NMR characterisation of a novel neamine antibiotic 12 and its interaction with a conserved 27mer RNA motif of 16S rRNA

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<td>(\Delta\omega)</td>
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## List of Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>1D</td>
<td>One Dimensional</td>
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<tr>
<td>2D</td>
<td>Two Dimensional</td>
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<tr>
<td>A</td>
<td>A-site (Adenosine)</td>
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<tr>
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<td>CDOSY</td>
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<td>DQF-COSY</td>
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<td>Deoxyribonucleic Acid</td>
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<td>EF</td>
<td>Emission Factor</td>
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<td>Free Induction Decay</td>
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<td>FnMDV</td>
<td>Forced Mutant Disease Virus</td>
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<td>G</td>
<td>Genome</td>
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<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>HSQC</td>
<td>Heteronuclear Single Quantum Coherence</td>
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<tr>
<td>HSQC-TOCSY</td>
<td>Heteronuclear Single Quantum Coherence-Total Overlap Spectroscopy</td>
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<td>IF</td>
<td>Inhibition Factor</td>
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<td>INEPT</td>
<td>Intrinsic Nuclear Enhanced by Polarization Transfer</td>
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<td>IRES</td>
<td>Internal Ribosome Entry Site</td>
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<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<td>MD</td>
<td>Molecular Dynamics</td>
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<td>Mr</td>
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<td>Nuclear Magnetic Resonance</td>
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<td>Nuclear Overhauser Effect Spectroscopy</td>
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<td>nanosecond</td>
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<td>GD</td>
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<td>Peptidyltransferase site</td>
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<td>Presently/Translome Centre</td>
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<td>RF</td>
<td>Release Factor</td>
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<td>Ribonucleic Acid</td>
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<tr>
<td>ROE</td>
<td>Rotating Frame Overhauser Effect</td>
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<tr>
<td>ROESY</td>
<td>Rotating Frame Overhauser Effect Spectroscopy</td>
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<td>Ribosomal Ribonucleic Acid</td>
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<td>Thymine</td>
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<td>TOCSY</td>
<td>Total Correlation Spectroscopy</td>
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<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
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<tr>
<td>μg</td>
<td>microgram</td>
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<td>μL</td>
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<tr>
<td>μs</td>
<td>microseconds</td>
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<tr>
<td>U</td>
<td>Unit</td>
</tr>
<tr>
<td>vdW</td>
<td>van der Waals</td>
</tr>
<tr>
<td>VT</td>
<td>Variable Temperature</td>
</tr>
<tr>
<td>WATGATE</td>
<td>Water Suppression by Gradient Taught Relaxation</td>
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Abstract

In recent times, the growing challenge of antibiotic resistance has prompted intense efforts to elucidate the mechanism of action of antibiotics at the molecular level, using techniques such as NMR spectroscopy. Blocking protein synthesis is an effective way of combating bacterial infection and many antibiotics function in this manner. Interest in the involvement of RNA in protein biosynthesis has increased following extensive studies on the binding of antibiotic drugs to specific target sites on ribosomal RNA. A systematic NMR study of a novel antibiotic derived from neamine and its interaction with a conserved and highly stable 27mer RNA motif of the A-site 16S rRNA was carried out. The antibiotic showed well resolved and dispersed resonances including exchange retarded amide protons suggesting a stable, folded conformation of the drug. The NMR results obtained provide a structural rationale to modify selected groups on the neamine 12 antibiotic to enhance the affinity for the RNA. The empirical Gibbs energy ($\Delta G = -10.70 \text{ kcal/mol}$) and secondary structure were predicted for the 27mer RNA by the Vienna RNAfold software exhibiting a good thermodynamic stability. Similarly, the 27mer RNA exhibited well resolved and stable exchangeable imino and amino resonances in the lowfield region of the $^1$H-NMR spectrum. The NMR spectra of the 27mer RNA-neamine 12 complex showed small but detectable changes in chemical shift and linewidth, indicating weak interaction between the neamine 12 and the 27mer RNA. These changes can be qualitatively interpreted as changes in the local conformation of the 27mer RNA and the neamine 12, arising from the formation of their complex. The NMR results obtained have laid a solid foundation to determine the three dimensional solution state structures of neamine 12 after necessary modification and the complex with the RNA to elucidate the mechanism of action of the antibiotic.
Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on presentation of Theses.
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I would like to thanks to Anthony, Mary and Stacey Murphy for having shared their house with me and for generous support in any situation.

I would like to express gratitude to my family for their continued financial support.
Chapter 1: Introduction

In this chapter the aim of the project, the background to the research area of aminoglycodide antibiotics, the phenomenon of antibiotic resistance and the significance of the project are described. The chapter also provides relevant background to cellular transcription and translation processes, nucleic acid structure and NMR spectroscopy as an analytical tool.

1.1 Aim of the project

The overall aim of the project is to elucidate interactions of an azidoglycoside (neamine derivative 12) with a highly conserved and thermodynamically stable 27mer RNA motif of the A-site 16S rRNA using 1D and 2D NMR spectroscopy. To achieve this, it is proposed to characterize:

1) The NMR spectra of the neamine 12 and build an unconstrained model of the antibiotic.

2) The NMR spectra of the 27mer RNA by assigning the imino and amino proton resonances of the RNA.

3) The NMR spectra of the 27mer RNA-neamine 12 complex by monitoring changes induced to the resonances of the RNA and neamine 12.

The results of the above NMR studies should enable the synthesis of neamine derivatives with enhanced affinity for the RNA and gain a better understanding of the specificity of interactions between the two molecular components.
1.2 Antibiotic action

Antibiotics are important therapeutic agents in the treatment of infections. The basis of treatments with antibiotics is that the drugs have selective toxicity to bacterial cells. Antibiotics work using a variety of modes of actions, which include inhibition of the bacterial cell wall synthesis, inhibition of the bacterial protein synthesis and inhibition of DNA synthesis. Antibiotics can be classified as bacteriostatic and bactericidal, the former prevent the bacteria growth, the latter kill bacteria. Bacteriostatic antibiotics are Spectinomycin, Sulphonamides, Chloramphenicol and Trimethoprim. Bactericidal antibiotics are Penicillins, Glycopeptides, Monobactams and Carbapenems.

1.3 Aminoglycoside antibiotics

Aminoglycosides are highly potent, broad-spectrum antibiotics used for treatment of life-threatening infections. Their history began in 1944 with streptomycin which was followed by a series of milestone compounds (kanamicin, getamicin and tobramycin) successfully used for the treatment of gram-negative bacillary infections. Unfortunately, neomycin cannot be directly used clinically mainly because of its nonspecific electrostatic interactions with RNA which largely causes the high toxicity. The neamine, which is a simplified neomycin mimic, keeps the same targeting site as neomycin but shows lower toxicity. The chemical structures of the neomycin and neamine are shown in Figure 1.3.1.

![Figure 1.3.1. Chemical structures of (a) the neomycin and (b) the neamine.](image-url)
1.3.1 Action of aminoglycoside antibiotics

Aminoglycosides act primarily by impairing bacterial protein synthesis through binding to prokaryotic ribosomes,\(^3\) inducing miscoding. Prokaryotic ribosomes are 10 to 15 times less sensitive to the antibiotic concentration than eukaryotic ribosomes. Translation fidelity is established by ribosomal recognition of the codon - anticodon interaction within its A-site. The ribosome must sense formation of codon - anticodon interaction through structure-specific, sequence-independent interactions.\(^4\)-\(^6\) However, Watson-Crick base pairing between anticodon and codon is insufficient to account for the fidelity of translation.\(^4\)-\(^6\) The interactions between N1 of the A•A mismatch of the 16S rRNA and two 2’-OH groups in mRNA could represent an initial step in communication between a codon - anticodon complex. Aminoglycoside antibiotics decrease the fidelity of translation by binding and protecting the A•A of the rRNA at N1 position by mRNA-dependent tRNA binding to the ribosome. The A•A mismatch of the 16S rRNA is crucial for mRNA-dependent tRNA binding in the A site. Mutations of these two universally conserved nucleotides are lethal in *E. coli* and decrease A-site binding affinity.\(^7\)-\(^8\) Aminoglycoside antibiotics act preferentially on prokaryotic organisms.\(^3\)
1.3.2 Antibiotic resistance

The emergence of resistant bacterial strains, such as tuberculosis strains and gram-negative bacteria, has somewhat reduced the potential of aminoglycosides in empiric therapies.\textsuperscript{9-10} There are three main mechanisms of aminoglycoside resistance: the first consists of a decreased drug uptake and/or accumulation of the drug in bacteria, due to likely a membrane impermeabilization. The second is due to alteration at the ribosomal sites, causing significant resistance to streptomycin only. The third is due to bacterial expression of enzymes that modify the antibiotic and therapy inactivating them.\textsuperscript{11-12}

1.4 Biosynthesis of RNA: Transcription\textsuperscript{13-17}

Transcription is the process by which the RNA is synthesized from a template DNA and translation is the process by which the genetic message in DNA that was passed on to mRNA is decoded and used to build proteins. DNA contains sequences of bases known as promoter sites.\textsuperscript{14}

An enzyme recognizes a promoter site and binds to it initiating the RNA synthesis. The DNA at the promoter site unwinds to give two single strands, exposing the bases. One of the strands is called the sense strand and the complementary stand is called antisense strand. A template strand is read in the $3'\rightarrow 5'$ direction, so that RNA can be synthesized in the $5'\rightarrow 3'$ direction (Figure 1.4.2). In eukaryotic organisms, transcription of DNA occurs in the nucleus, while translation of mRNA occurs in the cytoplasm. In prokaryotic organisms, DNA undergoes transcription and mRNA undergoes translation in undivided cell compartments. There are three kinds of RNA: messenger RNA (mRNA), whose sequence determines the sequence of amino acids in proteins, ribosomal RNA (rRNA), a structural component of ribosomes, and transfer RNA (tRNA), the carrier of amino acids for protein synthesis. Biosynthesis of proteins takes place in ribosome, which are composed of about 40% protein and 60% rRNA.
Ribosomes are made up of two subunits whose size depends on whether they are prokaryotic organisms or eukaryotic organisms (Figure 1.4.3). A prokaryotic ribosome is composed of a 50S subunit and a smaller 30S subunit that together form the 70S ribosome (S stands for sedimentation constants), while an eukaryotic ribosome has a 60S subunit and 40S subunit, that together form an 80S ribosome.
1.5 Biosynthesis of proteins: Translation\textsuperscript{13-19}

A protein is synthesized from its N-terminal end to its C-terminal by reading the bases along the mRNA strand in the 5$\prime$$\rightarrow$3$\prime$ direction and this takes place on the ribosome. For a prokaryotic organism, translation initiation begins on the 40S ribosomal subunit, the mRNA enters in the 40S ribosomal subunit from the entry channel and passes through the A, P and E sites, and leaves through the mRNA exit channel. A sequence of three bases in mRNA is called codon and specifies a particular amino acid that will be incorporated into a protein. The three bases at the bottom of the loop directly opposite the 5$\prime$- and 3$\prime$-end of the tRNA are called anticodon. Each tRNA can carry an amino acid bound as an ester to its terminal 3$\prime$-OH group. In most cellular mRNAs, recognition of the start site for protein synthesis occurs by ribosome scanning.\textsuperscript{15, 18} A model of the process is shown in Figures 1.5.4 and 1.5.5. In the first step, initiation, the Met-tRNA with the anticodon UAC binds the P site of the smaller unit of the ribosome, forming base pairing with the codon AUG of the mRNA. Thereafter, the large unit of the ribosome is added to form the structure shown in Figure 1.5.4 and catalyses the peptide bond formation.\textsuperscript{16}

![Diagram of translation process](image)

Figure 1.5.4. Illustration of the first step of the translation process, initiation. The Met-tRNA with the anticodon UAC binds the P-site of the smaller unit of the ribosome, forming base pairing with the codon AUG of the mRNA. A indicates the A-site, aminoacyl or attachment site, P indicates peptidyl transferase site and E indicates E-site, exit site of the small subunit of the ribosome.

