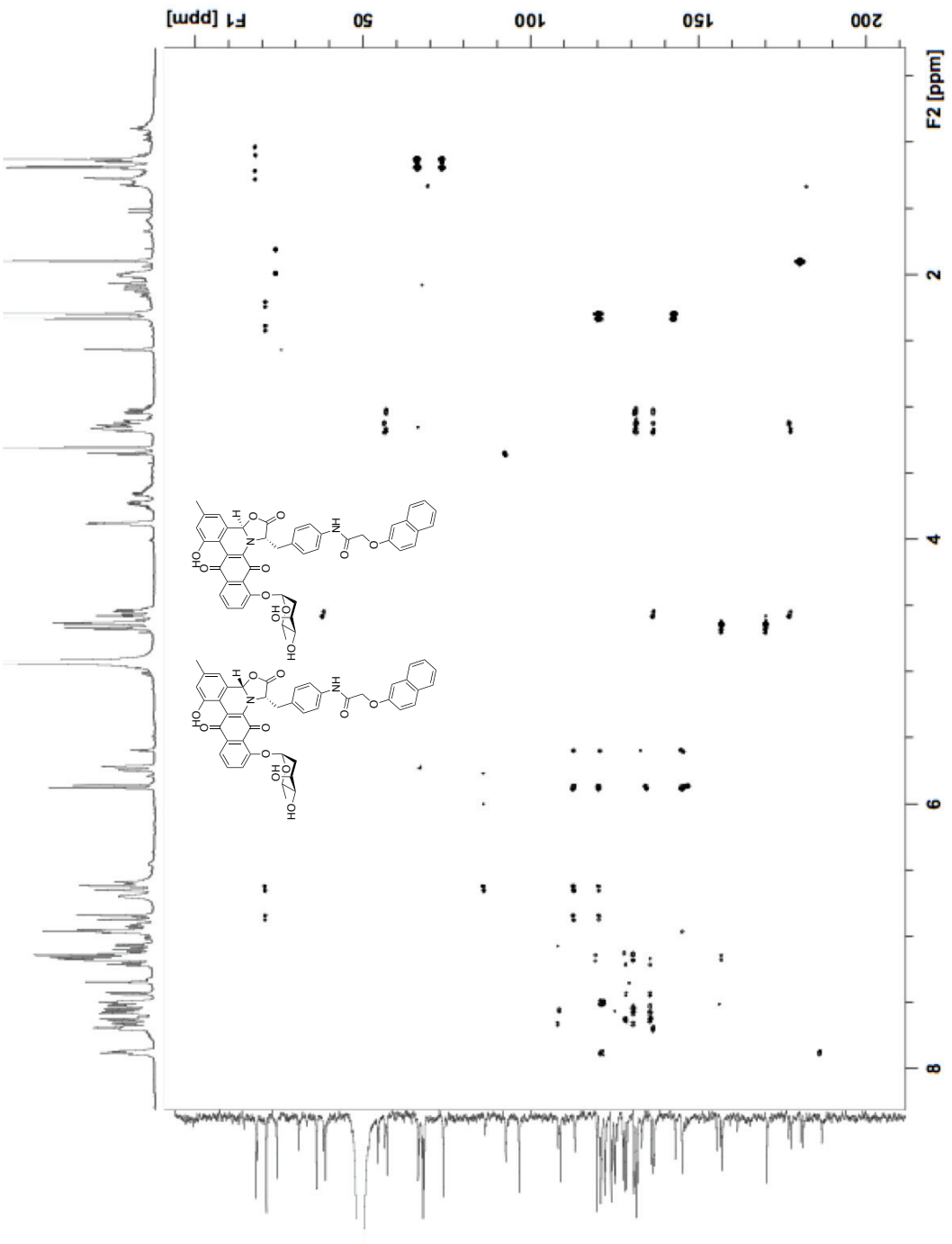


Figure S15. ^{1}H-^{13}C HMBC (25 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine naphthoxyacetylamide (diastereomeric mixture) in CD_3OD.

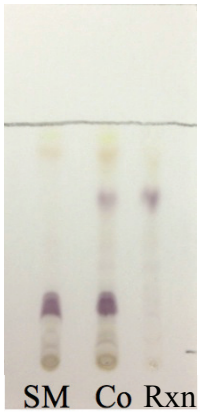


Figure S16. Normal phase silica TLC (5:5:1 CH_3CN:EtOAc:H_2O) comparing starting material (SM), reaction mixture between jadomycin 4-amino-L-phenylalanine and phenoxyacetic acid *N*-hydroxysuccinimide ester after one hour (Rxn), and a co-spot of both (Co).

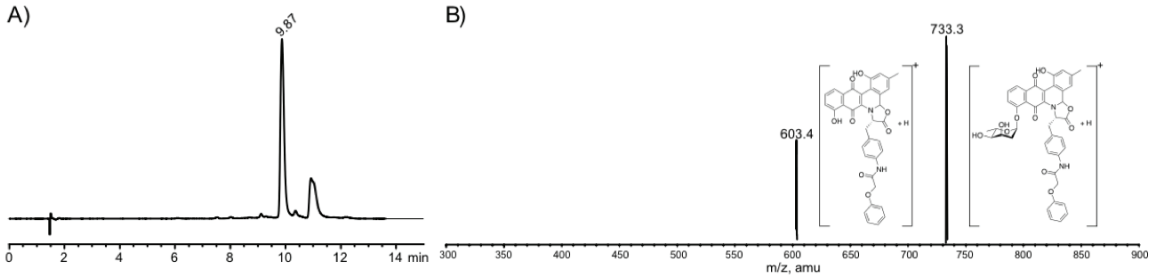


Figure S17. (A) HPLC trace and (B) mass spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetyl amide. The mass spectrum shows the parent compound and the loss of the L-digitoxose moiety.

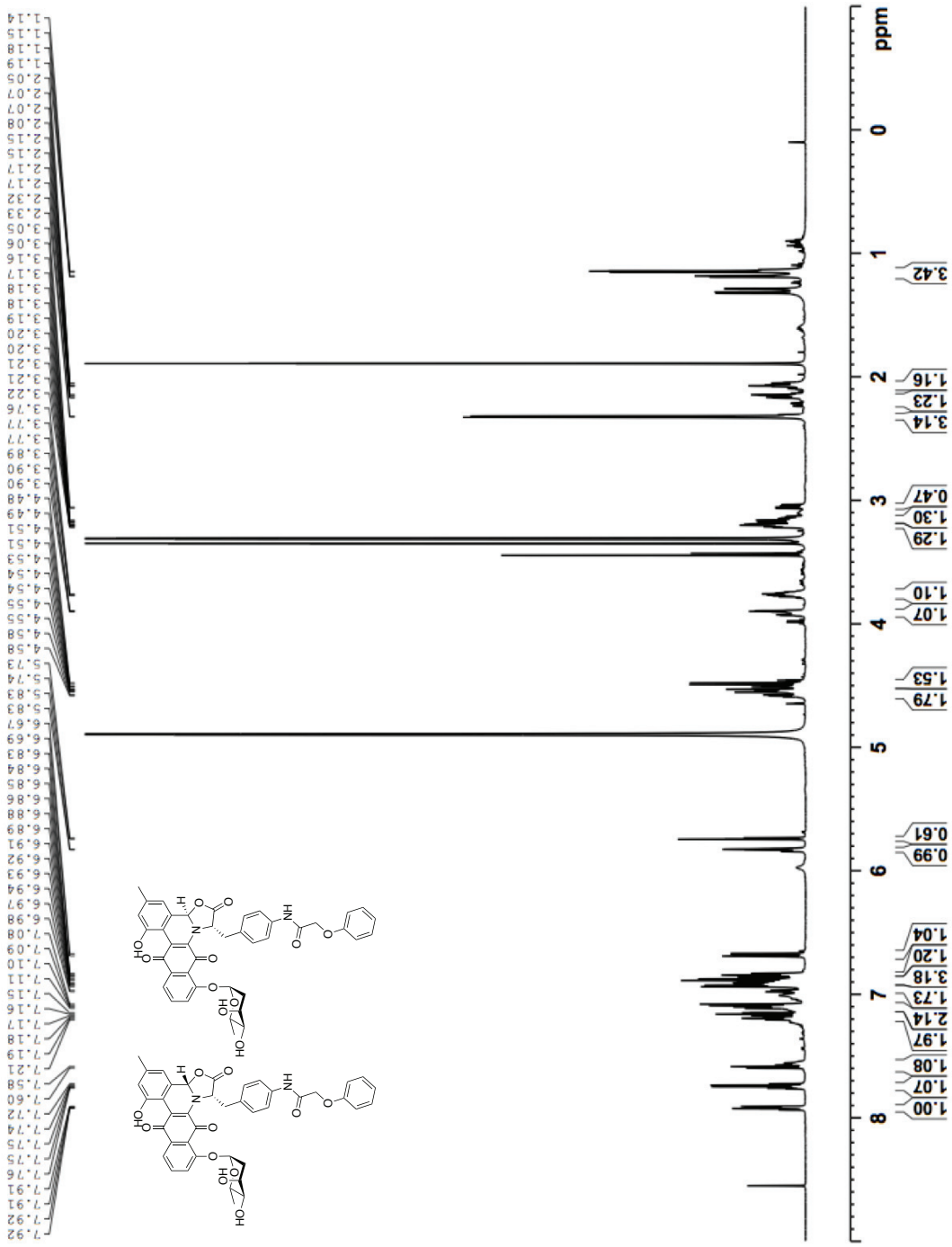


Figure S18. ^{1}H-NMR spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD (^{1}H: 700 MHz).

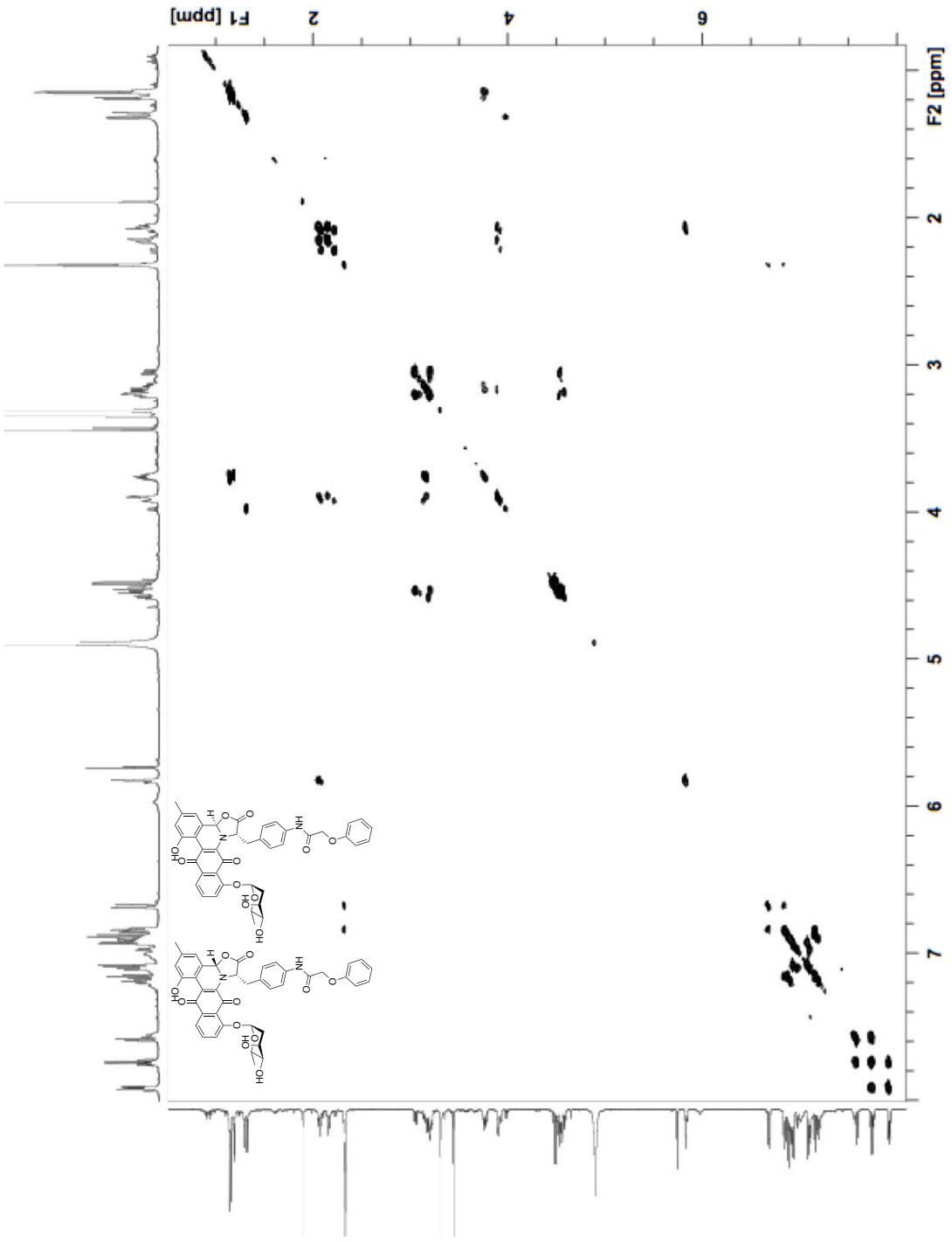


Figure S19. ^{1}H-^{1}H COSY spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD.

