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Cloning, purification and substrate conformations of aminoglycoside acetyltransferase (3)-IIIb

Michael Andrew Owston

To the Graduate Council:

I am submitting herewith a thesis written by Michael Andrew Owston entitled "Cloning, purification and substrate conformations of aminoglycoside acetyltransferase (3)-IIIb." I have examined the final electronic 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 Serpersu, Major Professor

We have read this thesis and recommend its acceptance:

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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CLONING, PURIFICATION AND SUBSTRATE CONFORMATIONS OF AMINOGLYCOSIDE ACETYLTRANSFERASE (3)-IIIb

A Thesis Presented for the
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Michael Andrew Owston

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Abstract

The aminoglycoside resistance protein aminoglycoside acetyltransferase (3)-IIIb (AAC3-IIIb) was successfully cloned, expressed and purified. This will allow many future experiments to be done using AAC3-IIIb. Using 2D NMR and molecular modeling, the conformations of two aminoglycosides, kanamycin and ribostamycin, were determined when bound to AAC3-IIIb. The structures determined for ribostamycin were divided into two conformers defined by the phi-psi angles of the glycosidic bonds as follows: Conformer 1 (11 total): Φ_{1A} = -22 +/- 3, Ψ_{1A} = -42 +/- 1, Φ_{1C} = -9 +/- 4, Ψ_{1C} = 51 +/-1 and for Conformer 2 (11 total): Φ_{1A} = -67 +/- 0.7, Ψ_{1A} = -59 +/- 0.8, Φ_{1C} = -9 +/-3, Ψ_{1C} = 49 +/- 1. This gives the C to B ring orientation with respect to one another to be the same in both conformers. For kanamycin there were three conformers determined using the phi-psi angles as follows: Conformer 1 (9 structures): Φ_{1A} = -11 +/- 3, Ψ_{1A} = - 45 +/- 3, Φ_{1C} = -9 +/- 4, Ψ_{1C} = 50 +/- 2; Conformer 2 (6 structures): Φ_{1A} = -8 +/- 3, Ψ_{1A} = 48 +/- 2, Φ_{1C} = -18 +/- 2, Ψ_{1C} = -42 +/-2; and Conformer 3 (5 structures): Φ_{1A} = -73 +/- 0.6, Ψ_{1A} = -57 +/- 0.2, Φ_{1C} = -14 +/- 0.5, Ψ_{1C} = 51 +/- 0.2. Because the B to C orientation was the same for Conformer 1 and Conformer 3, this gives 14 out of 20 structures with that conformation for these rings. The C ring appears to be more constrained when either aminoglycoside is used while the A ring is somewhat more flexible. However, a comparison of the two aminoglycosides gives the A ring in one orientation (Conformer 1 for both ribostamycin and kanamycin) more than the others. This suggests that this may be the proper orientation of this ring. It has been suggested that the A and B rings provide the most important contributions when binding to both other aminoglycoside

modifying enzymes and to RNA (Fourmy *et al.,* 1996; Yoshizawa *et al.,* 1998; Serpersu *et al.*, 2000). Comparisons superimposing these rings with other enzyme bound aminoglycosides as well as RNA bound aminoglycosides shows remarkable similarity among the conformations. This gives important conformational information that may be used in future drug and enzyme inhibitor design studies.

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Chapter I.

Introduction

The first antibiotic discovered was penicillin by Sir Alexander Fleming in 1928, when he noticed a *Pennicillium* mold inhibiting the growth of *Staphylococci*. Since then a large number of clinically relevant antibiotics have been discovered or synthesized, including the β-lactams like penicillin, aminoglycosides, tetracyclines, glycopeptides, sulfonamides, macrolides, and quinolones (Spratt, 1994; Chu, *et al.*, 1996). Each of these antibiotics has its own mode of action against the bacteria it is useful against. Antibiotics were thought to be miracle drugs, even though as early as 1940 an enzyme called penicillinase was discovered that could hydrolyze the β-lactam ring of penicillin, and it was noted that it could interfere with penicillin therapy (Abraham & Chain, 1940). Several other resistance mechanisms exist, but the most common is chemical modification of the antibiotic.

Aminoglycosides are one class of antibiotics that have broad-spectrum ability to kill bacteria (Davies, 1987). However, they also are known to have oto- and nephro-toxicity so their use is usually confined to a clinical setting (Mingeot-Leclercq *et al.*, 1995). Three groups exist, one with a 4, 6 disubstituted 2-deoxystreptamine ring, one with a 4, 5 disubstituted 2-deoxystreptamine ring, and those without these features. Figure 1 shows some 4, 6 and 4, 5 disubstituted deoxystreptamine aminoglycosides. They are characterized as having a 2-deoxystreptamine with amino sugar groups being connected through glycosidic linkages. With all of the amino groups, aminoglycosides tend to be highly cationic and water soluble. Members of the *Streptomyces* genus

produce most of these. For example, *S. kanamyceticus* produces the aminoglycoside kanamycin. However, due to resistance to many of the naturally occurring aminoglycosides, many semi-synthetic aminoglycosides have been produced. These usually consist of an aminoglycoside that has been modified with an extra side chain, such as amikacin and isepamicin (Figure 1; all figures are in the appendix). Despite these extra efforts, there is still a large amount of resistance occurring (Davies, 1987).

Aminoglycosides work by binding to the rRNA within the ribosome, and thus interfere with protein translation. Resistance can occur by either modifying the target (rRNA or ribosome), by expelling the aminoglycoside from the cells before it attaches to the ribosome, or by modifying the aminoglycoside itself. However, resistance to aminoglycoside antibiotics usually occurs by the presence of an enzyme that chemically modifies the aminoglycoside making it inoffensive to the bacteria. The primary method by which this occurs is by adding substituent groups to the aminoglycosides that prevents them from binding to their target rRNA. The substituents added are acetyl groups by acetyltransferases, phosphate groups by phosphotransferases, and nucleotide groups by nucleotidyltransferases. The standard nomenclature for the resistance enzymes is AAC, APH and ANT respectively. They are also named for the position that they modify, and for which ring they modify. Finally, the resistance enzymes are grouped by the resistance profile they confer, and given a letter designation based on what isozyme of that particular enzyme that it is. For example, AAC3-IIIb refers to aminoglycoside acetyltransferase that modifies at the "3" position on the B ring (the center 2-deoxystreptamine ring), has the resistance profile III and is the second (b) isozyme discovered. The resistance profile of AAC3-III is that which confers resistance to the

following: gentamicin, tobramycin, dibekacin, 5-episisomicin, sisomicin, kanamycin, neomycin, paromomycin, and lividomycin (Shaw *et al.,* 1993).

The method by which these enzymes confer resistance may be that the substituent either causes steric hindrance or ionic interference so that the aminoglycoside cannot bind correctly or it blocks a functional group that is necessary for specific rRNA binding. For instance, because the rRNA has many phosphates, the addition of a phosphate on the aminoglycoside may prevent it from binding rRNA even if the modified position is not necessary for aminoglycoside binding, due to ionic interactions. However, in the case of acetyl groups, this may block a necessary binding group on the aminoglycoside, or it could cause steric hindrance. This is the same principle that allows some of the semi-synthetic aminoglycosides to not be bound to some of the resistance enzymes.

An understanding of binding of aminoglycosides to both the RNA target and to the enzymes that inactivate them may give a better idea of how to combat resistance. Studies in one laboratory have determined the structures of gentamicin and paromomycin while bound to RNA (Fourmy *et al.,* 1996; Yoshizawa *et al.,* 1998). From this, key functional groups on the aminoglycosides and RNA that are involved in binding have been determined. In particular, the 6′ and 2′ positions, which are conserved as hydrogen bond donors across the aminoglycosides, appear to have key interactions with the RNA for the A (or "prime") ring. On the deoxystreptamine (B or unprimed) ring the 1-amino group makes hydrogen bond contacts with the RNA and the 3-amino group makes contact with a phosphate through hydrogen bonds or ionic interaction with the RNA. Because of the difference in the C ring (double prime), not as much can be said about its importance, but it does contribute a hydrogen bond with the 2″ OH with gentamicin.

With 4,6 disubstituted aminoglycosides, the 2″ OH is probably significant in binding to RNA, but for 4, 5 disubstituted aminoglycosides, it is thought that there is not necessarily one important group. In summary, the most important binding sites according to the RNA structure of gentamicin are the 6′, 2′, 1, 3, and 2″. The 3′ OH group, though not as important for binding, may be commonly modified because the presence of a phosphate may interfere with the hydrogen bonding of the 2′ group with the phosphate on the RNA.

Except for the 2″ OH, which is not modified or important for binding to RNA in the 4,5 aminoglycosides, the modified positions occur on the A and B rings. Also, the modification of the 1-amine position on the semi-synthetic aminoglycosides does not appear to interfere with (hydrogen) binding to RNA (and hence there are no common resistance enzymes with this ability), but the extra group does interfere with binding to some of the enzymes. Therefore, important groups for binding in both the 4, 5 and 4, 6 disubstituted aminoglycosides appear to be the 6′, 2′, (3′), and 3 positions. Recent studies have shown that the A and B rings appear to be the most important rings involved in binding to the aminoglycoside modifying enzymes due to less flexibility of these rings when bound and also similarities of the conformations of these rings when bound to different enzymes (Serpersu *et al.,* 2000; Ekman *et al.,* 2001). Conformations of aminoglycosides bound to APH3′-IIIa, ANT2″-1a, and AAC6′-Ii have already been determined and published (Ekman, *et al.*, 2001; Cox, *et al.,* 2000; Cox & Serpersu, 1995; Cox & Serpersu, 1997; Mohler, *et al.*, 1998; DiGiammarino *et al.*, 1998). However, no conformations of antibiotics while bound to an enzyme that modifies the center ring had yet been done. Therefore, AAC3-IIIb was selected to fill in this gap in knowledge.

The purpose of studying the aminoglycoside modifying enzymes and in particular, the conformations of bound substrates, is to understand more fully the method by which the enzymes bind and catalyze the reaction that renders the antibiotics inoffensive. By studying this, it is hoped that inhibitors of these enzymes can be designed through a rational drug design approach. Structure based rational drug design is becoming a greater technique whereby new antibiotics can be made that have no previous existence in nature, and therefore, hopefully, no enzymes that can confer resistance to them. The basic method of doing this is by the following steps: 1) Identifying the target of interest, 2) Determine the 3-dimensional structure of the target and/or its ligand (or substrate), 3) Predict compounds that should have the appropriate chemical and conformational requirements to binding the active site, 4) Test the compounds for biological activity (Bohacek *et al.,* 1996; Greer *et al.,* 1994). Different methods can be used to determine these 3-dimensional structures, such as crystallography or NMR. Basically, either the structure of the macromolecule must be known, at least the active site, or the structure of the ligand when bound must be known.

Four aminoglycoside modifying enzymes have been crystallized to date. These are the following: APH3′ (Hon *et al.,* 1997), AAC6′ (Wybenga-Groot *et al.,* 1999), ANT-4′-4″ (Sakon *et al.,* 1993), and AAC3 (Wolf *et al.,* 1998). However, only one of these (ANT-4′-4″) has been crystallized with the aminoglycoside substrate. Also, with this one, it was not possible to determine the orientation of the substrate within the active site, probably due to the dual modification sites (Sakon *et al.,* 1993; Penderson *et al.,* 1995). The precise active sites of the other enzymes are not known well either due to the absence of both substrates. Therefore, a study of the conformation of the aminoglycoside

substrate when bound using NMR proves useful when studying these enzymes and should be useful for inhibitor designs. Another possible future method may be to determine the active site using NMR. The purpose of the present study was to determine the conformations of a 4,5 and a 4,6 disubstituted aminoglycoside when bound to the enzyme AAC3-IIIb using NMR.

Chapter II.

Cloning, purification and substrate identification of AAC3-IIIb

A. Introduction

1. *Background*

The only previous work done on aminoglycoside acetyltransferase 3-IIIb (AAC3-IIIb) has been to sequence the gene and to determine the substrate profile to classify it (Shaw *et al.*, 1993). It was classified as an acetyltransferase that modifies on the 3-amine position, and is within substrate profile group III. It was the second enzyme found in this group so it is given the letter 'b' at the end to signify it as an isozyme. We were given a clinical isolate containing the gene for AAC3-IIIb from the authors of this original study.

The goal of this study was to clone, express, purify and then study this enzyme using NMR, specifically to determine substrate conformations. The reason why this enzyme was chosen is that previous studies, from this laboratory, have determined the substrate conformations of aminoglycosides bound to enzymes that modify either the A or the C rings (Cox & Serpersu, 1995; Cox & Serpersu, 1997; Mohler, *et al.*, 1998; DiGiammarino *et al.*, 1998 Cox, *et al.*, 2000; Ekman, *et al.*, 2001). AAC3-IIIb is different in that it modifies the center (B) ring of aminoglycosides. This ring is called the 2-deoxystreptamine (2-DOS) ring. Figure 1 shows the chemical structures of some aminoglycosides relevant to this study. The 2-deoxystreptamine ring can be substituted at different positions, most often the 4, 5 and 6 positions. In this study aminoglycosides modified at the 4,5 and 4,6 positions were studied to give a broader view of substrate binding than just using one aminoglycoside.

2. *NMR*

Several NMR experiments have been used with this study. These include 1D and 2D NMR. All of these were proton NMR experiments, although other atoms can be detected. The simplest NMR experiment is the one dimensional proton detection experiment. This consists of a 90° pulse, which flips the magnetic spin on the protons to a right angle to the external field (600MHz in this case). Upon relaxation, the chemical shifts of these protons are detected. Each proton has a chemical shift which is dependent upon its intrinsic chemical (and electro-magnetic) environment.