In the elongation step (Figure 1.5.5, a-d), next tRNA, carrying the second amino acid (Valine), binds the A-site. The amino group of the amino acid carried by tRNA, located
in the A-site, reacts in an enzyme-catalyzed nucleophilic acyl substitution reaction with the ester of the adjacent tRNA, located in the P-site, forming a dipeptide (Figure 1.5.5, c). In the translocation step, the tRNA carrying the growing peptide chain shifts from the A-site to the P-site, displacing the tRNA through the E-site (Figure 1.5.5, d). Subsequent, amino acids are brought one at a time in the same way, with the codon in mRNA specifying the amino acid to be incorporated by complementary base pairing with the anticodon of the tRNA (Figure 1.5.5, e). In the termination step, a stop or terminating codon (carried by the release factor) enters the A-site (Figure 1.5.5, f). At this point the translation will stop and the completed polypeptide will disconnect to be folded into protein. The rRNA will float off to be used in other translations and the mRNA in other transcriptions. Several initiation (IF-1, IF-2, and IF-3), elongation (EF-Tu, EF-Ts, EF-G) and release (RF-1/2, RF-3) factors take part during the process to promote correct binding at the corresponding step.
Figure 1.5.5. Illustration of the translation process for protein synthesis. The elongation step is shown in (a), (b), (c) and (d) illustrations. In the illustration (a), a next tRNA, carrying valine, binds the A-site. The illustrations (b) and (c) show the enzyme-catalyzed nucleophilic acyl substitution reaction with the ester of the adjacent tRNA, located in the A-site, forming a dipeptide. The illustration (d) shows the translocation step, the tRNA carrying the dipeptide shifts in the P-site, displacing the tRNA through the E-site, and a next tRNA, carrying phenylalanine, approaches the A-site. The illustration (e) shows the termination step, in which a stop or terminating codon (carried by the release factor), enters the A-site stopping the translation process. The red dots indicate the growing polypeptide after the addition of a number of amino acids. The illustration (f) shows the rRNA that floats off to be used in other translations and the mRNA in other transcriptions.
1.6 The structure of Nucleic Acids

Nucleic acids are polymers of nucleotides linked in long chains through phosphodiester bonds. The numbering of the carbon atoms runs clockwise, following IUPAC rules. Note the absence of the hydroxyl (-OH) group on the 2’ carbon in the deoxy-ribose sugar found in DNA as compared with the ribose sugar found in RNA (Figure 1.6.6). It determines differences in tertiary structure (A-form for RNA and B-form for DNA). Nucleic acids play an important role in the transmission and transcription of genetic information. Nucleotides are composed of three main components covalently bound together: which are a heterocyclic base, a ribose or deoxyribose pentose sugar and a phosphate residue.

![Figure 1.6.6](image)

Figure 1.6.6. (a) Illustration of the difference between 2’-deoxyribose found in DNA and ribose found in RNA, the H₃'' (red brackets) in DNA is replaced by 2’-hydroxyl (2’-OH) group in RNA (black box). The green box represents a phosphodiester bond linking two cytosine nucleotides from the 5’- to 3’-end and the red box represents the glycosidic bond between the heterocyclic base and the sugar. (b) Illustration of the difference between syn and anti orientations; for syn orientation distances between H1’ and H8, and H2’’ and H8 are about 2.5Å and 2.0Å, respectively. For anti orientation distances between H1’ and H8, and H2’’ and H8 are about 3.7Å and 4.0Å, respectively.

Heterocyclic bases can be characterised into two types; purines: adenine (A) and guanine (G) and pyrimidines: cytosine (C) and uracil (U). Adenine, guanine, uracil and cytosine bases are found in RNA. The structure of these bases is illustrated in Figure 1.6.7.
Figure 1.6.7. Structures of purine and pyrimidine bases found in RNA. Atoms are numbered according to the IUPAC convention.21

1.6.1 DNA and RNA base pairing and stacking17

Two main types of base pairing occur in RNA: canonical and non-canonical. Canonical base pairing is also known as Watson-Crick complementary base pairing and involves hydrogen bonding between G•C bases for both RNA and DNA, and A•U base pairing for RNA and A•T bases for DNA (Figure 1.6.1.8 a-c). The G•C base pairing involves three hydrogen bonds, while the A•U and A•T base pairings involves two hydrogen bonds. Non-canonical base pairing includes any type of base pairing that is not of the Watson-Crick type. Wobble base pairing is a common type of non-canonical base pairing that involves hydrogen bonding between G•U bases, forming two hydrogen bonds (Figure 1.6.1.8, d).
Figure 1.6.1.8. Illustration of canonical (a) A•U, (b) G•C, (c) A•T and non-canonical (d) G•U base pairings. Atoms are numbered according to the IUPAC convention.\textsuperscript{21}

1.6.2 Primary, secondary, tertiary and quaternary structure of nucleic acids\textsuperscript{17, 20}

The primary structure of nucleic acids consists of a linear sequence of nucleotides linked together by phosphodiester bonds. The sequence typically starts from the 5'-end to the 3'-end; an example of primary structure could be: 5’-GGCGUCACACCUCGUUGGUGAAGUCGCC-3’. The secondary structure consists of the two dimensional shape of the nucleic acids based on interactions between bases. An example of secondary structure would be the duplex with all the bases paired (Figure 1.6.2.9, a). There are more complicated secondary structures that do not follow the standard base pairing, such as hairpin, bulge, internal loop and 4-way junction (Figure 1.6.2.9, b-e).\textsuperscript{22}
Tertiary structure determines the location of the atoms in a three dimensional arrangement, taking into account the geometrical and steric constraints. The tertiary arrangement of RNA’s double helix in space is the A-form conformation, while in DNA, it includes A-form and B-form conformations (Figure 1.6.2.10). The tertiary structure is determined by the sugar puckers (C3’-endo in A-form and C2’-endo in B-form).

The quaternary structure refers to a higher-level of organization, including interactions of nucleic acids with other molecules.
1.6.3 Hairpin in RNA structural biology

Hairpins are among the most important secondary structure elements found in RNA (Figure 1.6.2.9, b). Hairpins are one of the building blocks of the secondary structure of ribosomal and messenger RNAs. Quite often, the size and sequence of hairpin loops are evolutionarily conserved. Stable hairpins may provide nucleation sites to direct the folding of RNAs with complex secondary and tertiary interactions. Among all hairpin loops, tetraloops are the most abundantly found in RNA.24 Single stranded oligonucleotides of small size (10-15 nucleotides) can fold into hairpins, RNA hairpins are well suited candidates for structural studies.

1.6.4 UUCG - tetraloop

The UUCG tetraloop has already been extensively studied by different biophysical methods in order to understand its unusual thermal stability at the molecular level. The tetraloop sequence is particularly stable with a high melting temperature of 74°C and is one of the most stable hairpin motifs in RNA. The UUCG tetraloop occurs often in ribosomal and other RNAs, and may serve as a nucleation site for RNA folding and as a protein recognition site. The 27mer RNA used as model system in this project (Figure 4.1.43) contains the UUCG tetraloop and adopts a well-defined structure composed of two conformational domains: the stem with a canonical helix conformation and the loop with a very unusual non-canonical conformation including the wobble G•U base pair, in the UUCG loop.25-27
1.7 Significance of the project

The recognition between mRNA and tRNA is an important step in the process of protein synthesis, and in bacteria the recognition occurs at the A site decoding region of 16S rRNA. The aminoglycoside antibiotics can bind potentially to the A site of 16S RNA and induce miscoding or inhibition of the bacterial protein synthesis, thus leading to bacterial cell death. Keeping with the growing trend of recent years, targeting RNA with small molecules have appeared to be an attractive strategy for new drug discovery. Many efforts have been made on taking 3D structure of RNA to design small molecules that selectively target RNA sites might be therapeutically useful. Using a structure-guided approach, Mobashery and coworkers took into account steric and electronic contributions to interactions between RNA and aminoglycosides (Figure 1.3.1, a) to make a random search of 273,000 compounds from the Cambridge structural database and the National Cancer Institute 3-D database of ribosomal aminoglycoside-binding pocket. However, their widespread use over the last decades has been significantly compromised by oto- and nephrotoxicity and the rapid emergence of bacterial resistance. To overcome the undesirable properties of parent structures, it is highly desirable to synthesize modified aminoglycosides that will possess higher RNA binding affinity, better selectivity, better antibacterial activity and stronger resistance against the aminoglycoside modifying enzymes compared to their parent structures.

A promising approach would be to optimize the structure of neamine (Figure 1.3.1, b) to overcome the undesirable properties of the parent structures. Neamine exhibits some antibacterial activities against Escherichia coli. The amino groups on neamine play an important role in their binding to rRNAs due to electrostatic interaction with the phosphate backbone. To elucidate the relationship between the structure and binding activity to 16S RNA, a series of derivatives of neamine–nucleoside conjugates were designed and synthesized, which contain the connection between neamine and nucleoside with different configurations. In this thesis work, the interactions of the neamine derivative 12 with 27mer RNA motif were investigated to monitor its binding activity.
1.8 NMR spectroscopy

Nuclear Magnetic Resonances (NMR) spectroscopy is a form of absorption spectroscopy, akin to UV or IR spectroscopy. In a typical NMR experiment, a NMR glass tube containing a solution of the chemical compound of interest is placed in a static magnetic field $B_0$ and then subjected to irradiation by a radio-frequency (rf) field, $B_1$. The energy adsorbed by the compound is detected by the receiver and plotted as a spectrum of resonance frequency.