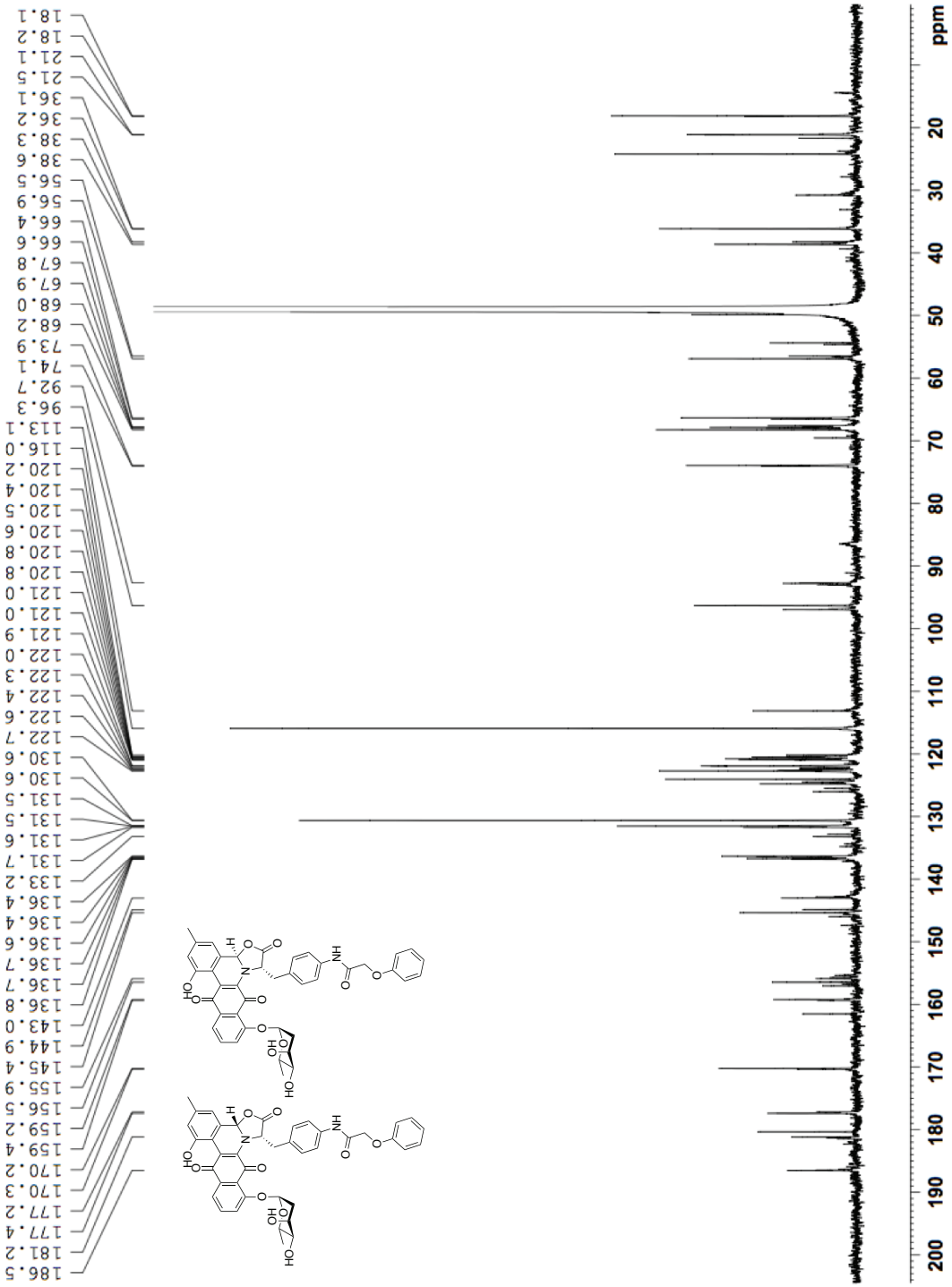


Figure S20. ^{13}C-NMR spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD (^{13}C: 176 MHz).

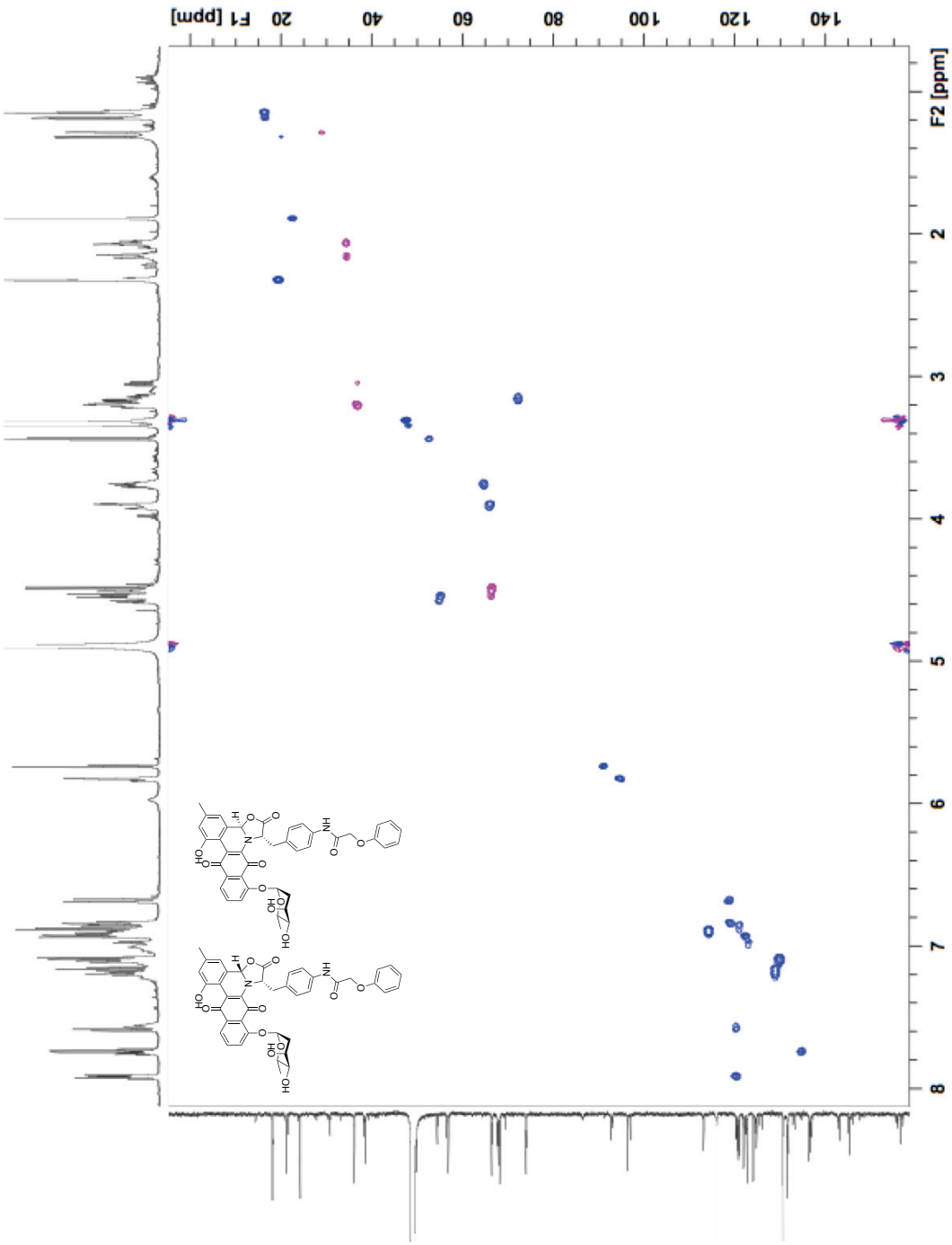


Figure S21. Edited-HSQC (^{1}H-^{13}C) spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD.

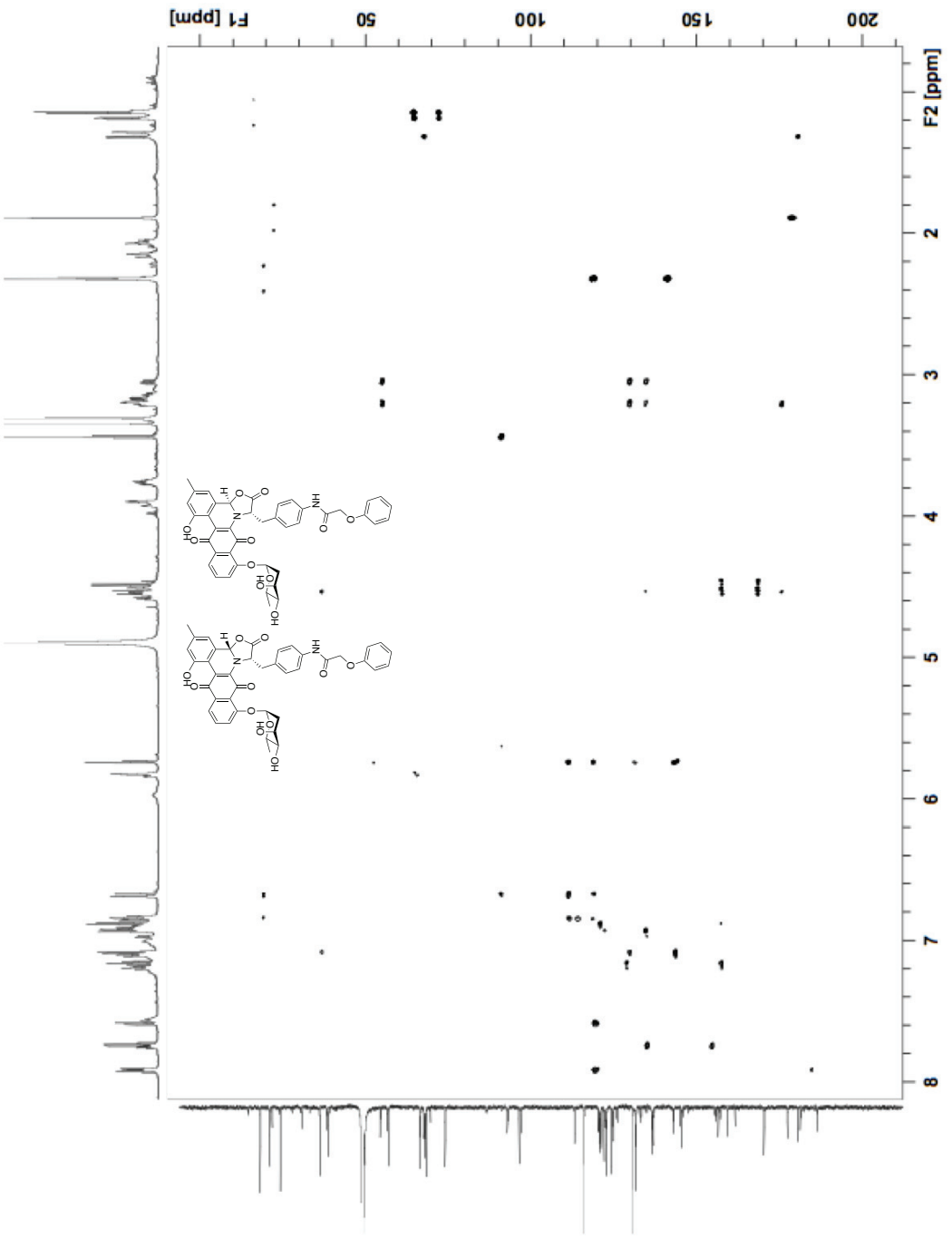


Figure S22. ^{1}H-^{13}C HMBC (6.5 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD.