Two dimensional experiments are used to detect interactions between two protons. The diagonal of a 2D proton to proton experiment is simply the 1D spectrum. A simple 2D NMR experiment is correlation spectroscopy (COSY). This detects through-bond coupling interactions between protons. An interaction will be shown as a crosspeak where one chemical shift of it will be the chemical shift of one interacting proton, and one will be the chemical shift of the other. Because COSY only tends to detect interactions through three bonds (H-C-C-H), total correlation spectroscopy (TOCSY) can be used to detect farther interactions. By increasing the time that magnetization transfer is allowed to occur (mixing time), the interactions can be followed through longer distances. A third very useful 2D NMR experiment is nuclear Overhauser effect spectroscopy (NOESY). The nuclear Overhauser effect is a transfer of magnetization from one nucleus to another nucleus through space when they are close. Two protons may not be close in terms of chemical bond distance but may be close through space due to molecular conformation. In order to measure this, three 90° pulses are used, separated by two time periods. After the first pulse, the time period is called the evolution time,

when the magnetizations are allowed to precess to back towards the initial state, but will not usually reach that state and different nuclei precess at different rates, and different times are used to get the second dimension. A second pulse is then applied which changes the direction of the spins again. This is followed by a mixing time, which is the same within one experiment, which allows the magnetization transfer to occur. The last pulse redirects the spins again, and immediately after, the free induction decay is collected. Because one nuclei transfers magnetization to another, this will be detected as well. Again, as in COSY and TOCSY these are detected as crosspeaks in the 2D spectrum.

B. Experimental Procedures

1. *Materials*

Growth media (Yeast extract, tryptone) was from Difco. The plasmid pET-22b+, Deep Vent DNA polymerase were from New England Biolabs. Restriction enzymes, and T4 DNA ligase were from Promega. Plasmid extraction kits were from Promega (Wizard Minipreps). isepamicin was a gift from Karen Shaw (Schering Plough) and G. Miller (formerly Schering Plough). D2O was from Cambridge Isotope Laboratories. All other chemicals used below were from Sigma.

2. Bacterial Strain

A clinical isolate of *Pseudomonas aeruginosa* was obtained from Dr. Karen Shaw of Schering Plough. The isolate contained resistance to aminoglycoside antibiotics with the gene AAC3-IIIb (henceforth referred to as AAC3).

3. Cloning

The R-plasmid containing the AAC3 gene was isolated from the clinical isolate using a standard kit (Promega) with no modifications in protocol. The gene was amplified by PCR and was cut with restriction endonucleases (*Nde*I and *Not*I) allowing the gene to subsequently be put in correct orientation into the plasmid pET-22b+ (which was cut with the same enzymes), and T4 DNA ligase was used to ligate the fragment into the vector. The presence and orientation of the gene was confirmed by sequencing analysis. *E. coli* strain BL21(DE3) was transformed with the pET22b-AAC3 plasmid, and the bacteria containing the plasmid was selected for on media containing kanamycin and ampicillin (25mg/L each).

4. Protein purification

The transformed cells were grown in LB media (37°) containing ampicillin and kanamycin (25mg/L each) to ensure selection for transformed cells and at approximately 0.4-0.6 O.D. the culture was induced with 3mM IPTG to activate the T7*Lac* promoter, thus overproducing the AAC3 gene product. Cells were allowed to grow overnight and were then spun in a centrifuge (6000g, GS-3), washed with STE (NaCl 50mM, TrisHCl 50mM, EDTA 5mM, pH 7.5), centrifuged again at 28000g (SA600), and then lysed using a French Press. The cell lysate was centrifuged at 28000g (SA600), and the pellet and supernatant were separated. The pellet contained AAC3 protein in inclusion bodies in

relatively large amounts compared to other proteins as visualized on SDS-PAGE (Figure 2). Therefore, this was used for subsequent purification.

Inclusion bodies were homogenized and washed with 1% Triton X-100 (v/v) in 50mM sodium phosphate buffer (pH 7.5) three times, and then washed three times in 50mM sodium phosphate buffer (pH 7.5) alone, with centrifuge spins at 28000g (SA600) after each wash. The pellet was then solubilized in 8M urea (pH 8.0), spun at 10000g (1.5 mL microfuge) to remove insoluble material, and dialyzed against 200mL of 5mM sodium phosphate buffer (pH 7.5), 1mM $MgCl_2$ and 5mM NaCl. After the first dialysis, DNase and RNase (0.1mg) were added. The enzyme solution was then dialyzed 3 more times against the same buffer. After the third dialysis, the enzyme solution was lyophilized. After this, protein was resolubilized in deuterated 5mM sodium phosphate buffer and purity was visualized by SDS-PAGE (figure 2). Also, protein concentration was determined using a HP Diode-array spectrophotometer, measured at 280nm with an extinction coefficient of 1.4 $M^{-1}cm^{-1}$). A gel analysis computer (LKB 2400 Gelscan) was used to determine purity of the protein, which was >85% of the total final protein based on densitometry.

5. Activity assay

A modification to a protocol used to determine activity of other acetyltransferases was used for assessment of AAC3 activity (Wright & Ladak, 1997; Williams & Northrop, 1978). AAC3 catalyses the exchange of an acetyl group from acetyl CoA to the 3-amine position on the center ring. In this activity assay, 4,4′-dithiodipyridine is

used to react with the free SH that is on Coenzyme A after the reaction occurs. The product, thiopyridine, absorbs light at a max of 324nm, with an extinction coefficient of 19,800$M^{-1}cm^{-1}$. In the reaction mixture were the following: 0.75mM 4,4'-dithiodipyridine, 25μM kanamycin, 25μM acetyl CoA, 25mM TrisHCl (pH 7.5), 0.5mM EDTA, with a final total volume of 1mL. The absorbance at 324nm is measured with a Beckman Du70 spectrophotometer, and the reaction is measured continuously. A range of substrate concentrations were used to determine kinetic parameters. Using the extinction coefficient of thiopyridine, the concentration of free Coenzyme A can be calculated based on a one to one ratio for this titration. Free Coenzyme A also equals the amount of modified aminoglycoside because the transfer of the acetyl group from acetyl CoA to the aminoglycoside is also one to one. Therefore, measuring the absorbance of the reaction at 324nm as the reaction occurs allows a quick way of measuring product formation, using a simple conversion where the absorbance is divided by 19.8 to give μmoles of product formed. Dividing by the time (minutes) and then by the protein concentration in mg gives the specific activity (S.A.) in μmol/min./mg. For AAC3 with the above concentrations of substrates (50μM each) this gives a S.A. of ~4 μmol/min/mg. Also, ribostamycin was substituted for kanamycin to determine the enzymatic activity when this aminoglycoside was present.

6. Deuteration for NMR

Deuteration of exchangeable protein protons was accomplished by a series of solubilizations in D_2O (with 5mM $NaPO_4$ buffer, pH 7.5 and 2mM $MgCl_2$) and

lyophilizations. 1D NMR showed the H_2O peak to be sufficiently low to allow subsequent experiments to be performed without interference from H_2O.

*7. **Product verification***

Acetyl-CoA and kanamycin were put into an NMR tube at high concentrations (2mM) in D_2O with 50mM sodium phosphate buffer and 5mM $MgCl_2$. All NMR experiments were done on a 600MHz Varian Inova instrument equipped with a single gradient axis and a triple resonance probe for the observation of proton, carbon, and nitrogen nuclei. For 2D NMR a total of 228-256 FIDs of 1408 complex data points were collected. The spectral width was 5479Hz, and 80-128 scans per FID were acquired. 1D and 2D NMR spectra (NOESY 400ms, TOCSY 90ms) were done. Then a small amount of AAC3 was added to the tube and the reaction allowed to proceed for four days. After this 1D and 2D spectra (NOESY 400ms, TOCSY 90ms) were again preformed to verify that the kanamycin was modified at the correct position. NMR data was analyzed with NMR data analysis software (Felix 95.0 or Felix 2000, Biosym) on a Silicon Graphics Indigo 2 workstation. The data was multiplied by a sine-squared window function in both dimensions before Fourier transformation. Similar parameters for NMR experiments were used later for the substrate conformation studies.

C. Results/Discussion

1. Cloning and Purification

As previously mentioned, AAC3 was successfully cloned and the protein product enriched to approximately 85-90% purity as can be seen on SDS-PAGE (figure 2) and as measured directly by a gel analyzer (LKB 2400 Gelscan). The use of the pET22-b+ plasmid for expressing large amounts of protein was successful, and about 20mg of enriched protein could be prepared from one liter of LB broth. The protein was in high concentrations in the pellet (inclusion bodies) and in the supernatant. However, because the inclusion bodies had much fewer other proteins the pellet was used for subsequent purification. Also, inclusion body purification is much more efficient in that it requires fewer steps, less time, and had greater ease of purification than a purification from the supernatant would require. AAC3 does have a need to keep the pH above 7.0. Any time that the pH drops below 7.0, AAC3 falls out of solution and will fail to be able to regain activity. Thus, in all parts of the procedure, buffers were used to keep the pH at 7.5. Also, phosphate buffer was used so that interference from buffer hydrogens would not be present in the NMR.

For activity parameter measurements, kanamycin was added last so that any background activity could be subtracted from the total. Activity measurements showed that the enzyme was active with a specific activity of about 5 units/mg of protein when using kanamycin. One unit equals 1 μmole of product produced per minute. The k_{cat} was

1.95 s^{-1} which is faster than AAC6 but within the range (0.1-100 s^{-1}) measured for other aminoglycoside modifying enzymes (Gates and Northrop, 1988). The Km for kanamycin was determined to be 14 +/- 2.8 μM and for ribostamycin Km is about 26 μM. In the table below are approximate values for Km for different substrates in μM.

Lividomycin	Paromomycin	Butirosin	Ribostamycin	Amikacin	Kanamycin	Isepamicin	Acetyl CoA
9	6	180	26	370	14	500	94

Figure 3 shows a kinetic plot for kanamycin with varying concentrations of acetyl CoA which was used to calculate the Km for kanamycin and acetyl CoA.

2. Product Verification

The reaction that AAC3 catalyses is the conversion of an aminoglycoside to an acetylated product using acetyl-CoA as an acetyl donor. The modification is made at the 3-amine position on the center (B) ring. This has been based upon profiles of antibiotic resistance. However, some aminoglycoside modifying enzymes have been shown to modify at more than one position, so it was checked to see if AAC3 did this as well. It was found through NMR that only the B ring 3-amine position was modified, within the limits of the NMR spectroscopy under the reaction conditions given. Any other product that might be present would represent less than 5% of the main product. It was found through 1D NMR that the acetyl group was shifted from 2.28 to 1.89 ppm. Figure 4 shows a comparison between the initial acetyl CoA, kanamycin mixture, and the mixture after catalysis was allowed to occur. The 1D NMR shows that the initial position of the acetyl group (peak A) shifts to another position (peak B) with apparently none left in the

original position, showing that the reaction went to completion. Also, several other peaks shifted as well. This indicates a change in the chemical composition of the molecules. This change is confirmed to be the expected position with 2D NMR techniques. 2D NMR verified the major component of the acetyl group shift to be the B ring 3-amine position on kanamycin A, shown by the presence of NOEs to other parts of the aminoglycoside, and a very large downfield shift of the hydrogen at position 3 on the center ring from 3.06 to 3.8 ppm, showing the deshielding effects of the carbonyl group. On Figure 3 it can also be seen that another peak (C) shifts from its position at 2.95 ppm to peak D at 2.75 ppm. This shift corresponds to the known positions for this peak in acetyl CoA and CoA respectively which is a CH_2 group on the pantothenic acid tail.

Chapter III.

Substrate conformations of aminoglycosides bound to AAC3-IIIb

A. Introduction

The ability to obtain substrate conformation on an enzyme with more hydrogens than the substrate and still observe the substrate hydrogens requires a special method. This is called transferred nuclear Overhauser effect spectroscopy (TRNOESY). The purpose of TRNOESY is to detect through space magnetization transfers (cross relaxation) between nuclei on the ligand or substrate (Clore & Gronenborn, 1982; Ni & Scheraga, 1994). This method has been used for a wide variety of protein-bound ligands, including antigens with antibodies, nucleotides with regulatory proteins, peptides with phospholipids, hormones with receptors, and substrates with enzymes (Campbell and Sykes, 1991). It is especially useful when the substrate cannot be co-crystallized with its enzyme, as is the case with the aminoglycoside modifying enzymes with aminoglycosides to date, except for ANT4′-4″(Sakon *et al.,* 1993). The intensity of the crosspeaks observed in 2D TRNOE spectra can be measured, and are inversely related to the sixth power of the distance between the two interacting nuclei, and therefore they are used to estimate distances between the hydrogens with which the NOEs are associated.

The reason this method works is that small molecules have a much shorter correlation time (~10^{-10}s) than much larger proteins (>10^{-8}s). Correlation time is a measure of the tumbling rate of a molecule in solution where it equals the amount of time it takes for a molecule to rotate through one radian (Sanders & Hunter, 1992). This will

obviously vary based on such things as size, shape, and viscosity. The importance of this is that short correlation times are characterized with slow cross relaxation rates and longer correlation times are characterized with shorter cross relaxation. Thus when the experiments are actually done, NOEs can be detected with a bound substrate with a much shorter mixing time (duration allowed for magnetic exchange) than with a substrate free in solution. Also, the NOEs for the bound state give crosspeaks of opposite phase than NOEs gained from those free in solution. Therefore, no information from the substrates free in solution are obtained when using the shorter mixing times used in the TRNOESY experiment.

The other requirement for this method is that the small molecule must be in fast chemical exchange with the protein to which it binds. This may seem to negate the effect mentioned in the above paragraph, but the molecules that have been bound to the protein and undergo magnetization transfer keep that information provided that the spin lattice relaxation is slow with respect to the exchange rate. The process just mentioned is necessary because the protein has many more hydrogens than the small molecule and so if they were in a one to one complex, the proton signals of the small molecule would be buried under the large signals of the protein protons. Thus the smaller molecule is in large excess (~10X) of the protein, and if fast chemical exchange compared to spin lattice relaxation occurs, this allows information about the bound state to be collected, though most of the molecules will be free in solution.

One problem with TRNOESY is that protein hydrogens near the substrate hydrogens can interfere with magnetization transfer between substrate hydrogens. They may cause facilitated magnetization transfer by an indirect route so that the distance

between two protons appears much shorter than it really is. This is caused by transfer of magnetization of a protein hydrogen from one substrate hydrogen to another one, which is called spin diffusion. A method for minimizing the effect of spin diffusion is to use a pulse sequence known as QUIET-NOESY (Vincent *et al.*, 1997). The QUIET-NOESY sequence selectively inverts selected regions of the spectra halfway through the mixing time. The result of this is that the direction of magnetization flow through selected hydrogens is reversed halfway through the mixing time, and this results in a complete cancellation of the effect of spin diffusion caused by the hydrogens outside of the selected area. Thus their contribution to the observed transfer of magnetization is effectively eliminated.