1.8.1 Magnetic properties of nuclei

An atomic nucleus is specified by three numbers: the atomic number ($Z$), the mass number ($A$) and the nuclear spin quantum number ($I$). The atomic number ($Z$) specifies the number of protons inside the nucleus, while the mass number ($A$) specifies the total number of neutrons and protons. The combination of odd or even atomic number ($Z$) and odd mass number ($A$) leads to the half-integer nuclear spin quantum number $I= 1/2, 3/2, 5/2$, etc. The combination of odd atomic number ($Z$) and even mass number ($A$) leads to the whole integer nuclear spin quantum number $I=1, 2, 3$, etc. The combination of even atomic number ($Z$) and even mass number ($A$) leads to the nuclear spin quantum number $I=0$. Nuclei with the same atomic number but different mass numbers are called isotopes. NMR is mainly concerned with stable isotopes, such as $^1$H, $^{13}$C, $^{15}$N, $^{19}$F, $^{31}$P, etc. Other properties of nuclear isotopes are: gyromagnetic ratio, $\frac{\gamma}{2\pi} (10^7 \text{ rad s}^{-1}\text{T}^{-1})$, natural abundance and Larmor frequency, $\frac{\alpha_0}{2\pi} (\text{MHz})$. In the absence of an external magnetic field, the spin polarizations are uniformly distributed, pointing in all possible directions in space. The total magnetic moment of the sample is very close to zero, since approximately the same number of spins point towards a given direction as against it. A nuclear state with spin $I$ is $(2I+1)$-fold degenerate. If a magnetic field is applied, the degeneracy is broken, leading to a splitting between nuclear spin levels (nuclear Zeeman splitting). A nucleus of spin $I$ has $2I+1$ possible orientations, which are given by the magnetic quantum number ($m_I$) that has values $-I, -I+1, ..., I-1, I$. 


1.8.2 Excitation of spins $\frac{1}{2}$ nuclei

If the magnetic field is present, the spin polarization vectors steadily precess around the magnetic field at the Larmor frequency ($\omega_0$), forming a double cone, and are distributed in two different states at thermal equilibrium (Figure 1.8.2.11). The Larmor frequency is the frequency of precession of a nucleus, it is equal to:

$$\omega_0 = -\gamma B_0$$

(1)

For nuclei with $I=1/2$, the upper energy state is indicated by $N_\beta$ and the lower energy is indicated by $N_\alpha$. Under the influence of the magnetic field $B_0$, all the spins are polarized and the net macroscopic magnetization $M_0$ in the direction of $B_0$ since $N_\alpha$ is greater than $N_\beta$.

![Illustration of spin polarization vectors](image)

**Figure 1.8.2.11.** Illustration of the spin polarization vectors, precessing at the Larmor frequency around the magnetic field (Black arrows). The green arrow represents the net macroscopic magnetization $M_0$ towards the direction of the magnetic field $B_0$ (Red arrow). $N_\alpha$ and $N_\beta$ are the number of population of spins in the $\alpha$- and $\beta$-states.

There are two possible orientations of the nuclear spin, given by $m_I = \pm 1/2$. The lower state $\alpha$ is more stable than the higher state $\beta$ and therefore, it is the most populated. There are two allowed transitions $\alpha \rightarrow \beta$, which corresponds to absorption of energy, and $\beta \rightarrow \alpha$, which corresponds to emission of energy. The excess of population in the lower state
determines the probability of a transition to the higher state, \( \alpha \rightarrow \beta \). For detection of this transition energy, radiation given by \( \Delta E = h\nu_1 \) must be applied and the *resonance condition* is obtained in Equation 2.

\[
\omega_0 = \nu_1 = \frac{\gamma}{2\pi} B_0
\]  

(2)

For NMR, frequency \( \nu_1 \) occurs in the radiofrequency (rf) region, and the NMR signal obtained is directly proportional to the number of nuclei producing it. The system tends to return to the thermodynamic equilibrium situation as the magnetization decays with time (Figure 1.8.2.12).

![Figure 1.8.2.12. Illustration of the resonance condition, the green arrow represents the transition from the lower level to the higher level, \( \alpha \rightarrow \beta \). The red arrow represents the transition from the higher level to lower level, \( \beta \rightarrow \alpha \).](image)

The corresponding *Free Induction Decay* (FID) induces an electric current in a *detection coil* and is recorded during a period of approximately 1s, and then the NMR spectrum is obtained by *Fourier Transformation* (FT) of these data. Relaxation time is the time needed to relax the nuclei back to thermal equilibrium. This is often a long time of several seconds or sometimes minutes. Since the energy difference \( \Delta E = h\nu_1 \) is very small compared with the average energy \( K_B T \) of the thermal motions, \( h\nu_1 \ll k_B T \), where \( k_B \) (1.380 \( \times \) \( 10^{-23} \) m\(^2\) Kg s\(^{-2}\)) represents the Boltzmann constant, \( T(K) \) is the temperature and \( h \) (6.626 \( \times \) \( 10^{-34} \) m\(^2\) Kg s\(^{-2}\)) is the Planck constant. Consequently the populations of the energy levels are nearly equal (Equation 3).
\[ \frac{N\alpha}{N\beta} = e^{-\left(\frac{h\nu}{k_B T}\right)} = 1 - \left(\frac{h\nu}{k_B T}\right) \]  

(3)

The gap between the two energy states increases with the magnetic field strength, \( B_0 \) (Figure 1.8.2.13).

Figure 1.8.2.13. Illustration of the gap between the two energy states that increases with the magnetic field strength, \( B_0 \).
1.8.3 $T_1$ and $T_2$ relaxation times

The longitudinal macroscopic magnetization vector $M_z$, along the z-axis, is made up of the sum of the microscopic vectors of individual nuclear spins. After the application of the rf pulse, the net magnetization vector is rotated on the y axis, and gives $M_x$ and $M_y$ components (Figure 1.8.3.14, a). This net magnetic vector perpendicular to the magnetic field is called transverse magnetization ($M_{x,y}$). Transverse magnetization in the $x, y$ plane precess under the influence of the static magnetic field $B_0$ at the Larmor frequency, $\omega_0$. In this state, the equilibrium is perturbed, and nuclei are restored to an equilibrium state via two different mechanisms known as longitudinal relaxation time or spin-lattice relaxation time ($T_1$) and transverse relaxation time or spin-spin relaxation time ($T_2$).

The Longitudinal relaxation time or spin-lattice relaxation time ($T_1$) describes the rate at which the transverse magnetization $M_{x,y}$ returns to the thermodynamic equilibrium orientation along $B_0$ after a rf 90° pulse (Figure 1.8.3.14, b-d, black arrows). In this and to ensure the full recovery of the nuclear magnetization, it is necessary to wait for a period of time of $5T_1$. For a proton, this time usually ranges from 0.5-1.0 s, whilst for carbon it is ranged about 2.0 s-1.0 min. $T_1$ is correlated with the overall rotational tumbling of the molecule in solution and may be further affected by intramolecular mobility.

The transverse relaxation time or spin-spin relaxation time ($T_2$) describes the decay rate of the effective magnetization observed in the $x, y$ plane after 90° pulse (Figure 1.8.3.14, b-d, green arrows). The $T_2$ is a constant and is shorter than or equal to $T_1$. Differences between $T_1$ and $T_2$ arise because of loss of phase coherence among the individual spins that precess in the $x, y$ plane at different rates, due to spin-spin coupling and chemical shift differences. $T_2$ is correlated with dynamic processes in the molecule under study and is affected by intra- and inter-molecular interactions. Therefore, it could be useful to study dynamic process and molecular structure since it decreases with increasing molecular size.
Figure 1.8.3.14. Illustration of the 90° rf pulse of suitable chosen duration on the net magnetization vector, $M_z$. (a) The rf pulse rotates $M_z$ into the $x$, $y$ plane perpendicular to $B_0$. Both (b) and (c) describe the recovery of the nuclear magnetization, in (b) $M_z$ is shorter than $M_{x, y}$ and in (c) $M_z$ is longer than $M_{x, y}$. In (d) $M_z$ is equal to $M_{x, y}$. The black arrows represent $T_1$ and the green arrows represent $T_2$.

1.8.4 Chemical shift

As electrons circulate around a nucleus, a local magnetic field is generated $B_{\text{loc}}$, which is opposed to the external magnetic field, $B_0$. Therefore, the nucleus is shielded from $B_0$ and is exposed to an effective magnetic field, $B_{\text{eff}}$(Equation 4).

$$B_{\text{eff}} = B_0 - B_{\text{loc}}$$

A more accurate description of the resonance condition takes into account this effect by introducing the shielding constant, $\sigma$ (Equation 1.8.4.5). Its value is proportional to the degree of shielding by the electron cloud. At a given value of $B_0$, the effective frequency at resonance is less than the applied frequency $\nu_1$ (Equation 5).

$$\nu_{\text{eff}} = \frac{|\nu|}{2\pi} |(1 - \sigma)B_0$$

The degree of shielding of a proton on a carbon atom will depend on the inductive effect of other groups attached to the carbon atom.
The chemical shift (δ) is the resonance frequency of a nucleus to a standard and is defined as the nuclear shielding divided by the applied oscillator frequency (Equation 6). The chemical shift is only a function of the nucleus and its environment.

$$\delta \text{ (ppm)} = \frac{\nu_{\text{reference}} - \nu_{\text{sample}}}{\text{Oscillator frequency (Hz)}} \times 10^6 \quad (6)$$

For samples containing $^1$H and $^{13}$C, the reference compound used is usually tetramethylsilane (TMS), since it is inert, soluble in most organic solvents and appears as a sharp singlet due to its twelve identical protons. By definition its chemical shift is assigned a value of 0.00ppm.

### 1.8.5 Spin-spin coupling constant (J)

Spin-spin coupling constant (J) characterize scalar interactions (through-bond) between nuclei linked via a small number of covalent bonds in a chemical structure (usually 1-5 bonds away). J is field independent and is customarily quoted in Hz, and it depends on both the number of bonds separating nuclei and their conformation. For example, considering two spins A and B (I=$\frac{1}{2}$), connected to each other by three bonds. In the presence of $B_0$, A can exist in two states $\alpha$ and $\beta$; in the $\alpha$ state its magnetic moment is aligned towards the magnetic field $B_0$ and in the $\beta$ state its magnetic moment is aligned against the magnetic field $B_0$. Since the two orientations are equally probable, the A peak is split into a doublet with a spacing of $^3J_{A,B}$; the same applies for B (Figure 1.8.5.15, a). The spin-spin coupling constant (J) is also correlated to the dihedral angle, $\varphi$. The relationship between the dihedral angle and $^3J_{H,H}$ coupling constant for vicinal protons is shown in Equation 7, where $X$, $Y$ and $Z$ are constants dependent on the bond types present.

$$^3J = (X \cos^2 \varphi - Y \cos \varphi + Z) \quad (7)$$

An example of a Karplus curve is illustrated in Figure 1.8.5.15, b.
Figure 1.8.5.15. (a) Illustration of the spin-spin coupling, using a typical $^1$H-NMR spectrum for two protons $H_A$ and $H_B$ that are three bonds apart. In the absence of any spin-spin coupling, two single peaks are observed for $H_A$ and $H_B$ protons at $\nu_A$ and $\nu_B$, respectively. In the case of spin-spin coupling, the proton $H_A$ is split into a doublet by $H_B$ (According to the rule $2I+1$). The same effect is observed for the proton $H_B$. (b) Illustration of a Karplus curve.