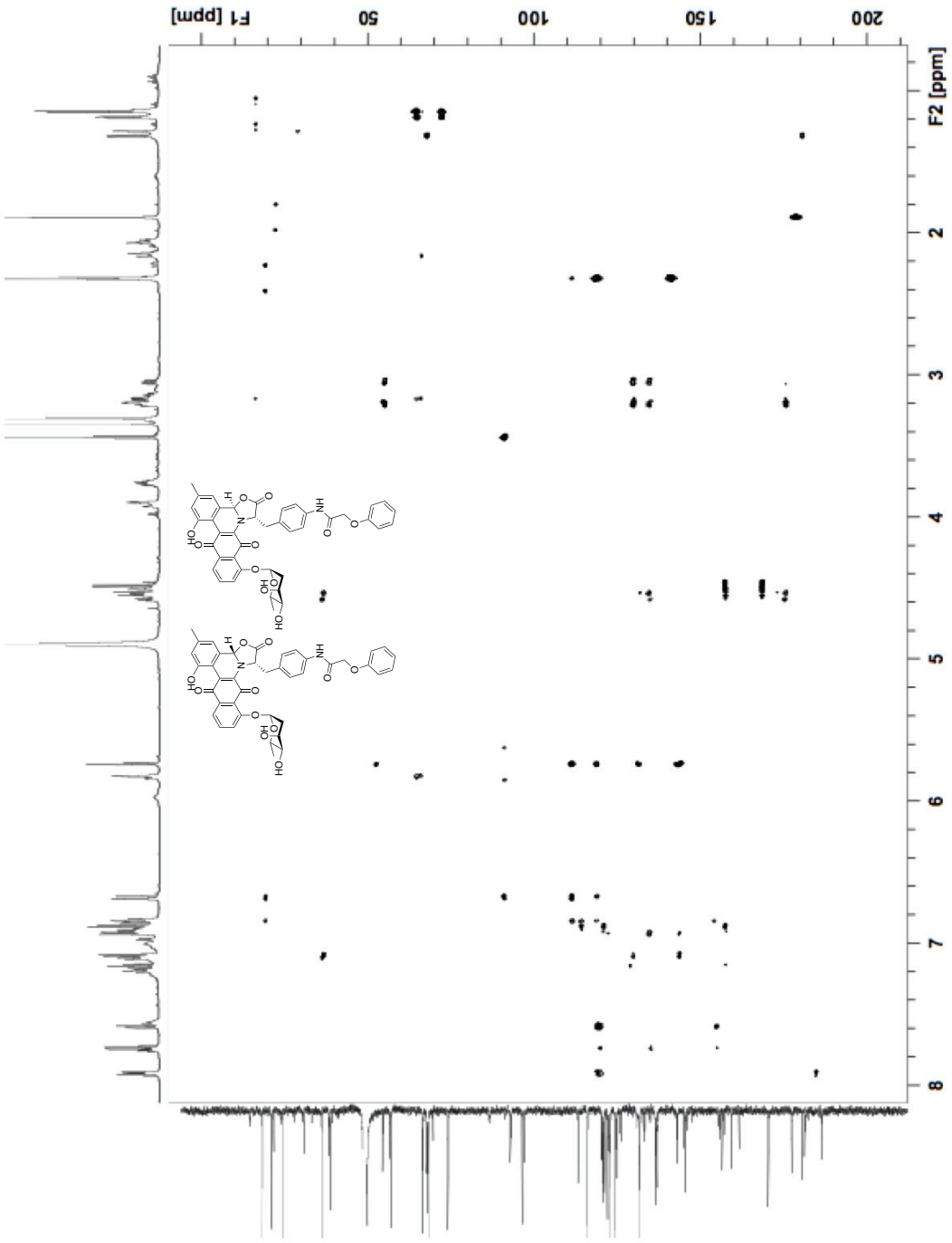


Figure S23. ^{1}H-^{13}C HMBC (25 μs mixing time) spectrum of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (diastereomeric mixture) in CD_3OD.

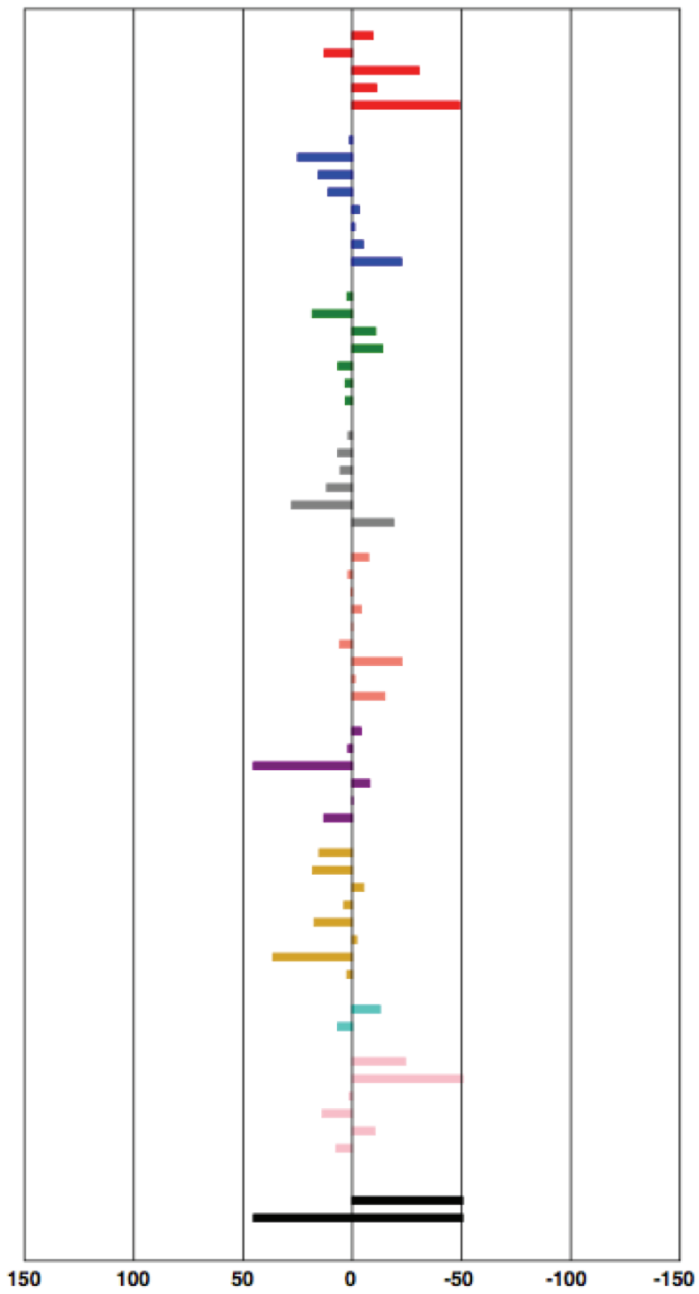
Table S1. One dose NCI-60 tumour cell line screen of jadomycin 4-amino-L-phenylalanine phenoxyacetylamide (1×10^{-5} M).

Developmental Therapeutics Program

One Dose Mean Graph

NSC: D-778284 / 1	Conc: 1.00E-5 Molar	Test Date: Dec 02, 2013
Experiment ID: 1312OS99		Report Date: Oct 07, 2014

Panel/Cell Line	Growth Percent
Leukemia	
CCRF-CEM	92.55
HL-60(TB)	114.79
K-562	71.44
MOLT-4	90.99
SR	53.06
Non-Small Cell Lung Cancer	
A549/ATCC	103.06
HOP-62	126.79
HOP-92	117.43
NCI-H226	112.94
NCI-H23	98.94
NCI-H322M	101.04
NCI-H460	97.06
NCI-H522	79.57
Colon Cancer	
COLO 205	104.33
HCC-2998	120.25
HCT-116	91.38
HCT-15	88.12
HT29	108.41
KM12	105.20
SW-620	105.15
CNS Cancer	
SF-268	103.79
SF-295	108.52
SF-539	107.34
SNB-19	113.62
SNB-75	129.70
U251	83.09
Melanoma	
LOX IMVI	94.65
MALME-3M	103.81
M14	102.48
MDA-MB-435	97.91
SK-MEL-2	101.90
SK-MEL-28	107.66
SK-MEL-5	79.29
UACC-257	100.92
UACC-62	87.12
Ovarian Cancer	
IGROV1	98.06
OVCAR-3	103.85
OVCAR-5	147.18
OVCAR-8	94.11
NCI/ADR-RES	101.83
SK-OV-3	114.94
Renal Cancer	
786-0	117.05
A498	120.14
ACHN	96.82
CAKI-1	105.72
RXF 393	119.38
SN12C	100.05
TK-10	138.18
UO-31	104.40
Prostate Cancer	
PC-3	89.43
DU-145	108.56
Breast Cancer	
MCF7	77.77
MDA-MB-231/ATCC	51.46
HS 578T	103.19
BT-549	115.65
T-47D	91.74
MDA-MB-468	109.27
Mean	101.98
Delta	50.52
Range	95.72



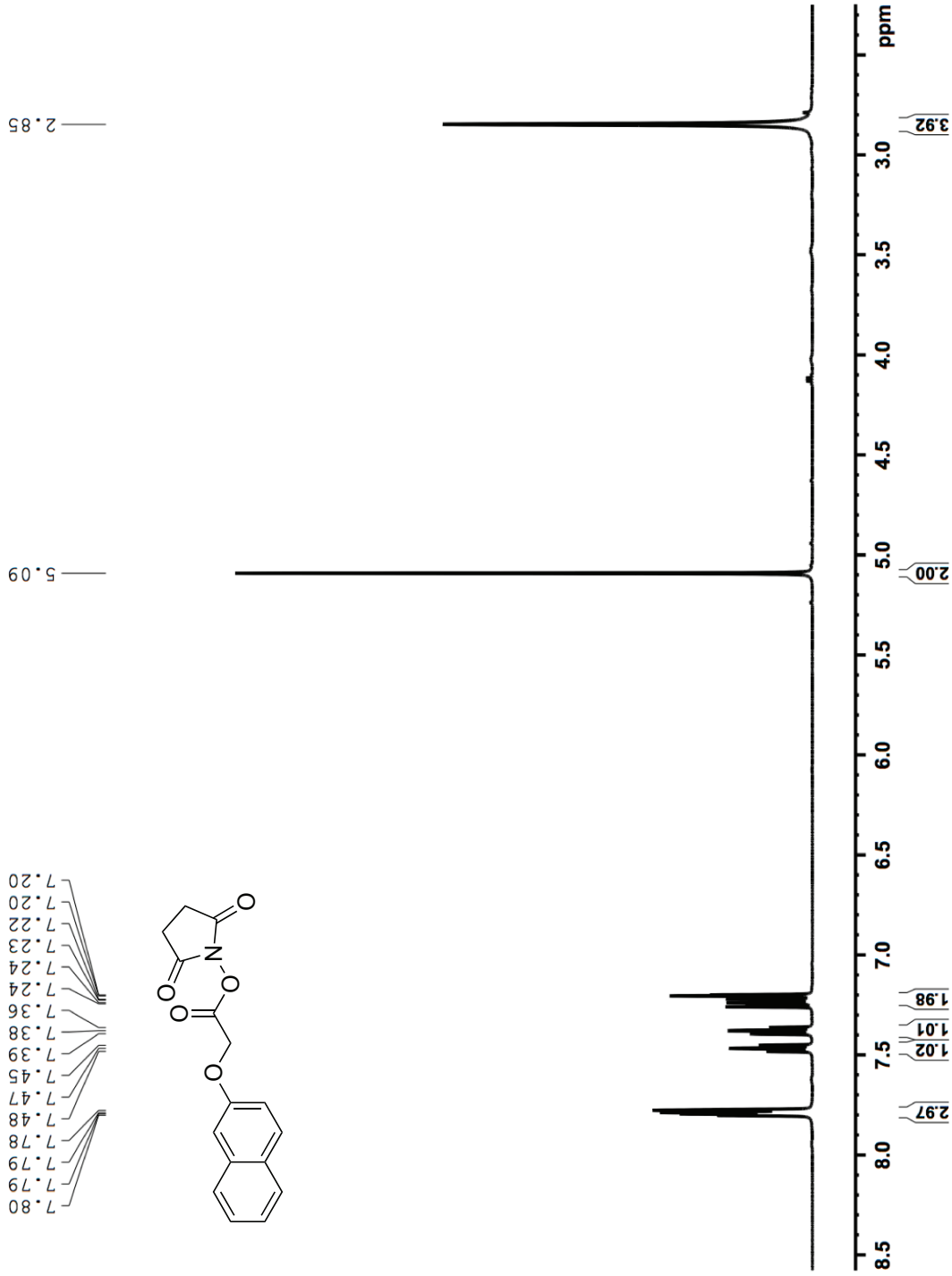


Figure S24. ^{1}H-NMR spectrum of (2-naphthoxy)acetic acid *N*-hydroxysuccinimide ester in $CDCl_3$ (^{1}H: 500 MHz).

