Isolation and Characterization of Gentamycin C_1

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Abstract

Aminoglycoside antibiotics have been used as an effective means of treating bacterial infections for many years. However, these drugs have been over prescribed, which led to the development of resistance in pathogenic bacteria. The resistance has been conferred to the bacteria primarily through R plasmids, and the organisms can now produce proteins that effectively disarm an aminoglycoside.

The focus of this project has been to characterize one component of the gentamycin complex, gentamycin C_1. After its isolation from the complex, the gentamycin C_1 was subjected to a series of NMR experiments including one-dimensional proton experiments and two-dimensional gCOSY, TOCSY, NOESY, and ROESY experiments. The data from these experiments allowed for the assignment of its NMR spectrum and for obtaining its lowest energy conformation or solution.

Introduction

The fight against bacterial infections has been going on for many years, and with the advent of antibiotics, man supposedly made a significant step toward conquering bacterial illnesses. The first antibiotic was penicillin followed by the discovery of streptomycin in 1944, and since then a host of related medicines has followed. Unfortunately resistant organisms also followed. In fact multi-resistant bacterial strains began to be isolated in increasing numbers throughout the 1950s and 1960s. The resistance patters arose from clinical antibiotic use and genetic resistance. The resistant bacteria were selected for by antibiotic use, and the R plasmids that generally carried the resistance allowed the bacteria to gain resistance to newly introduced antibiotics. This

resistance has also proven to be a larger problem because it occurs in all enteric bacteria without species limitations. (Mitsuhashi, 1982)

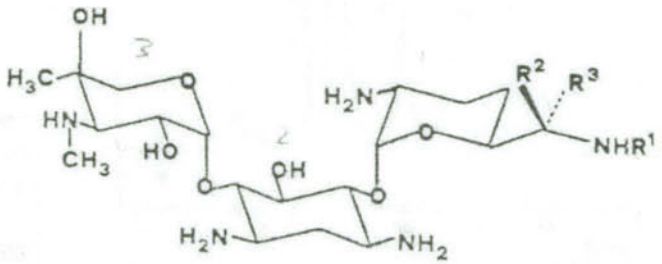
As mentioned previously, the second antibiotic to come into the forefront was streptomycin, which is a member of the aminoglycoside family of antibiotics. Aminoglycosides are characterized by the presence of amino sugars, and because of their structural similarities they have similar chemical properties such as their good water solubility, poor solubility in organic solvents, and their ability to be very effective at treating gram-negative bacterial infections. (Reden, 1979) Eukaryotic cells are mostly unaffected by aminoglycosides, but they kill bacteria by binding to the ribosome's 16S RNA subunit. (Tanaka, 1982) This either inhibits protein synthesis or increases codon misreading such that an affected bacterium dies. Unfortunately, resistance to this class of antibiotics is increasing, and the R plasmids conferring resistance to aminoglycosides has a propensity to carry multiple resistance. (Mitsuhashi, 1982, & Cox, 1977)

The mode of inactivation conferred by these R plasmids is enzymatic. Enzymes are produced that acetylate, phosphorylate, or add a nucleotide to the active functional groups (amines and hydroxyls) of the aminoglycoside, and because of the structural similarities of aminoglycosides cross-resistance has also been observed. (Cox, 1977, Mitsuhashi, 1982, & Reden, 1979)

Among the aminoglycosides, gentamycin, discovered by Weinstein in 1963, has proven to be very effective. (Cox, 1977) In fact it is hailed as one of the most powerful, and the gentamycin C complex, in particular, has been referred to as "the most important aminoglycoside in modern therapy." (Reden, 1979) It exhibits significant effectiveness against gram-negative bacteria including "*Pseudomonas, Proteus, E. coli, Enterobacter,*

Serratia, and *Klebsiella."* (Reden, 1979) Furthermore, the gentamycin C complex is among the largest selling antibiotics as a mixture. (Hooper, 1982) Unfortunately, gentamycin resistant organisms are also becoming more prevalent. (Cox, 1977) Their widespread use and clinical importance therefore makes the gentamycin C complex a primary target of study for information about the mechanism of resistance and for obtaining data toward the design of new drugs. This is the reason for the isolation of gentamycin C_1 – the largest component of the gentamycin C complex. (See Figure 1)

Figure 1 (Seidl, 1988)



	R_1	R_2	R_3
C_1	CH_3	CH_3	H
C_{1a}	H	H	H
C_2	H	CH_3	H
C_{2a}	H	H	CH_3
C_{2b}	CH_3	H	H

Fig. 1
Structures of gentamicin components.

Materials and Methods

The gentamycin sulfate from Sigma was converted into its free base form using Amberlite CG-50 according to the method outlined by Vanderhaeghe (Vanderhaeghe, 1984) and modified according to Marquez (Marquez, 1972). The free base was then concentrated down using a rotary evaporator and redissolved into a solution of 1:2:1 isopropanol, chloroform, and 17% ammonium hydroxide.

