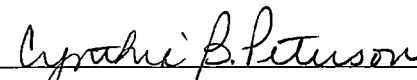
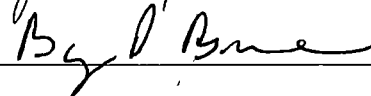


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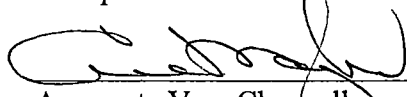
I am submitting herewith a thesis written by Drew Ekman entitled "Isolation and Substrate Binding Studies of Aminoglycoside Nucleotidyltransferase 2"-Ia " I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biochemistry and Cellular and Molecular Biology


Engin H. Serpersu Ph D, Major Professor

We have read this thesis and
recommend its acceptance

Accepted for the Council


Associate Vice Chancellor and
Dean of the Graduate School

ISOLATION AND SUBSTRATE BINDING STUDIES
OF AMINOGLYCOSIDE
NUCLEOTIDYLTRANSFERASE 2"-Ia

A Thesis Presented for the
Master of Science Degree
The University of Tennessee, Knoxville

Drew Robert Ekman
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ABSTRACT

Aminoglycoside nucleotidyltransferase (2'')-Ia (ANT) was cloned from *P. Aeruginosa* and purified from overexpressing *E. coli* BL21(DE3) cells. The high yields obtainable from this system will permit in-depth structural studies in the future. In addition, the conformation of the aminoglycoside antibiotic isepamicin, a pseudo-tri-saccharide, bound to aminoglycoside nucleotidyltransferase 2''-Ia has been determined using NMR spectroscopy. Molecular modeling employing experimentally determined inter-proton distances resulted in two different enzyme-bound conformations (conformer 1 and conformer 2) of isepamicin when comparing the structures using the 2-deoxystreptamine ring (B ring) and the D-garosamine ring (C ring). Conformer 1 was by far the major conformer defined by the average glycosidic dihedral angles $\phi_{BC} -65.26 \pm 1.63$ and $\psi_{BC} -54.76 \pm 4.64$ (20 structures). Conformer 1 was further subdivided into one major (conformer 1a) and two minor components (conformer 1b and 1c) based on the comparison of ϕ_{AB} and ψ_{AB} . Conformer 1a and conformer 2 have almost identical ϕ_{AB} and ψ_{AB} values. In addition, further comparisons with the A and B rings were made with the conformations of isepamicin bound to both a phosphotransferase (APH (3')-IIIa) and an acetyltransferase (AAC (6')-II) again.

resulting in striking similarities. It has been suggested that aminoglycosides use both electrostatic interactions as well as hydrogen bonds in binding to these enzymes and that the contacts made by the A and B rings to active site residues are the most crucial (Serpensu et. al , 2000). The similarities in isepamicin bound to three different aminoglycoside modifying enzymes lends further support to this theory. This information should aid in the design of effective inhibitors possessing a broad range of aminoglycoside modifying enzymes as targets.

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Chapter I

Introduction

A. Antibiotic Resistance

One of the greatest discoveries of the modern world was the finding that biological compounds exist which can be used to halt or prevent bacterial growth. This was of course first observed by Sir Alexander Fleming in 1928 when he noticed the amazing ability of a mold (*Penicillium notatum*) to restrict the growth of *Staphylococci*. Thereafter, penicillin came into clinical use and the world witnessed the life-saving power of antibiotics. Since 1928 a myriad of other compounds with antimicrobial properties have been discovered and developed. The presence of this vast array of antibiotics has made many diseases (e.g. tuberculosis, dysentery, and cholera) that were once fatal now routinely curable. In fact their success was so great that U.S. Surgeon General William Stewart declared before Congress in 1969 that we could “close the book on infectious disease.”

However, the security that these drugs provided was not to last long. As

early as the 1940s, resistance mechanisms were beginning to be identified that threatened the effectiveness of these life-saving compounds (Abraham and Chain, 1940). In the 60's and 70's resistance was widespread such that many pharmaceutical companies focused their efforts toward these resistant strains. Antibiotic research has continued up to the present time resulting in an enormous variety of these compounds. However, despite the presence of these drugs, patients with untreatable infections still suffer due to resistant pathogens. This is not a problem that will be solved in the foreseeable future. Circumventing deleterious factors in the environment to ensure survival is a natural evolutionary response inherent to the design of life. Thus, the presence of antimicrobials will always produce resistance. To make matters worse, once a strain has become resistant it can often transfer this ability to others, giving them the resistant phenotype. Furthermore, when bacteria acquire a gene for resistance it is usually retained even in the absence of selective pressure (Levy, 1995). Over time this can produce microbes which are resistant to many different drugs simultaneously.

Three major mechanisms have been discovered that allow bacteria to elude the harmful effects of antibiotics. The first, inhibition of entry, can be achieved by either preventing passive diffusion through the outer-cell membrane (Nikaido, 1988), stopping active transport processes that aid entry (Hayes, 1990), or by forcing the drug back out of the cell via an efflux pump (Chopra, 1992). The second mechanism involves alteration of the target for

the drug. By preventing the antibiotic from binding effectively to its intended target, an organism can tolerate high levels of the drug (Hayes and Wolf, 1990 and Jacoby and Archer, 1991) Many different targets can be altered by the bacteria to evade the drugs Some of the alterations reported include. amino acid changes in enzyme targets (Wolfson and Hooper, 1989), as well as changes in cell wall proteins (Shlaes et. al., 1989), ribosomal RNA (Benveniste and Davies, 1973), and penicillin-binding proteins (Chambers, 1988 and Georgopapadakou, 1993) The third, and most common mechanism involves the use of antibiotic inactivating enzymes These enzymes chemically alter the antibiotic such that its intended activity is lost (Bush, 1989) The β -lactams, chloramphenicol, and the aminoglycosides are particularly susceptible to this mechanism The two types of alteration known are breakage of essential bonds and covalent modification of specific sites on the molecule In the case of the β -lactam antibiotics, enzymes called β -lactamases exist which hydrolyze the lactam ring This action destroys the properties that are necessary for the drug to affect its target. Many different types of these enzymes exist such as penicillinases, cephalosporinases and others including broad spectrum β -lactamases which are able to hydrolyze a variety of β -lactam antibiotics More significant to this work are the aminoglycoside inactivating enzymes or the aminoglycoside modifying enzymes These enzymes covalently attach a specific functional group (e g phosphate, acetyl, or a nucleotide monophosphate) to specific sites on the molecule thus rendering it inoffensive

to the cell. There are three major types of these enzymes known: the phosphotransferases, the acetyl transferases, and the nucleotidyl transferases (Davis, 1986). These enzymes are responsible for the presence of widespread resistance to this important class of antibiotics.

B. The Aminoglycosides

Primarily produced by the actinomycetes, the aminoglycoside antibiotics are a diverse class of water-soluble, cationic molecules possessing a broad spectrum of antimicrobial activity. They can be structurally classified as those which have a 2-deoxystreptamine ring and those which do not. Among those which possess this structural feature, there are two patterns of substitution: at the four and the five positions (4,5-disubstituted) of this ring or at the four and the six positions (4,6-disubstituted). The glycosidic ring attached to the four position is designated the A or prime (') ring while that which is attached at either the five or six position is referred to as the C or double prime (") ring (**figure 1**¹). These compounds typically are positively charged at physiological pH due to the pKa values (~ 7-9) of their amine groups, and it appears as

¹All figures can be found in appendix I on page 50

though these charges are important for binding of the aminoglycoside to its target. Unfortunately for the design of novel aminoglycosides however, the charged groups that are necessary for binding to the ribosome are also used for binding by the resistance enzymes (Gates and Northrop, 1988).

The ribosome is the primary target of the aminoglycosides. This has been discovered by the presence of resistance resulting from the introduction of point mutations into both ribosomal RNA and ribosomal proteins. It is known that the majority of aminoglycosides bind to the 16S rRNA of the 30S subunit where the tRNA first enters the ribosome (i.e. the A-site). While the processes leading up to cell death are not exactly known, tempting theories exist to explain the observations that have been made. It is thought that after diffusing through the outer membrane and periplasmic space in Gram-negative organisms, and the cell wall assembly in Gram-positives, the antibiotic binds to the cell membrane via electrostatic interactions. The positively charged aminoglycoside then is transported through the cell membrane by an unidentified membrane protein (possibly a porin). Upon entering the cell the drug then binds to the ribosome causing mistranslation of cellular proteins. These aberrant proteins are then thought to target the cell membrane (Davis et al, 1986) where they cause its degradation producing pores through which additional drug molecules may enter the cell. Once a large enough concentration of aminoglycoside is present in the cell, the extent of protein translation interruption eventually causes death (Davis, 1987). A better

understanding of the bactericidal mechanism may lead to the discovery of novel targets or to ways in which to modify the existing molecules to enable them to be more effective

As mentioned above, resistance can occur through mutations in the targets for the aminoglycosides. However, the most common, and more importantly the most clinically relevant mechanism by which resistance is conferred is through the use of aminoglycoside modifying enzymes which are widespread in the environment and diverse in their spectra of activity.

C. The Aminoglycoside Modifying Enzymes

The aminoglycoside modifying enzymes are a large family of enzymes. There have been at least 50 different enzymes identified and most of these enzymes individually have the ability to confer resistance to a wide range of aminoglycosides. These enzymes are often obtained through transfer of an R-plasmid although the genes can exist within the genome as well. The enzymes confer resistance by covalently modifying the antibiotic molecule, thus rendering it inoffensive to the cell. Covalent modification can be achieved in three ways: via phosphate transfer, acetyl transfer, or nucleotide transfer.

The established nomenclature (Shaw et al., 1993) uses a three letter

designation for each type of enzyme ANT for the nucleotidyltransferases, AAC for the acetyltransferases, and APH for the phosphotransferases The specific acronym is then followed by a number in parentheses which identifies the specific site of modification on the antibiotic molecule Beyond this number is a roman numeral which describes the enzyme's resistance profile and a lower case letter identifying the specific gene encoding the enzyme. For example, the nomenclature for the enzyme studied in this work is ANT (2'')-Ia Thus it can be inferred that this enzyme belongs to the class of nucleotidyltransferases and that it modifies the antibiotic at the 2'' position (the number two position on the C ring) It shares the same resistance profile of other enzymes that have a roman numeral one in their nomenclature and it is a specific gene among the other members of its class

It is thought that several different sources are responsible for the presence of aminoglycoside resistance genes. One source is the organisms which produce the aminoglycosides (e g the actinomycetes) (Benveniste and Davies, 1973) There are sequence relationships as well as biochemical evidence in support of this theory (Davies, 1997) Another source appears to be mutation or overexpression of normal cellular genes. For example, phylogenetic relationships between the phosphotransferases and cellular protein kinases have been observed (Piepersberg, 1988)

The aminoglycoside modifying enzymes are kinetically characterized as having K_m values in the low to sub-micromolar level and the observed k_{cat}

values tend to vary between 0.1-100 s⁻¹. These two factors result in highly efficient enzymes showing specificity constants (k_{cat}/K_m) of 10⁶-10⁸ M⁻¹ s⁻¹ (Mckay et al., 1994). Interestingly, kinetic studies have shown that the minimum inhibitory concentration is positively correlated with the V_{max} to K_m ratio (Davies and Smith, 1978). Thus, it appears as though these enzymes are designed to inactivate low concentrations of aminoglycosides at optimal rates (Davies and Wright, 1997).