B. Materials/Methods

1. NMR

Sample was prepared as mentioned in Chapter II under "Protein Purification" and "Deuteration for NMR." Coenzyme A and an aminoglycoside were titrated in an NMR tube with 250 μM protein so that the substrate protons became visible above the proton signals of the protein on 1D H-NMR. The final concentrations of CoA and aminoglycoside were equal to one another and in 10 times excess of the protein (2.5 mM each). Three aminoglycosides were used in these studies: isepamicin, kanamycin, and ribostamycin. All NMR experiments were done on a 600MHz Varian Inova instrument equipped with a single gradient axis and a triple resonance probe for the observation of proton, carbon, and nitrogen nuclei. For 2D NMR a total of 228-256 FIDs of 1408

complex data points were collected. The spectral width was 5479Hz, and 80-128 scans per FID were acquired. Several NMR experiments were done. First, 1D NMR was used upon addition of substrates. Then a series of 2D experiments were performed. These are as follows: TOCSY at 60 and 90 ms mixing times, TRNOESY at 60, 90, and 120 ms mixing times, and QUIET-NOESY at 90 and 120 ms mixing times. For QUIET-NOESY, a Gaussian Cascade Q3 pulse was used in the middle of the mixing period, selectively inverting portions of the spectrum. Enzymatic activity was tested before and after the experiments, and no significant loss was found after the experiments were done.

2. Data analysis

NMR data was analyzed with NMR data analysis software (Felix 95.0 or Felix 2000, Biosym) on a Silicon Graphics Indigo 2 workstation to determine position and size of crosspeaks. The data was multiplied by a sine-squared window function in both dimensions before Fourier transformation. The assignments of the peaks of kanamycin and ribostamycin were from previous studies (Cox & Serpersu, 1994; Cox *et al.*, 2000). Crosspeak intensities observed in NOE experiments were divided into Strong, Medium, and Weak, and were used to determine distance restraints to be used in structure determination. Peak strengths were based upon a comparison to the crosspeak volume between the hydrogens at positions 1 and 2 on the A ring which has a known distance (H1A to H2A distance= 2.38 Å) and divided into relative strength categories. Inter-proton distance restraints used were as follows: strong: 2.0-2.7, medium: 2.0-3.6, weak: 2.0-4.5 Å. Using a structure analysis and dynamics program (Insight II 95, Discover 95,

Biosym/Molecular Simulations), NMR derived restraints were applied to the aminoglycosides and a series of dynamics and minimization steps were performed. First, random structures were created by doing unrestrained molecular dynamics at 600K and recording a structure every 10000 iterations. Because there was a wide variation in the conformations obtained, these were considered random. Then the NMR derived distance restraints mentioned above were applied and a series of dynamics used decreasing temperatures 400K, 350K, 300K, 200K for 500 ps, each followed by a minimization (until RMS <0.01 kcal/Å) to simulate annealing. The force constant for the distance restraints was 50 kcal/mol $Å^{-2}$. The dielectric constant used in the above experiments was 4.0. Using this simulated annealing method 20 or more structures that are consistent with the NOE derived distances were obtained, and the AAC3-bound structures of the aminoglycosides kanamycin and ribostamycin were determined by superpositioning of these structures.

C. Results/Discussion

1. NMR analysis

Isepamicin was the first aminoglycoside that was used to try to get a substrate conformation. While isepamicin would seem to be an ideal choice for structure determination when bound to AAC3, because of previous studies done with other enzymes. However, as this work showed, this was not possible with AAC3. The conformation of isepamicin bound to APH3, AAC6, and ANT2 have been determined

previously (Cox *et al.*, 2000; Digiammarino *et al.*, 1998; Ekman *et al.*, 2001). However, upon analysis of the NMR data with AAC3, it was determined that the limited number of NOEs (a total of 12) and weakness of them, especially interring NOEs, would not allow sufficient restraints to be imposed for a reasonably good structure determination. Strong NOEs and higher numbers of restraints allow for a good structure determination, but with isepamicin this did not seem feasible. However, the two other aminoglycosides used (kanamycin and ribostamycin) did give enough NOEs (38 and 18 respectively). The reason for this probably has to do with the strength of binding. Isepamicin may not bind as well, prohibiting it from forming NOEs that will be detectable by the NMR instrument, due to the randomness of the molecular movements. This is reflected in the higher Km (~500μM) for isepamicin, which shows that it binds to the enzyme weakly. The most likely reason for this is that isepamicin has a 3 carbon substituted side chain ((S)-3-amino-2-hydroxypropionyl) on the 1 position of the center ring. It is this group that allows isepamicin to be a useful antibiotic where other aminoglycosides fail, by preventing binding of the drug to some aminoglycoside modifying enzymes. This group prevents or slows down binding of isepamicin to aminoglycoside binding enzymes preventing them from modifying the antibiotic, probably mainly through steric hindrance. The group does not prevent binding of isepamicin to RNA so that it is still useful as an antibiotic. This may be confirmed in the kinetics data for the aminoglycosides in that isepamicin has a much lower reaction rate than kanamycin or ribostamycin. It may also be surmised that there is an increasing selection pressure on the bacteria to have only aminoglycoside modifying enzymes that bind the minimal structure of aminoglycoside

that can bind to RNA, due to the increase of aminoglycosides with extra substituents attached.

Unlike isepamicin, the conformations of kanamycin and ribostamycin were determined. As mentioned above, several different NMR experiments were used to do this. Comparison of TRNOESY spectra with Quiet-NOESY is intended to rule-out the interference of spin-diffusion by protein protons that are near the aminoglycoside protons. Because none of the crosspeak volumes were affected to the point that they were put into a different strength grouping (strong, medium, or weak), this shows that spin diffusion effects were minimal and not significant. An example of the crosspeak comparisons of the TRNOESY and QUIET-NOESY spectra for both kanamycin and ribostamycin is given in figures 5 and 6, showing some important NOEs observed. The most important NOEs are those that are inter-ring. Inter-ring NOEs that were used to determine the structure of ribostamycin are as follows: H1C-H5B and H1A-H5B; and for kanamycin: H1C-H6B and H1A-H3B.

2. Ribostamycin conformations

A total of 22 acceptable structures were obtained for ribostamycin. The structures were divided into two primary conformers. The structures were divided based on the measured differences in the glycosidic dihedral angles defined as the following: Φ_{1A} (H1′-C1′-Oα-C4), Ψ_{1A} (H4-C4-Oα-C1′), Φ_{1C} (H1″-C1″-O$_\beta$-C5), and Ψ_{1C} (H5-C5-O$_\beta$-C1″). Using these angles, the structures fell into two conformations (Conformer 1 with 11 structures and Conformer 2 with 11 structures). The average angles (+/- S.D.) for

Conformer 1 of ribostamycin are as follows: Φ_{1A} = -22 +/- 3, Ψ_{1A} = -42 +/- 1, Φ_{1C} = -9 +/- 4, Ψ_{1C} = 51 +/-1 and for Conformer 2: Φ_{1A} = -67 +/- 0.7, Ψ_{1A} = -59 +/- 0.8, Φ_{1C} = -9 +/-3, Ψ_{1C} = 49 +/- 1. It can be seen from these angles and from overlaying the structures on the B ring that there are two conformations (see figure 7 and also figure 8 with overlaid structures). Figure 8 shows that even by visual inspection an overlay of all 22 structures puts them into two conformations. Figure 9 shows two stereo views of two of these structures, one from each conformation. However, all of the structures have the same conformation for the C ring based on the phi and psi angles. By overlaying the A and B rings of ribostamycin it does appear that the two conformations are similar (though not identical) with an RMSD of 0.546 (figure 9, top). However, by overlaying the B and C rings, it can be seen that the conformation of the B and C rings of ribostamycin is identical for both conformers with an RMSD of 0.102 (Figure 9, bottom). Thus it is the A ring that divides these structures into two conformations. This may suggest that the A ring is more flexible when bound to enzyme, while the C ring is more constrained when bound. The inability for the C ring to move while bound seems unlikely due to the fact that while the A and B rings are the basic structure of aminoglycosides, the C ring varies greatly, and may even be at different positions on the B ring (4 or 5 position). This means that aminoglycoside modifying enzymes like AAC3 must be able to bind a variety of C rings, and may do this in a number of ways. One way might be to leave the C ring not bound to the enzyme, which would probably allow the ring to rotate freely. This is not what is seen here. Another way for enzymes to bind the extra ring would be to provide a number of different binding sites (and various amino acid side chains) allowing many different types of rings to bind. This may allow rings to be bound in a large

number of orientations, but also may provide a highly stable binding site for some aminoglycosides. This may be what is occurring here. That is, perhaps ribostamycin happens to fit into a binding site well, allowing it to bind with strong interactions, keeping the ring constrained. This may actually be due to steric hindrance rather than binding of specific groups. The fact that the A ring appears more flexible may indicate that this ring may not be as vital for binding, which is unlikely, or at least not bind as tightly as the C ring, which seems more reasonable. This is more reasonable because the A and B rings are the basic aminoglycoside structure necessary for catalysis for a number of aminoglycoside modifying enzymes, and are the most similar rings between different ones as well. Because of this, the A and B rings alone should be necessary for efficient binding and bind in an adequate way for the enzyme to catalyze the reaction. However, because different functional groups may be present on the A ring and the B ring, there is also a need to provide some flexibility in binding and that may be what is being seen here with the greater number of conformations for these rings. It is also possible that both structures represent structures that can bind and catalyze sufficiently, because the center ring is the modified ring, and the enzyme may be able to bind more than one orientation for the other rings. However, the data cannot exclude the fact that one of the orientations may be consistent with the NOE restraints but may not occur in the active site.

3. Kanamycin conformations

For kanamycin, 20 acceptable structures were obtained. kanamycin, unlike ribostamycin had a greater number of NOEs but also a greater number of conformations. This shows that the number of NOEs is not necessarily indicative of how many structures there will be after the restrained simulated annealing procedure. On the basis of the Phi-Psi angles, the structures were divided into three conformers. The average dihedral angles (+/- S.D.) are as follows: for Conformer 1 (9 structures): Φ_{1A} = -11 +/- 3, Ψ_{1A} = -45 +/- 3, Φ_{1C} = -9 +/- 4, Ψ_{1C} = 50 +/- 2; for Conformer 2 (6 structures): Φ_{1A} = -8 +/- 3, Ψ_{1A} = 48 +/- 2, Φ_{1C} = -18 +/- 2, Ψ_{1C} = -42 +/-2; and for Conformer 3 (5 structures): Φ_{1A} = -73 +/- 0.6, Ψ_{1A} = -57 +/- 0.2, Φ_{1C} = -14 +/- 0.5, Ψ_{1C} = 51 +/- 0.2 (figure 10).

A comparison of Conformers 1 and 2 of kanamycin overlaying on the center (B) ring shows that the conformers appear quite different (figure 11), as the dihedral angles show for both the A and C rings, with an RMSD of 1.2. Overlaying Conformers 1 and 3 shows that the C ring is in the same orientation (figure 11, blue and pink), with an RMSD of 0.031 when overlaying the B and C rings. This is also seen in the comparison of the Phi and Psi dihedral angles for ring C, with Conformer 1 having an average Φ_{1C} = -9 +/- 4, Ψ_{1C} = 50 +/-2 and for Conformer 3 Φ_{1C} = -14 +/- 0.5, Ψ_{1C} = 51 +/-0.2 (figure 10). Adding these together gives 14 out of the 20 that have the C ring in this conformation. This is reminiscent of ribostamycin, which also had the C ring with fewer conformations. It might be deduced from this that the binding site for the C ring on the enzyme allows less flexibility, at least for these two aminoglycosides. Again, this may be due to additional residues used for binding a variety of substrates tightly enough to allow

catalysis to occur, or due to steric hindrance. This varied binding is necessary because the C rings of kanamycin and ribostamycin are significantly different, in that kanamycin has a hexose ring and ribostamycin has a pentose ring.

Nine structures had the A ring in the Conformer 1 (C1) position, 6 had ring A in the conformer 2 (C2) position, and 5 had it in the Conformer 3 (C3) position. As mentioned previously, it has been thought that the A and B rings are the most important rings for binding since they are present in all aminoglycosides and are the basic structure necessary for catalysis in many of the enzymes. Also, previous studies have shown that across a wide variety of aminoglycoside modifying enzymes these rings often have similar binding conformations. However, because there is a greater number of the C1 structures than either of the other two, this may be the actual bound structure. However, because there are a significant number of other conformations (11 total compared to 9 of C1), this may indicate that the A ring does not bind as tightly as the C ring which appears more constrained. The C2 and C3 conformations could represent a poorly binding structure, and the C1 the actual catalytic state. Also, because the modification of the aminoglycoside occurs at the center (B) ring, it may be that any of the structures can bind and undergo catalysis effectively, allowing the A ring to move more freely. Also, the other conformations could represent structures that satisfied all distance restraints but does not exist in the bound state. Additionally, it may be that there were simply not appropriate number and/or strength of NOEs to determine the structure adequately. This may have been due to residues on the enzyme interfering with NOEs.

4. Comparisons between ribostamycin and kanamycin conformations

Conformer 1 of kanamycin and conformer 1 of ribostamycin both have very similar conformations of the A and B rings which can be seen in figure 12 (top) and by the dihedral angles. ribostamycin has angles of Φ_{1A} = -22 +/- 3, Ψ_{1A} = -42 +/- 1, and kanamycin has angles of Φ_{1A} = -11 +/- 3, Ψ_{1A} = -45 +/- 3. An RMSD value of 0.15 when overlaying these two rings on the different aminoglycosides confirms this. However, when comparing Conformer 1 of kanamycin or ribostamycin, with Conformer 2 of the other or by comparing Conformer 2 of both, there is little similarity (RMSD >0.5). Because C1 of ribostamycin and C1 of kanamycin are the most numerous of all of the structures for both aminoglycosides, this may indicate that with regards to the orientation of the A and B rings, C1 of kanamycin and C1 of ribostamycin would be the most accurate bound conformation. This also shows that using multiple substrates on one enzyme using NMR may give a better indication of the true bound structure. Conformer 3 of kanamycin and conformer 2 of ribostamycin are also very similar with an RMSD when overlaying the A and B rings of 0.028 (figure 12, bottom). Due to the small number of C3 kanamycin structures, this may not be significant, but it may represent another bound structure. Because the C ring of ribostamycin is at the 5 position on the B ring and in kanamycin is at the 4 position, as well as the rings being quite different themselves, this does not allow a direct comparison of the conformations of these. However, because the C rings of both ribostamycin and kanamycin are more constrained, this suggests that the active site of AAC3 for this ring is more flexible and allows good complementarity for both substrates.