A silica gel slurry in this solvent system was packed into a 38cm x 3cm glass column. The gentamycin free base was then loaded onto this column and eluted with 1:2:1 isopropanol, chloroform, and 25% ammonium hydroxide. After checking the fractions by TLC using 1:1:1 ethanol, acetone, and concentrated ammonium hydroxide and ninhydrin for staining, the fractions containing purified gentamycin C_1 were dried, redissolved in water, and, finally, lyophilized. (Medina, 1995, & Wilson 1973)

The gentamycin C_1 was then redissolved in D_2O for one-dimensional proton experiments and two-dimensional gCOSY, TOCSY, NOESY, and ROESY proton experiments with a Varian 600MHz NMR.

The results of the two-dimensional proton experiments were used to assign the one-dimensional proton spectra of gentamycin C_1. The NOESY spectrum was then used in conjunction with the Discover software to determine the solution structure of gentamycin C1 by simulated annealing. The Discover program was used for the simulated annealing process with the Amber forcefield and a dielectric constant of 4.0. The consecutive conjugate minimizations and dynamics simulations were done at 400K, 350K, 300K, 250K, and 200K to allow for sufficient scanning of the energy surface with the NOESY constraints in place. These constraints were applied based on the intensity or strength of the proton-proton interactions observed in the NOESY spectrum as 1.8A – 2.8A for strong interactions and 1.8A – 4.5A for weak interactions. After the simulated annealing was completed, the restraints were removed, and dynamics was run at 100K followed by minimization to 0.001 rmsd to obtain the lowest energy structure within the energy minimum obtained by the simulated annealing process.

Results

The spectra obtained using the Varian 600MHz NMR allowed for the assignment of the one-dimensional proton spectrum seen below. These assignments are summarized in Table 1a, 1b, and 1c.

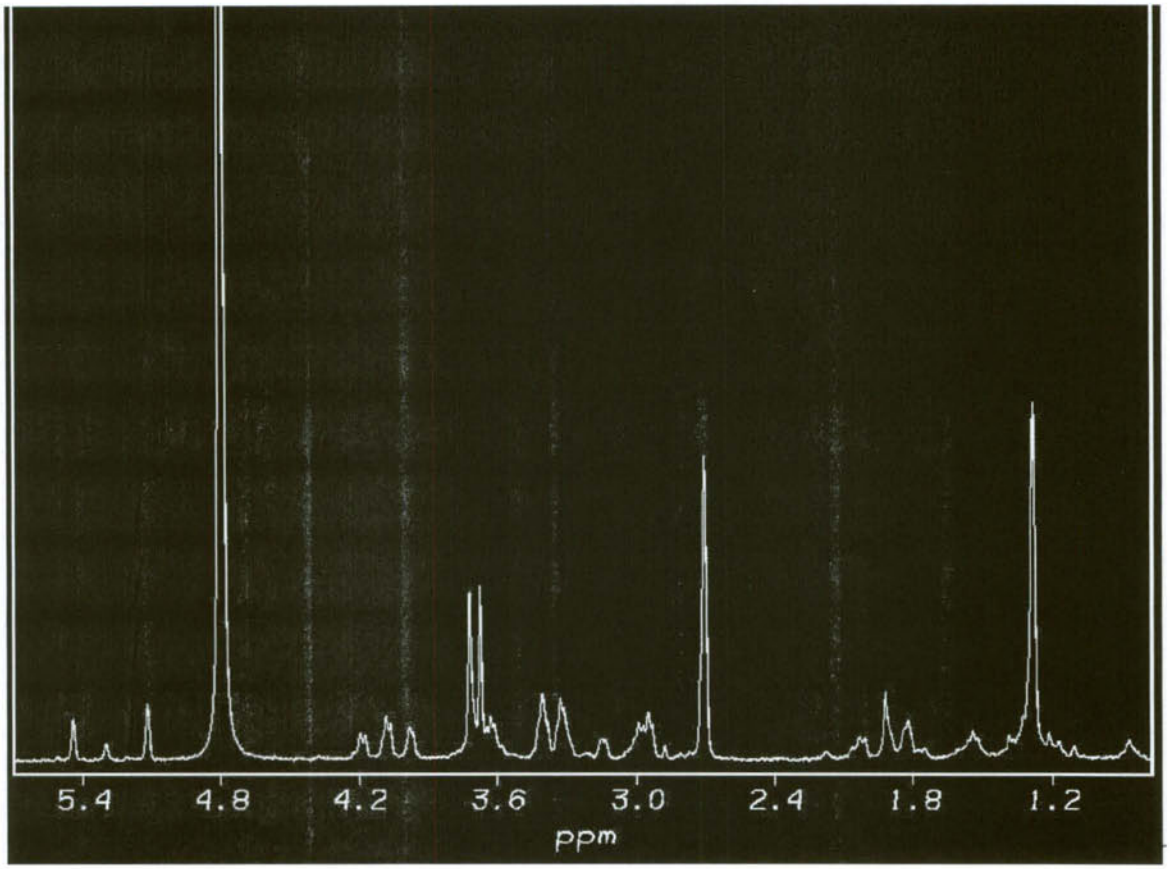


Table 1a – A ring Proton Assignments

Proton	**Chemical Shift (ppm)**	**Proton**	**Chemical Shift (ppm)**
H1'	5.441	H5'	3.327
H2'	3.141	H6'	3.627
H3'	1.821	H7'	1.284
H4'	1.920		