The need for structural information about these enzymes is crucial to better understanding the enzymatic mechanisms of modification. So far the crystal structures of a nucleotidyl transferase ANT (4',4'')-Ia have been solved (Sakon et al., 1993, Penderson et al., 1995). The three-dimensional structure of ANT (4',4'')-Ia was solved with the aminoglycoside substrate and a nucleotide triphosphate analog. Interestingly, the electron density map of the antibiotic (kanamycin) is unclear. This would help to explain the variety of structurally different aminoglycosides that this enzyme can modify. As for the nucleotide triphosphate analog, the majority of interactions with the protein were seen for the phosphate groups and not for the nitrogenous base. This might explain the lack of discrimination that has been observed for the nucleotide triphosphate substrates that ANT (4',4'')-Ia can utilize (the ability to utilize a variety of nucleotide triphosphates has also been observed for ANT (2'')-Ia). The crystal structure of a phosphotransferase, APH (3')-IIIa, has also been solved (Hon et al., 1997). In support of Piepersberg's phylogenetic

evidence for similarity of the phosphotransferases to eukaryotic protein kinases (Piepersberg, 1988), the three-dimensional crystal structure reveals significant structural similarity between the two types of enzymes (Hon et al , 1997). It is hoped that through advances in three-dimensional NMR and X-ray crystallography that more structural information will be obtained for the aminoglycoside modifying enzymes so that an in-depth understanding of their binding modes and catalytic mechanisms can be established and used in combating their effects.

D. Aminoglycoside Nucleotidyltransferase 2"-Ia

Formerly known as gentamicin adenyltransferase, aminoglycoside nucleotidyltransferase 2"-Ia (i.e. ANT) is recognized as being among the most frequently found aminoglycoside modifying enzymes in the *Enterobacteriaceae* (Miller et al , 1997) In addition, Shimizu et al in 1985 determined it to be the predominant aminoglycoside antibiotic resistance mechanism among nosocomial pathogens in the United States. It possesses a diverse resistance profile enabling the pathogens expressing it to evade a variety of

aminoglycosides (**table 1**²) and can utilize a variety of ribonucleotide cofactors including ATP, GTP, CTP, UTP, and TTP, and the deoxyribonucleotide cofactors dGTP, dATP, and dCTP (Gates and Northrop, 1988). ANT (2'')-Ia is also thought to be located in the periplasmic space where it can intercept and inactivate any aminoglycosides that attempt to enter the cell

Gates and Northrop have determined that ANT uses a Theorell-Chance kinetic mechanism where the magnesium chelate of a nucleotide triphosphate binds first followed by aminoglycoside binding. Catalysis then occurs followed by the release of magnesium pyrophosphate and finally the nucleotidylated aminoglycoside (**figure 2**). It has been shown that release of modified aminoglycoside is the rate-limiting step, as opposed to the actual catalytic event itself. It has also been found that binding of a second aminoglycoside to the active site can cause inhibition by preventing release of the modified antibiotic (Gates and Northrop, 1988). K_m values range from the low millimolar to micromolar range for the aminoglycosides. Similar values have been observed for the nucleotide triphosphates. ANT is known to modify the aminoglycosides exclusively at the 2'' hydroxyl group and this modification is presumed to occur via nucleophilic attack on the alpha phosphorous of the magnesium-nucleotide triphosphate by the aminoglycoside 2'' hydroxyl

Although important in studying the problem of resistance to the

²all tables can be found in appendix II on page 64

aminoglycosides, problems in the purification and stabilization of this enzyme have prevented its in-depth study. This is due in part to the lack of proper expression systems but also to the poor solubility properties that ANT possesses. It has been found extremely unstable in past studies (Bongaerts and Molendijk, 1983) and purity above 82% has not been achievable (Van Pelt et al , 1983) This work describes both the high-level expression of ANT and its purification to over 95%. These accomplishments now allow in-depth structure and function studies to be performed with this enzyme that will provide detailed information concerning this specific mechanism of resistance

E. Rational Drug Design

The method of drug discovery used prior to the 20th century was one entirely based upon "trial and error" Many of the drugs in use were of plant origin and knowledge of their beneficial effects existed solely as a result of the successes of past generations. However, this approach to drug discovery changed in the early to mid 1900's when scientists such as Paul Ehrlich (a medicinal chemist responsible for the synthesis of salvarsan, the first chemical treatment for syphilis) deliberately synthesized, in the laboratory, a specific drug compound The discovery of antibiotics followed with the finding that

microbes could produce useful therapeutics as well. As the effectiveness of the earlier antibiotics began to wane in the 60's and 70's due to resistance, chemists sought to give them renewed potency by modifying their chemical structures. This effort led to the existence of the "semisynthetic antibiotics" (e.g. amikacin, isepamicin, and netilmicin) which are still in use today.

Drug design evolved slowly from then on until the emergence of biotechnology in the last half of the 20th century which greatly increased the sophistication of drug development by allowing greater amounts of drug targets (e.g. enzymes, receptors, etc.) to be obtained for study. This, in addition to greater efforts in chemical synthesis and screening by pharmaceutical companies, increased the intensity of the drug discovery process. These developments have led to greater interest (and success) in the rational design of drugs (Smith, 1992)

Advances in NMR and X-ray crystallography now give high resolution structural information making rational drug design infinitely more feasible than before. In combination with computational tools this structural information can be used to dock ligands into receptors and potential inhibitors into enzyme active sites. However, the main impediment to widespread successful rational drug design is in fact the lack of detailed structural information of intended targets and the inadequacy of computational models (although intense interest in the subject is constantly improving the situation)

This thesis presents the continuation of an attempt at utilizing both

structural data (via NMR spectroscopy) and computational tools to rationally design enzyme inhibitors. By elucidating the similarities in the same aminoglycoside (isebamycin) binding to the active sites of these enzymes and then searching molecular databases for compounds possessing the required properties, potential inhibitors should emerge for use against these resistance enzymes. Reported here is the conformation of isepamicin bound to the active site of a nucleotidyltransferase (ANT (2'')-Ia) and comparisons of this conformation of the same molecule bound to the other two types of modifying enzymes.

Chapter II

Expression and Purification of ANT (2'')-Ia

A. Introduction

The gene for ANT (2'')-Ia was cloned from a *Psuedomonas aeruginosa* clinical isolate using the polymerase chain reaction. It was then inserted into the pET-22b(+) expression vector designed for use in genetically engineered *E. coli*. This provided excellent yields of recombinant ANT (2'')-Ia. The gene was cloned in two fashions: a full-length construct and a truncated construct which uses an identified second start-site (this work was done by Enrico L. Digiammarino, a previous lab member). The latter construct proved to be more stable and was thus used in all of the experiments.

One of the reasons for attempting the overexpression and purification of ANT (2'')-Ia is its relevance to human health. As mentioned in the previous chapter, these enzymes are dispersed throughout the environment and are frequently seen in hospital settings. However, certain enzymes are seen with greater frequency and are thus more of a threat. One in particular, ANT (2'')-Ia,

is widespread among all gram-negative bacteria and is responsible for the aminoglycoside resistance most common in the United States (Shaw et al , 1993, Shimizu et al., 1985). These facts being known, it seemed appropriate to pursue one of the enzymes most likely to cause harm.

The other reason we sought to study this enzyme was in the interest of rational drug design. It is our hope that by finding effective inhibitors of these enzymes we will be able to re-establish the potency of the aminoglycoside antibiotics and thus re-introduce a class of antibiotics that are becoming obsolete due to bacterial ingenuity and human misuse. Having already determined the conformations of an aminoglycoside (isepamicin) bound to the other two types of modifying enzymes, (a phosphotransferase and an acetyltransferase) (Cox et al , 1997 and Digiammarino et al., 1998), the next step was to determine the aminoglycoside's conformation when bound to the third type of modifying enzyme, a nucleotidyltransferase. Using this strategy it is hoped that similarities will be observed in the binding of the antibiotic that can be exploited to create an effective inhibitor to all three types of modifying enzymes.

B. Experimental Procedures

1. Materials

E. coli strain BL21 (DE3) and plasmid pET 22b(+) were obtained from Novagen

Materials for growing and expressing strain BL21 (DE3)

Yeast extract, tryptone, and bacto-agar were obtained from Difco
Sodium chloride and IPTG (isopropyl β -D-thio-galactopyranosyl)
were obtained from Sigma

Recovery and purification of ANT (2'')-la

Glycerol, ethylene glycol, DTT, urea, KCl, magnesium acetate,
EDTA, hepes, and Triton X-100 were obtained from Sigma
Ultrafiltration units using polyethersulfone membranes were
obtained from Pall-Gelman

Assay for enzymatic activity

Phospho-molybdate was obtained from Avocado Research Chemicals Ltd. Sulfuric acid was obtained from Aldrich Chemical Company Inc. KCl, Pyrophosphatase, Trizma base and ascorbic acid were obtained from Sigma. Samples were assayed spectrophotometrically on a Beckman DU-70 spectrophotometer.

Purification of kanamycin-AMP

ATP, KCl, DTT, hepes, magnesium acetate sephadex 25A, sepharose G10, potassium acetate, and TEAB were obtained from Sigma.

2. *Expression of ANT (2'')-Ia*

ANT (2'')-Ia was expressed as prescribed by the manufacturer of the pET expression system (Novagen) except that the length of time used for induction was changed to eight hours.

3. *Washing of ANT (2'')-Ia inclusion bodies*

After lysis via french press and centrifugation (30,000 g) the supernatant was removed and the remaining pellet consisting of ANT (2'')-Ia and cell debris was washed three times in a solution of Tris-HCl pH 8.0, EDTA, and Triton X-100. The Triton was then washed away with the same solution lacking in Triton.

4. *Resolubilization and Reactivation of ANT (2'')-Ia*

One hundred milligrams of ANT (2'')-Ia inclusion bodies were resolubilized using one milliliter of 8M urea followed by extensive vortexing and incubation at 37°C for 30 minutes. After the incubation time, the sample was centrifuged for 10 minutes at maximum speed (13,500 g) and the supernatant was drawn off. This process was repeated two more times so that all insoluble material was removed. Subsequently, the resolubilized enzyme was recovered/reactivated in a solution containing 10 mM DTT, 0.125 mM EDTA, 10 mM HEPES, 1 mM Magnesium Acetate, 50 mM KCl, 1% glycerol, and 0.5 % ethylene glycol at pH 8.0. The enzyme was always recovered such that the final concentration of protein was no more than 0.25 mg/mL. In addition, the enzyme in urea was always added slowly to the recovery medium with gentle stirring at room temperature. Immediately upon recovery the enzyme was concentrated

using ultrafiltration and stored at 4° C. A typical preparation would provide approximately eight or nine milligrams of enzyme.