5. Comparisons to other enzyme bound aminoglycosides

It has previously been reported in our laboratory that the A and B rings may be the rings important for binding, based on similarities between bound conformations on different enzymes (Serpersu *et al.*, 2000; Ekman *et al.*, 2001). Therefore comparisons have also been done between these two aminoglycosides bound to AAC3 and aminoglycosides bound to other enzymes.

It can be seen that when centered on the B and C rings, few of the aminoglycosides show similarities to the conformations found with AAC3. For example, AAC6 bound isepamicin conformer 1 has an RMSD of 0.489 when these rings are overlaid with AAC3 bound kanamycin. isepamicin conformer 2 of AAC6 does not show similarity to AAC3 bound kanamycin C1 (RMSD = 0.609), though it is similar to the C3 of AAC3 bound kanamycin (A and B rings). However, when AAC6 bound isepamicin C1 (figure 11) and other enzyme bound aminoglycoside conformations are compared to conformer 1 of kanamycin and conformer 1 of ribostamycin overlaying the A and B rings, it can be easily seen that the structures appear similar. See figure 13 with AAC6 bound isepamicin (RMSD = 0.064), ANT 2 bound isepamicin (RMSD = 0.131), APH3 bound isepamicin (RMSD = 0.175), and APH3 bound ribostamycin (RMSD = 0.0716). RMSD values shown are for the A and B rings overlaid, and confirm that these conformations are similar. It is very interesting and possibly quite significant that the structures across a wide range of enzymes are so similar.

6. Comparison to RNA bound aminoglycosides

The RNA bound conformations of gentamicin and paromomycin show a similar orientation of the A and B rings as well. When compared to the kanamycin and ribostamycin Conformer 1, there is a good similarity between the A and B rings but not the C rings (figure 14). Again, this leads to the conclusion that the A and B rings, because they are the minimal aminoglycoside structure may be the rings most important for binding across a broad spectrum on enzymes and even RNA. Figure 15 shows the combination of all of the similar enzyme bound structures, as well as the RNA bound structures. A comparison of these structures has been presented previously (Ekman *et al.*, 2001; Serpersu *et al.*, 2000). The reasons why these are so similar are unknown. It is known that the A and B rings make the most important contacts between the RNA and the aminoglycosides. In particular, the 2′, 6′, and 3-amine positions are thought to provide important hydrogen bonds or ionic interactions with phosphates in the RNA strand of the 16S rRNA (Fourmy *et al.*, 1996; Yoshizawa *et al.* 1998). These are modification sites for some resistance enzymes. The C (double prime) rings of 4,5 and 4,6 substituted aminoglycosides bind in different ways but may also have important contacts (Fourmy *et al.*, 1996; Yoshizawa *et al.* 1998). It is thought by analogy and because this is the minimum aminoglycoside structure that the same functional groups are responsible for binding to the enzymes as well (Serpersu *et al.*, 2000).

Chapter IV.

Conclusion and future studies

These results show that the A and B rings are quite likely the rings important for binding both to enzymes and to RNA. In fact, the A and B rings together form the minimum structure necessary for catalysis for many enzymes. Assuming that the conformation in which the aminoglycosides bind the enzymes is important, this has great implications for future drug design. Because these identical conformations appear across different types of enzymes (APH3′, ANT2″, AAC6′ and AAC3) with different modification sites and different co-reactants, this may allow a drug or drugs to be designed that have a broad spectrum inhibition of aminoglycoside modifying enzymes. The designed drug may also have antibiotic activity by itself. This study has allowed for the final evidence of this in that this was the first B ring modifying enzyme to have its enzyme bound aminoglycoside structures to be determined. Because of this, future work in the laboratory regarding substrate conformations may be focused towards design of inhibitors using the conformations obtained from this and previous studies, as well as focusing on aminoglycoside modifying enzyme structures.

Future work with this enzyme may include many experiments. First, the 3D structure of AAC3 may be determined by NMR using uniformly labeled C13 and N15 protein. Due to the large size of the protein, for NMR work, this may be difficult. To aid in this work, selective isotope labeling of specific amino acid types may be used as well. For example, N15 labeled histidine is available and could be used in growth media to label the enzyme with only N15 histidine. It will also be important to increase the purity

of the enzyme for future work as well. This will probably only require a minimum number of steps because the enzyme is already ~85% pure. Extra purity may allow the crystal structure of the enzyme to be solved as well. Also, any other distance information that can be obtained through other means may be used. Experiments that give information about the active site may also give distance measurements that could be used in overall structure determination.

Several different experiments could be done to determine active site structure. For instance, the use of a spin-labeled coenzyme A may be useful in determining amino acid residues that are close in proximity to this substrate analog, as well as providing information that would allow the arrangement of the substrates (aminoglycoside and CoA) in the active site to be determined. Another useful experiment would be to use N15 or N15 and C13 labeled aminoglycoside (kanamycin or ribostamycin) to determine protein residues that are involved in binding the substrate. If the enzyme binds to a divalent ion in a one-to-one ratio, this would allow a paramagnetic metal ion to be used to determine the residues important for binding this, as well as determining substrate to metal distances. All of these experiments would also be useful in the design of inhibitors to the enzymes as well.

Aminoglycoside modifying enzymes in general are also useful for understanding basic principles in enzyme binding and kinetics, so different experiments could be performed with this in mind too. These enzymes are scientifically interesting and useful in this regard because there are a large number of substrates that are modified by a single enzyme and this would allow individual contributions in binding of functional groups to be measured, for instance. Also, because there are a large number of similar enzymes

with the same or similar substrates, this may allow experiments to be designed to discover the residues important for binding and catalysis. It may also be possible to mutate these enzymes to make one have a different substrate specificity, or even change one to catalyze a different reaction. This would allow delving deep into the fundamentals of ligand binding and enzyme catalysis that may in the future allow for the creation of "designer" enzymes.

LIST OF REFERENCES

References

Abraham, E. P. & Chain, E. (1940). An Enzyme from Bacteria Able to Destroy Penicillin. *Nature* **146**, 837.

Benveniste, R. and Davies, J. (1973). Aminoglycoside Antibiotic-inactivating Enzymes in Actinomycetes Similar to Those Present in Clincal Isolates of Antibiotic-resistant Bacteria. *Proc. Natl. Acad. Sci.* USA **70**, 2276-2280.

Benveniste, R., and Davies, J. (1973). Mechanisms of Antibiotic Resistance in Bacteria. *Annu. Rev. Biochem.* **42**, 471-506.

Bongaerts, G. P. A., & Molendijk, L. (1983). Relation Between Aminoglycoside 2"-*O*-Nucleotidyltransferase Activity and Aminoglycoside Resistance. *Antimicrob. Agents and Chemother.* **25**, 234-237.

Bush, K. (1989). Characterization of β-lactamases. *Antimicrob Agents Chemother.* **33** (3), 259-263.

Campbell, P. A., Sykes, B. D. (1991). Theoretical Evaluation of the Two-Dimensional Transferred Nuclear Overhauser Effect. *Journal of Magnetic Resonance* **93**, 77-92.

Chambers, H. F. (1988). Methicillin-resistant Staphylococci. *Clin Microbiol Rev.* **1** (2), 173-186.

Chen, P. S., Toribara, T. Y., Jr., Warner, H. (1956). Assay of Inorganic Phosphate. *Anal. Chem.* 28, 1756-1760.

Chopra, I. (1992). Efflux-based Antibiotic Resistance Mechanisms: The Evidence for Increasing Prevalence. *J Antimicrob Chemother.* **30** (6), 737-739.

Clore, G. M., and Gronenborn, A. M. (1982). Theory and Applications of the Transferred Nuclear Overhauser Effect to the Study of the Conformations of Small Ligands Bound to Proteins. *Journal of Magnetic Resonance* **48**, 402-417.

Cox, J. R., & Serpersu, E. H. (1994). The Complete 1H NMR Assignments of Aminoglycoside Antibiotics and Conformational Studies of Butirosin A Through the Use of 2D NMR Spectroscopy. *Carbohydrate Research* **271**, 55-63.

Cox, J. R., & Serpersu, E. H. (1997). Biologically Important Conformations of Aminoglycoside Antibiotics Bound to an Aminoglycoside 3'-Phosphotransferases as Determined by Transferred Nuclear Overhauser Effect Spectroscopy. *Biochemistry* **36**, 2353-2359.

Cox, J. R., Ekman, D. R., DiGiammarino, E. L., Akal-Strader, A., Serpersu, E. H. (2000). Aminoglycoside Antibiotics Bound to Aminoglycoside-Detoxifying Enzymes and RNA Adopt Similar Conformations. *Cell Biochemistry and Biophysics* **33**, 297-308.

Davies, J. E. (1986). Aminoglycoside-aminocyclitol Antibiotics and Their Modifying Enzymes, in "Antibiotics in Laboratory Medicine, 2nd ed., " V. Lorian, ed., Williams & Wilkins, Baltimore.

Davis, B. D. (1987). Mechanism of Action of Aminoglycosides. *Microbiol. Rev.* **51**, 341-350.

Davis, B. D., Chen, L. L., Tai, P. C. (1986). Misread Protein Creates Membrane Channels: An Essential Step in the Bactericidal Action of Aminoglycosides. *Proc. Natl. Acad. Sci.* USA **83**, 6164-6168.

Davies, J. & Wright, G. D. (1997). Bacterial Resistance to Aminoglycoside Antibiotics. *Trends in Microbiol.* **5** (6), 234-240.

Davies, J. and Smith, D. I. (1978). Plasmid-determined Resistance to Antimicrobial Agents. *Annu. Rev. Microbiol.* **32**, 469-518.

Digiammarino, E. L., Draker, K., Wright, G. D., & Serpersu, E. H. (1998). Solution Studies of Isepamicin and Conformational Comparisons between Isepamicin and Butirosin A when Bound to an Aminoglycoside 6'-N-Acetyltransferase Determined by NMR Spectroscopy. *Biochemistry* **37**, 3638-3644.

Ekman, D. R., DiGiammarino, E. L., Wright, E., Witter, E. D., Serpersu, E. H. (2001). Cloning, Overexpression and Purification of Aminoglycoside Antibiotic Nucleotidyl Transferase (2")-Ia: Conformational Studies With Bound Substrates *Biochemistry* **40**(24), 7017-24.

Fourmy, D., Recht, M. I., Blanchard, S. C., Puglisi, J. D. (1996). Structure of the A Site of *Escherichia coli* 16S Ribosomal RNA Complexed with an Aminoglycoside Antibiotic. *Science* **274**, 1367-1371.

Georgopapadakou, N. H. (1993). Penicillin-binding Proteins and Bacterial Resistance to β-lactams. *Antimicrob. Agents Chemother.* **37** (10), 2045-2053.

Hayes, J. D., and Wolf, C. R. (1990). Molecular Mechanisms of Drug Resistance. *Biochem J* **272** (2), 281-295.

Homans, S. W. (1990). A Molecular Mechanical Forcefield for the Conformational Analysis of Oligosaccharides: Comparison of Theoretical and Crystal Structures of Manα 1-3 Manβ1-4GlcNAc. *Biochemistry* **29**, 9110-9118.

Hon, W., McKay, G., Thompson, P., Sweet, R., Yang, D., Wright, G., & Berghuis, A. (1997). Structure of an Enzyme Required for Aminoglycoside Antibiotic Resistance Reveals Homology to Eukaryotic Protein Kinases. *Cell* **89**, 887-895.

Jacoby, G. A., and Archer, G. L. (1991). New Mechanisms of Bacterial Resistance to Antimicrobial Agents. *New Engl J Med* **324** (9), 601-612.

Levy, S. B. (1995). In *Antimicrobial Resistance: A Crisis in Health Care* (Jungkind, D. L., Mortensen, J. E., Fraimow, H. S., and Calandra, G. B.) chapter 1, pp 1-13. Plenum Press, New York and London.

Lian, L. Y., Barsukov, I. L., Sutcliffe, M. J., Sze, K. H., Roberts, G. C. K. (1994). Protein-Ligand Interactions: Exchange Processes and Determination of Ligand Conformation and Protein-Ligand Contacts. *Meth. Enzymol.* **239** (C), 657-699.

McKay, G. A., Thompson, P. R., and Wright, G. D. (1994). Broad Spectrum Aminoglycoside Phosphotransferase Type III from *Enterococcus*: Overexpression, Purification, and Substrate Specificity. *Biochemstry* **33**, 6936-6944.

Miller, G. H., Sabatelli, F. J., Hare, R. S., Glupczynski, Y., Mackey, P., Shlaes, D., Shimizu, K., Shaw, K. J., and Aminoglycoside Resistance Study Groups. (1997). The Most Frequent Aminoglycoside Resistance Mechanisms–Changes with Time and Geographic Area: A Reflection of Aminoglycoside Usage Patterns? *Clin. Infect. Dis.* **24**, S46-S62.

Mohler, L. M., Cox, J. R., Serpersu, E. H., (1998). Aminoglycoside phosphotransferase(3')-IIIa (APH(3')-IIIa)-bound conformation of the aminoglycoside lividomycin A characterized by NMR. *Carbohydr. Lett.* **3**, 17-24.

Nikaido, H. (1988). Bacterial Resistance to Antibiotics as a Function of Outer Membrane Permeability. *J Antimicrob Chemother* **22** (Suppl A), 17-22.

Northrop, D. B., and Gates, C. A. (1988). Substrate Specificities and Structure-Activity Relationships for the Nucleotidylation of Antibiotics Catalyzed by Aminoglycoside Nucleotidyltransferase 2"-I. *Biochemistry* **27**, 3820-3825.