Table 1b – B ring Proton Assignments

Proton	Chemical Shift (ppm)	Proton	Chemical Shift (ppm)
H1	?	H4	3.404
H2a	2.020	H5	3.716
H2e	1.544	H6	4.069
H3	2.963		

Table 1c – C ring Assignments

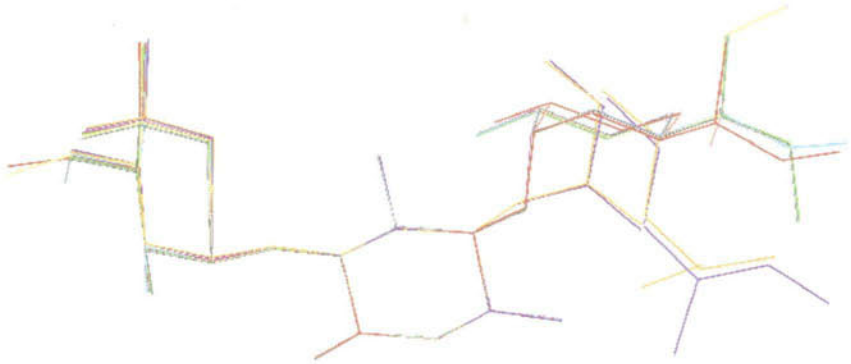
Proton	Chemical Shift (ppm)	Proton	Chemical Shift (ppm)
H1"	5.119	H4"-methyl	?
H2"	3.969	H5"	?
H3"	2.963		

The structure of gentamycin C1 was drawn into Discover, and nine random structures were created by running dynamics on the basic gentamycin C1 structure at 600K. These structures were then subjected to simulated annealing to obtain their lowest energy conformations based on the proton-proton interaction constraints from the NESY experiment. These constraints are summarized in Table 2, and Figure 2 shows random structures two through nine superimposed after the simulated annealing process.

Table 2 – NOESY Constraints

Protons	Interaction Strength	Protons	Interaction Strength	Protons	Interaction Strength
H1' – H4	Strong	H1' – H2'	Weak	H2 – H6	Weak
H4 – H6	Very Strong	H1'' – H2''	Weak	H3 – H4	Weak
H7' – H5'	Strong	H2'' – H3''	Weak	H4 – H5	Weak

Figure 2



Discussion

The purpose of purifying gentamycin C_1 from the other components of the gentamycin complex was to assign its proton spectrum and use that data to obtain its solution structure. The NOESY constraints used to find the lowest energy conformation from the nine random structures of gentamycin C_1 that were generated revealed two distinct conformations. The major conformation was exhibited by six of the nine conformations where the B ring and C ring exhibited great agreement between structures. The A ring of these six structures also showed good alignment, and this indicates one major structure. Tow of the other conformations also had good alignment of the B and C rings; however, these two conformations had the A ring aligned perpendicular to the

other conformer. The final structure exhibited almost no agreement with the other eight conformers, and its B ring appeared to be in a chair-flip relative to the other conformers.

The presence of a major and a minor conformation along with the agreement of each of the rings within these conformations is not entirely unheard of; however, these results are not definitive. Many more random structures of gentamycin C_1 would need to be subjected to simulated annealing for the assertion/assumption that there are two primary conformers to be proven. Current data cannot be considered to be statistically significant.

After creating a larger pool of structures to determine the solution structure of gentamycin C_1, this data will be used to compare the free structure to an enzyme bound structure of gentamycin C_1. The NOESY constraints for the enzyme bound structure will then be used in drug-design. Gentamycin was chose for this instead of one of the more easily purified aminoglycoside antibiotics because it binds more tightly with the detoxifying enzymes, and data from more tightly binding species are needed to gather data for drug-design. Fast-exchange NMR cannot be used, and because of gentamycin's stronger binding properties, it is an ideal candidate for enzyme bound NMR studies and drug design.

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UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - PROSPECTUS

Name: Sarah Elizabeth Trotman

College: Arts & Sciences Department: BCMB

Faculty Mentor: Dr. Engin Serpersu

PROJECT TITLE: Isolation and Characterization of Gentamycin C_1

PROJECT DESCRIPTION (Attach not more than one additional page, if necessary):

Projected completion date: 5-9-00

Signed: Sarah Elizabeth Trotman

I have discussed this research proposal with this student and agree to serve in an advisory role, as faculty mentor, and to certify the acceptability of the completed project.

Signed: Engin Serpersu, Faculty Mentor

Date: 5/9/00

Return this completed form to The University Honors Program, F101 Melrose Hall, at the beginning of your Senior Project Seminar.

Appendix D - UNIVERSITY HONORS PROGRAM
SENIOR PROJECT - APPROVAL

Name: Sarah Elizabeth Teatman

College: Arts and Sciences Department: BCMB

Faculty Mentor: Dr. Engin Serpersu

PROJECT TITLE: Isolation and Characterization of Gentamycin C_2

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

Signed: Engin Serpersu, Faculty Mentor

Date: 5/9/00

Comments (Optional):