5. Assay for Enzymatic Activity of ANT (2'')-la

The activity of ANT (2'')-la was assessed using a colorimetric phosphate assay (Chen et al., 1956). The enzyme was first incubated for 30 minutes at 37°C in a solution containing 2.5 mM ATP, 1mM MgCl₂, 10mM DTT, 2 mM Tris-HCl pH 8.5, 50 mM KCl, and 2.5 units of pyrophosphatase brought to a final volume of 400 µL with deionized water. After the incubation period, an equal volume (400 µL) of 8% HClO₄ was added to the solution followed by centrifugation at 5000 rpm. Then 300 µL of this solution was added to 700 µL of a solution containing sulfuric acid, ammonium molybdate and 10% ascorbic acid. The resulting solution was then incubated at 45° C for 20 minutes and its absorbance was read at 820 nm.

6. Purification of kanamycin-AMP

Five milligrams of purified enzyme was incubated with 10 mM ATP, 100 µM kanamycin A, 4 mM MgCl₂, 50 mM Tris-HCl (pH 8.0), and 1 mM DTT at 37° C.

overnight. An additional two milligrams of enzyme was added the following morning to replace any that might have become inactive and the reaction was incubated for another four hours. After the incubation period, the enzyme was precipitated using 4% HClO_4 and then put on ice. Any KCl that precipitated was pelleted and then the sample was concentrated down to three milliliters. Half of this was then applied to a Sepharose G-10 column equilibrated with 5 mM potassium acetate at pH 6.0. The first 30 milliliters were discarded before beginning fraction collection. Each fraction was analyzed for the presence of nucleotide by measuring its absorbance at 260 nm. Those fractions showing an absorbance were then applied to a Sephadex 25A ion-exchange column equilibrated with a 50 mM TEAB (triethyl ammonium bicarbonate) buffer at pH 7.8. A linear gradient was run using 150 mM TEAB at pH 7.8 as the final concentration. Fractions were collected and analyzed again for the presence of nucleotide. Two distinct peaks were observed in the elution profile. Fractions within each peak were analyzed using 1D ^1H -NMR and those showing aminoglycoside resonances were combined and concentrated.

C. Results and Discussion

Utilization of bacterial strains that were genetically engineered to produce high expression levels of recombinant proteins allowed for large quantities of ANT (2'')-1a to be obtained. However, the majority of the enzyme was expressed in an insoluble form so that a specific strategy was required to bring the protein into solution and return it to an active state. It was found that a high concentration of denaturant was able to solubilize the enzyme. Once the enzyme was made soluble, conditions for refolding and reactivating it were developed through rigorous experimentation. Each reagent in the recovery solution was found to serve specific purpose. For example, if ethylene glycol is missing from the recovery solution the protein is stable in solution but shows no activity. Without glycerol present the enzyme can't be kept in solution. The presence of both a monovalent salt (KCl) and a reducing agent aided in increasing yields and maintaining solubility and activity. Interestingly, increasing the concentration of DTT ten fold doubled the amount of enzyme that could be recovered. With seven cysteines present in the primary sequence of ANT, the reducing agent probably prevents re-aggregation through intermolecular disulfide bonds. Other factors were also found to be important for stability. For instance, the enzyme could be stored in an active state for at least 30 days if kept at 4°C. Conversely, if stored at room temperature the enzyme lost activity.

within two or three days. The type of membrane used in ultrafiltration also had a drastic effect on stability. Using a polyethersulfone membrane as opposed to one made of cellulose produced almost ten times as much soluble enzyme. This may be due to the enzyme's affinity for carbohydrates which could have allowed cellulose to increase aggregation by immobilizing enzyme molecules in close proximity to one another.

In contrast to solubilization and recovery, the task of purifying ANT was simple. After lysis and centrifugation, the resulting pellet was washed with Triton X-100 so that much of the cell debris was removed. The denaturant also selectively solubilized ANT away from other insoluble matter so that after recovery the enzyme was over 95% pure (**figure 3**). This is a common advantage to the expression of proteins as inclusion bodies. The high expression levels exhibited by genetically engineered *E. coli* systems result in a crude pellet consisting of a high percentage of the target protein. In addition, much of the pellet will solubilize to a different extent upon exposure to denaturants and detergents. This gives one the advantage of a relatively easy purification scheme.

The activity of the resolubilized and purified enzyme was confirmed using a highly sensitive modified phosphate assay that produces an absorbance at 820 nm. Using this assay, the K_m for kanamycin was determined to be 200 μ M which is similar to that reported in an earlier study (Gates and Northrop, 1988). The measured k_{cat} of 1.45 s⁻¹ also agrees well with previously reported values.

Hence, this procedure provides pure and active enzyme with relative ease

Once the conditions necessary for recovery were determined, the point of modification by ANT (2'')-1a was confirmed using NMR spectroscopy. This was done to ensure that the stereochemistry was exclusive to the 2'' hydroxyl since originally the site of modification was determined using 3',4'-dideoxy kanamycin. In order to do this, the product (kan-AMP) had to be purified away from the unmodified substrates (ATP and kanamycin) using gel filtration chromatography and ion-exchange chromatography. The one dimensional spectrum for this purified product shows the presence of AMP and kanamycin A in equal concentrations suggesting a single modification point on kanamycin (**figure 4**) This is significant considering that in the original reaction ATP was in abundance to kanamycin by a factor of 100. This spectrum was excellent evidence for the presence of the purified kanamycin-AMP complex. Further proof was provided by a two dimensional COSY spectrum of the complex. Using this spectrum and the previously determined assignments of kanamycin (Cox and Serpersu, 1994), significant changes in chemical shift were observed for protons near to the site of modification (**table 2**). These changes would be expected as a result of the covalent attachment of an AMP moiety (**figure 5**). Due to the presence of a nearby phosphate group, the 2'' and 3'' protons exhibit a down field shift which is typical for protons exposed to electronegative atoms such as oxygen. It is interesting that a greater shift was observed for the 3'' proton than for the 2'' proton even though the site of modification is 2''. This is probably due to the

orientation of the phosphate group relative to the double prime ring. If the phosphate group is placed in such a way that the oxygens are in closer spatial proximity to the 3" proton than to the 2" proton, a more drastic change in chemical shift will result. This orientation seems quite possible for kanamycin-AMP modified at the two double prime position and thus gives further evidence for this site of modification. The chemical shift changes observed here are also consistent with earlier findings (Van Pelt et al., 1990)

Chapter III

ANT (2'')-Ia Bound Conformation of Isepamicin

A. Introduction

In the design of specific inhibitors to enzyme targets, knowledge of the three dimensional structure of the bound substrate is essential. Once obtained, this information can then be used to search databases in order to find other molecules exhibiting similar spatial conformations. A proven method for determining these conformations is through the use of NMR, specifically transfer nuclear overhauser effect spectroscopy or TRNOESY. This technique has gained in popularity over the past twenty years because of its many applications to interactions involving smaller molecules binding to much larger ones, such as antigens with antibodies, nucleotides with regulatory proteins, peptides with phospholipids, hormones with receptors, and as used in this work, substrates with enzymes (Campbell and Sykes, 1991). Specifically important for interactions which elude crystallization, the TRNOE experiment conveys cross-relaxation information obtained in the bound state to the free state where it can

be easily observed. The binding induced cross-relaxation enhancements experienced by the ligand are a result of the longer correlation times (τ_c) of the macromolecule with which it interacts. This correlation time is a measure of the average time taken for a molecule to rotate through one radian (Sanders and Hunter, 1992) and thus is dependent upon many factors including the molecular weight and the shape of the molecule as well as the viscosity and temperature of the solvent. Therefore, smaller molecules are generally characterized by shorter correlation times ($\tau_c < 10^{-10}$ sec) whereas larger molecules exhibit longer correlation times ($\tau_c > 10^{-8}$ sec) (Clare and Gronenborn, 1982). Once the ligand binds to the macromolecule, cross-relaxation between protons in close spatial proximity (i.e. 4-5 Å) occurs much more efficiently. The ligand is then released from the macromolecule and returns to the free state where it once again assumes a shorter correlation time. However, the "memory" of the bound state is retained and can be easily observed. The major requirement for this type of NMR experiment is that the ligand be in fast chemical exchange with the macromolecule and that this rate is significantly faster than the spin-lattice relaxation rate (R_1 or $1/T_1$) of the free state. In this way, ligands displaying the bound state geometries can accumulate in solution providing the concentration needed to obtain reasonable spectra. Once obtained these distances can then be used as restraints in molecular modeling computer programs to determine the enzyme-bound conformation of the ligand. Use of this technique is described for determining the conformation of isepamicin (an aminoglycoside antibiotic)

bound to ANT (2'')-Ia (described previously)

One problem encountered when analyzing TRNOESY spectra is the effects that nearby protein resonances can have on the observed ligand resonances. Two ligand protons (A and X) which are in reality further apart in space can be made to appear closer if an active site residue proton (P) is positioned between them. In this situation, longitudinal magnetization can migrate from the starting ligand proton (A), through the proton attached to the residue (P), and on to the target proton (X) on the ligand. This is referred to as spin diffusion. However, by inverting the spins of the ligand resonances in the middle of the mixing time (τ_m), the direct flow of magnetization between spins A and X is not perturbed but the indirect flow through P is reversed in sign and almost perfectly canceled at the end of the mixing time. In this way the complications that spin diffusion can produce in a spectrum can be minimized or eradicated. This technique is called QUIET-NOESY (Vincent et al., 1997) and has been employed here to validate the TRNOEs that were observed.

Isepamicin was chosen for this work for three reasons. First, its bound conformations to two other aminoglycoside modifying enzymes (APH (3')-IIIa and AAC (6')-II) have already been determined (Cox et al., 1997 and Digiammarino et al., 1998). This will allow us to look for similarities in binding to all three types of enzymes which can hopefully be exploited to produce potent inhibitors possessing wide ranges of activities. The second reason for choosing isepamicin is that for some reason this aminoglycoside is still effective in a

clinical setting. Discovery of why this might be will also aid in improved inhibitor design. The third and final reason for using this antibiotic is the technical feasibility of the TRNOESY experiments. As mentioned above, in order for the TRNOESY spectrum to be effective the ligand to be bound must exchange rapidly between the enzyme active site and the bulk solution. Isepamicin possesses this property and so it is an excellent candidate for this type of structural work.

B. Experimental Procedures

1. Materials

Isepamicin (sulfate salt) was a gift from Drs. George Miller and Karen Shaw (Schering Plough Research Institute). Calcium chloride and ATP were purchased from Sigma. D₂O was from Cambridge Isotope Laboratories and deuterated Tris (Tris-d₁₁), deuterium chloride, sodium deuterioxide, deuterated DTT, deuterated ethylene glycol, and deuterated glycerol were from Isotec, Inc.