Pendersen, L. C., Benning, M. M., & Holden, H. M. (1995). Structural Investigation of the Antibiotic and ATP-Binding Sites in Kanamycin Nucleotidyltransferase. *Biochemistry* **34**, 13305-13311.

Piepersberg, W. et al., (1988). *Actinomycetologics.* **2**, 83-98.

Propst, C. L., and Perun, T. J. (1989). In *Computer Aided Drug Design: Methods and Applications.* Chapter 1, pp. 1-15. Marcel Dekker, Inc., New York and Basel.

Sakon, J., Liao, H. H., Kanikula, A. M., Benning, M. M., Rayment, I., & Holden, H. M. (1993). Molecular Structure of Kanamycin Nucleotidyltransferase Determined to 3.0-A Resolution. *Biochemistry* **32**, 11977-11984.

Sanders, T and Hunter, S. (1992). In *Modern NMR Spectroscopy: A Guide for for Chemists.* chapter 7, pp. 225-226. Oxford University Press, Oxford, New York, and Toronto.

Serpersu, E. H., Cox, J. R., Digiammarino, E. L., Mohler, M. L., Acal, A., Ekman, D. R., Owston, M. (2000). Conformations of Antibiotics in Active Sites of Aminoglycoside-Detoxifying Enzymes. *Cell Biochem. & Biophys.* **33,** 309-321.

Shaw, K. J., Rather, P. N., Hare, R. S., and Miller, G. H. (1993). Molecular Genetics of Aminoglycoside Resistance Genes and Familial Relationships of the Aminoglycoside-modifying Enzymes. *Microbiol. Rev.* **57**, 138-163

Shlaes, D. M., Bouvet, A., Devin, C., Shlaes, J. H., al-Obeid, S., and Williamson, R. (1989). Inducible, Transferable Resistance to Vancomycin in *Enterococcus faecalis. Antimicrob Agents Chemother.* **33** (2), 198-203.

Shimizu, K., Kumada, T., Hsieh, W.-C., Chung, H.-Y., Chong, Y., Hare, R. S., Miller, G. H., Sabatelli, F. J., & Howard, J. (1985). Comparison of Aminoglycoside Resistance Patterns in Japan, Formosa, and Korea, Chile, and the United States. Antimicrob. *Agents Chemother.* **28**, 282-288.

Smith, C., Van Pelt, J. E., Mooberry, E. S., Frey, P. A. (1990). ^{1}H, ^{13}C, and ^{31}P Nuclear Magnetic Resonance Spectral Assignments for Tobramycin, 2"-

(Adenosine-5'-phosphoryl)-tobramycin and 2"-(Adenosine-5'-thiophosphoryl)-tobramycin. *Archives of Biochemistry and Biophysics* **280** (2), 284-291.

Van Pelt, J. E., & Northrop, D. B. (1983). Purification and Properties of Gentamicin Nucleotidyltransferase from Escherichia coli: Nucleotide Specificity, pH Optimum, and the Separation of Two Electrophoretic Variants. *Arch. of Biochem. and Biophys.* **230** (1), 250-263.

Vincet, S. J. F., Zwahlen, C., Post, C. B., Burgner, J. W., Bodenhausen, G. (1997). The Conformation of NAD+ Bound to Lactate Dehydrogenase Determined by Nuclear Magnetic Resonance with Suppression of Spin Diffusion. *Proc. Natl. Acad. Sci. USA* **94**, 4383-4388.

Weiner, S. J., Kollman, P. A., Nguyen, D. T., & Case, D. A. (1986). An All Atom Forcefield for Simulations of Proteins and Nucleic Acids. *J. Comput. Chem.* **7**, 230-252.

Wolf, E., Vassilev, A., Makino, Y., Sali, A., Nakatani, Y, Burley, S. K. (1998) Crystal structure of a GCN5-related N-acetyltransferase: Serratia marcescens aminoglycoside 3-N-acetyltransferase. *Cell* **94**(4) 439-49.

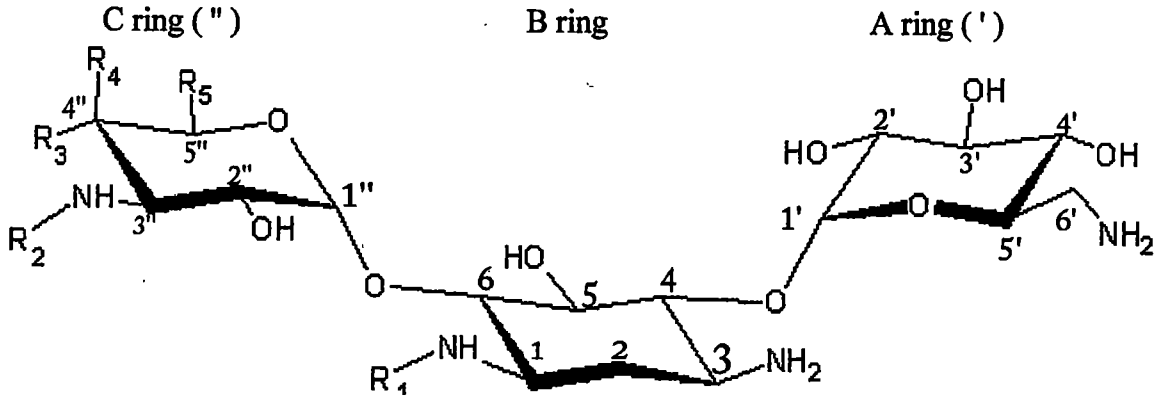
Wolfson, J. S., and Hooper, D. C. (1989). Fluoroquinolone Antimicrobial Agents. *Clin Microbiol Rev.* **2** (4), 378-424.

Wybenga-Groot, L. E., Draker, K., Wright, G. D., Berghuis, A. M. (1999). Crystal strucutre of an aminoglycoside 6'-N-acetyltransferase: defining the GCN5-related N-acetyltransferase superfamily fold. *Structure Fold Des.* **7**(5), 497-507.

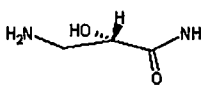
Yoshizawa, S., Fourmy, D., and Puglisi, J.D. (1998). Structural Origins of Gentamicin Antibiotic Action. *EMBO* **17**(22), 6437-6448.

Zwahlen, C., Vincent, S. J. F., Di Bari, L., Levitt, M. H., and Bodenhausen, G. (1994). Quenching Spin Diffusion in Selective Measurements of Transient Overhauser Effects in Nuclear Magnetic Resonance. Applications to Oligonucleotides. *J. Am. Chem. Soc.* **116**, 362-368.

APPENDIX (Figures)



	R1	R2	R3	R4	R5
Kanamycin A	H	H	OH	H	CH2OH
Isepamicin	Y	CH3	CH3	OH	H
Amikacin	Z	H	OH	H	CH2OH

Y= 

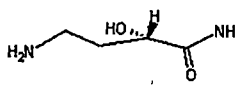
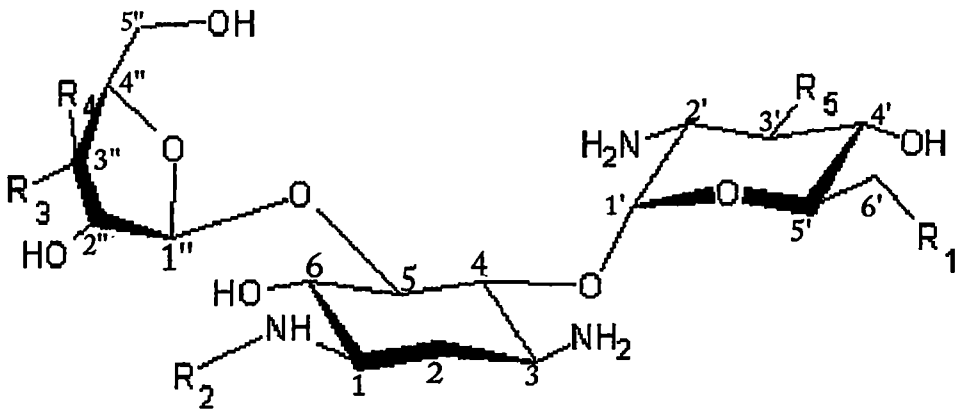
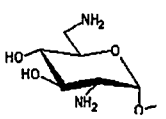
Z= 

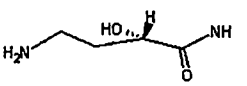
Figure 1. 2-DOS aminoglycosides. A) 4,6 disubstituted.
The center (B) ring is the 2-deoxystreptamine ring. The 4 position is where the A ring attaches and the 6 position is where the C ring attaches. The table below shows different substituent groups that are at different positions for the selected aminoglycosides.

C ring (") B ring A ring (')



	R1	R2	R3	R4	R5
Butirosin A	NH2	Z	OH	H	OH
Ribostamycin	NH2	H	H	OH	OH
Paromomycin	OH	H	H	Y	OH
Lividomycin A	OH	H	X	H	H

Y= 

Z= 

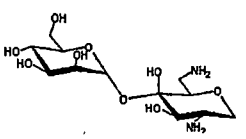
X= 

Figure 1 (continued). 2-DOS aminoglycosides. B) 4,5 disubstituted.
The center (B) ring is the 2-deoxystreptamine ring. The 4 position is where the A ring attaches and the 5 position is where the C ring attaches. The table below shows different substituent groups that are at the different positions for the selected aminoglycosides.

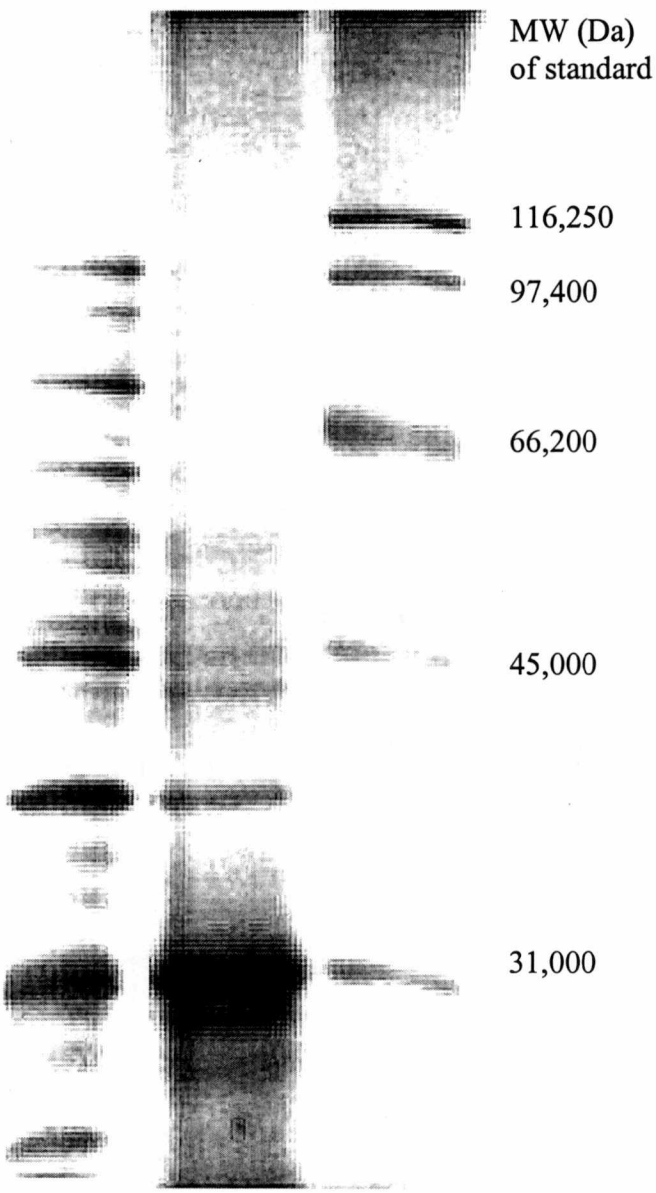


Figure 2: SDS-PAGE (10% SDS) of AAC3 (lane 1: crude pellet, lane 2: final "pure" AAC3, lane 3: MW standard)

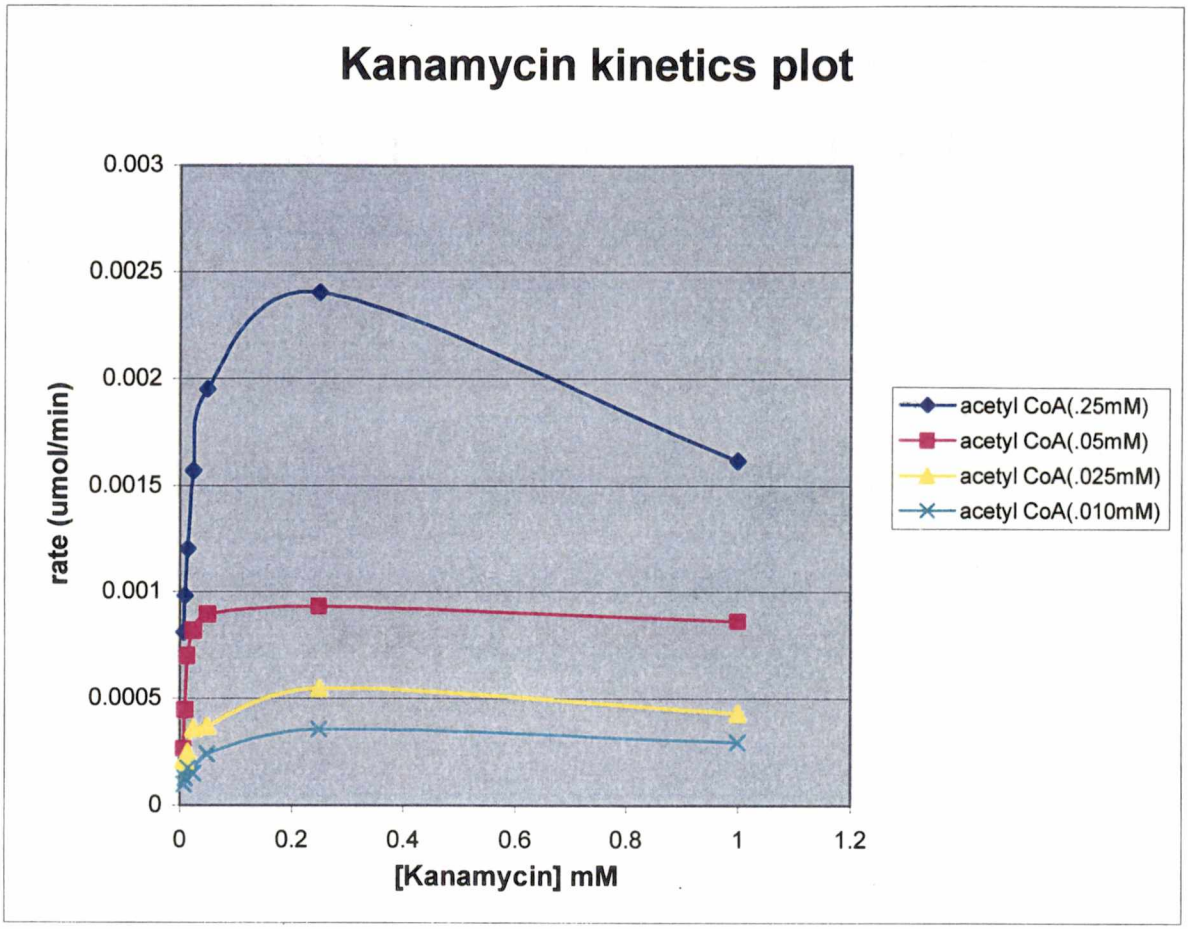


Figure 3. Kanamycin kinetics. Plot of rate vs. kanamycin concentration in mM at different acetyl CoA concentrations. Data from these experiments was used to calculate Km for kanamycin (14 +/- 2.8 μM)