1.8.6 Linewidth and line intensity

The resonance linewidth in a NMR spectrum determines the spectral resolution that can be attained at a given field strength, $B_0$. Assuming a Lorenzian line shape, the linewidth is presented as the half-width at the half-height of the line $\Delta \nu_{1/2}$, in Hz units. In a NMR experiment with a kinetically stable system, the linewidth is simply related to $T_2$ and it is denoted $\Delta \nu_{1/2} = 1/2\pi T_2$. Therefore, a short $T_2$ indicates fast relaxation that leads to broad NMR lines, whilst a long $T_2$ indicates slow relaxation and sharp NMR lines.$^{29}$ Linewidth is drastically affected by exchange processes and experimental artefacts, such as inhomogeneity of the static magnetic $B_0$. Such artefacts affect the experimentally observed, apparent $T_2$ relaxation time, $T^*_2$, and are denoted by $T^{+}_2$ (Equation 8).

\[
1/T^*_2 = 1/T_2 + 1/T^{+}_2 \quad (8)
\]
The *line intensity* is another important parameter in NMR. In a $^1$H-NMR spectrum, the relative line intensity of NMR resonances corresponds to the number of protons giving rise to the signal.

**1.8.7 NMR time scale**

A qualitative appreciation of NMR time scales is indispensable for designing NMR experiments and analysing NMR spectra. Furthermore this provides a survey of the dynamic processes that may be studied by NMR. Molecular rate processes with characteristic times in the range from approximately $10^{-7}$ to $10^{-12}$ s are thus directly manifested in the relaxation parameters $T_1$ and $T_2$ (including the overall rotational tumbling of the molecules in solution). An important message is that a particular NMR experiment may cover largely different time scales, depending on the chemical shift differences between the exchanging nuclei. Corresponding to short relaxation times $T_2$, the NMR lines for macromolecules are broadened compared to small molecules. Further *line broadening* may result from aggregation or increase in viscosity, which are common in concentrated solutions of biopolymers.
1.8.8 Rotational correlation time ($\tau_c$)

Rotational motions are characterised by a correlation time ($\tau_c$) that is equal to $\omega_0^{-1}$. Typically, correlation time ($\tau_c$) for molecular motions, both rotational and translational, is ranged $10^{-12}$- $10^{-11}$ ns for small molecules (Figure 1.8.8.16). In this situation of extreme notional narrowing, $T_1=T_2$ and both relaxation times decreases proportional to $\tau_c$. For small molecules, the frequencies of rotational tumbling are high relative to the resonance $\omega_0$ at a fixed magnetic field strength, $B_0$. If $\tau_c$ becomes larger, $T_1$ increases proportionally to $\tau_c$ and $T_2$ decreases proportional to $\tau_c$. For macromolecules the frequencies of rotational tumbling are slow relative to $\omega_0$ at a fixed magnetic field strength ($B_0$).

![Figure 1.8.8.16. Illustration of the dependence of $T_1$ and $T_2$ on the rotational correlation time, $\tau_c$.]
1.8.9 Nuclear Overhauser Enhancement (NOE)

The Nuclear Overhauser Enhancement (NOE) experiments provide data on internuclear distances. This data can be more directly correlated with the molecular conformation. The NOE is a consequence of modulation of the dipole-dipole coupling between different nuclear spins by the Brownian motion of the molecules in solution. The NOE intensity is a function of the distance ($r$) between preirradiated and observed spin and the correlation time, $\tau_c$ (Equation 9).

$$\text{NOE} \propto \frac{1}{r^6} \cdot f(\tau_c) \quad (9)$$

If a pair of protons AX, close in space and not $J$-coupled, is now considered. Such a system has four energy states, corresponding to the $\alpha\alpha$, $\alpha\beta$, $\beta\alpha$, and $\beta\beta$ spin states (Figure 1.8.9.17). Interaction among protons will cause $T_1$ relaxation between the spin states with the transition probabilities $W_1$ (Single quantum relaxation $\alpha\alpha\leftrightarrow\alpha\beta$, $\alpha\alpha\leftrightarrow\beta\alpha$, $\alpha\beta\leftrightarrow\beta\beta$ and $\beta\alpha\leftrightarrow\beta\beta$), $W_2$ (Double-quantum relaxation $\alpha\alpha\leftrightarrow\beta\beta$) and $W_0$ (Zero-quantum relaxation $\alpha\beta\leftrightarrow\beta\alpha$). Initially, the spins are at equilibrium in the ground $\alpha\alpha$ state ($N/4+\Delta$ population), which is the most populated. The $\beta\beta$ state ($N/4-\Delta$ population) is the least populated, and the $\alpha\beta$ and $\beta\alpha$ state ($N/4$ populations) are equally populated.

Figure 1.8.9.17. Illustration of the energy level diagram for a NOE experiment. $N$ is the total number of spins and $\Delta$ is the excess in population.
If X-transitions of the X nucleus are irradiated (A nucleus is unaffected), populations of \( \alpha\alpha \) and \( \alpha\beta \), and \( \beta\alpha \) and \( \beta\beta \) are equalized (Saturated) through \( \alpha\alpha \rightarrow \beta\alpha \) and \( \alpha\beta \rightarrow \beta\beta \) (Figure 1.8.9.18, 1). In this situation the equilibrium is perturbed, subsequently the different relaxation mechanisms compete with each other to restore equilibrium (Figure 1.8.9.18, 2). The \( W_1 \) process re-establishes initial population condition, X nucleus relaxes \( \beta \rightarrow \alpha \) through \( \beta\beta \rightarrow \alpha\beta \) and \( \beta\alpha \rightarrow \alpha\alpha \) transitions (Figure 1.8.9.18, 3 and 4, a). In the \( W_0 \) process, the A nucleus undergoes a \( \alpha \rightarrow \beta \) when the X nucleus relaxes \( \beta \rightarrow \alpha \) through \( \alpha\beta \rightarrow \beta\alpha \) transition (Cross-relaxation) (Figure 1.8.9.18, 3 and 4, b). This process dominates when the molecular motions are slower than \( \omega_0 \) and gives a negative NOE since the spin population of A decreases. In the \( W_2 \) process, both the X nucleus and the A nucleus relax \( \beta \rightarrow \alpha \) through \( \beta\beta \rightarrow \alpha\alpha \) transitions (Figure 1.8.9.18, 3 and 4, c). This process gives a positive NOE since the spin population of A increases and dominates when the molecular motions are faster than \( \omega_0 \). To summarise, in the case \( W_2 > W_0 \), the NOE will be positive. Conversely, in the case \( W_0 > W_2 \), the NOE will be negative and if \( W_2 = W_0 \) then there is no NOE.
Figure 1.8.9.18. Illustration of the scheme of the transitions probabilities $W_0$, $W_1$, and $W_2$. 
1.8.9.1 Chemical exchange

Chemical exchange processes are very significant in NMR spectroscopy since they can provide information on dynamic processes. Linewidth and line intensity of NMR resonances are very sensitive to chemical exchange processes. If a molecule is in equilibrium between two conformations, A and B, the two nuclei will experience different chemical environments (Figure 1.8.9.1.19). As the chemical shift depends on the chemical environment, the effect on the resonance positions will depend on the rates of interconversion $k_A$ and $k_B$. There are three types of exchange regimes:

1) Slow exchange on NMR timescale, whereby $k_A$ and $k_B \ll \Delta \nu$ ($\Delta \nu$ is the difference in chemical shift in Hz). In this situation two distinct peaks, corresponding to the two different conformations of A and B, are observed.

2) Intermediate exchange on NMR timescale, the interconversion rate increases, whereby $k_A$ and $k_B \sim \Delta \nu$. In this exchange regime, there is a partial averaging of the two chemical shifts that lead to a broadening of both resonances peaks.

3) Fast exchange on NMR timescale, whereby $k_A$ and $k_B \gg \Delta \nu$. In this regime, the effects of the chemical shifts are completely averaged and one single resonance peak is observed between the positions of the A and B resonances.
Figure 1.8.9.1.19. Illustration of the chemical exchange between two conformations A and B of the same nucleus.
1.9 NMR experiments

One-dimensional and two-dimensional homo- and heteronuclear NMR experiments are used to help in assignment of the RNA and the drug. The following section describes the 1D and 2D NMR experiments used.

1.9.1 1D NMR experiment

A general 1D NMR experiment includes three successive time periods.

- Relaxation delay \( (d_1) \)
- \( 90^\circ \) rf pulse \( (90^\circ x) \)
- Acquisition time \( (t_2) \)

The relaxation delay consists of a delay time \( (d_1) \), during which thermal equilibrium is attained, followed by one \( 90^\circ \) rf pulse to create phase coherence among like or unlike spins. After the equilibration and the rf pulse \( (90^\circ x) \), delay time and acquisition of the order of seconds (Figure 1.9.1.20, a) occur. This sequence is repeated until a satisfactory signal/noise ratio is obtained. The information about chemical shift and spin-spin coupling are developed during the acquisition time \( (t_2) \) along the \( x, y \) plane and then detected by the receiver. Subsequently, the detected time domain signal called the free induction delay (FID) is Fourier transformed to generate a one dimensional frequency domain spectrum.

(a)                                                                (b)

Figure 1.9.1.20. Illustration of the comparison between (a) a normal \( ^1\text{H}-\text{NMR} \) experiment and (b) \( X \{ ^1\text{H} \} \) decoupled pulse sequence, where \( X \) indicates any heteronucleus.
The sequence in the X channel is exactly the same as the sequence in the $^1$H channel (Figure 1.9.1.20, b), but the proton decoupler is tuned on during experiments, such as $^{13}$C-NMR and $^{31}$P-NMR. The protons are decoupled from X nuclei by irradiating the protons with a stronger 90°rf pulse, whilst the X nucleus is observed with a normal 90°rf pulse. This broadband decoupling technique results in effective decoupling of all protons, simplifying the 1D spectrum and eliminating overlapped peaks and multiplets. Furthermore, an enhancement in sensitivity is obtained for the insensitive nuclei.

1.9.2 Variable Temperature (VT) experiment

Variable Temperature (VT) experiments involve 1D NMR experiments at different temperatures, allowing the observation of the temperature-dependent effects in NMR spectra. A stack plot of 1D experiments can be used to monitor changes in chemical shift and resonance peak intensity, providing information on the stability of the drug and RNA base pairings.

1.9.3 2D NMR experiment

A general scheme for a 2D NMR experiment includes four successive time periods:

- Relaxation delay ($d_1$)
- Evolution time ($t_1$)
- Mixing time ($\tau_m$)
- Acquisition time ($t_2$)

The advantage of 2D NMR experiments is that overlapped signals can be spread along the second dimension. In a 2D NMR experiment, the relaxation delay is followed by two pulses with an intervening time interval. This “evolution” period is inserted between the pulses and is termed evolution time ($t_1$). The evolution time ($t_1$) is systematically increased in increments ($t_1 + \Delta t$) after each pulse sequence. Varying the evolution period interval over many different experiments, the resulting FIDs are collected in one overall experiment. This first series of Fourier transformations of these FIDs, results in the second frequency axis, F2 of the 2D NMR spectrum, derived from the acquisition time ($t_2$) of each FID. Data are then turned by 90°, and a second Fourier transformation is
carried out. This second Fourier transformation results in the first frequency axis, F1 of the 2D NMR spectrum which is a function of the evolution time \( t_1 \), incremented in each successive FID. A general scheme is shown in Figure 1.9.3.21. The NMR spectrometer has a set of independent frequency channels, each of which may be tuned on/off to examine a single narrow frequency window. For example, a two-channel spectrometer may be used to examine narrow frequency windows around the \(^1\text{H}\) and \(^{13}\text{C}\) Larmor frequencies.