2. Preparation of ANT (2'')-Ia and substrates for NMR experiments

ANT (2'')-Ia was concentrated using ultrafiltration and then protonated. Solute and water were replaced with deuterated versions through repeated dilutions and concentrations. Isepamicin, calcium chloride, and ATP were all dissolved in D₂O and then brought to a pH of 8.0 with either sodium deuterioxide or deuterium chloride. Samples for the TRNOESY experiments contained 2 mM isepamicin, 2 mM ATP, and 2 mM CaCl₂.

3. NMR spectroscopy

All experiments were performed on a 600 MHz Varian Inova spectrometer with a single gradient axis. A total of 228 FIDs of 704 complex data points were collected. The spectral width was 5479 Hz and 128 scans per FID were acquired. Mixing times of 30, 60, and 90 ms were used in TRNOESY experiments. The data were multiplied by a sine² window function in both dimensions before Fourier transformation. Similar parameters were used in the QUIET-TRNOESY experiments except for a Gaussian Cascade Q3 pulse used for the selective inversion of substrate protons (Vincent et al., 1996).

4. Molecular Modeling of ANT (2'')-la bound isepamicin

The proton assignments of ANT-bound isepamicin were determined based upon those reported previously for isepamicin in solution (Digiammarino et al, 1998). Using these assignments cross peaks were then identified and their volumes were measured at three different mixing times: 30 ms, 60 ms, and 90 ms. Based on its volume, each crosspeak was labeled either strong, medium or weak. These categories were each assigned specific distance limits based on the volume of the H1_A to H2_A crosspeak whose distance is already known to be 2.38 Å. The distance limits are as follows: strong (2.0-2.7 Å), medium (2.0-3.6 Å), and weak (2.0-4.5 Å) (**table 3**).

Isepamicin was constructed with Insight II (1995 version) (BIOSYM/Molecular Simulations) operating on a Silicon Graphics Indigo-2 workstation. All calculations were carried out using the AMBER forcefield (Weinger et al, 1986) interfaced with the molecular mechanics software DISCOVER (BIOSYM/Molecular Simulations) on the same workstation. The atom potential types and charges were set according to Homan's potential types for carbohydrates (Homans, 1990) in the AMBER forcefield. The amine groups of isepamicin were given a formal charge of +1.00 consistent with the pK_a values reported previously (Digiammarino et al., 1998).

A process called restrained simulated annealing was used to obtain

possible ANT (2'')-Ia bound conformations of isepamicin. Prior to simulated annealing, a set of random structures was generated by subjecting an isepamicin molecule to unrestrained dynamics at 600 K for 10 0 ps. The distance restraints obtained through TRNOESY were then imposed upon the randomized structures and they were carried through a restrained simulated annealing scheme using restrained conjugate gradient minimizations (500 iterations) followed by restrained molecular dynamics (10.0 ps) at 450 K. This was then repeated at temperatures of 400, 350, and finally 300 K followed by a final restrained conjugate gradient minimization until the r.m.s. derivative was less than 0 001 kcal/Å. The restraints were then removed and the structure was subjected to molecular dynamics at 100 K (10 0 ps) followed by a final conjugate gradient minimization until the r m s. derivative was less than 0 001 kcal/Å. All simulations were carried out using a dielectric constant of 4.0 *in vacuo* which was prescribed by the Discover module for structure calculations of carbohydrates with the AMBER/Homans forcefield. An energetic penalty of 50 kcal/mol Å⁻² was used for violation of the distance restraints.

C. Results and Discussion

A total of 25 TRNOEs were observed for isepamicin bound to ANT (2'')-1a. Of these 25, 21 were intra-ring and served to define the conformations of the individual rings, while the remaining four TRNOEs were inter-ring and thus provided information about the relation of the rings to one another (**table 3**). Particularly important for defining the conformation of the rings with respect to one another were the inter-ring TRNOEs that were observed across the glycosidic bonds (**figure 6A**). To ensure that the observed inter-ring TRNOEs (shown in figure 6A) were valid ones and not a product of spin diffusion, the QUIET-TRNOE technique described above was used. As seen in **figure 6B**, no differences were observed in the spectra resulting from either normal TRNOESY or QUIET-TRNOESY.

Use of the TRNOEs in the simulated annealing scheme mentioned above resulted in two conformers of isepamicin (conformer 1 and conformer 2) about the B and C rings (**figure 7**). The conformers were quantitatively distinguishable from one another by comparing the pairwise r.m.s. derivative (RMSD) values

from superimposition about the B and C rings and by measurement of the glycosidic dihedral angles, Φ ($H1_C-C1_C-O_\alpha-C6_B$) and Ψ ($H6_B-C6_B-O_\alpha-C1_C$) that determine the orientation of the C ring relative to the B ring (**table 4 and figure 8**) Conformer 1 was determined to be the major conformer containing 20 structures while conformer 2 was considered to be the minor conformer containing only three. After this distinction was made, it was found that conformer 1 could be subdivided into three different conformers (conformer 1a (12 structures), conformer 1b (5 structures), and conformer 1c (3 structures)) based on the orientations of the A ring (**figure 9**) relative to the B ring After subdividing, the average RMSD values dropped significantly from those seen for all structures in conformer 1 (**table 4**)

To confirm that the two conformers observed were in fact distinct conformers, unrestrained molecular dynamics at 300K was used for a duration of one nanosecond starting from one of the final structures representing each conformer A trajectory of the ϕ and ψ angles defining the B and C rings was obtained over this time period to determine if the two conformers could interconvert. **Figure 10** shows the results of this trajectory. Starting from conformer 1a, the Ψ glycosidic angle was not seen to convert to that of conformer 2 (the ϕ angle differences between conformer 1a and conformer 2 were small compared to the differences in Ψ angles) This would imply that the energetic barrier is too great for interconversion In support of this idea, when starting from conformer 2 the Ψ angle relatively quickly converts into the value

recognized for conformer 1a and then doesn't convert back. In order to further explore this phenomenon the energies of the final structures were examined to see if conformer 1a could represent a lower energy structure and thus a potential energy well. Interestingly, the vast majority of values for the structures in conformer 1a did in fact display lower energy values when compared to those that were measured for conformer 2. Using the rationale that when binding two molecules with similar substituent orientations but different energies the enzyme will choose the antibiotic with the lower energy, it would seem reasonable that conformer 2 represents a structure which satisfies the restraints but does not necessarily represent the bound conformation. It should be mentioned however that given that the time allowed for the simulation (≤ 1 nanosecond) is much shorter than the time required for a single catalytic turn (~ 1 second), it may still be possible for conformer 2 to convert into conformer 1a while in the active site of the enzyme and for catalysis to occur. Greater computational time will be required to examine this situation.

Another possibility for the existence of two conformations about the C ring is that only one of them presents the correct orientation for catalysis to occur while the other conformer binds but in an unproductive manner. It has been suggested that binding to aminoglycoside modifying enzymes is primarily determined by the A and B rings (Serpensu et al., 2000). Further evidence for this theory may be the ability of a specific conformation to form intra-molecular hydrogen bonds which could act to stabilize the structure in the active site.

Upon searching for sites of potential hydrogen bonding within the molecule, the A and B ring conformation of conformer 1a displayed a hydrogen bond between the number two hydroxyl of the A ring to the number five hydroxyl of the B ring in the majority of structures (**figure 11**) Conformer 1b also displayed hydrogen bonding between the A and B rings but from the number 6 amine on the A ring to the number 3 amine on the B ring None were observed for conformer 1c This may be evidence for the major A and B ring conformation observed being the preferred orientation in the active site and therefore a more representative bound structure With this in mind, superimposition of conformer 1a and conformer 2 about these rings would place the C ring of each conformer into a different position Thus although both conformers may bind to ANT (2'')-1a in a similar fashion with respect to their A and B rings, the orientation of the C ring in one of them may not be sufficient for catalysis to occur. This ability of isepamicin to bind to the enzyme in an unproductive manner may be the key to its clinical efficacy If the antibiotic binds in a manner unsuitable for catalysis it may decrease the enzyme's efficiency by spending a greater amount of time occupying its active site and allowing other unmodified drugs to move into the cell unaffected

Another explanation is that both conformers can bind in a manner sufficient for catalysis With this assumption, conformer 2 and conformer 1a (the dominant conformer 1 structure) were superimposed about both the A and B rings to compare placement of the C ring In this situation the reactive hydroxyl

groups are positioned exactly 2.12 Å apart. This may be sufficient for the residue or residues in the active site to create a good nucleophile out of the 2'' oxygen atoms on both conformers such that catalysis can occur. However, additional structural data will be required to discern if this is a possibility or not.

Chapter IV

General Conclusions

A. Introduction

The process used for drug discovery in the past has typically been one involving the screening of a large number of synthetic chemical compounds or natural products for the desired activity. This approach has been successful for the most part and has produced many of the essential drugs in existence today. However, this purely random process is not marked by efficiency, nor is it regarded as cost effective. Unfortunately, without structural information about the drug and its target there are not many alternatives.

Newer methods are evolving that give hope to a faster, more controlled, and rational approach to drug design. These methods are inherently dependent upon structural information about the mechanisms involved in drug interaction. Due to advances in technology, both X-ray crystallography and NMR are the major sources of this structural information. These techniques allow the researcher to study diseases at the molecular level making the identification of

the pathways and molecules responsible for them accessible. This in addition to advances in cloning and expression systems are allowing the concept of rational drug design to become a feasible approach

In the last chapter, enzyme-bound conformations of the antibiotic isepamicin were revealed and discussed with the overall intent of identifying potential inhibitors of the aminoglycoside modifying enzymes. This chapter provides further discussion on that topic through comparisons of the results presented in this work to those reported previously for this class of antibiotics

B. Comparisons of Enzyme-Bound Conformations

The purpose of this work is to elucidate similarities in binding of the aminoglycoside antibiotics to their resistance enzymes. With the conformations of isepamicin bound to the two other types of enzymes (an acetyltransferase and a phosphotransferase) already determined, its conformation bound to a nucleotidyltransferase provides information for the third type of modifying enzyme. Now that this data has been acquired, we can begin to look for similarities in the structures to figure-out which aspects of the molecule may be most crucial to effective enzyme binding. These characteristics may then be exploited to find an inhibitor that can provide these requirements but with an

increased affinity such that binding is prolonged allowing the actual drug to remain in its active state and able to affect its target.

As mentioned in the previous chapter, conformer 1a was the major conformer that emerged from restrained simulated annealing. Upon comparison of this structure to the enzyme-bound structures of AAC (6')-II and APH (3')-IIIa, distinct similarities were observed for the orientations of the A and B rings. Superimpositioning of these rings produces striking agreement in their conformations (**figure 12**) with an RMSD value measuring 0.0865 ± 0.025 (**table 3**). These results present further evidence for the important inter-molecular binding contacts that the A and B rings may contribute. The orientations of the A and B rings probably allows specific positioning of the potential hydrogen bond donors and acceptors that may often be required among modifying enzymes. The fact that ANT (2'')-Ia modifies at the C ring as opposed to the A ring and yet the A ring still adopts a similar conformation lends evidence to this idea. There are hydroxyl groups at positions 2, 3, and 4 on the A ring of isepamicin and an amine group at position 6. These all most likely present necessary binding contacts (possibly electrostatic in the case of the amine) to the enzymes and thus must orient correctly. In addition, the amine at position 3 of the B ring, common to many of the aminoglycosides, is also probably utilized in binding and so must be positioned in a similar manner to the three different enzymes.