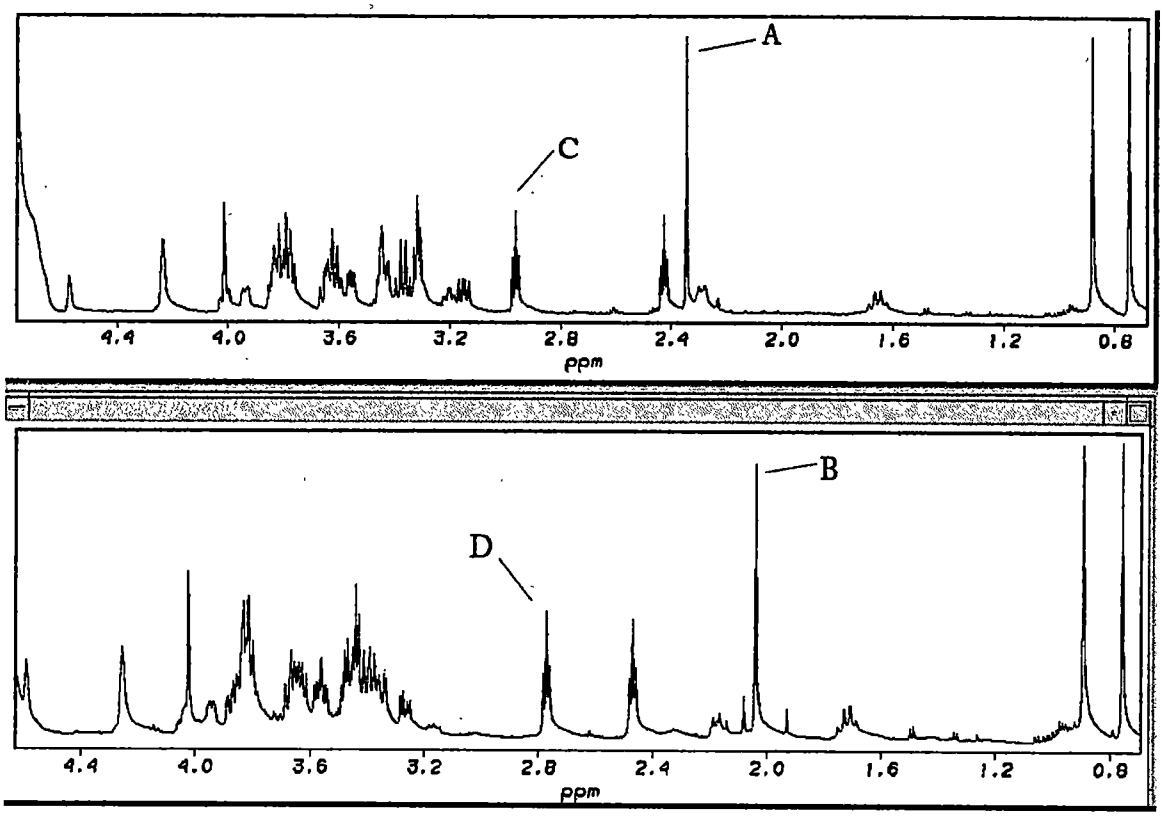


Figure 4: 1D comparison of Acetyl-CoA, kanamycin to acetylated kanamycin and CoA. Peak A is the methyl of the acetyl of acetyl-CoA, peak B is this group transferred to kanamycin. Peak C is a CH_2 group of Acetyl-CoA, and peak D is this same group shifted to the CoA position. Other positions, such as the H at the 3 position on the B ring, shifted as well but are not as visible in the 1D spectrum.

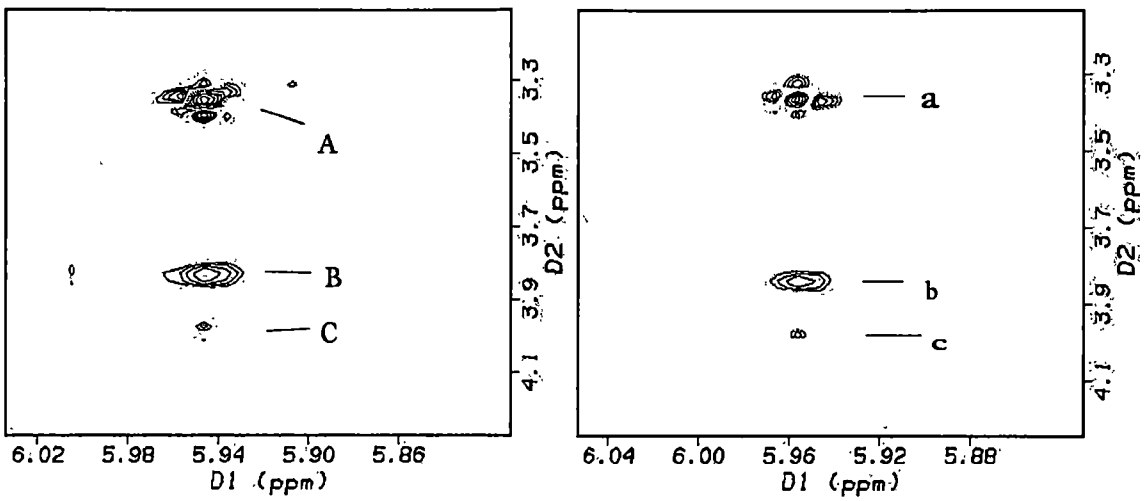


Fig. 5. Comparison of part of 120ms TRNOE and QUIETNOE spectrum of ribostamycin. Left frame is TRNOE, and right frame is QUIETNOE spectrum. Some crosspeaks are marked as follows: H1A-H2A (A, a), H1A-H5B (B, b), H1A-H3A (C, c). Refer to figure 1B for naming of hydrogens.

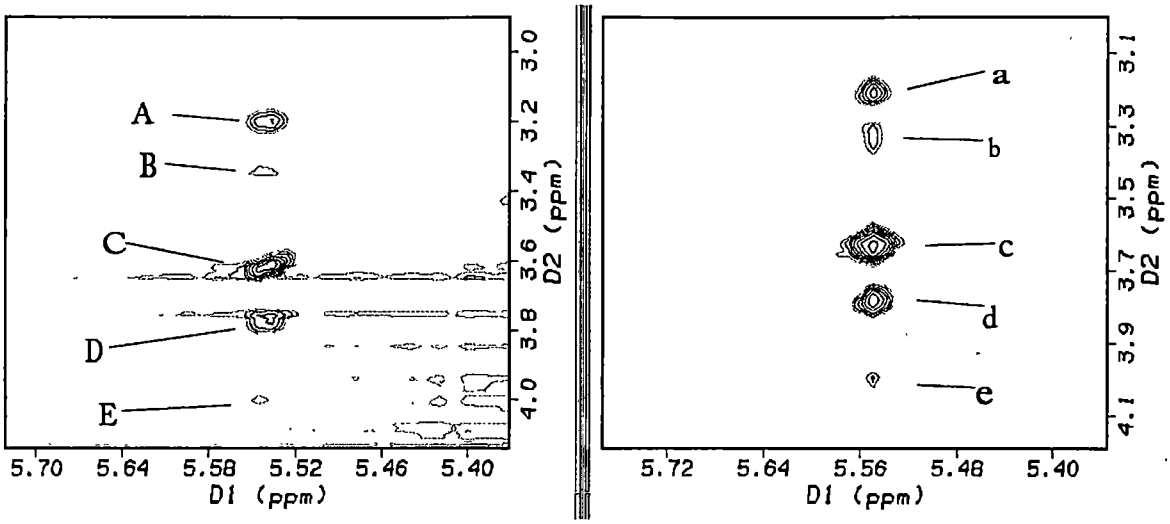


Fig. 6. Comparison of part of 120ms TRNOE and QUIETNOE spectrum of kanamycin. Left frame is TRNOE, and right frame is QUIETNOE spectrum. Some crosspeaks are marked as follows: H1A-H3B (A, a), H1A-H4A (B, b), H1A-H2A (C, c), H1A-H3A (D, d), H1A-H5A (E, e). Refer to figure 1A for naming of hydrogens.

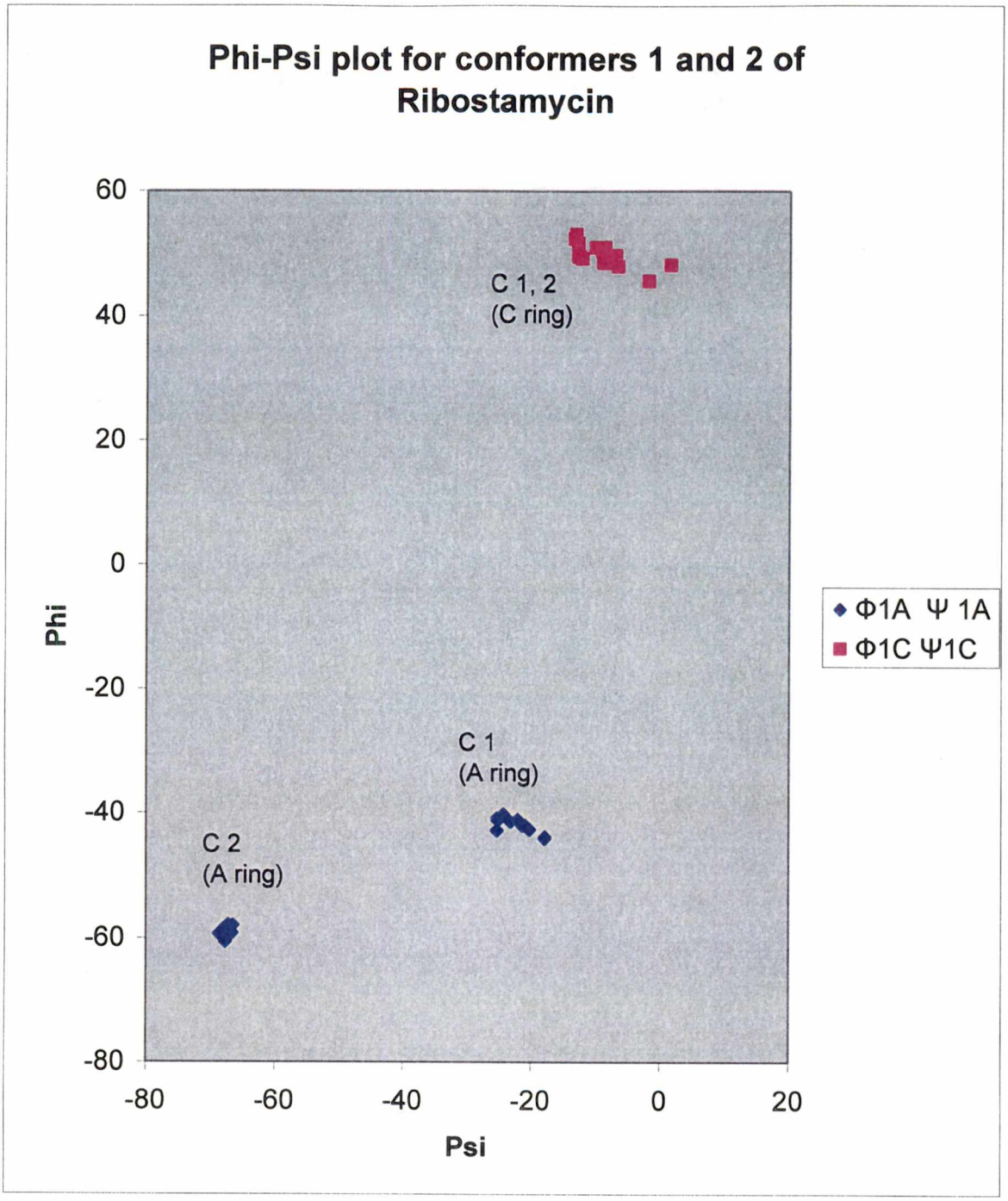


Figure 7. Phi-Psi plot for comparison of AAC3 bound ribostamycin structures. Blue represents A ring to B ring Phi-Psi angles and pink represents C ring to B ring. Notice that all of the C ring phi-psi angles cluster (for both conformations) whereas the A ring is divided into two regions, defining the two conformers.