![Figure 1.9.3.21. General scheme for a 2D NMR experiment.](image)

2D NMR experiments for delineating through-bond, scalar coupling connectivities, include COSY and DQF-COSY. 2D NMR experiments for delineating through-space, scalar coupling connectivities, include NOESY and ROESY. A more detailed description of the 1D and 2D NMR experiments is reported in materials and methods.
Chapter 2: Materials and Methods

Chapter 2 describes the materials used and methods adopted to acquire, process and analyze all the NMR data related to neamine 12 and 27mer RNA. All the NMR experiments were set up with the assistance of the technical staff at the School of Chemistry, University of Manchester. All the data processing, analysis and interpretation were carried out by the author of this thesis. All the diagrams were drawn using MS software packages PowerPoint, UCSF Chimera, PyMOL and ChemDraw Ultra 7.0.

2.1 Sample preparation for NMR studies

Three main samples were prepared for NMR investigations; the sample of neamine derivative 12, the sample of 27mer RNA and the sample of its complex with the neamine 12.

2.1.1 Neamine 12 sample preparation for NMR studies

The neamine 12 antibiotic was synthesized and kindly supplied by the State Key Laboratory for Natural and Biomimetic Drugs, Peking University, Beijing. Figure 2.1.22 shows the synthesis strategy of the neamine derivative 12 (II) from the azidodisaccharide (I). Azidodisaccharide (I) was obtained by the known procedures in good yield to give Azidodisaccharide–nucleoside conjugate (neamine 12 (II)). The reaction conditions for step a were: (i) trifluoromethanesulfonic anhydride (TF₂O), pyridine, CH₂Cl₂; (ii) NaNO₂, 15-crown-5, DMF, rt, and for b were: (i) dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, DMF, 0°C to rt; (ii) K₂CO₃, CH₃OH, rt.
Figure 2.1.1.22. Illustration of the synthesis strategy of the neamine derivative 12 (II) from the azidodisaccaride (I). B=uracil and n=2.

The NMR sample was prepared by dissolving of 2.2mg of neamine in 0.6mL of $^1$H$_2$O, the final concentration was 5.2 mM.
2.1.2 RNA sample preparation for NMR studies

The single stranded 27mer RNA bearing the sequence 5′-GGCGUCACACCUCGUUGGAAGUCGCC-3′ was chemically synthesised with HPLC purification and obtained commercially (Metabion, Germany). The NMR samples were prepared by dissolving the RNA in approximately 1mL phosphate buffer (20mM K$_3$PO$_4$ and 20mM NaCl, pH 6.2). The samples were then annealed by heating to 80°C followed by slow cooling. The samples were then dissolved by the addition of 90% filtered Q-water (H$_2$O) and 10% deuterium oxide (D$_2$O) and transferred into a 5mm NMR tube with a total volume of 600μL. The final concentration of 27mer RNA was 5.0μM. For UV absorbance measurements, the diluted RNA samples were placed in a clean sterile cuvette containing 1000μL filtered Q-water prepared as “blank”. RNA absorbs at 260nm producing a sharp peak observable in the spectrum. The absorbance at this wavelength was used to calculate the concentration of the sample using the molecular weight and the molar extinction coefficient. These values were obtained using an oligonucleotide calculator online RiboTask. The UV absorbance melting experiment (A$_{260}$ versus Temperature) was carried out over 20°C to 90°C @ 1°C per minute using an Agilent spectrophotometer, the resulting UV melting temperature was 75.1°C. The 27mer RNA-neamine 12 complex was prepared by addition of 0.44mM of neamine 12 to one molar equivalent (0.44mM) of 27mer RNA to give a 1:1 complex.

2.2 FT-NMR spectrometers

All the NMR experiments (H, C and P) were carried out using a Bruker Avance 400MHz NMR spectrometer at the School of Chemistry of the University of Manchester. The Bruker Avance 400MHz spectrometer are equipped with a broadband (N-P) inverse detection probe to detect H, C, N and P nuclei and electronic temperature control units. All the 1D and 2D NMR experiments were carried out using manufacture supplied pulse programs.
2.3 NMR experimental parameters

Several NMR experimental parameters were considered when performing 1D and 2D NMR experiments using FT-NMR spectrometers. The most important experimental parameters taken into account before setting up a 1D NMR experiment were the pulse sequence (PULPROG), the Pulse Width (PW), Spectral Width (SW), acquisition time (ac), Number of Scan (NS), number of data points (TD), relaxation delay (d1), carrier frequency (O1), temperature (TE), solvent, and the spectrometer operating frequency (SFO1). For 2D NMR experiments, in addition to the above parameters, mixing time ($\tau_m$) for NOESY and spin lock mixing time ($SL_m$) for TOCSY, HSQC-TOCSY and ROESY must be set. In a typical NMR acquisition, the pulse sequence was chosen according to the 1D or 2D NMR experiment to be performed (all the pulse sequences used are described in this section). The pulse sequence was repeated many times in order to improve the signal-to-noise ratio (S/N), which increases as the square root of the number of scans (ns), according to the Equation 10.\(^{32}\)

$$S/N = \sqrt{ns} \quad (10)$$

The spectrometer operating frequency was 400.232 MHz for $^1$H-NMR experiments and 100.7 MHz for $^{13}$C-NMR experiments at the external magnetic field strength of 9.40 T, according to Equation 11.

$$\nu_f = \frac{\gamma}{2\pi} B_0 \quad (11)$$

$\nu_f$ = resonance frequency (MHz)

$\gamma$ = gyromagnetic ratio (10\(^7\) rad s\(^{-1}\)T\(^{-1}\))

$B_0$ = external magnetic field (T)

The number of scans ranged from 128-512 for the $^1$H-NMR experiments and for the $^{13}$C-NMR experiments was 1024, while it ranged from 64-256 for 2D experiments. The spectral width used in $^1$H-NMR experiments depends on the solvent. Consequently, a larger spectral width range of 20-24 ppm was used for samples in $^1$H\(_2\)O, while a smaller
spectral width of about 12 ppm was used for samples in $^2$H$_2$O. The carrier frequency was always set on the water signal frequency for both 1D and for 2D experiments. The total number of data points represents the number of points along the exponential FID decay. In general, the more points used to acquire the FID, the higher the resolution of the NMR spectrum. Hence, the number of points (TD) acquired was 65536 for 1D NMR experiments, while it was set to 4096 (TD2) and 512 (TD1) for 2D experiments. The relaxation delay was set at 2.0 s for 1D NMR experiments and 1.0s for 2D NMR experiments. The acquisition time is the time required to acquire the FID and was set according to Equations 12 and 13, where res indicates the digital resolution:

$$ac = \frac{np}{2sw} \quad (12)$$

$$res = \frac{1}{ac} = \frac{2sw}{np} \quad (13)$$

The mixing time for NOESY experiments was set at 250 ms and the spin lock mixing time for ROESY, TOCSY and HSQC-TOCSY experiments were set at 250 ms, 75 ms and 75 ms, respectively. The WATERGATE (described in 2.5.1) water suppression technique was used in order to suppress the large, intense water signal for samples prepared in $^1$H$_2$O.
2.4 NMR data processing and analysis

The 1D NMR data were processed by SpinWorks.\textsuperscript{54} The program uses a Lorentzian apodisation function, the line broadening was adjusted to reduce the noise in the spectrum. The 2D raw NMR data were processed using the program NMRPipe, which is a UNIX based command line program and has a graphical user interface called NMRDraw.\textsuperscript{55} The initial processing was based on a typical conversion script and then modified to correct the phase and baseline and remove water suppression. A standard data processing script is shown in Appendix I. A typical NMRPipe script is composed of three parts. The first part converts the .fid file from Bruker to NMRPipe format, allowing NMRPipe to read acquisition parameters. These parameters, such as acquisition mode and carrier frequency, could be checked and/or altered. Echo-Anti Echo acquisition mode was set for HSQC and HSQC-TOCSY experiments and States-TPPI was set for TOCSY, ROESY, NOESY and DQF-COSY experiments. The carrier frequency was positioned on the water peak, which was calibrated according to the temperature of the NMR experiment. The second part of the script involves methods of data processing, which includes solvent filter, baseline correction, window function, zero filling, Fourier transformation and phase correction. Typically, 2D data processing included zero filling, a Gaussian apodisation function in the first dimension and zero filling and adjustable sine apodisation function in the second. The third part of the script converts the processed data file into a .ucsf format, which can be visualised by the program Sparky. The processed 2D data were then analysed using the graphical program Sparky.\textsuperscript{56}
2.5 NMR techniques

Several strategies for elucidation of the nucleic acids structure were developed exploiting well established two-dimensional NMR techniques, such as phase-sensitive double-quantum-filtered correlated spectroscopy, relayed coherence transfer spectroscopy, double quantum spectroscopy and nuclear overhauser spectroscopy.\(^5\) Due to the improved resolution in phase-sensitive spectra, the fine structure of cross peaks could be exploited as a powerful source of information for establishing \(^1\)H-\(^1\)H connectivities.

2.5.1 \(^1\)H-NMR experiments with solvent suppression

To suppress the sharp water resonance at 5.0 ppm, which is much more intense than the peaks of the solute of interest, several methods have been developed. The common water suppression techniques include presaturation and water suppression by Gradient Tailored Excitation (WATERGATE).\(^5\)\(^8\)\(^-\)\(^9\) Presaturation is a simple two pulse experiment that utilizes a long, low power 90° rf pulse to selectively saturate a specific frequency (water frequency), and a non-selective 90° pulse to excite the desired resonance (Figure 2.5.1.23, a). This method significantly reduces the intensity of the water peak and enhances the sensitivity of the sample. But it has one disadvantage, this method also affects the intensity of the peaks close to the water frequency. This technique is particularly important for biological macromolecules, since the concentration of the solute is generally 1mM or less, while the concentration of the protons is 110M.

![Figure 2.5.1.23. Illustration of (a) the presaturation pulse sequence and (b) WATERGATE pulse sequence.](image)

The WATERGATE pulse was designed to provide high selectivity and effective water suppression, using a combination of tailored excitation with pulsed magnetic field gradients
(Figure 2.5.1.23, b). It consists of a non-selective 90° pulse that excites uniformly all the protons, followed by an echo section of pulse sequence formed by two short magnetic field gradient pulses. The first magnetic field gradient dephases all the proton resonances. The 180° selective pulse acts on all the protons except for the water protons. The second magnetic field gradient refocuses all the coherences dephased by the first magnetic gradient. The water proton resonance keeps being dephased since it is unaffected by the 180° selective pulse. Subsequently, the FID is acquired with the water signal significantly suppressed.\(^ {60-62}\)

### 2.5.2 2D Double-Quantum Filtered Correlation Spectroscopy (DQF-COSY)

One of most basic 2D NMR experiments is the homonuclear COSY (COrelated Spectroscopy) experiment (Figure 2.5.2.24, a).\(^ {63-64}\) The COSY technique is used to determine basic connectivity between protons within the same scalar coupled spin system separated by three chemical bonds. However, crosspeaks are difficult to see when they occur close to the diagonal. Consequently, the Double-Quantum Filtered (DFQ) COSY experiment is employed to reduce the intensity of the diagonal peaks by filtering the double quantum transitions and subsequently to resolve any cross peaks originally obscured by the broad diagonal in a standard COSY experiment (Figure 2.5.2.24, b).\(^ {22, 48, 57}\) Single quantum transitions are suppressed by selecting coherence transfer between the evolution and detection periods. The signal detected during \(t_2\) derives from double-quantum coherence present between the second and the third 90° pulses, separated by a fixed delay time (\(\tau_0\)), typically of a few milliseconds.