It should also be mentioned that ribostamycin, a 4,5-disubstituted aminoglycoside, shows a similar A and B ring orientation to those mentioned

above. Thus it may be that aminoglycosides with a 4,5-disubstituted deoxystreptamine ring also bind in a similar fashion despite potential steric differences. It may be that any additional contacts that the C ring can make with the active site serve to enhance binding but are not entirely necessary.

C. Comparisons of Enzyme-Bound Conformations to RNA-bound Conformations

From comparisons of the enzyme-bound conformations to RNA-bound conformations of aminoglycoside antibiotics it appears as though the aminoglycoside-modifying enzymes may have evolved their active site to emulate the chemical environment of the aminoglycoside-binding site on the 16S rRNA target. Superimposition about the A and B rings of enzyme-bound isepamicin to both paromomycin and gentamicin bound to the 16S rRNA reveal distinct similarities in their conformations (**figure 13**). It therefore seems possible that the same contacts responsible for RNA binding are utilized in enzyme binding. In both the gentamicin and paromomycin RNA-bound structures specific interactions are seen between the hydroxyl and amine groups of the antibiotic and the hydrogen bond donors and acceptors on the nitrogenous bases and phosphate groups of the nucleotide bases (Yogishawa et

al., 1998 and Fourmey et.al , 1996) The importance of similar hydrogen bond donors and acceptors as well as similar electrostatic interactions between the rRNA binding site and the enzyme binding sites have been observed in earlier studies For example the amine groups present on aminoglycosides have been rated according to their importance in conferring antibacterial activity in the following manner 2',6'-diamine > 6'-amine > 2'-amine > no amine (Benveniste and Davies, 1973). It was also found that the gentamicins containing both the 2' and 6' amino groups were better substrates of ANT (2'')-Ia (Gates and Northrop, 1988) In addition, the majority of enzymatic modification sites are located on the A and B rings This would give the advantage of creating electrostatic or steric hindrances where they would be most likely to detract from binding to the target. It may be simply that the A and B ring orientations that we and others have observed are energetically favorable conformations that can bind favorably to both the RNA binding sites and the enzyme active sites and at the same time place the hydrogen bond donors and acceptors within favorable positions within the drug itself At any rate, these results paint a bleak picture for the development of resistant aminoglycoside antibiotics It seems as though a drug that is effective at evading modification by decreased enzyme binding will also display decreased binding to the rRNA target On the other hand, an antibiotic that binds tightly to the resistance enzyme may also bind tightly to the RNA This situation could possibly provide enough antibacterial effects in the face of resistance to be effective therapeutically. More structural information of the

enzymes themselves will be of great help in completing this picture and in producing more effective therapeutics

D. Future Studies

Although conformations of enzyme-bound aminoglycosides gives significant insight toward the design of inhibitors, structural information about the enzyme target itself would greatly enhance our understanding of the mechanism used in binding and catalysis. With this consideration, the pursuit of the three dimensional solution structure of ANT (2'')-Ia is the next logical choice for the future of this research. This information will allow the elucidation of which residues are present in the active site and specifically which are responsible for binding and catalysis. Presently, few three dimensional structures are known for the aminoglycoside modifying enzymes. This makes the need for the structure of ANT (2'')-Ia even greater. The more structural information known about these diverse and adaptive enzymes, the better our chances are for preventing the return to a pre-antibiotic state of existence.

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APPENDICES

APPENDIX I

(Figures)

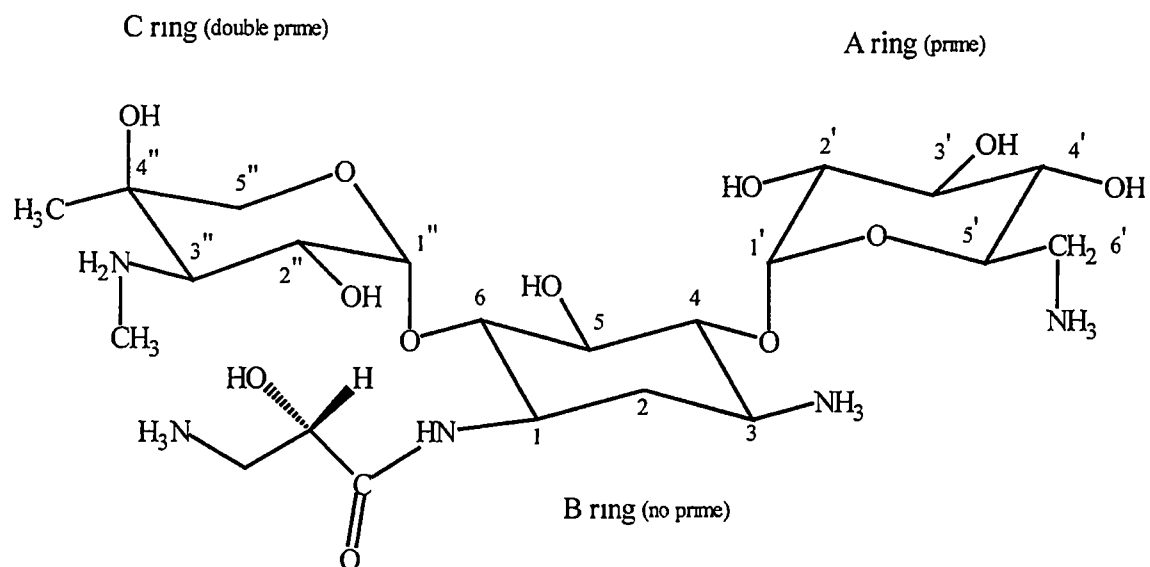


Figure 1 The structure of isepamicin Notice the nomenclature used for the rings and their substituents.

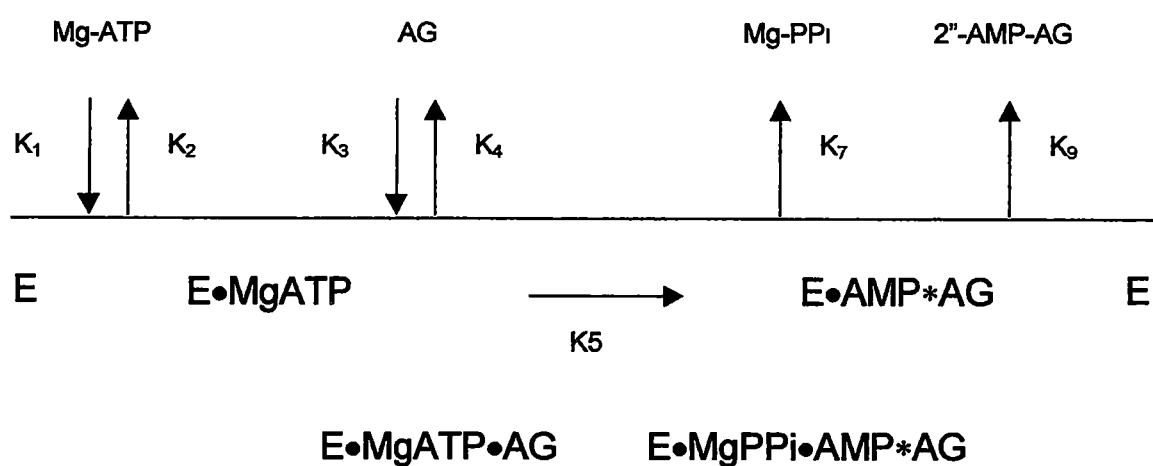


Figure 2. Proposed kinetic mechanism of ANT (2'')-1a. Based on kinetic studies a Theorell-Chance kinetic mechanism like the one drawn above has been proposed for ANT (2'')-1a.

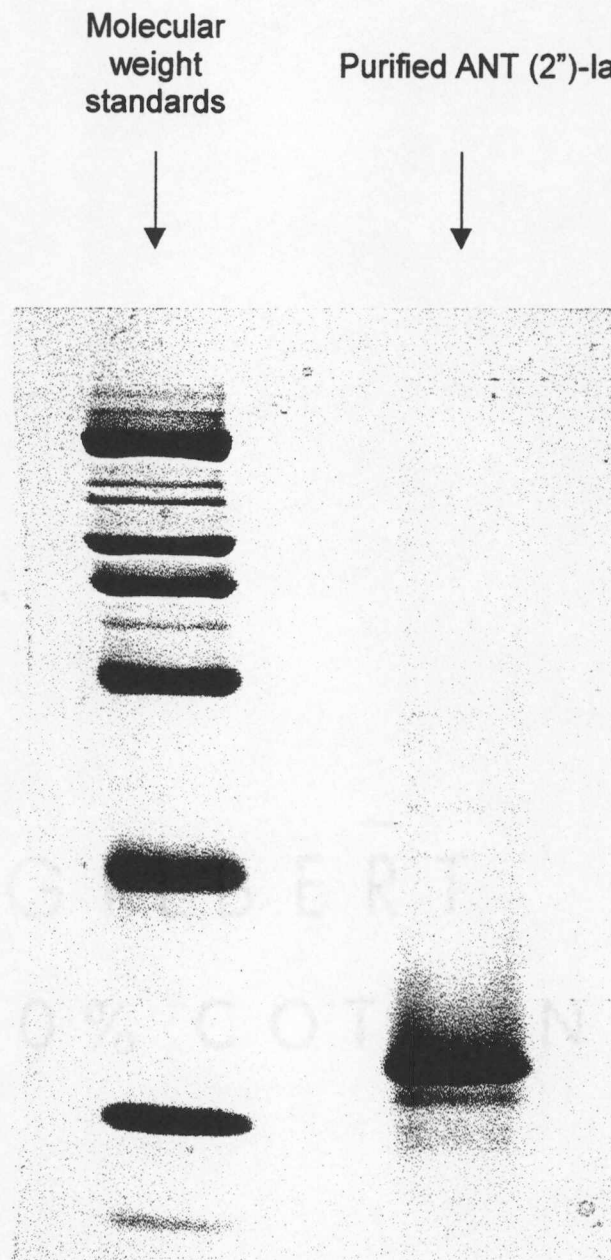


Figure 3. SDS-PAGE of soluble and active ANT (2'')-Ia. After washing the inclusion bodies with 1% Triton and then using the resolubilization and recovery scheme ANT (2'')-Ia is more than 91% pure (as measured by densitometry).