APPENDIX II: NMR, ETC FOR CHAPTER 3

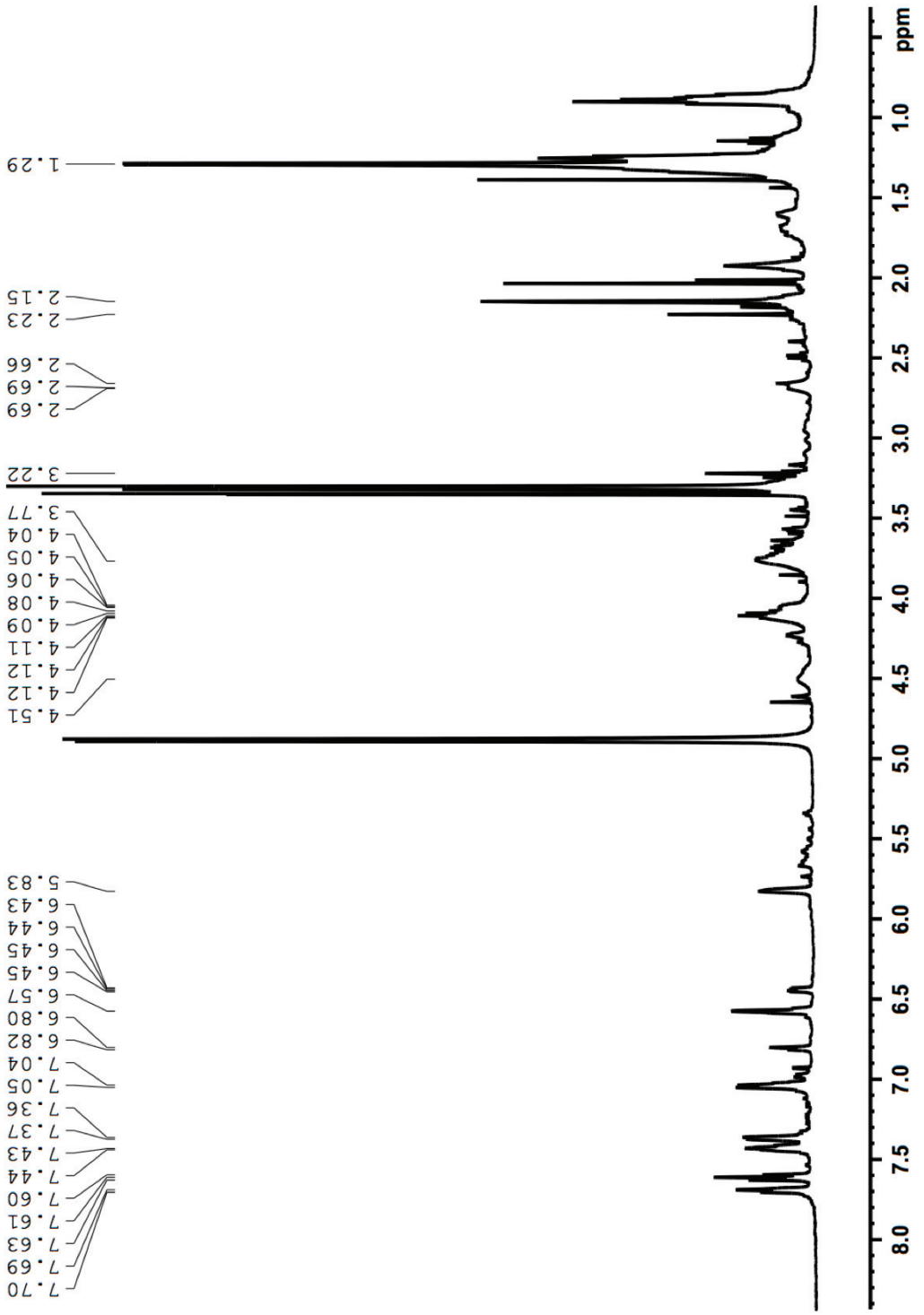


Figure S25. ^{1}H-NMR spectrum of jadomycin 4-borono-DL-phenylalanine (diastereomeric mixture) in CD_3OD (^{1}H: 700 MHz).

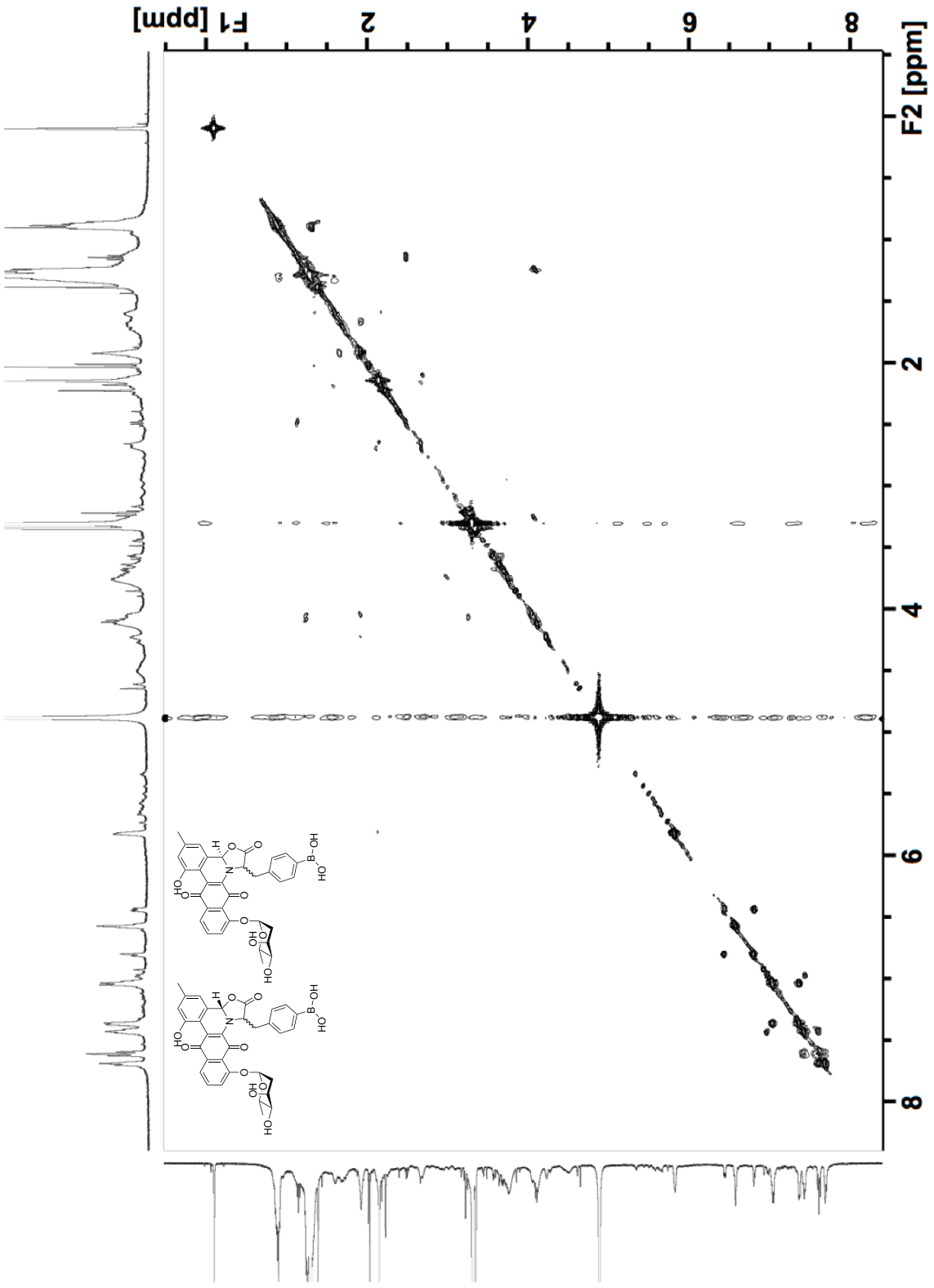


Figure S26. ^{1}H-^{1}H COSY spectrum of jadomycin 4-borono-DL-phenylalanine (diastereomeric mixture) in CD_3OD.

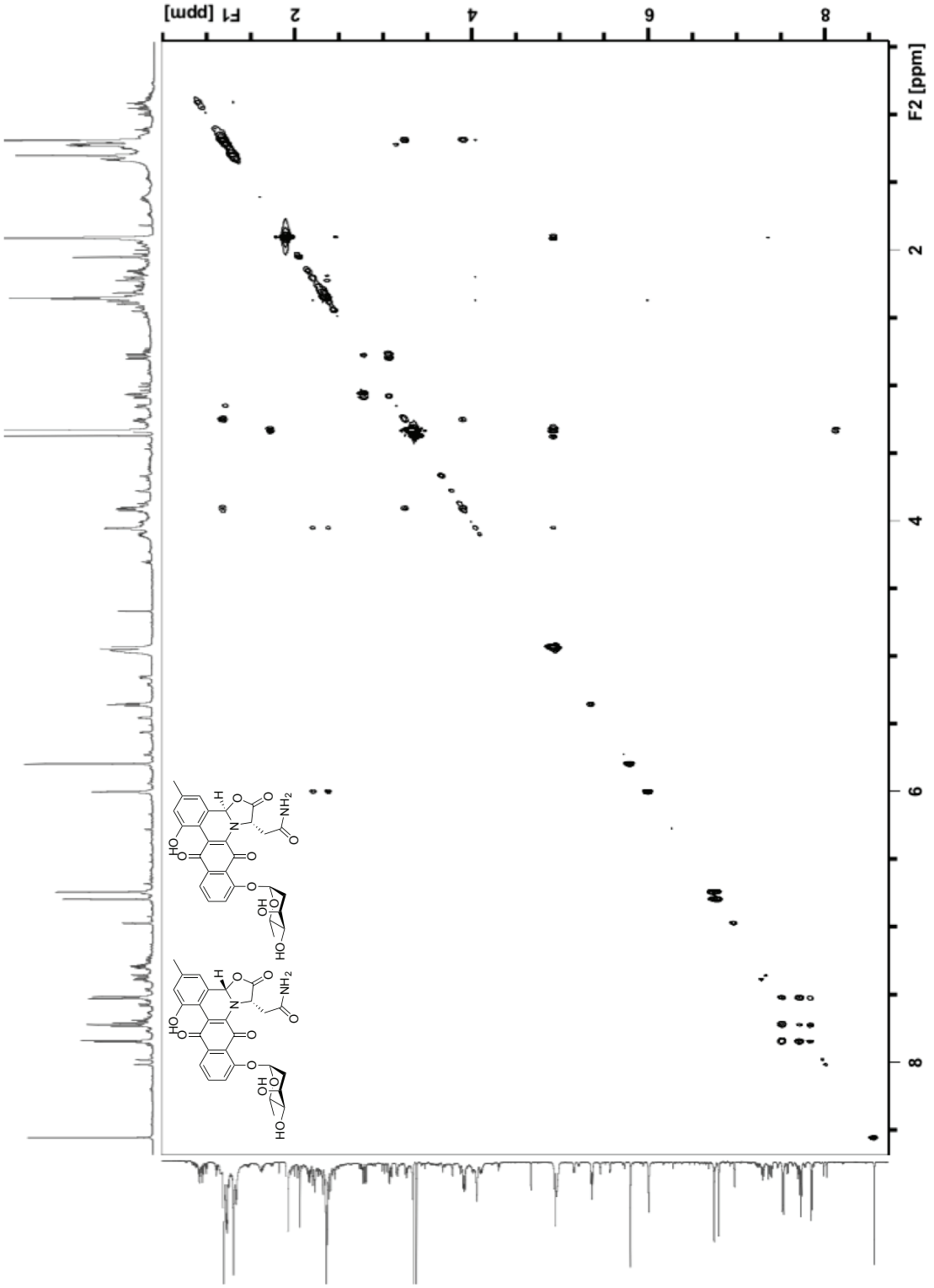


Figure S27. TOCSY (60 ms mixing time) spectrum of jadomycin L-asparagine purified following current methodology in CD_3OH (^1H: 700 MHz).