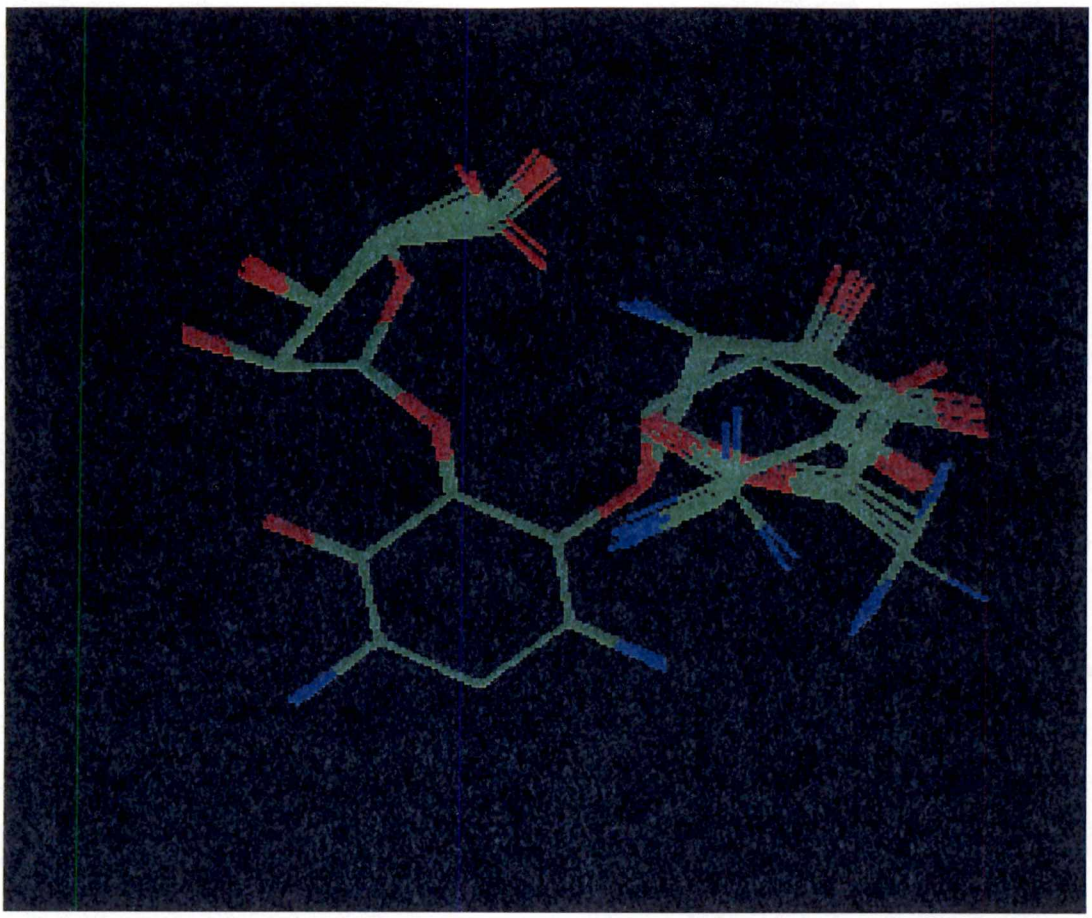


Figure 8. Comparison of 22 ribostamycin structures. This figure shows a comparison of all 22 structures of ribostamycin found by simulated annealing. By visual inspection alone, it can be seen that there are two conformations.

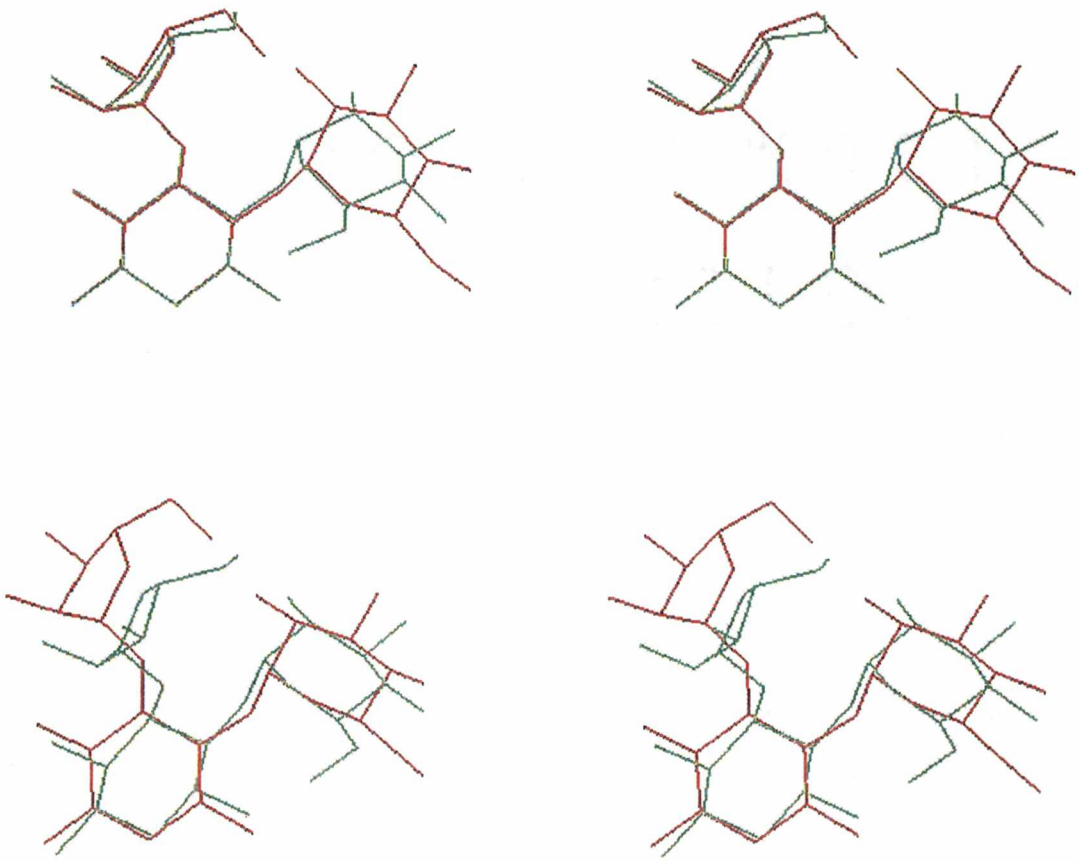


Figure 9. Comparison of AAC3 bound conformations of ribostamycin. Stereo views.
Conformer 1 (red), Conformer 2 (green).
Top: A and B rings overlaid, Bottom: B and C rings overlaid.

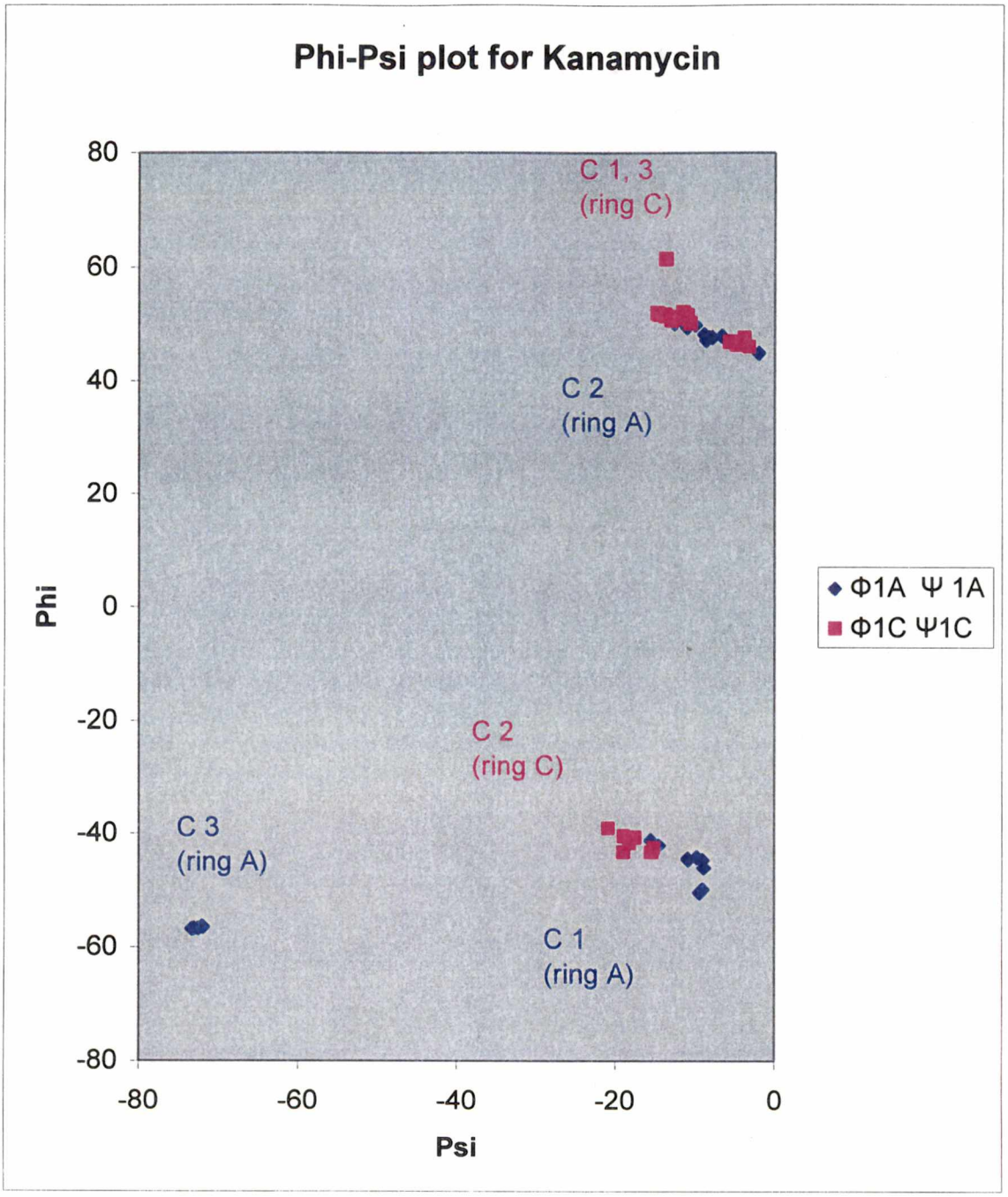


Figure 10. Phi-Psi plot for comparison of AAC3 bound kanamycin structures. Blue represents A ring to B ring phi-psi angles and pink represents C ring to B ring phi-psi angles. Notice that conformers 1 and 3 have clustering together for the C ring whereas conformer 2 is separate from them. For the A ring, there are three distinct phi-psi angle clusterings.

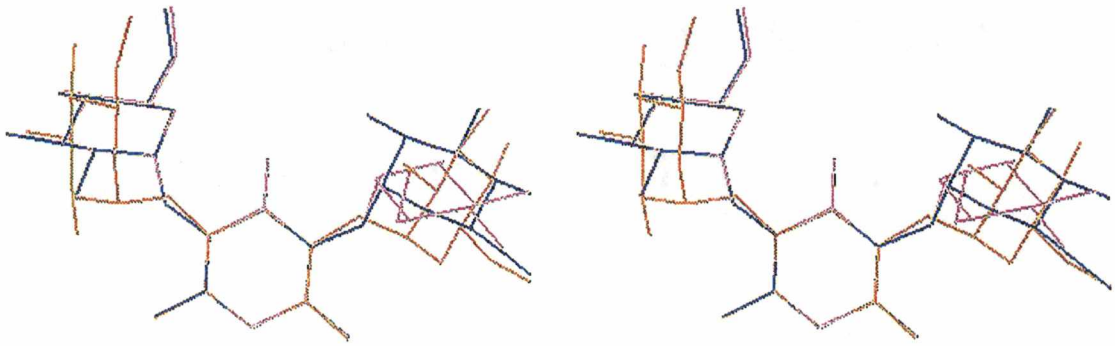


Figure 11. Comparison of AAC3 bound conformations of kanamycin A. Stereo view. Conformer 1 (blue), conformer 2 (orange), conformer 3 (pink). It can be seen that conformer 2 is very different than the other two conformers, while conformers 1 and 3 are similar with respect to the B and C rings.

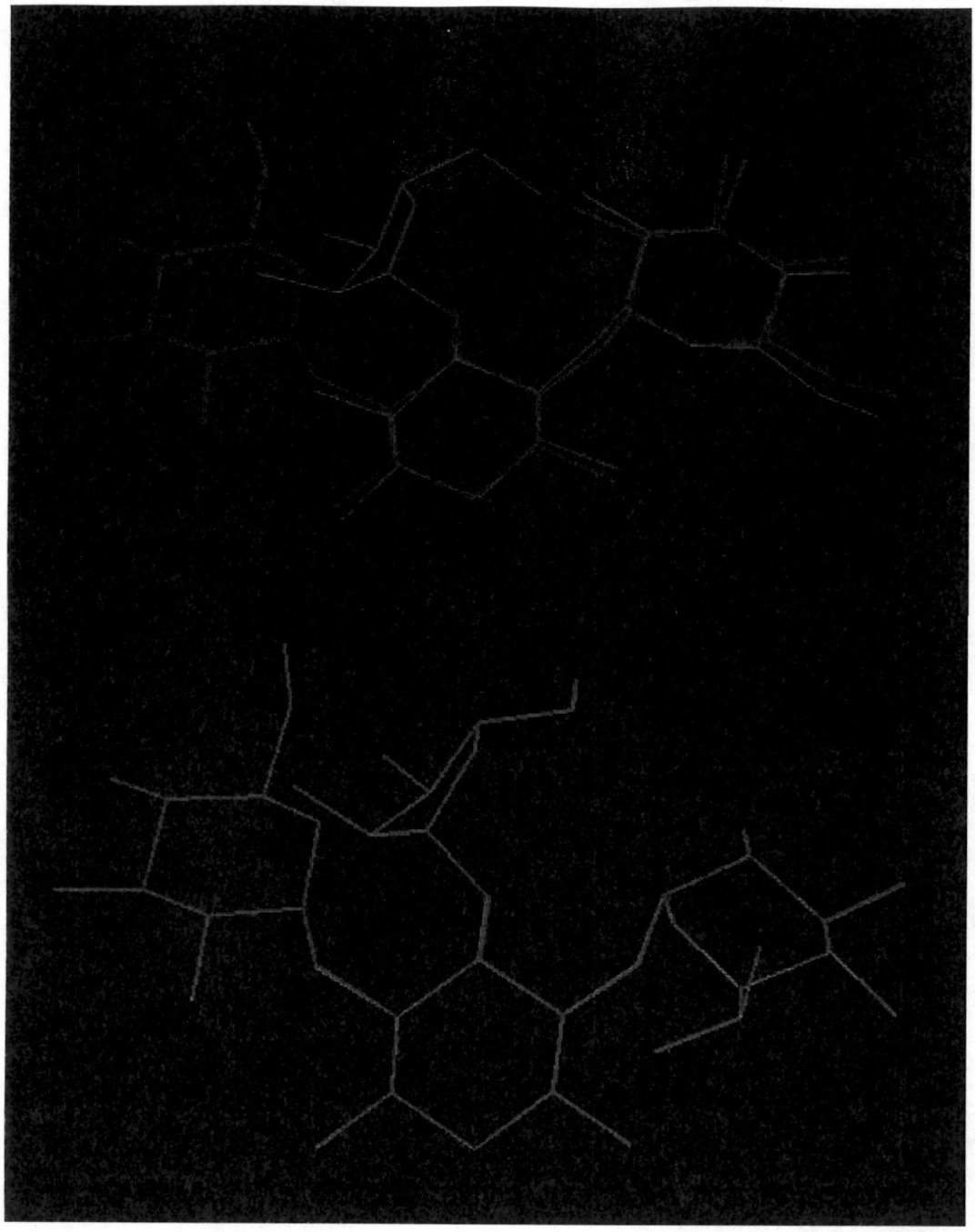


Figure 12. Comparison of AAC3 bound structures of kanamycin and ribostamycin.
Top: Ribostamycin conformer 1 (red), kanamycin conformer 1 (blue);
Bottom: Ribostamycin conformer 2 (green), kanamycin conformer 3 (pink).