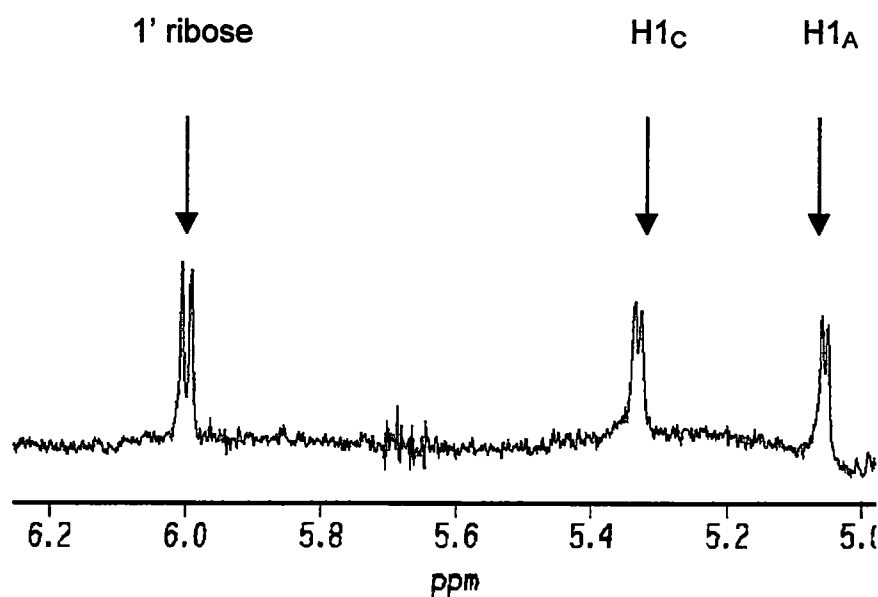


Figure 4. one dimensional spectrum of kanamycin modified at the two position of ring C. In the original reaction mixture ATP was in thirty-fold excess to kanamycin. However, as shown here after purification the two substrates are in approximately one to one relative concentrations. This spectrum gives good evidence that the purification of modified kanamycin was successful.

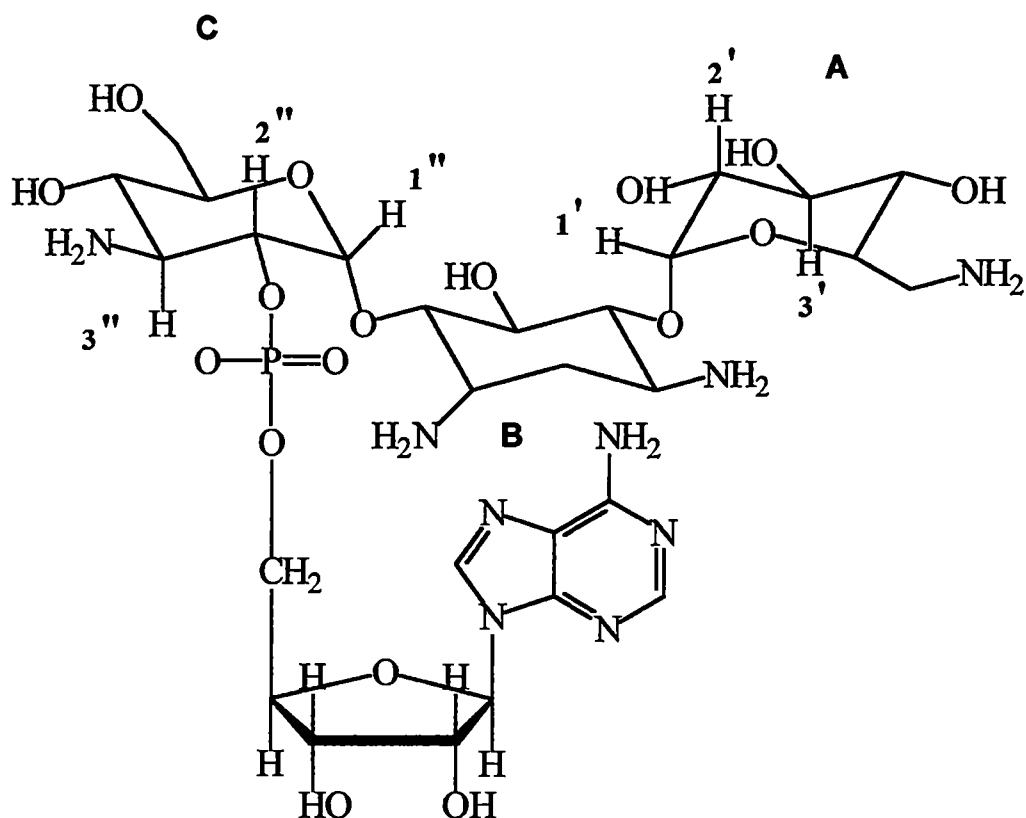


Figure 5. The structure of adenylylated kanamycin. Protons near to the site of modification are in close proximity to the oxygens of the alpha phosphate. This would explain the chemical shift changes that were observed.

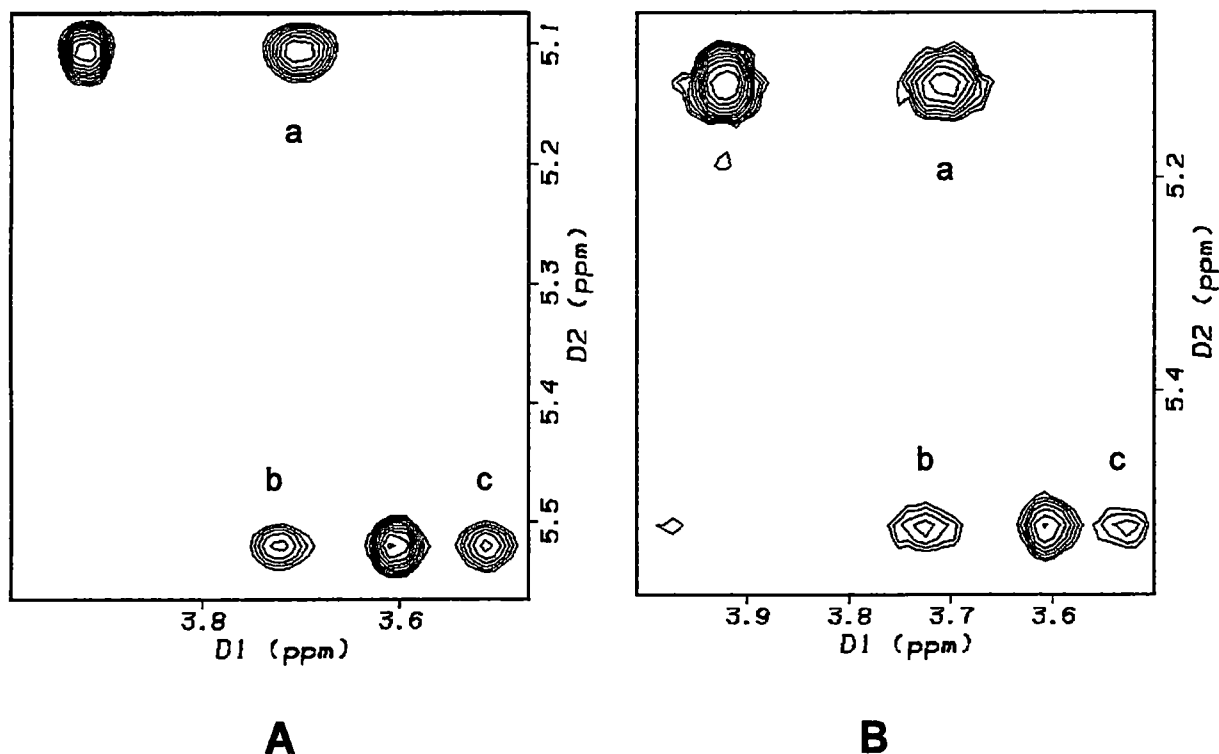


Figure 6. (A) TRNOESY spectrum displaying the crosspeaks resulting from interactions across both glycosidic bonds of isepamicin. (B) Quiet-TRNOESY spectrum displaying the same region as depicted in A. The similarity of these spectra indicate that the TRNOEs observed for these important interactions are genuine and not simply products of spin diffusion. The interactions depicted are as follows: (a) H1_C-H6_B, (b) H1_A-H4_B, (c) H1_A-H3_B.

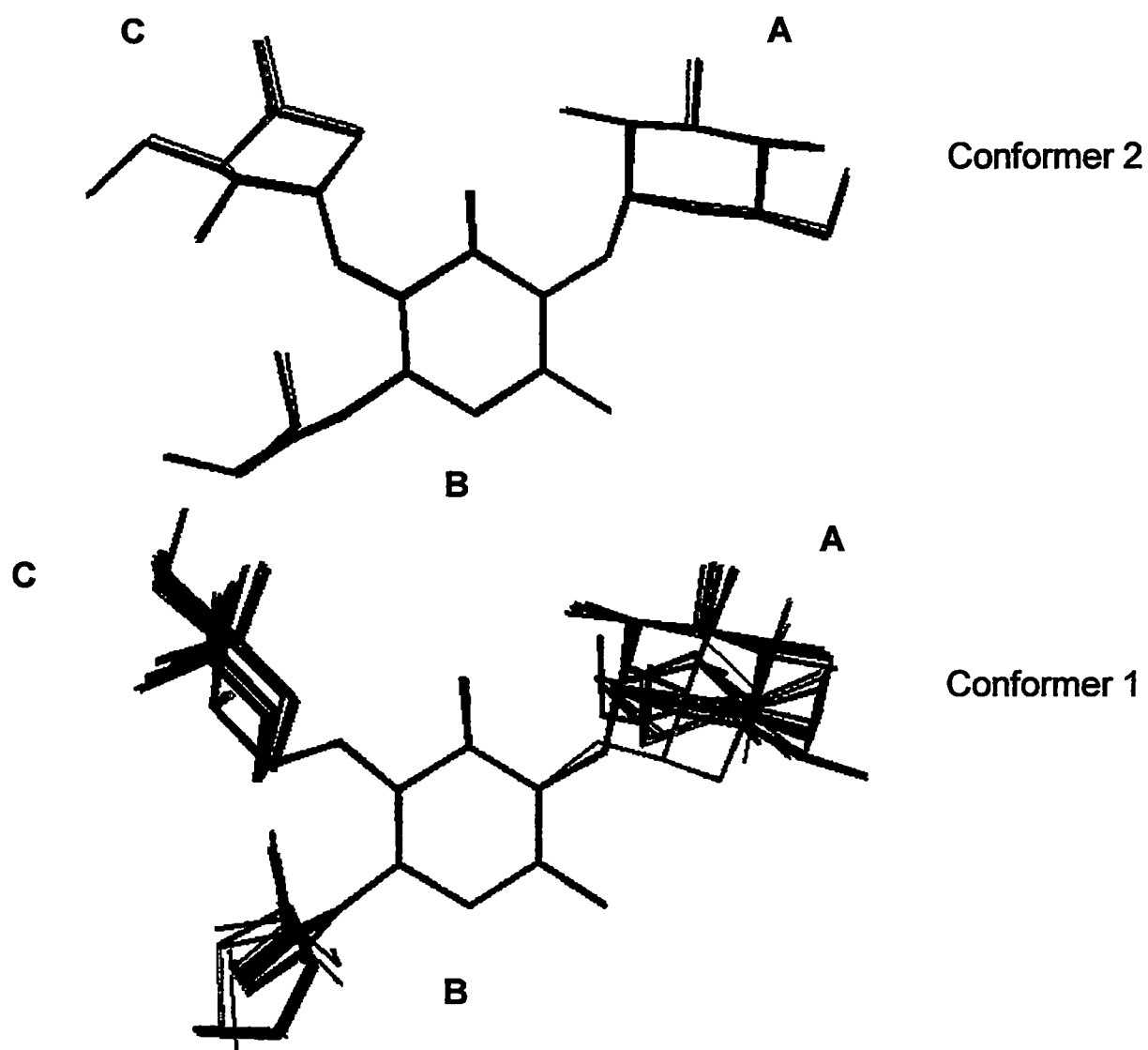


Figure 7. Enzyme-bound conformations of isepamicin (conformer 1 and conformer 2). Superimposition about the B and C rings resulted in two distinct conformations.