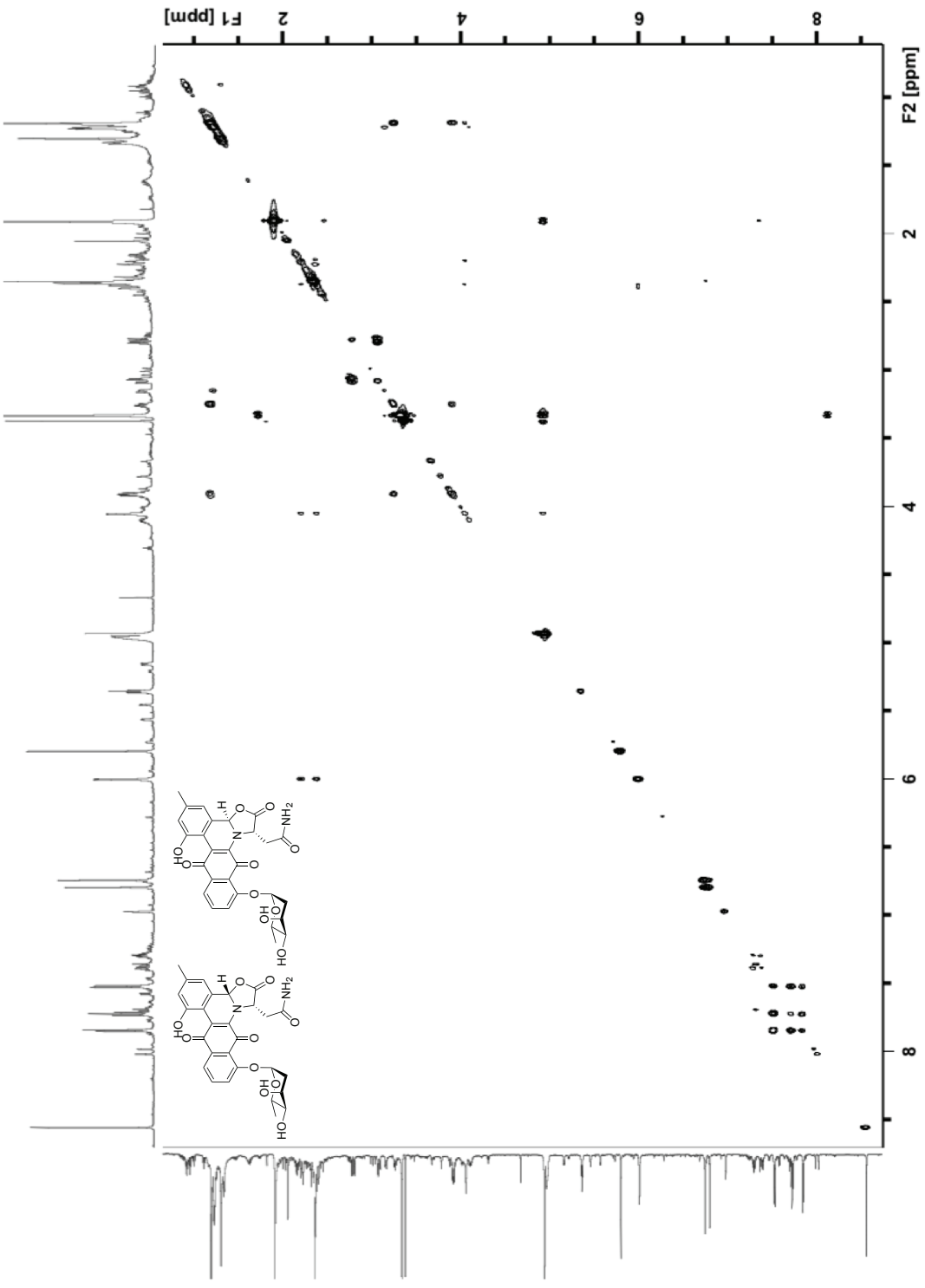


Figure S28. TOCSY (120 ms mixing time) spectrum of jadomycin L-asparagine purified following current methodology in CD_3OH (^1H: 700 MHz).

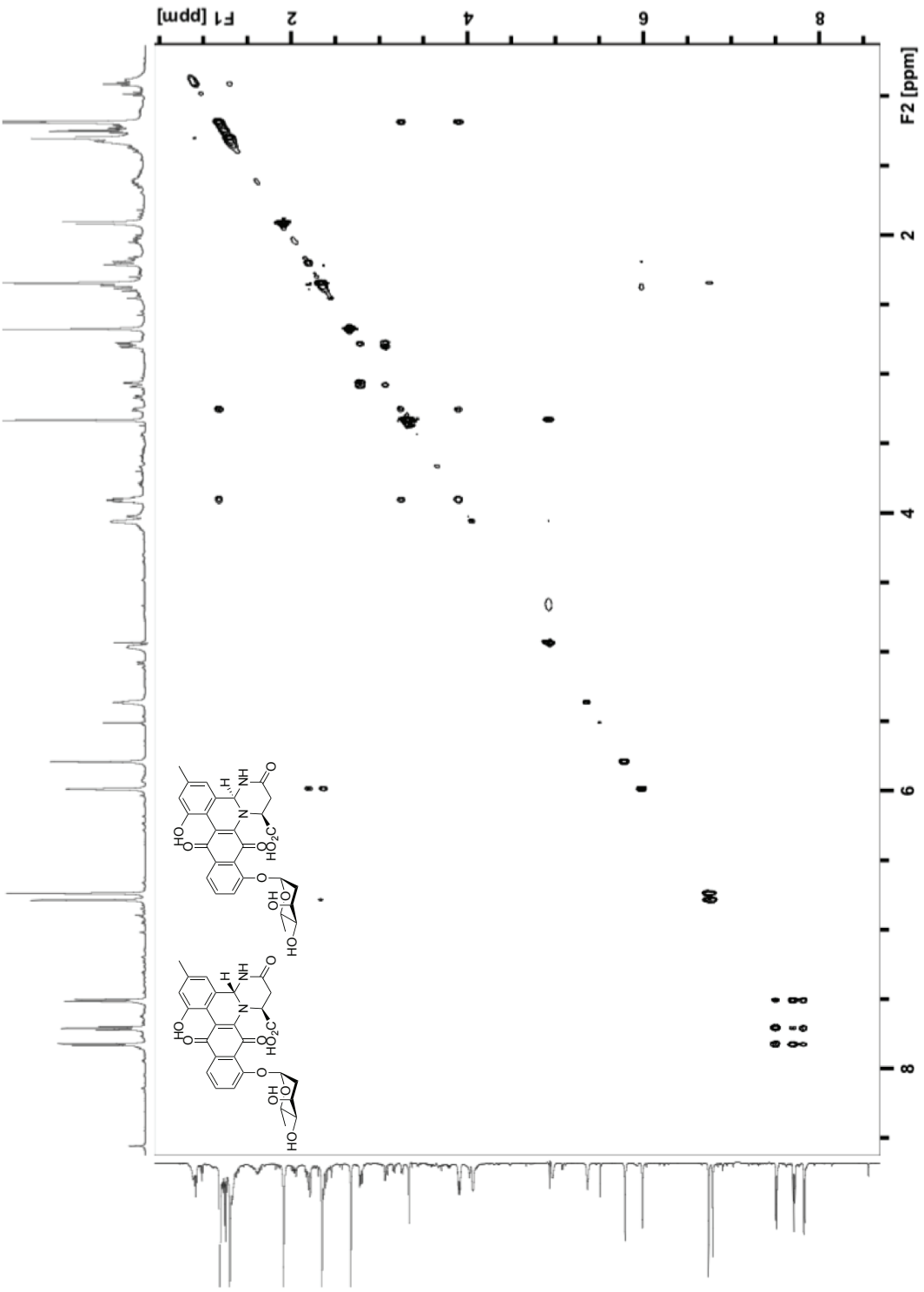


Figure S29. TOCSY (60 ms mixing time) spectrum of jadomycin L-asparagine purified following literature methodology in CD_3OH (^1H: 700 MHz).

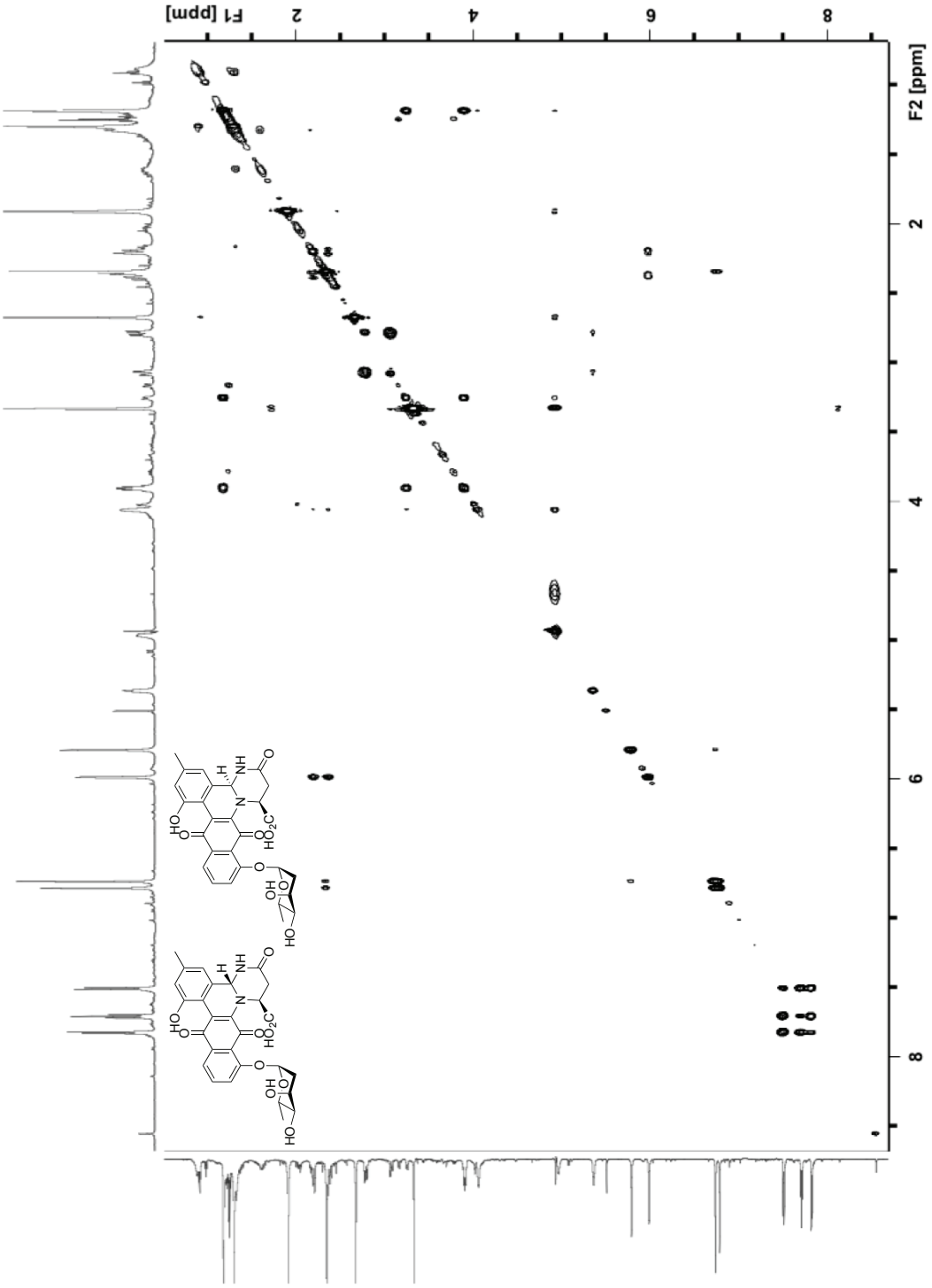


Figure S30. TOCSY (120 ms mixing time) spectrum of jadomycin L-asparagine purified following literature methodology in CD_3OH (^1H: 700 MHz).