![Figure 2.5.2.24](https://example.com/figure25224.png)

**Figure 2.5.2.24.** Illustration of (a) the COSY and (b) the DQF-COSY pulse sequences.
2.5.3 2D Total Correlation Spectroscopy (TOCSY)

The homonuclear 2D TOCSY (TOTal Correlation Spectroscopy) experiment is similar to the COSY experiment (Figure 2.5.3.25). The TOCSY pulse sequence differs from the COSY pulse sequence with the addition of the isotropic spin lock mixing time ($SL_m$). During this mixing time, magnetization transfer is possible between coupled protons within the same spin system, even if they are more distant from one another (>3 bonds). The extent of the magnetization transfer depends on the length of the mixing time, $SL_m$. Consequently, cross peaks found in TOCSY spectra correspond to correlations between protons belonging to the same coupled spin system. In a TOCSY experiment, it is possible to observe correlations among adjacent protons at shorter mixing times, while longer mixing times allow correlations among more distant nuclei to be observed.

![Figure 2.5.3.25. Illustration of the TOCSY pulse sequence.](image)

Figure 2.5.3.25. Illustration of the TOCSY pulse sequence.
2.5.4 2D Heteronuclear Single Quantum Coherence (HSQC)

The HSQC (Heteronuclear Single Quantum Coherence) experiment is used to determine couplings between $^1$H-X nuclei that are directly attached, $^1J_{H,X}$ (Figure 2.5.4.26).\textsuperscript{66-67} In the 2D heteronuclear NMR experiments, there are problems related to the lower gyromagnetic ratio of the $^{13}$C nuclei, as compared with $^1$H, which reduces the sensitivity of $^{13}$C nuclei. To enhance the sensitivity of $^{13}$C nuclei, the INEPT (Intensive Nuclei Enhanced by Polarization Transfer) pulse sequence is incorporated into the HSQC experiment.

![Figure 2.5.4.26. Illustration of the HSQC pulse sequence, where $\tau$ represents delay times that depend on the value of $^1J_{H,X}$ typically a value equal to $\frac{1}{2}T_1$ is used.](image)

The experiment starts with a 90° pulse in the $^1$H channel, which is followed by the first INEPT step where proton anti-phase magnetization is refocused by two 180° pulses. After refocusing, the magnetization is transferred to the heteroatom (X), which evolves with the chemical shift of the heteroatom. Proton coupling is removed by a 180° pulse during the $t_1$ period. Thereafter, INEPT step is carried out, which transfers magnetization back to the proton. When magnetization is back on the proton, it is then refocused by the double 180° pulses. The magnetization of the proton is detected during $t_2$. 
2.5.5 2D Heretonuclear Single Quantum Coherence-Total Correlation Spectroscopy (HSQC-TOCSY)

The 2D $^1$H-$^{13}$C/$^{15}$N HSQC-TOCSY experiment, is a combination of the HSQC and TOCSY experiments (Figure 2.5.5.27).\textsuperscript{52-53, 68} The HSQC-TOCSY experiment starts with the same pulse sequence of the HSQC, a spin-lock mixing time is inserted after the 180° pulse in the $^1$H channel. The HSQC pulse sequence correlates protons with their directly bonded heteroatom ($^1J_{H,X}$), followed by the TOCSY sequence, which then transfers the magnetization throughout the spin system. The extent of magnetization transfer depends on the length of the mixing time of the TOCSY experiment. A good resolution of the $^1$H-$^1$H and $^1$H-X couplings can be obtained in this experiment.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{hsqc-tocsy-pulse-sequence.png}
\caption{Illustration of the HSQC-TOCSY pulse sequence, where $\tau$ represents delay times that depend on the value of $^1J_{H,X}$, typically a value equal to $\frac{1}{4J}$ is used.}
\end{figure}
2.5.6 2D Nuclear Overhauser Effect Spectroscopy (NOESY)

The Nuclear Overhauser Effect Spectroscopy (NOESY) is the most important technique used for the structure determination of large molecules, such as nucleic acids and proteins (Figure 2.5.6.28). This homonuclear experiment is normally used to determine dipolar coupling within a molecule. Therefore, in NOESY experiments, it is possible to observe proximity of nuclei through space when they are less than approximately 5.0 Å apart. The pulse sequence of the NOESY experiment consists of three 90° x pulses. There is an evolution period \( t_1 \) between the first and the second 90° x pulses and a mixing time \( t_m \) between the second and the third 90° x pulses, followed by acquisition time \( t_2 \). It is possible to vary the mixing time, this is very useful for the structure determination. The magnitude of the cross relaxation depends on the distance between spins in space and also on the correlation time of the molecule \( \tau_c \).

![Figure 2.5.6.28. Illustration of the NOESY pulse sequence.](image)
2.5.7 2D Rotational Overhauser Effect Spectroscopy (ROESY)

The homonuclear Rotational Overhauser Effect Spectroscopy (ROESY) experiment is very similar to the NOESY (Nuclear Overhauser Effect Spectroscopy) experiment (Figure 2.5.7.29). The ROESY pulse sequence consists of a 90° pulse followed by the evolution period ($t_1$). After the evolution period a series of 180° pulses is applied, followed by the acquisition time, $t_2$. This technique is known as rotating NOE experiment since ROEs develop on the transverse $x, y$ plane. It is used to study NOEs of smaller molecules with an average mass between 700-1200 Dalton. In ROESY spectra, positive cross peaks that arise from protons that are close to each other in space (dipolar coupling), can be observed. Consequently, ROESY spectra provide information about $^1$H-$^1$H through-space proximity. The ROE build-up depends on the molecular correlation time, $\tau_c$. For large molecules a longer mixing time is used and for small molecules a shorter mixing time is used.

![Illustration of the ROESY pulse sequence](image)

Figure 2.5.6.29. Illustration of the ROESY pulse sequence, where P2 and P3 represent a series of 180° pulses.
2.6 Assignment strategies

A suite of 2D NMR experiments were carried out, in support of 1D NMR experiments, to assign the resonances of the neamine 12 and 27mer RNA and to investigate their interactions.

2.6.1 Neamine 12 assignment strategy

The 1D $^1$H-NMR spectrum of neamine 12 in $^1$H$_2$O exhibited a wide dispersion of signals arising from the various groups. To assign the protons of neamine 12, a suite of $^1$H-$^1$H and $^1$H-$^{13}$C correlated 2D NMR experiments carried out in support of the $^1$H-NMR spectra. Therefore, through-bond and scalar connectivities were first established in the DQF-COSY experiment and then spin systems within the neamine 12 were identified in the TOCSY experiment. Thereafter, the HSQC and HSQC-TOCSY techniques were used to assign the $^{13}$C nuclei. Following the resonance assignment, to elucidate through-space (dipolar) interactions among protons of neamine 12, a $^1$H-$^1$H correlated ROESY experiment was carried out. The protocol used for the assignment of the neamine 12, is shown in Table 2.6.1.1.

<table>
<thead>
<tr>
<th>Assignment strategy</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Through–bonds connectivities</td>
<td>400MHz $^1$H-$^1$H DQF-COSY in $^1$H$_2$O at 2°C</td>
</tr>
<tr>
<td>Identification of spin systems</td>
<td>400MHz $^1$H-$^1$H TOCSY(75ms) experiment in $^1$H$_2$O at 2°C</td>
</tr>
<tr>
<td>Assignment of $^1$H nuclei</td>
<td>400MHz $^1$H-NMR, $^1$H-$^1$H DQF-COSY, $^1$H-$^1$H TOCSY(75ms) experiments in $^1$H$_2$O at 2°C</td>
</tr>
<tr>
<td>Assignment of the $^{13}$C nuclei</td>
<td>400MHz $^1$H-$^{13}$C HSQC, $^1$H-$^{13}$C HSQC-TOCSY experiments in $^1$H$_2$O at 2°C</td>
</tr>
<tr>
<td>Through-space connectivities</td>
<td>400MHz $^1$H-$^1$H ROESY (250ms) experiment in $^1$H$_2$O at 2°C</td>
</tr>
</tbody>
</table>

Table 2.6.1.1. Protocol used for NMR assignment of the neamine 12. The boxes on the left indicate the assignment obtained from the corresponding experiment(s). The boxes on the right indicate information about the NMR experiment(s) performed, the magnetic field strength, the solvent used and the temperature.
2.6.2 RNA Assignment strategy \(^{25, 37, 72}\)

NMR spectroscopy is a powerful technique used to study the structure and dynamic of RNA. To assign the structure of RNA, 1D and 2D NMR experiments were carried out. The NMR assignment strategy of RNA is already established and usually follows a standard methodology.\(^{72}\) Furthermore, 1D and 2D NMR experiments provide information about base pairing, local conformation, secondary and tertiary structure of RNA. To provide the assignment of the RNA, three main steps should be followed. The first step is the identification of the base protons (NH, NH\(_2\), H2, H5, H6 and H8). The second step is to identify the sugar proton spin systems (H1’, H2’, H3’, H4’, H5’ and H5’’). The third step is to correlate the base proton to sugar protons using intra- and inter-nucleotide NOE connectivities. The protocol used for the assignment of the 27mer RNA, is shown in Table 2.6.2.2.

<table>
<thead>
<tr>
<th>Assignment strategy</th>
<th>Experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Identification of exchangeable (NH, NH(_2)/NH(_2^*))</td>
<td>400MHz (^1)H-NMR and (^1)H-(^1)H NOESY (250ms) experiments in (^1)H(_2)O at 2(^\circ)C</td>
</tr>
<tr>
<td>Assignment of exchangeable (NH, NH(_2)/NH(_2^*)) protons</td>
<td>400MHz (^1)H-(^1)H NOESY (250ms) in (^1)H(_2)O at 2(^\circ)C</td>
</tr>
<tr>
<td>Identification non-exchangeable (H5/ H6/ H8/ H2) protons</td>
<td>600MHz (^1)H-(^1)H NOESY (250ms) in (^2)H(_2)O at 25(^\circ)C</td>
</tr>
<tr>
<td>Assignment of (H5, H6, H8 and H2) aromatic protons</td>
<td>600MHz (^1)H-(^1)H NOESY (250 ms), 400MHz (^1)H-(^1)H DQF-COSY, 400MHz (^1)H-(^1)H TOCSY, 400MHz (^1)H-(^1)C HSQC experiments in (^1)H(_2)O at 25(^\circ)C</td>
</tr>
<tr>
<td>Sequential assignment H6/H8-H1’ and H6/H8-H2’</td>
<td>600MHz (^1)H-(^1)H NOESY (250 ms), 400MHz (^1)H-(^1)C HSQC in (^2)H(_2)O at 25(^\circ)C</td>
</tr>
<tr>
<td>Sugar (C1-H1’, C2-H2’, C3-H3’, C4’-H4’) scalar cross peak</td>
<td>400MHz (^1)H-(^1)C HSQC in (^1)H(_2)O at 25(^\circ)C</td>
</tr>
</tbody>
</table>

Table 2.6.2.2. Protocol used for NMR assignment of RNA. The boxes on the left indicate the assignment obtained from the corresponding experiment(s). The boxes on the right indicate information about the NMR experiment(s) performed, the magnetic field strength, the solvent used and the temperature.