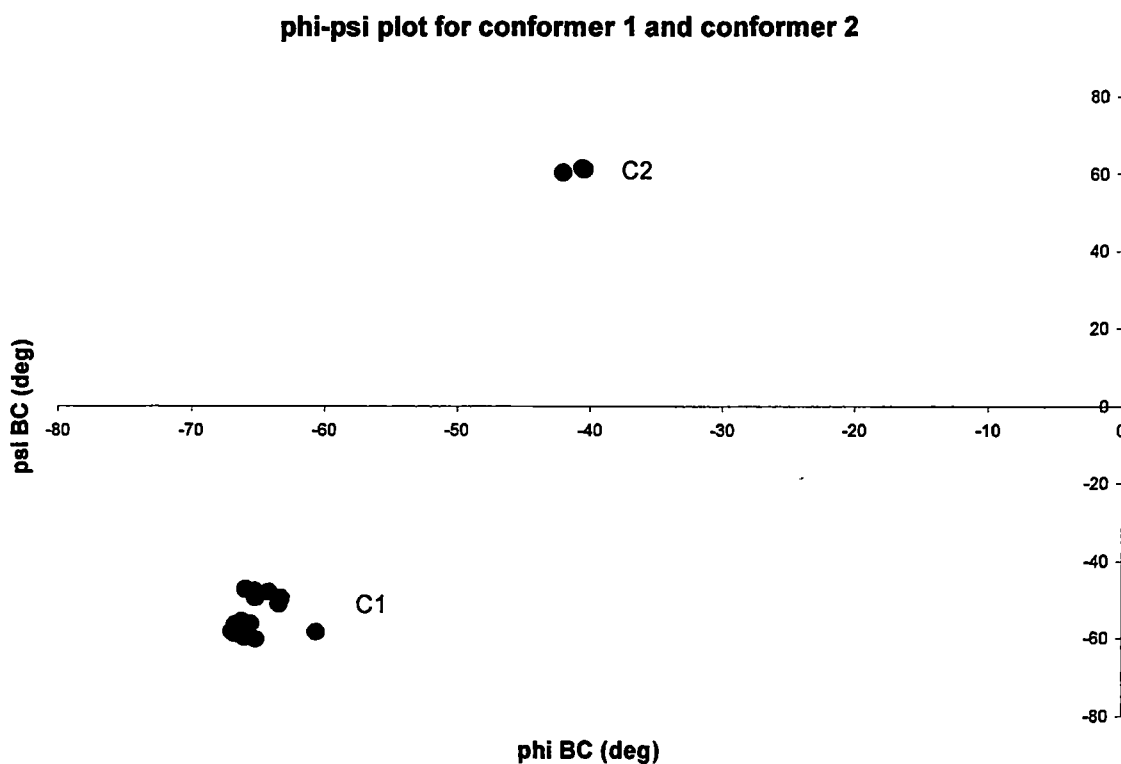


Figure 8. ϕ_{BC} vs. ψ_{BC} plot for conformer 1 and conformer 2. The angles that define the glycosidic bond between the B and C rings is distinct for each conformer.

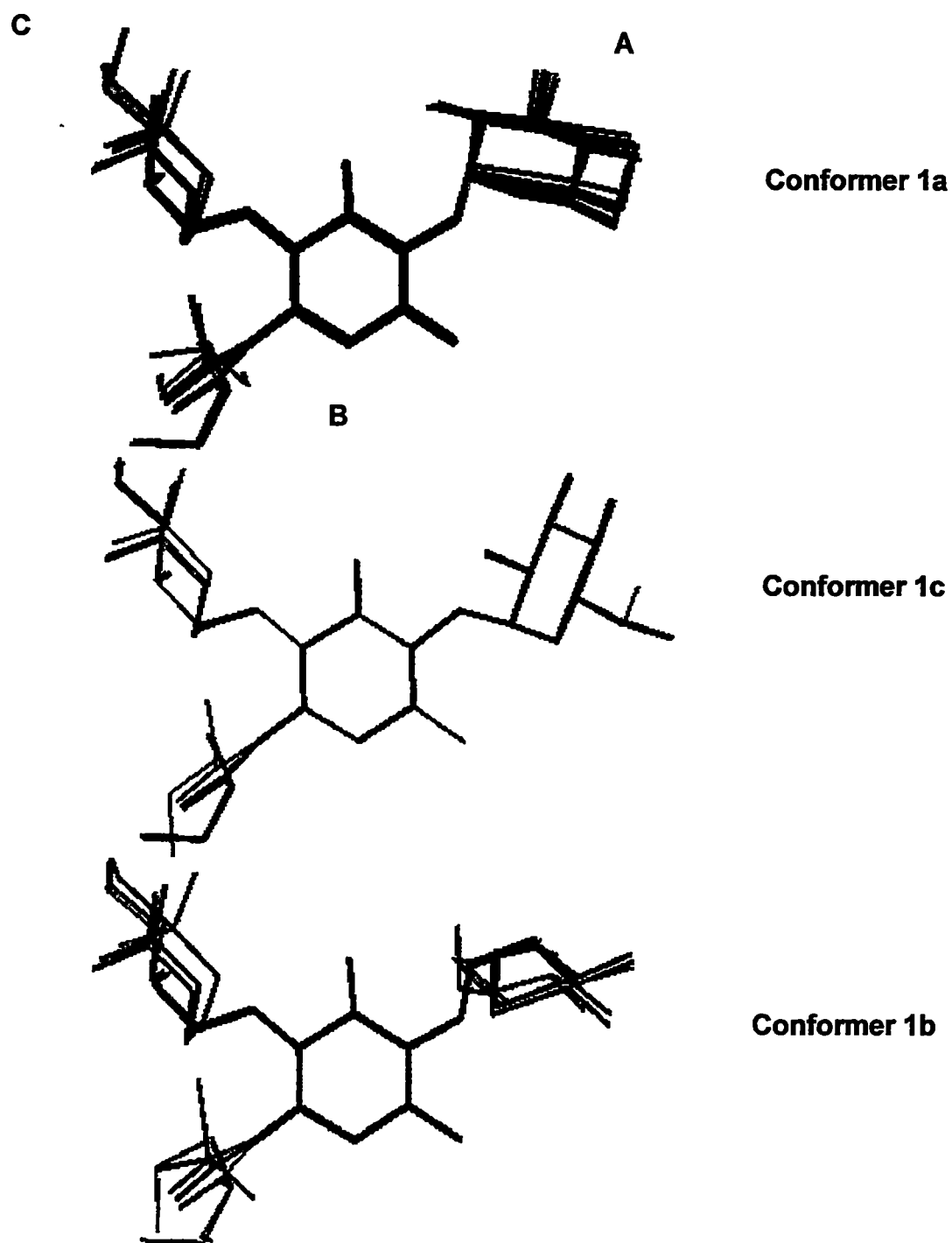


Fig 9. The three conformers within conformer 1 resulting from superimposition about the A and B rings.

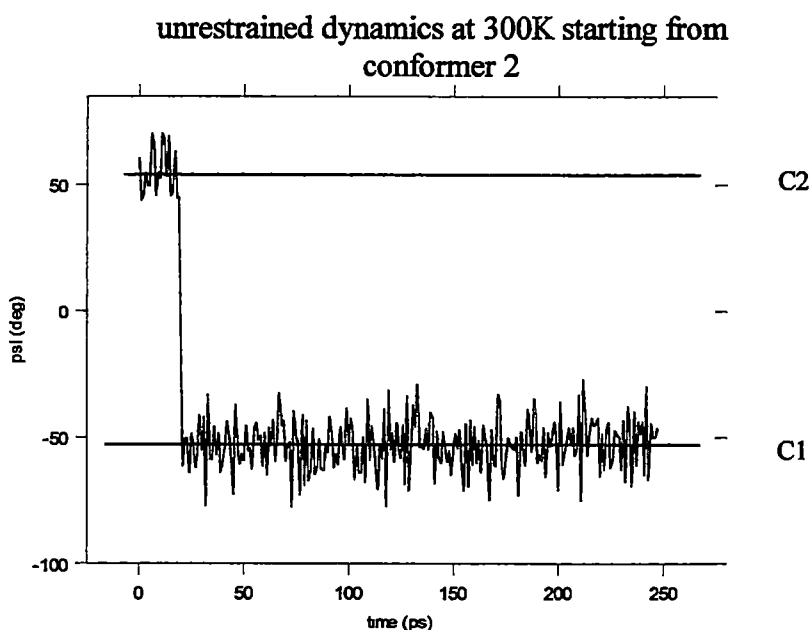
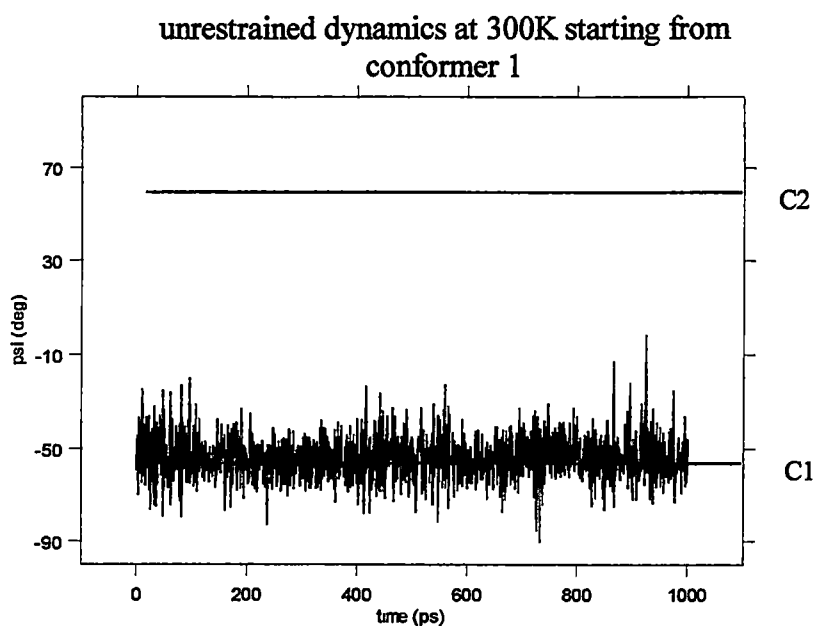


Figure 10. Trajectory plots for the B and C ring glycosidic angles of enzyme-bound isepamicin during unrestrained dynamics. The psi angle of conformer 1 was maintained throughout the one nanosecond simulation while the same angle in conformer 2 quickly converted to that of conformer one early in the short 237 picosecond simulation.