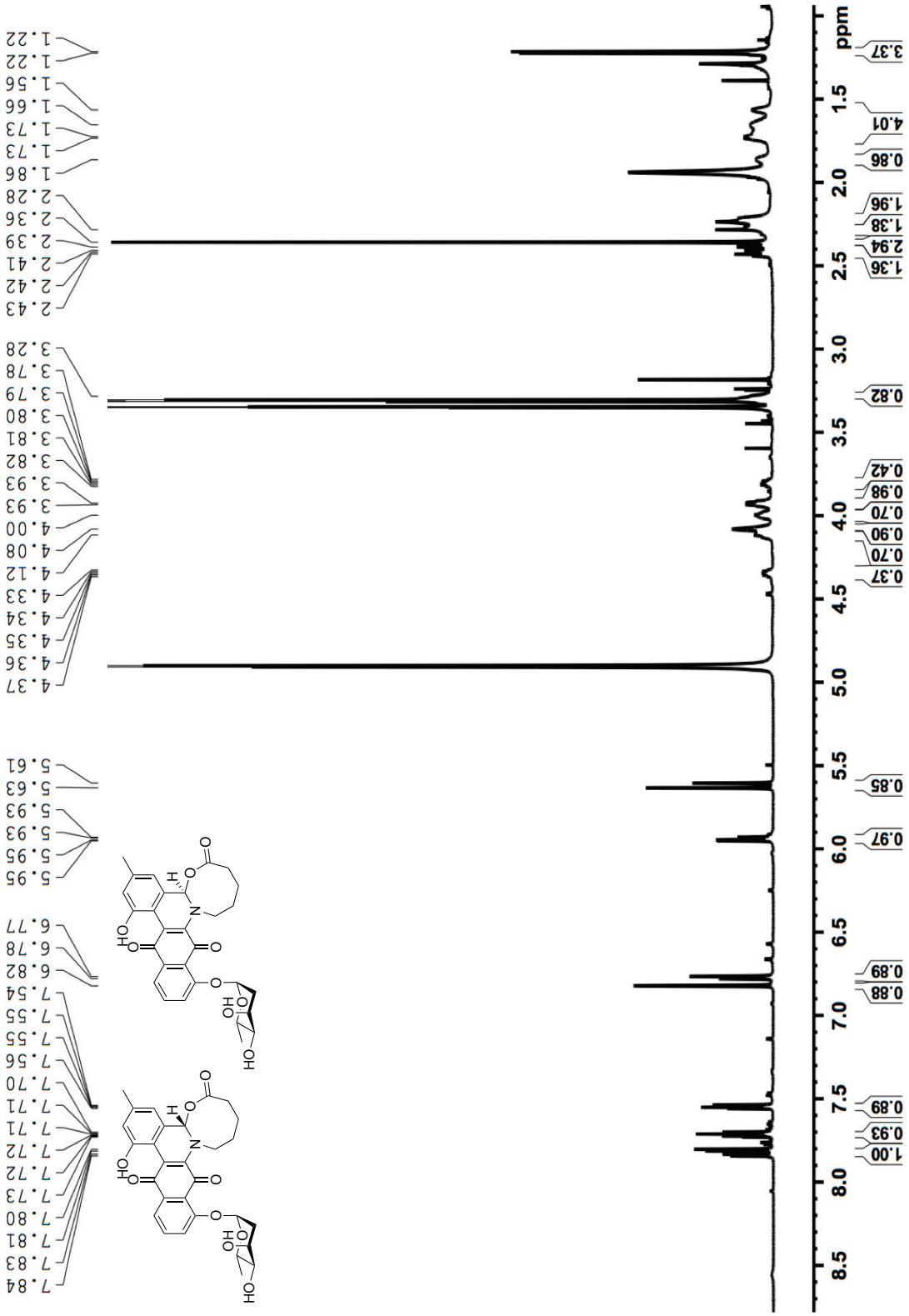


Figure S31. ^{1}H-NMR spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD (^{1}H: 700 MHz).

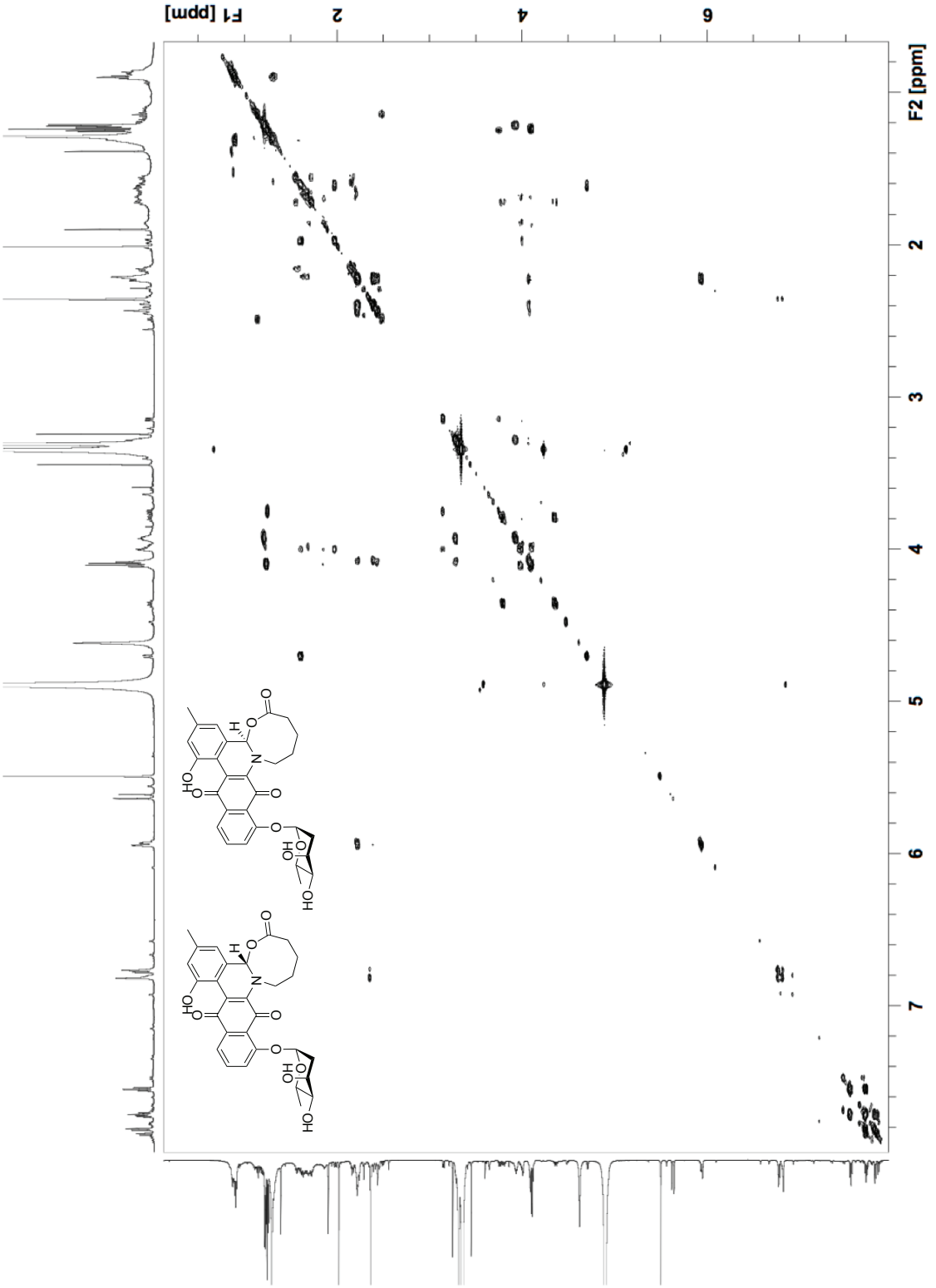


Figure S32. ^{1}H-^{1}H COSY spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD.

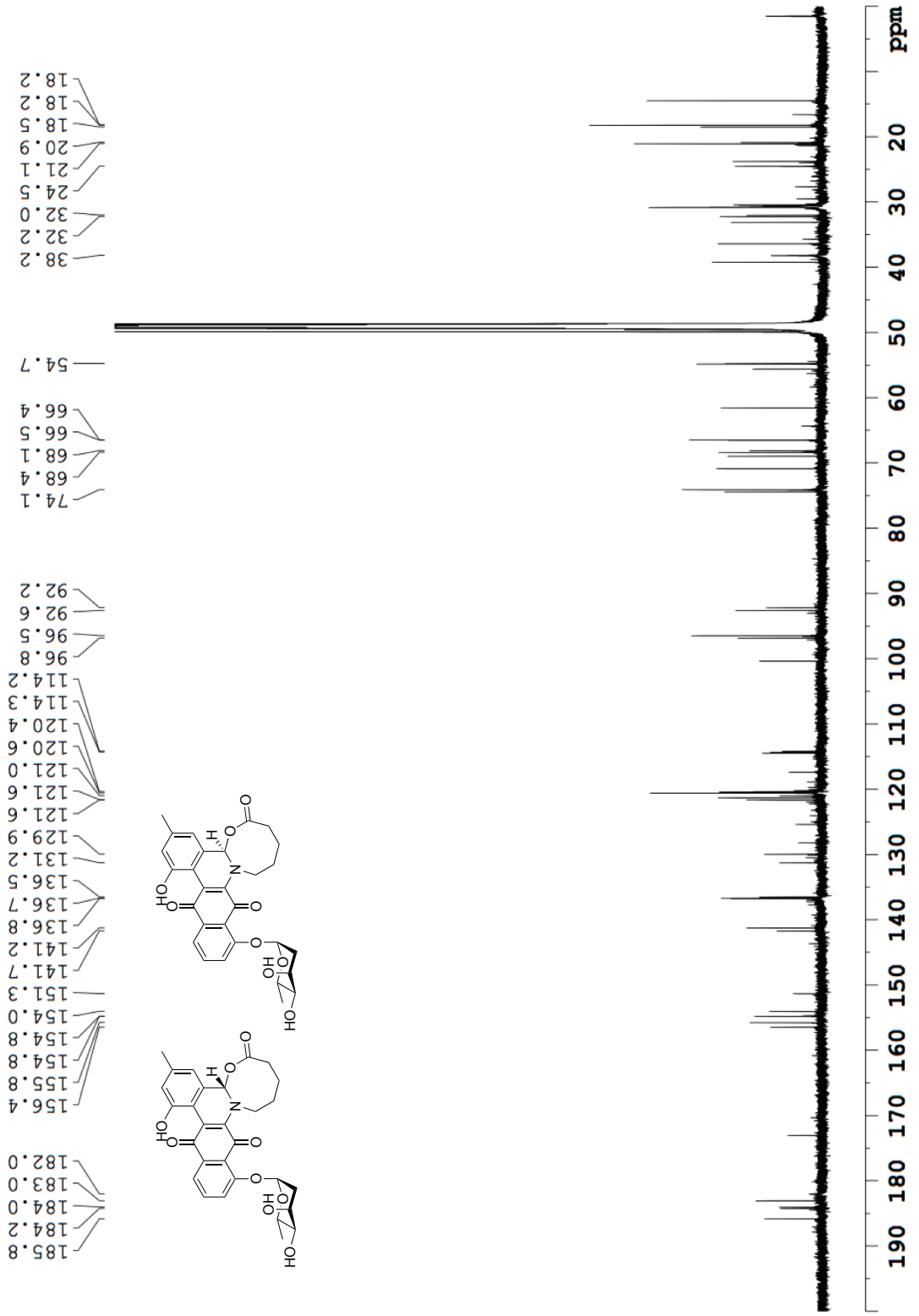


Figure S33. ^{13}C-NMR spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD (^{13}C: 176 MHz).