To identify the exchangeable (NH, NH\(_2\)) protons, 1D \(^1\)H-NMR and NOESY experiments in \(^1\)H\(_2\)O at 2\(^\circ\)C were carried out. Subsequently, the imino (NH) protons were assigned following imino-imino connectivities. Thereafter, the imino-amino crosspeaks were identified and then the (NH\(_3\)/ NH\(_2^*\)) protons were assigned in the same NOESY spectrum. The NOESY experiment in \(^2\)H\(_2\)O at 25\(^\circ\)C was also performed to assign H5-H6
crosspeaks and to establish a sequential H6/H8-H1’/H2’ intra- and inter-nucleotide assignment. The $^1$H-$^{13}$C HSQC experiment allowed the observation of the one bond $^1J_{C,H}$ coupling, which meant that proton chemical shifts could be clearly identified due to the large dispersion of the $^{13}$C chemical shifts. Consequently, to aid in the assignment of the crosspeaks in the NOESY spectrum in $^2$H$_2$O at 25°C, the HSQC spectrum in $^1$H$_2$O at 25°C was used to identify the C6-H6, C8-H8 and C1’-H1’ crosspeaks. The main use of the DQF-COSY technique was to identify the H1’-H2’ and H3’-H4’ crosspeaks, which provide information about sugar pucker conformation of nucleotides. To aid the assignment of the NOESY and HSQC spectra, the standard observable chemical shifts of some $^1$H and $^{13}$C nuclei present in RNA polynucleotides were utilised. Chemical shifts are characterised by specific ranges, which correspond to particular atoms within the RNA structure. The $^1$H and $^{13}$C chemical shifts of the peaks generally observed in the NOESY and $^1$H-$^{13}$C HSQC spectra are shown in Table 2.6.2.3.

<table>
<thead>
<tr>
<th>Proton</th>
<th>$^1$H(ppm)</th>
<th>Carbon</th>
<th>$^{13}$C(ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1’</td>
<td>5.0-6.0</td>
<td>C1’</td>
<td>89-95</td>
</tr>
<tr>
<td>H2’</td>
<td>4.0-5.0</td>
<td>C2’</td>
<td>70-80</td>
</tr>
<tr>
<td>H3’</td>
<td>4.0-5.0</td>
<td>C3’</td>
<td>70-80</td>
</tr>
<tr>
<td>H4’</td>
<td>4.0-5.0</td>
<td>C4’</td>
<td>81-86</td>
</tr>
<tr>
<td>H5/H5’</td>
<td>3.5-5.0</td>
<td>C5’</td>
<td>62-70</td>
</tr>
<tr>
<td>H2</td>
<td>6.5-8.5</td>
<td>C4</td>
<td>145-155</td>
</tr>
<tr>
<td>H5</td>
<td>5.0-6.0</td>
<td>C5</td>
<td>95-105</td>
</tr>
<tr>
<td>H6</td>
<td>7.0-8.0</td>
<td>C6</td>
<td>137-140</td>
</tr>
<tr>
<td>H8</td>
<td>7.0-8.0</td>
<td>C8</td>
<td>133-140</td>
</tr>
<tr>
<td>NH$_2$O (non-H-bonded)</td>
<td>5.5-6.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_2$C (non-H-bonded)</td>
<td>7.0-7.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_2$A (non-H-bonded)</td>
<td>6.0-6.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_2$O (H-bonded)</td>
<td>8.0-9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_2$C (H-bonded)</td>
<td>8.0-9.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH$_2$A (H-bonded)</td>
<td>7.5-8.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH G (in GC)</td>
<td>12.0-13.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH U (in UA)</td>
<td>13.0-15.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NH G (in GA)</td>
<td>10.0-11.0</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.6.2.3. Summary of $^1$H and $^{13}$C chemical shifts observed in the NOESY and $^1$H-$^{13}$C HSQC spectra of RNA.
2.7 Identification of the base protons

2.7.1 Identification of the exchangeable protons

There are two different types of protons of RNA that fall in the exchangeable category, namely, imino (-NH) and amino (-NH₂). Imino protons are found in uracil and guanine bases. The amino groups are found in adenine, cytosine and guanine bases. Imino proton resonances can be unambiguously assigned using NOE crosspeaks between imino-imino protons in the NOESY spectrum in ¹H₂O. An example of imino-imino connectivities, is shown in Figure 4.2.2.45.

The imino-imino proton distances between neighbouring bases are usually at least 3.5-4.0Å, while cross-strand imino-imino distances are 5.0Å, in an A-form RNA. Uracil and guanine imino resonances are distinguishable in a canonical base pairing. Uracil imino resonances occur in the range of 15.0-13.0 ppm and guanine imino resonances occur in the range of 12.0-14.0 ppm, respectively. The weaker hydrogen bonding of A•U base pair, compared to G•C base pair, results in uracil imino protons being more exposed to the external magnetic field than guanine imino protons and lowshield shifted in the NMR spectrum. Imino resonances belonging to unpaired bases and noncanonical base pairing occur highfield shifted in the range of 10-12 ppm. Paired bases are involved in base stacking interactions between the aromatic rings of adjacent bases. When an external magnetic field is directed perpendicular to the plane of the base aromatic rings, a secondary magnetic field called ring current is induced by π electrons.³² This results in a lowfield shift of the imino protons due to a deshielding effect because the induced magnetic field is in the same direction as the external magnetic field. Imino resonances of the bases not involved in bases stacking appear more highfield shifted.

Amino resonances are found highfield of the imino resonances due to the increased diamagnetic shielding provided by the lone pair of electrons of the –NH₂ group. The two NH₂ proton resonances occur at different chemical shifts, as one is involved in hydrogen bonding to make a base pairing. The hydrogen bonded proton resonance is found lowfield of its non-hydrogen bonded germinal proton. This is due to deshielding effect of the electronegative oxygen hydrogen bond acceptor. The NH₂ proton resonances of the
cytosine base can be easily identified in the NOESY spectrum, compared to guanine and adenine, due to slower exchange rates of the former with solvent proton. An example of imino-amino connectivities, is shown in Figure 4.2.3.46. Thus, the exchangeable proton correlations provide information about base stacking and base pairing.
2.8 Molecular Mechanics (MM)\textsuperscript{73-76}

Molecular Mechanics (MM) is a branch of molecular modelling, which uses classical/Newtonian mechanics as the basis of its calculations. Molecular modelling consists of theoretical and computational techniques to simulate the behaviour of molecules. Different force field and set parameters are employed, which define the energy of the system as a function of the atomic position. The software HyperChem Professional v8.0\textsuperscript{77} was used to carry out the molecular modelling of the neamine 12, 27mer RNA and 27mer RNA-neamine 12 complex. Geometry optimization and molecular dynamics simulations were performed using this software. The force field consists of a number of equations designed to define a set of energy terms (Equation 14), where $E_{\text{bond}}$ is the energy between covalent bonds, $E_{\text{bond angle}}$ is the energy between bond angles, $E_{\text{improper}}$ indicates planarity in aromatic rings, $E_{\text{dihedral}}$ is the energy of non-bonded interactions of atoms separated by three bonds, $E_{\text{vdW}}$ is the energy due to van der Waals interactions and $E_{\text{electrostatic}}$ is the energy due to electrostatic interactions.\textsuperscript{78}

$$E_{\text{empirical}} = E_{\text{bond}} + E_{\text{bond angle}} + E_{\text{improper}} + E_{\text{dihedral}} + E_{\text{vdW}} + E_{\text{electrostatic}} \quad (14)$$

The parameter set is a list of parameters to be used for the energy terms for a given atom type. Together force field and parameter sets are designed to reproduce certain properties of the molecule, such as molecular geometry. A good force field will ensure an accurate geometry optimization and molecular dynamic simulation, the force field chosen for all the simulations was AMBER 3 (Assisted Model Building and Energy Refinement).\textsuperscript{78} These simulations were performed to obtain results which are as close as possible to experimental data.
2.8.1 Geometry optimization

The geometry of a molecule determines many of its chemical and physical properties. Computational methods are employed to find the most stable structure that presents the most accurate geometry. Geometry optimization is a method to find the best atomic arrangements of the structure and consequently, the most stable structure with the lowest energy value. A geometry optimization samples single points on the potential energy surface, searching for a minimum. The technique used to search for the minimum is called the optimization algorithm (Polak-Ribiere). To carry out the geometry optimization, HyperChem was used with an initial set of Cartesian coordinates for a molecule and tries to find a new set of coordinates with a minimum of energy. Unfortunately, since geometry optimization calculations cannot cross or penetrate potential energy barriers, the molecular structure found during an optimization may be a local and not a global minimum. However, the minimum represents the potential energy closest to the starting structure of a molecule (Figure 2.8.1.30).

![Figure 2.8.1.30](image-url) Illustration of the potential energy surface. The optimization algorithm samples single points searching for a minimum.
2.8.2 Simulated annealing

In this simulation, molecular dynamics was used to anneal the system to obtain a lower energy minimum. During simulated annealing, the initial structure is heated to a very high temperature, increasing the energy of the atoms involved in base pairs. Subsequently, bases become unstacked from their original positions and move randomly through space. In the next step, the temperature is reduced slowly in order to increase the chance of finding the low energy conformation. A dynamic run has three optional phases: heat, run and cool. The first phase occurs over a simulation period of heat time, using the starting temperature of 100K to set initial velocities with rescaling of velocities at temperature increments of 30K to reach the simulation temperature of 300K. A short heat time of 0.1 picoseconds is used to raise the temperature from a starting of 100K to the simulation temperature of 300K incrementing by a temperature step of 30K. In the middle phase, velocities are not rescaled. The final phase occurs over a simulation period of cool time with rescaling temperature increments to reach the final temperature. All the simulations were performed firstly in vacuo and then in periodic boundary conditions. The periodic boundary conditions place the molecular system in a box and simulate a continuous system with a constant density of water molecules.
2.8.3 Molecular Dynamics (MD)

Molecular Dynamics (MD) simulation provides macroscopic information about the time-dependent motion of molecular systems. It involves the addition of kinetic energy to above potential energy surface and the subsequent motion of the molecular system over the potential surface. Normally, the classical total energy (sum of the kinetic energy and the potential energy) is conserved and the motion is faster near minima in the potential surface. If a set of initial conditions is defined (initial velocities and a particular point on the potential surface), then Newton’s laws cause the molecular system to evolve along a path that is referred to as the molecular dynamic trajectory. Molecular dynamics simulation allows molecules to explore conformational space in the region defined by atomic positions and velocities. Unlike energy minimization calculations, the MD calculation accounts for thermal motion, providing thermal energy to molecules that can cross the potential energy barriers. MD simulations require a force field, which calculates the potential energy of the system and describes the terms by which the particles in the simulation will interact. MD simulations calculate the future positions and velocities of atoms based on their current positions and velocities. One disadvantage of molecular dynamic simulations is that the timescale of the simulations are much shorter than the timescale of chemical and physical processes, which occur in nanoseconds or longer. Therefore, long-term processes cannot be studied as they would take too much computational time.
Chapter 3: NMR studies of the neamine antibiotic 12

Chapter 3 describes the full assignment of the $^1$H and $^{13}$C resonances of the neamine derivative 12 using a range of NMR techniques. Additionally, some aspects of the dynamics of neamine 12 are also discussed. The results of the NMR studies of the neamine 12 can provide structural information to modify its selected groups to enhance its affinity for the RNA.