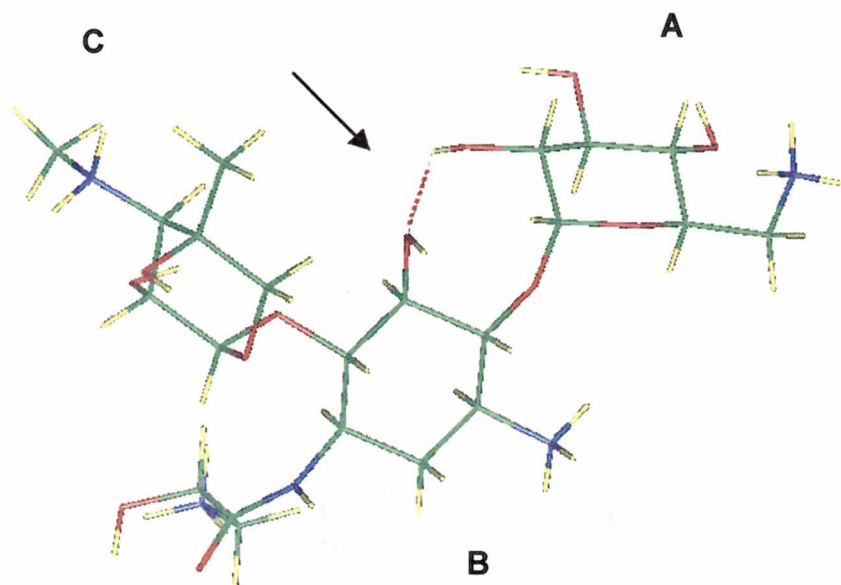


Figure 11. Potential intra-molecular hydrogen bond for enzyme-bound isepamicin. A potential hydrogen bond was observed to occur between the hydrogen on the A ring number two carbon and the B ring number five hydroxyl oxygen. This bond may serve to stabilize this conformation of the A and B rings in the active site of the enzyme.

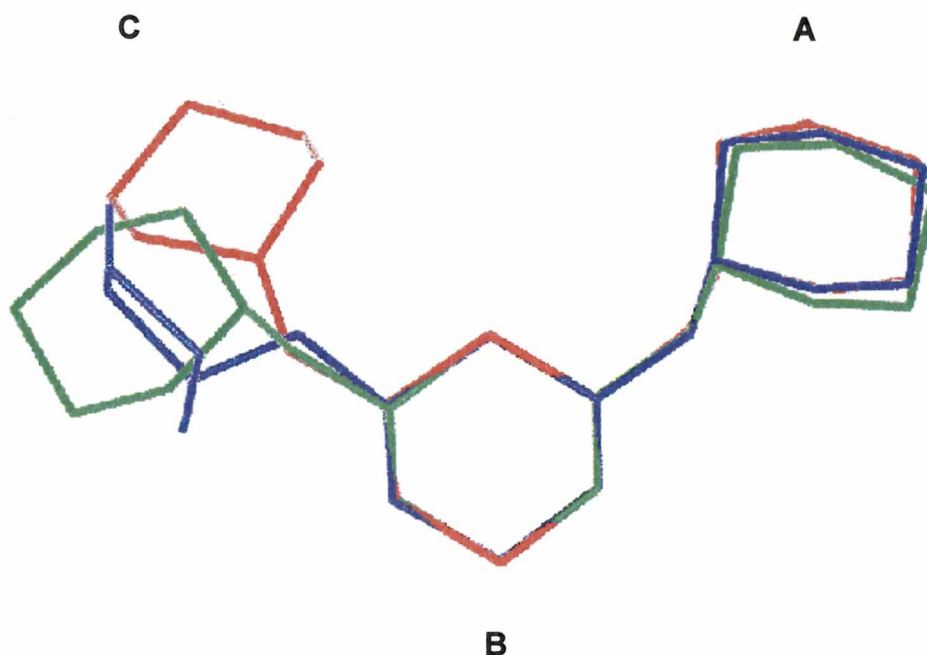


Figure 12. Conformations of isepamicin bound to three different types of modifying enzymes. Isepamicin bound to ANT (2'')-Ia (conformer 1a, blue), APH (3')-IIIa (red), and AAC (6')-Ii (green). The structures were all superimposed about the A and B rings. This figure suggests a common binding mode for the A and B rings of isepamicin for all three enzymes.

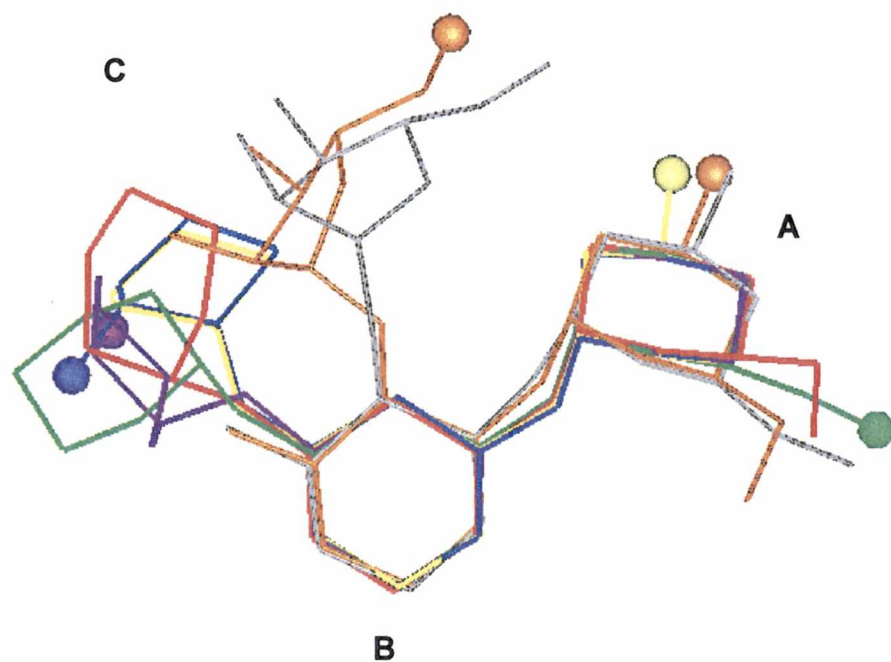


Figure 13. Comparisons of the A and B ring orientations for both enzyme-bound and RNA-bound aminoglycosides. Structures are colored as follows: ANT (2'')-Ia-bound isepamicin conformer 1a (purple) and conformer 2 (blue), AAC (6')-Ii-bound isepamicin (green), APH (3')-IIIa-bound isepamicin (yellow), APH (3')-IIIa bound ribostamycin (orange), RNA-bound gentamicin (red), RNA-bound paramomycin (grey). Modification sites are indicated as spheres.

APPENDIX II

(Tables)

Table 1

Resistance Profile for ANT (2'')-Ia

Gentamicin C1a	Kanamycin A
Gentamicin C2	Amikacin
Gentamicin C1	Kanamycin B
Sisomicin	Tobramycin
5-epiSisomicin	Nebramycin 4
Netilmicin	
Gentamicin A	
Gentamicin B	
Gentamicin B1	

Table 2

Chemical Shift Changes Observed for Specific Protons of Kanamycin
A as a Result of Modification

H ¹	Kanamycin ^a	Adenylated Kanamycin ^a	Δppm Kanamycin	Δppm Tobramycin ^b
H1 _A	5.49	5.45	0.04	0.01
H2 _A	3.59	3.62	0.03	0.11
H3 _A	3.72	3.80	0.08	N/A
H1 _C	5.04	5.08	0.04	0.06
H2 _C	3.67	3.95	0.28	0.35
H3 _C	3.18	3.80	0.62	0.03

^a Reported in ppm

^b As reported by Van Pelt et. al., 1990

Table 3

TRNOEs and Distance Groupings Used in Structure Calculations

Observed TRNOEs for ANT (2'')-Ia Bound Isepamicin

H1 _C -H2 _C H1 _A -H2 _A H1 _C -H6 _B H3 _A -H6 _A H3 _C -METH3 _C	STRONG (2.0-2.7 Å)
H5 _A -H6 _A H2 _A -H4 _A H2 _A -H3 _A H5 _{Cax} -METH4 _C H3 _C -METH4 _C	MEDIUM (2.0-3.6 Å)
H2 _{Beq} -H5 _B H2 _C -H3 _C H3 _C -H5 _{Cax} H2 _D -H3 _D H1 _A -H3 _A H1 _A -H3 _B METH3 _C -METH4 _C H3 _A -H4 _A H2 _D -H3 _D H4 _A -H5 _A H5 _{Ceq} -METH4 _C H5 _A -H6 _A H2 _{Beq} -H1 _B H2 _{Bax} -H4 _B H1 _A -H4 _B	WEAK (2.0-4.5 Å)

Table 4

Statistics of NMR Data and Structures

Distance Restraints	
Total restraints	25
Inter-ring	4
Strong (2.0-2.7 Å)	5
Medium (2.0-3.6 Å)	5
Weak (2.0-4.5 Å)	15
Pairwise r.m.s.d. among Final Structures	
Rings B and C heavy	
All structures	0.240 ± 0.419
Conformer 1	0.06475 ± 0.051
Conformer 2	0.047 ± 0.0127
Rings A and B heavy	
All structures	0.370 ± 0.383
Conformer 1	0.427 ± 0.3925
Conformer 1a	0.0488 ± 0.0283
Conformer 1b	0.066 ± 0.0828
Conformer 1c	0.016 ± 0.0026
Conformer 2	0.037 ± 0.013
Representatives of Conformer 1a and Conformer 2	0.166
Isepamicin bound to three different enzymes	0.0865 ± 0.025
Glycosidic (BC) angles for both conformers	
φ _{BC} (deg)	
Conformer 1	-65.26 ± 1.63
Conformer 2	-41.05 ± 0.88
ψ _{BC} (deg)	
Conformer 1	-54.76 ± 4.64
Conformer 2	61.15 ± 0.616

VITA

Drew Robert Ekman was born in Hartford, Connecticut on June 13, 1975. He completed his B.S. with a major in biochemistry at the University of Georgia in June, 1997. In that same year he was accepted to the graduate program in biochemistry at the University of Tennessee, Knoxville. He worked for three years to complete an M.S. in biochemistry focusing the majority of his efforts on the problem of bacterial resistance to antibiotics.