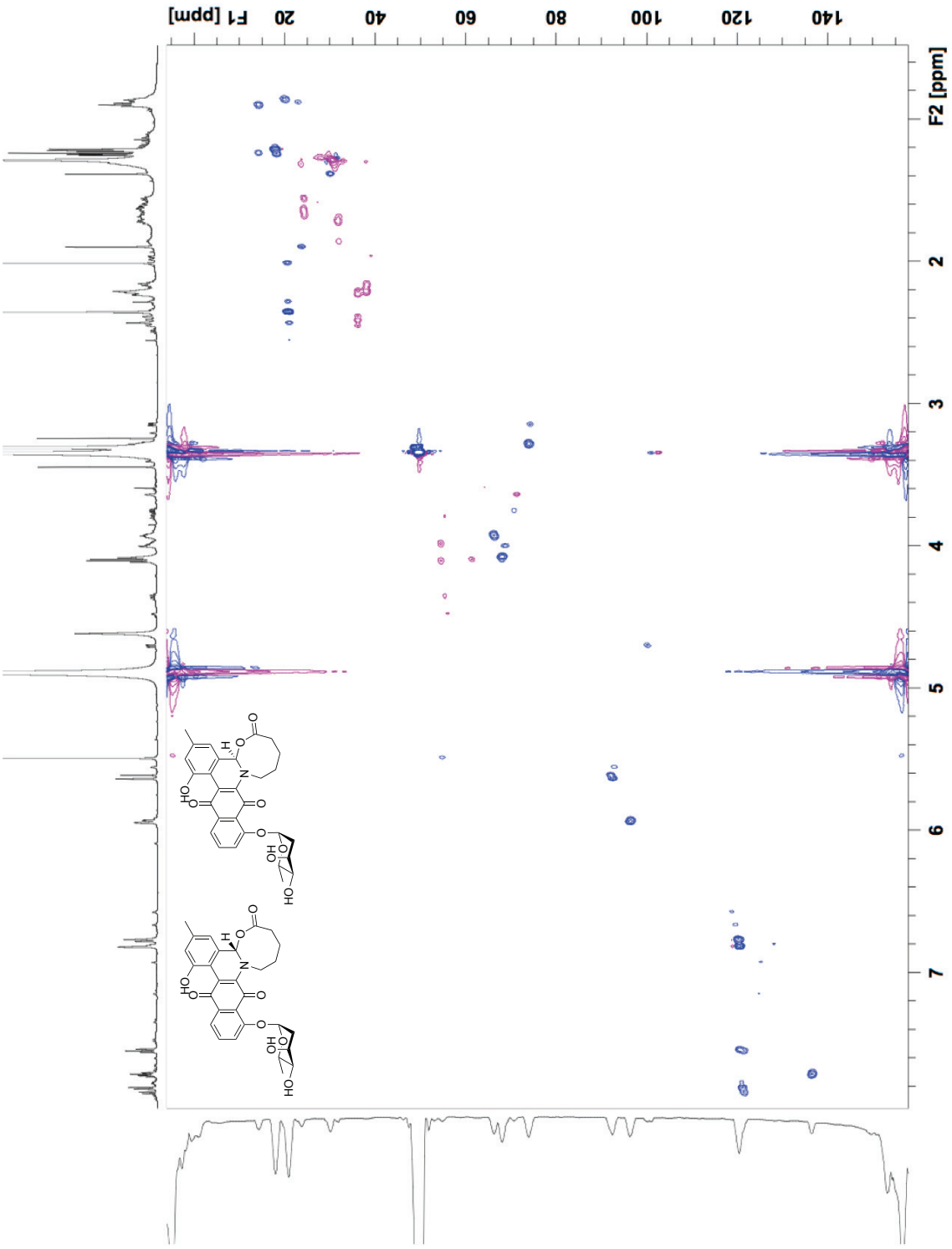


Figure S34. Edited-HSQC (^{1}H-^{13}C) spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD.

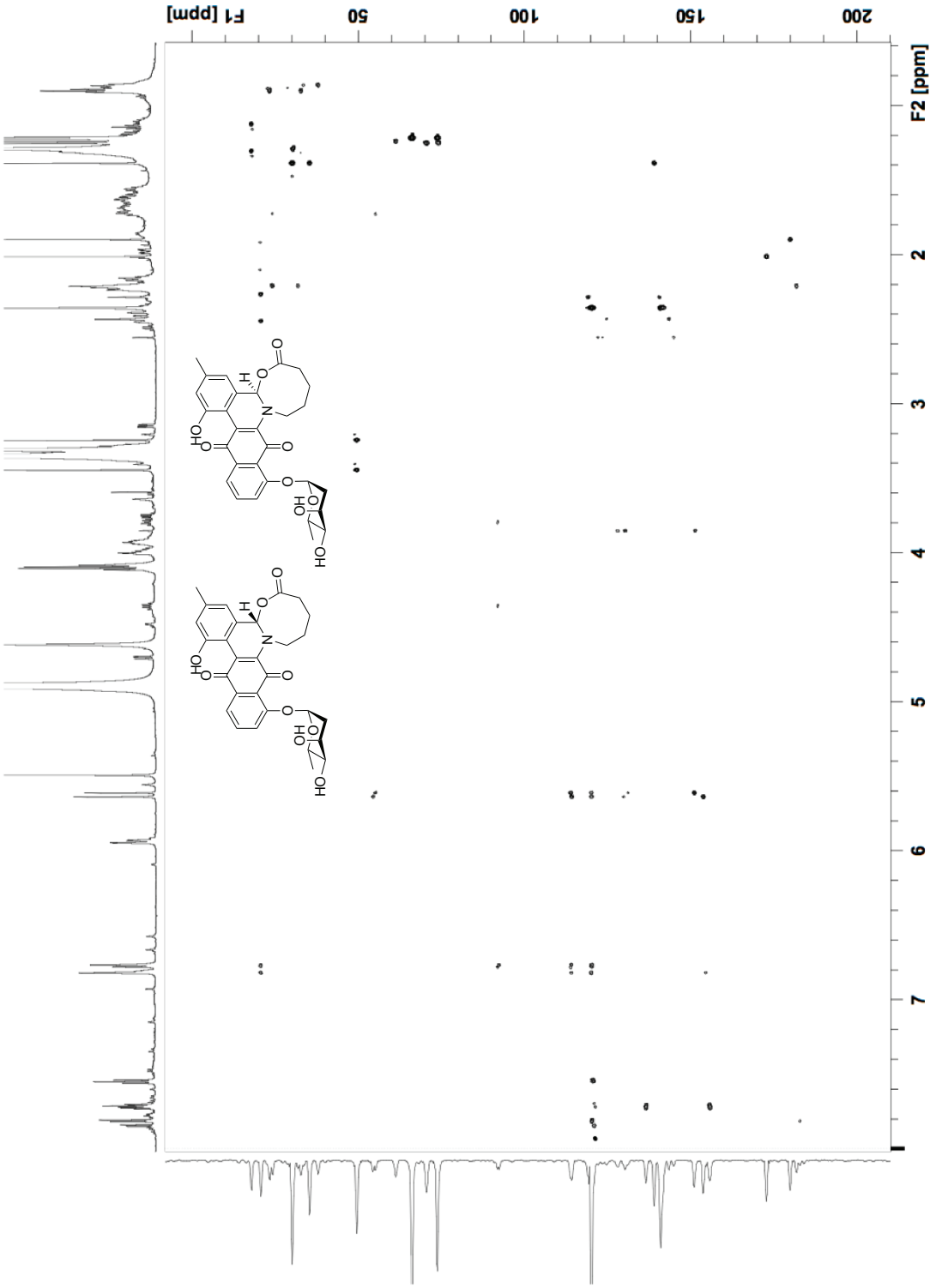


Figure S35. ^{1}H-^{13}C HMBC (6.5 μs mixing time) spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD.

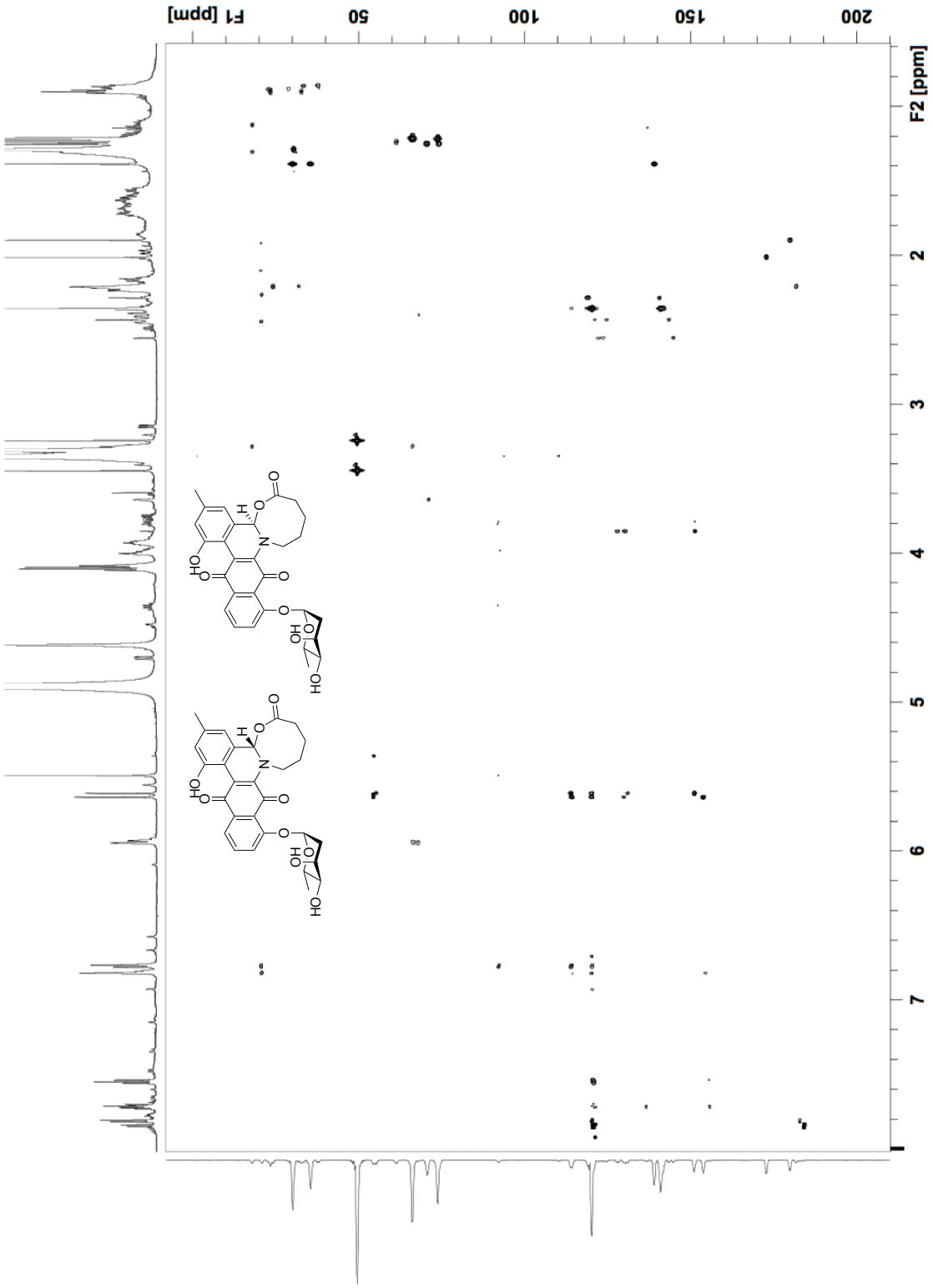


Figure S36. ^{1}H-^{13}C HMBC (25 μs mixing time) spectrum of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD.