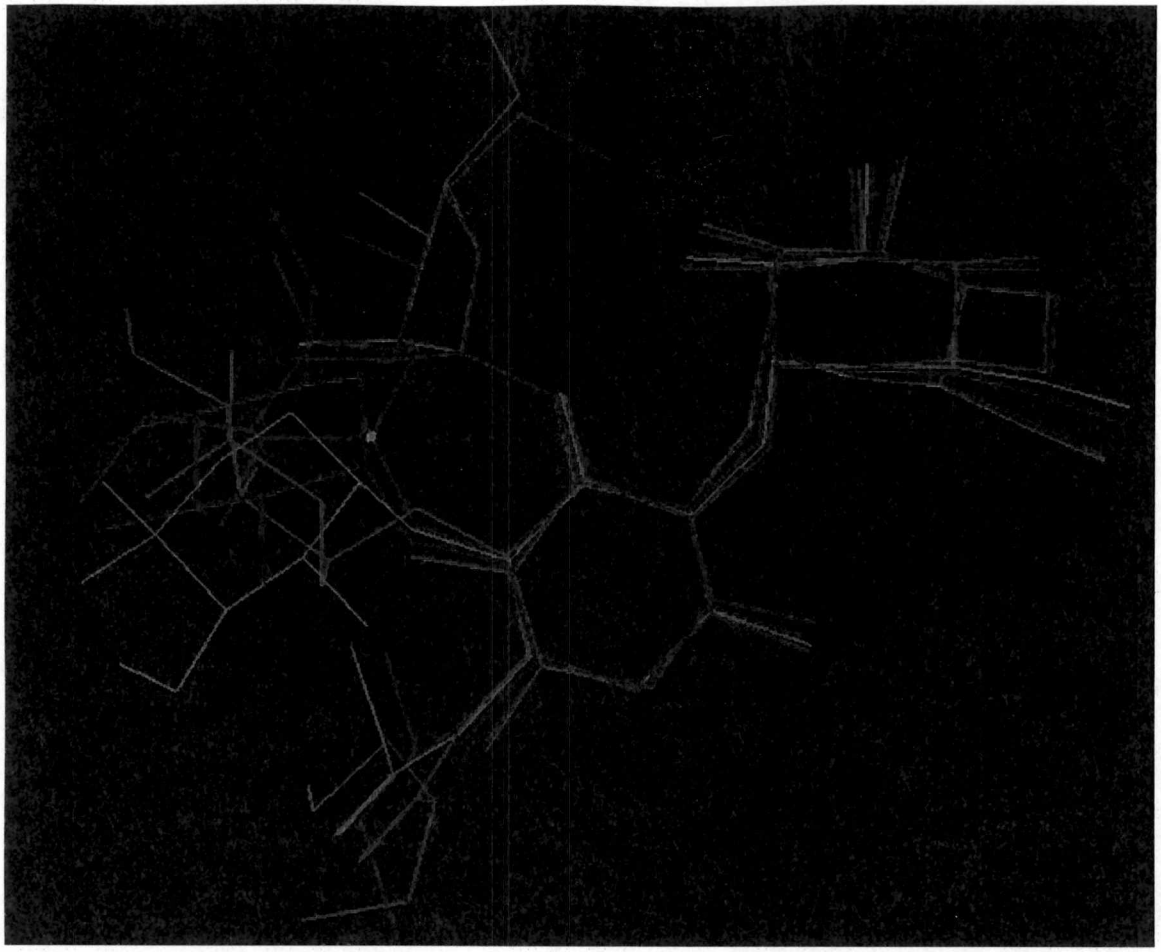


Figure 13. Comparison of AAC3 bound structures with aminoglycosides bound to other enzymes. AAC3 bound ribostamycin (C1: red), AAC3 kanamycin (C1: blue), APH3 bound ribostamycin (C1: light blue), AAC6 isepamicin (C1: yellow), ANT2 isepamicin (C2: green), APH isepamicin (C1: purple).

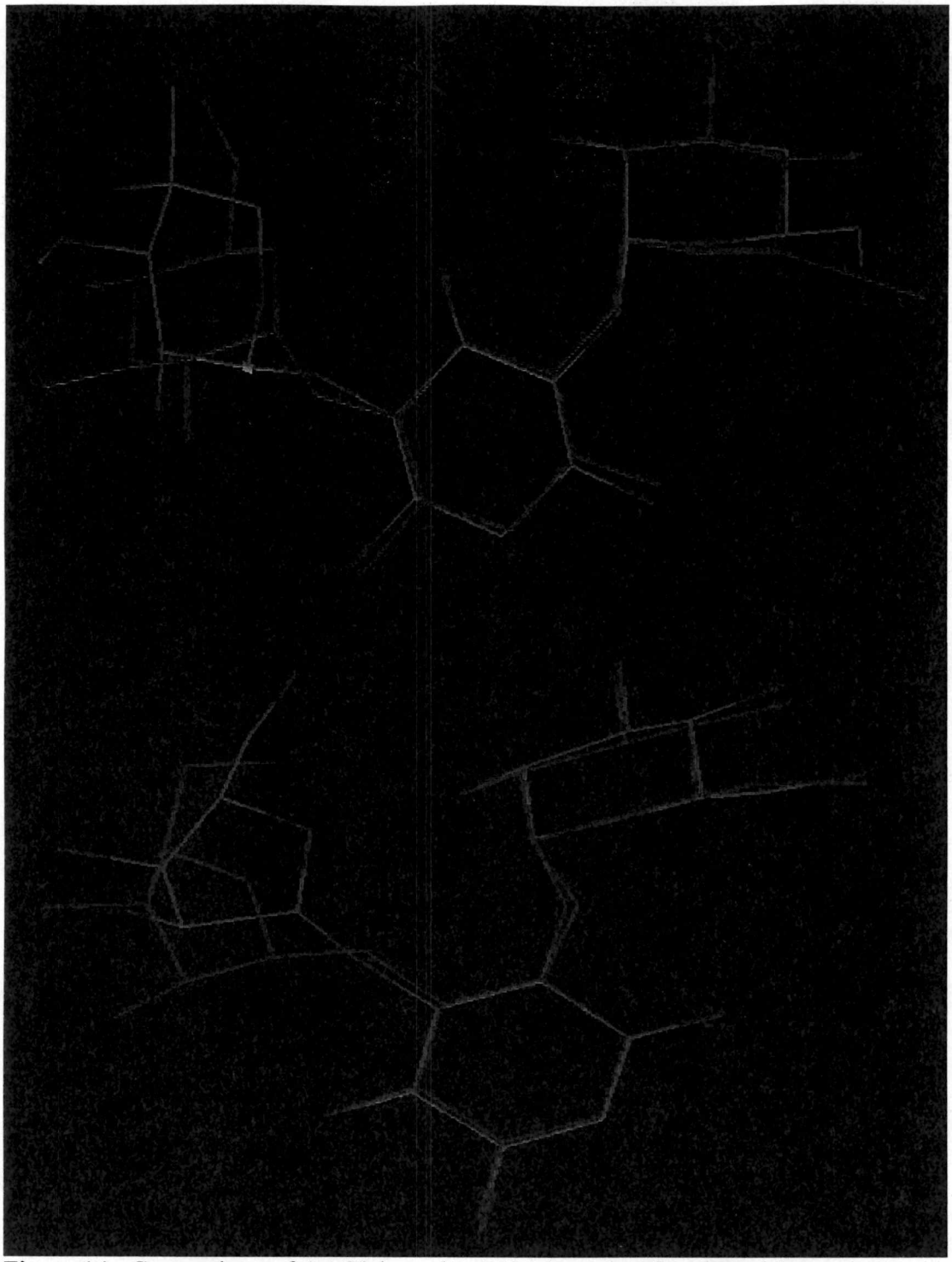


Figure 14. Comparison of AAC3 bound to RNA bound aminoglycosides. Comparison of AAC3 bound kanamycin (C1) to RNA bound gentamicin (top), and AAC3 bound ribostamycin (C1) to RNA bound paromomycin (bottom).

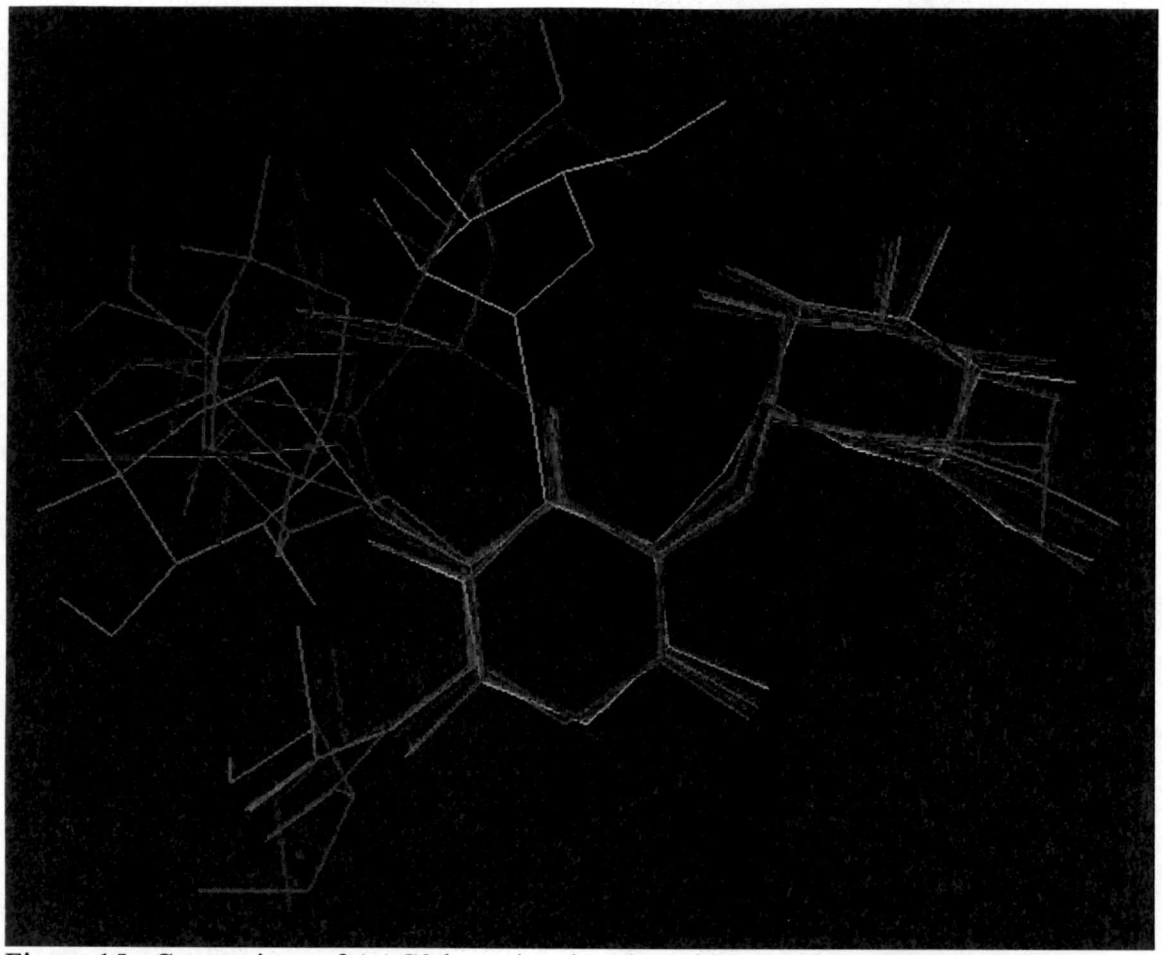


Figure 15. Comparison of AAC3 bound aminoglycosides to other enzyme and RNA bound aminoglycosides. AAC3 bound ribostamycin (C1: red), AAC3 kanamycin (C1: blue), APH3 bound ribostamycin (C1: light blue), AAC6 isepamicin (C1: yellow), ANT2 isepamicin (C2: green), APH isepamicin (C1: purple), RNA paromomycin (white), RNA gentamicin (orange).

Vita

Michael Owston was born in Logan, West Virginia on June 6, 1977. He moved to Kingsport, Tennessee where he attended public school and graduated from Sullivan South High School in May 1995. He entered East Tennessee State University (ETSU) in Johnson City, TN in August 1995, and received a B.S. degree in May 1998 with a major in Biology and a concentration in Biochemistry. He also received a Graduate Certificate in Business Administration from ETSU in May 2000, a while after completion of earlier coursework. While completing that program, he started a graduate program in Biochemistry, Cellular and Molecular Biology at the University of Tennessee, Knoxville in August 1998. He received his M.S. in Biochemistry, Cellular and Molecular Biology in August 2001, after an eight month period in the Doctor of Veterinary Medicine (DVM) program at the University of Tennessee College of Veterinary Medicine (UTCVM). He is currently completing the DVM program at UTCVM, and is expected to receive that degree in May 2004.