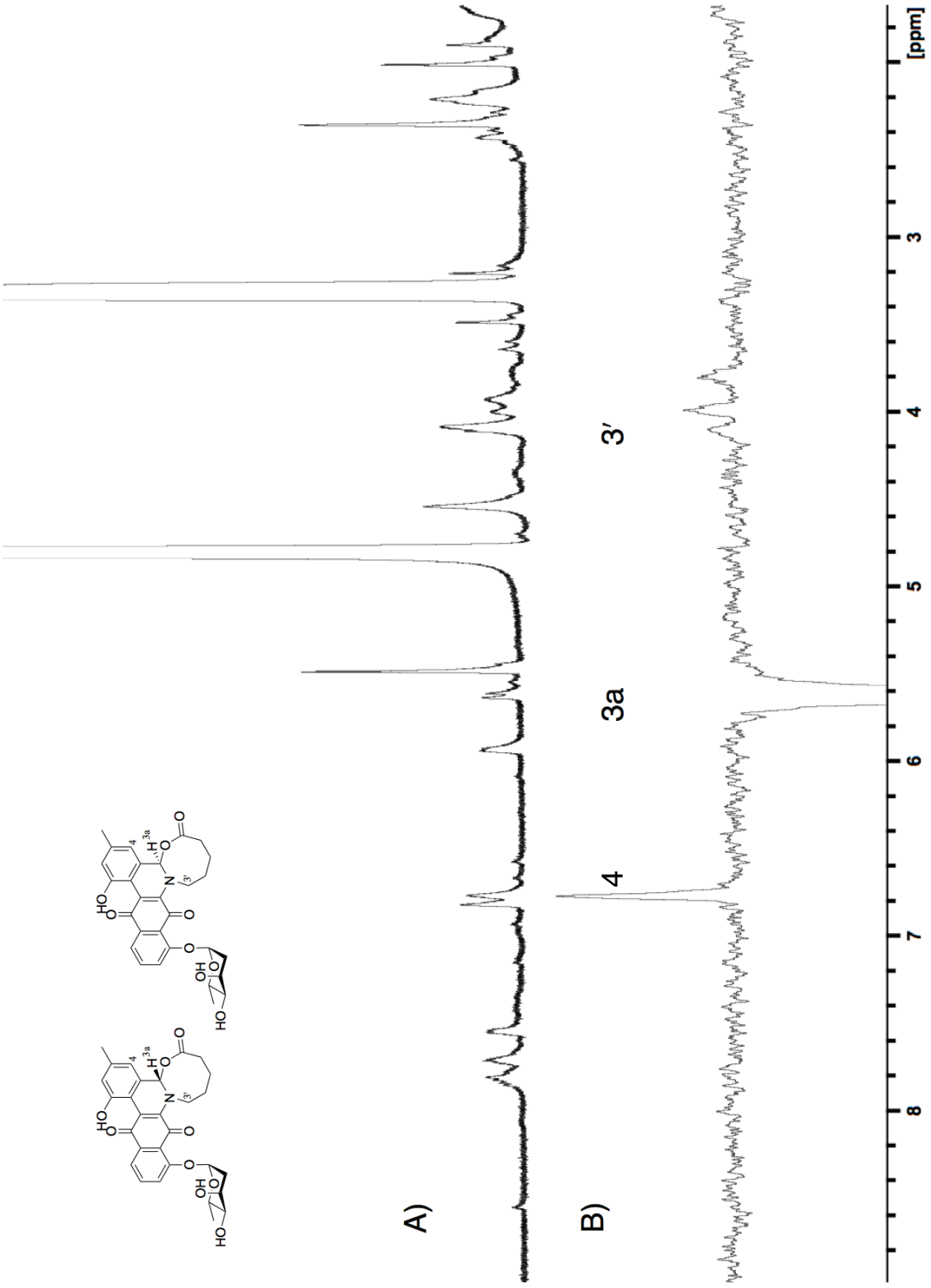


Figure S37. (A) ^{1}H-NMR spectrum and (B) NOESY NMR irradiating 3aH of jadomycin 5-aminovaleric acid (diastereomeric mixture) in CD_3OD (^{1}H: 500 MHz).

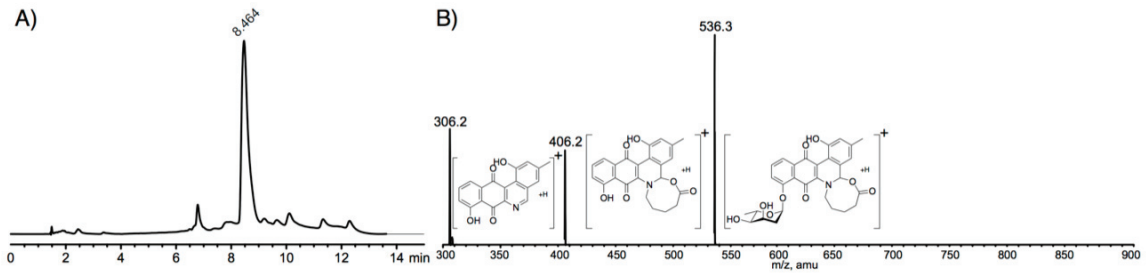


Figure S38. (A) HPLC trace and (B) mass spectrum of jadomycin 5-aminovaleric acid. The mass spectrum shows the parent compound, the loss of the L-digitoxose, and phenanthroviridin.

APPENDIX III: K_d DETERMINATION DATA FOR CHAPTER 4

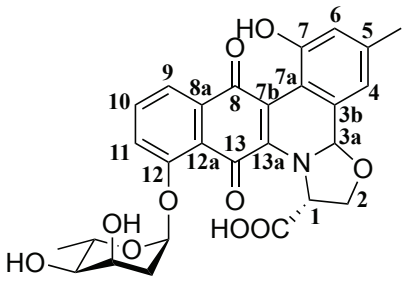


Figure S39. Fully labeled structure of jadomycin DS.

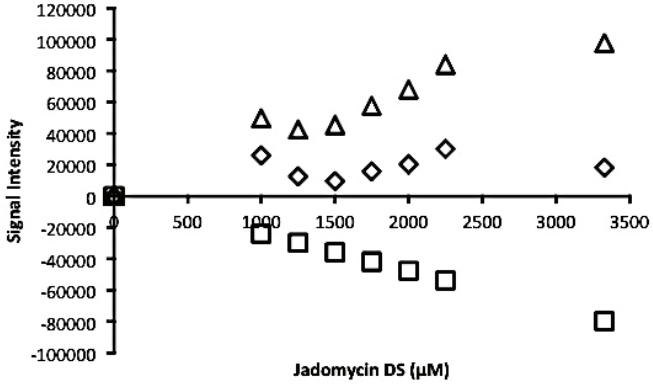


Figure S40. Compiled WaterLOGSY NMR K_d determination data compiled for 3aH showing the observed binding curve (◇), the negative standard curve (□), and the corrected values (Δ).

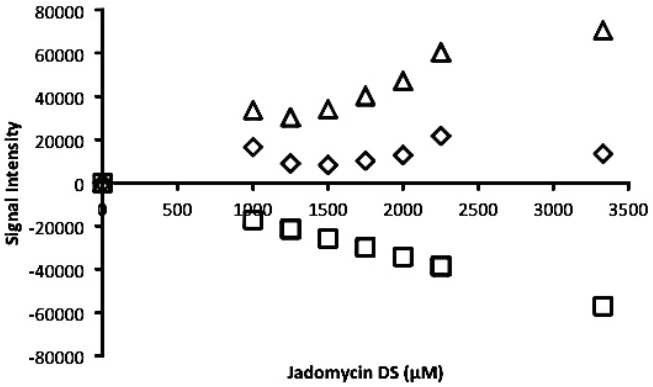


Figure S41. Compiled WaterLOGSY NMR K_d determination data compiled for 9H showing the observed binding curve (◇), the negative standard curve (□), and the corrected values (Δ).

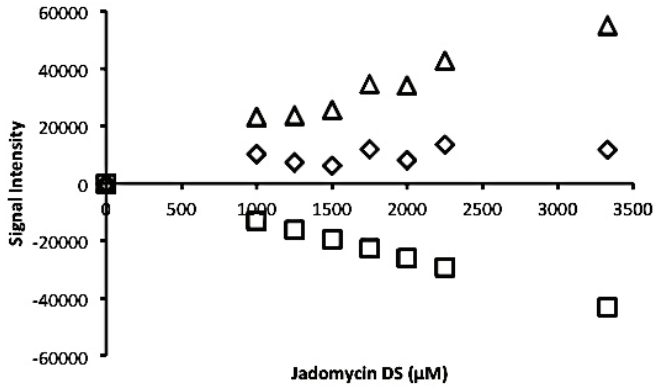


Figure S42. Compiled WaterLOGSY NMR K_d determination data compiled for 10H showing the observed binding curve (◊), the negative standard curve (□), and the corrected values (Δ).

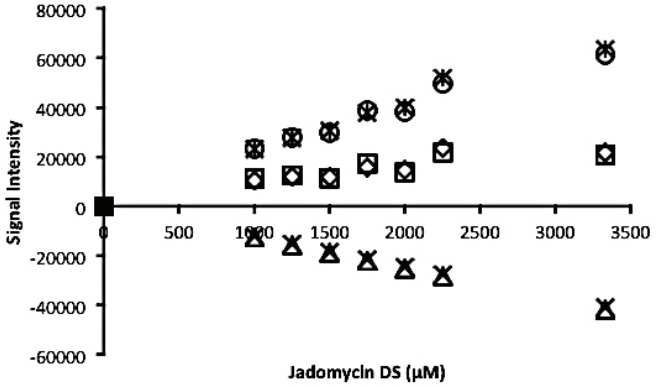


Figure S43. Compiled WaterLOGSY NMR K_d determination data compiled for 11H showing the observed binding curve for the left signal (◊) and right signal (□), the negative standard curve for the left signal (Δ) and the right signal (×), and the corrected values for the left signal (⁕) and the right signal (**O**).

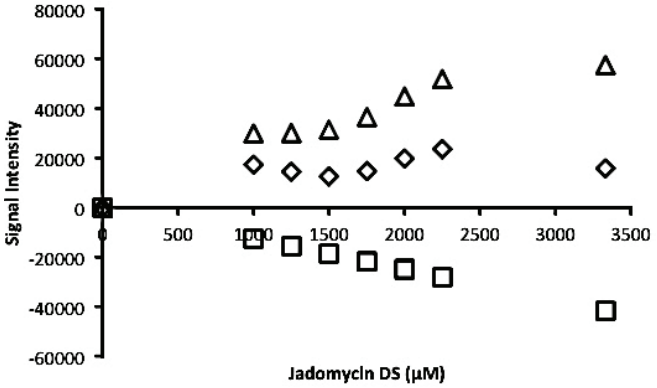


Figure S44. Compiled WaterLOGSY NMR K_d determination data compiled for 1″H showing the observed binding curve (◊), the negative standard curve (□), and the corrected values (Δ).

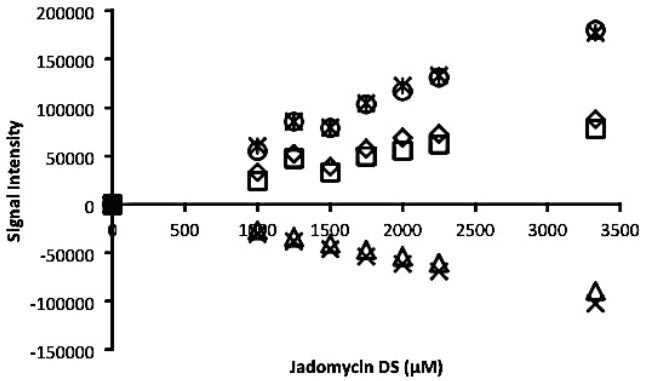


Figure S45. Compiled WaterLOGSY NMR K_d determination data compiled for 5″-CH_3 showing the observed binding curve for the left signal (◊) and right signal (□), the negative standard curve for the left signal (Δ) and the right signal (×), and the corrected values for the left signal (∗) and the right signal (O).

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Publication: Journal of the American Chemical Society

Publisher: American Chemical Society

Date: Feb 1, 2015

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