3.1 Structure of the neamine antibiotic 12

The chemical structure of the neamine 12, C$_{23}$H$_{32}$N$_{16}$O$_{11}$ is shown in Figure 3.1.31 and the assigned $^1$H-NMR spectrum in $^1$H$_2$O is shown in Figure 3.2.1.32. The neamine 12 consists of one uracil group (U), one ribose ring (R) connected to two six-membered azo sugars (A, B) via a two carbon linker chain. Both A and B residues are six-membered ring azo-sugars, they are linked to each other by a glycosidic linkage. In six-membered rings, bond angles are close to the tetrahedral angle of 109.5°. Therefore, there is no angular strain. To prevent steric strain, these sugars prefer the chair conformation with all of the hydroxyl groups and azide groups arranged in the equatorial position (sticking out of the plane of the ring) and with all of the less bulky-H atoms in the axial orientation lying above or below the plane of the ring. However, at room temperature there is a rapid interconversion between the axial and equatorial forms.

Figure 3.1.31. Chemical structure of neamine 12, C$_{23}$H$_{32}$N$_{16}$O$_{11}$. It consists of one uracil group (U), one ribose ring (R) connected to two six-membered azo sugars (A, B) via a two carbon linker chain. All the protons attached to C, N of the structure are labelled with unique numbers to distinguish their different chemical environments. The proton labels with suffixes α and β indicate geminal protons.
3.2 Assignment of the neamine 12

To assign the protons and carbons of neamine 12, a suite of 2D NMR experiments viz DQF-COSY, TOCSY, HSQC-TOCSY, HSQC, and ROESY experiments were performed. Thus, connectivities between protons were first established in the DQF-COSY spectrum and then the spin systems were identified in the TOCSY and HSQC-TOCSY spectra. After having assigned $^1$H resonances, all $^{13}$C resonances were assigned in the HSQC-TOCSY and HSQC spectra.

3.2.1 Assignment of the $^1$H-NMR spectrum of the neamine 12

The one-dimensional $^1$H-NMR spectrum of neamine 12 in $^1$H$_2$O (Figure 3.2.1.32) exhibits a wide dispersion of signals arising from the various groups of the molecule. Well resolved resonances corresponding to the aromatic protons were observed lowfield shifted (6.0 - 10.0 ppm) in the $^1$H-NMR spectrum, while the aliphatic protons appeared highfield shifted (0 - 6.0 ppm). To start at least one peak must first be identified by making certain assumptions. Standard chemical shifts can be used to unambiguously assign exchangeable and non-exchangeable aromatic proton resonances and anomic sugar proton resonances. Accordingly, the lowfield shifted exchangeable aromatic H1 (9.20 ppm) and non-exchangeable H2 (8.31 ppm) and H3 (6.22 ppm) protons of the uracil group and the anomic H4 (5.89 ppm) and H18 (5.51 ppm) sugar proton resonances were identified and assigned. The two H2 and H3 aromatic resonances of the uracil group appeared as sharp doublets due to the spin-spin coupling with each other. The H4 and H18 sugar protons appear each as a doublet due to the spin-spin coupling with the single H5 and H19 neighbouring protons. Thereafter, the H5 and H6 resonances were split into triplets due to the coupling with H4 and H6, and H5 and H7 protons, respectively. The H7 proton was split into a doublet by the H6 proton, and its resonance appears at 4.42 ppm. The H14, H15α/β and H16 resonances were observed in the range 1.2 - 2.0 ppm. The standard chemical shift of the H8 amide resonance was assigned to 8.62 ppm. The H19, H20, H21 and H22 proton resonances of sugar A and the H13 and H17 proton resonances of sugar B appeared overlapped in the range 3.2 - 4.2 ppm. At this stage, the 1D $^1$H-NMR spectrum did not provide unambiguous
assignment of the overlapped resonances, therefore a suite of 2D NMR experiments \textit{viz.} DQF-COSY, TOCSY, ROESY, HSQC and HSQC-TOCSY experiments were carried out.

![Figure 3.2.1.32](image) 400 MHz 1D $^1$H-NMR spectrum of neamine 12 in $^1$H$_2$O at 2 °C. The chemical shifts are calibrated with respect to the protons of the water signal which resonate at 5.03 ppm at 2 °C. Most of the resonances identified in the spectrum are labelled according to the labelling scheme shown in the chemical structure (Figure 3.1.31). A few protons are not labelled because overlapped in the 2.8-4.2 ppm region.

Connectivities between protons were first established using the DQF-COSY spectrum and then the spin systems were identified in the TOCSY spectrum. Finally, the individual protons of these spin systems will be assigned. The full assignment is shown later in Table 3.3.4.
3.2.2 Assignment of the DQF-COSY spectrum of the neamine 12

The through-bond $^3J_{\text{H-H}}$ correlations found in this DQF-COSY spectrum allowed adjacent protons within the same spin system to be systematically identified and assigned. All the $^1\text{H}$ resonances were assigned by following the $^1\text{H}$ to $^1\text{H}$ sequential connectivities in the spectrum (Figure 3.2.2.33).

![Diagram showing the DQF-COSY spectrum of the neamine 12](image)

Figure 3.2.2.33. Illustration of the identification of the $^1\text{H}-^1\text{H}$ connectivities (black lines) in the DQF-COSY spectrum of the neamine 12. 400 MHz DQF-COSY spectrum in $^1\text{H}_2\text{O}$ at 2°C, showing the $^1\text{H}-^1\text{H}$ cross peaks.

The cross peak between the H2 (8.31ppm) and H3 (6.22ppm) aromatic protons was found lowfield shifted in the spectrum. The H4-H5 and H5-H6 crosspeaks were identified by following the $^1\text{H}$ to $^1\text{H}$ sequential connectivities from the H4 resonance to the H6 resonance. The H6-H7 cross peak was not found in the DQF-COSY spectrum; the $^1\text{H}-^13\text{C}$
HSQC spectrum (described later) was used to assist in the identification of the H7 proton. The identified H18 proton chemical shift (5.51 ppm) was used to identify and assign the remaining protons belonging to sugar A spin system. Subsequently, the H18-H19 and H19-H20 cross peaks were identified in the DQF-COSY spectrum. The identified amide H8 resonance at 8.62 ppm helped to identify the H9α/β and the H10α/β aliphatic resonances. Subsequently, the crosspeaks between the H9α/β and the H10α/β protons were identified and the H9α/β and the H10α/β proton chemical shifts were assigned. The remaining crosspeaks in the DQF-COSY were associated with the resonances of sugar B. Strong correlations from H15α/β to H14 and H16 were found, and then the H14-H13, H16-H17 and H13-H12 crosspeaks were found. Consequently, the H15α/β, H14, H16, H13 and H12 protons were assigned in the DQF-COSY spectrum.
3.2.3 Assignment of the TOCSY spectrum of the neamine 12

The inspection of the TOCSY spectrum (Figure 3.2.3.35) showed $^3J_{HH}$ correlated crosspeaks. Five distinct coupled spin systems ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) within neamine 12 were identified in the TOCSY spectrum and these are highlighted by different coloured lines (Figure 3.2.3.34). Figure 3.2.3.34 illustrates the structure of the neamine 12 and the five spin systems. Every proton showed scalar correlations to every other proton belonging to the same spin system. The spin systems ($\alpha$, $\beta$, $\gamma$, $\delta$, and $\varepsilon$) were firstly identified before the assignment of individual proton resonances.

Figure 3.2.3.34. Illustration of the five spin systems within the neamine 12. The $\alpha$ spin system represents protons belonging to the uracil group (U), the $\beta$ spin system represents protons belonging to the ribose group (R), the $\gamma$ spin system represents protons belonging to the linker chain, the $\delta$ spin system represents protons belonging to the six-membered azo sugar B and the $\varepsilon$ spin system represents protons belonging to the six-membered azo sugar A.
Figure 3.2.3.35. Illustration of the identification of the spin systems of the neamine. 400 MHz TOCSY spectrum in $^1$H$_2$O at 2°C, showing five distinct spin systems. The yellow lines represent correlations within the spin system $\alpha$, the red lines represent correlations within the spin system $\beta$, the black lines represent correlations within the spin system $\gamma$, the green lines represent correlations within the spin system $\delta$ and the blue lines represent correlations within the spin system $\epsilon$.

**Assignment of spin system $\alpha$**

Spin system $\alpha$ was first assigned, it contains the aromatic H2 and H3 resonances belonging to the uracil group (Figure 3.2.3.35, yellow lines). The two aromatic resonances were easily assigned as these appear at characteristic aromatic proton chemical shifts. The H2 proton was shifted low field from the H3 proton, this is due to the C2 carbon being directly bonded to an electronegative nitrogen atom, which acts to deshield the H2 proton. Thus, H2 and H3 were assigned to the resonances at 8.31 and 6.22 ppm.
Assignment of the spin system β

The spin system β consists of the H4, H5, H6 and H7 resonances belonging to the ribose group (Figure 3.2.3.35, red lines). To confirm the assignment, the H4 resonance showed two crosspeaks to the H5, H6 and H7 (H5 and H7 resonances are overlapped) of its spin system β, and H5 showed a crosspeak to the adjacent H6 resonance. The H4 resonance was shifted lowfield from the H5, H6 and H7 resonances which are in a very similar chemical environment. This is due to the C4 carbon being directly bonded to a nitrogen atom and an oxygen atom, acting to deshield the H4 proton. The H5 and H7 protons resonate in the overlapped region around 4.42 ppm, this was expected as these two protons are both in very similar chemical environments. Thus the resonances at 4.42 and 4.45 ppm were assigned to H5 and H7 protons, respectively. This is due to the C7 carbon being directly attached to an oxygen atom, a CO carbonyl group and the C6 carbon, whilst the C5 carbon is only attached to one hydroxyl group and C4 and C6 carbons.

Assignment of the spin system γ

The spin system γ is composed of the H9α/β and H10α/β aliphatic protons, the H8 amide proton and the H11 proton, belonging to the linker chain connecting the ribose and the azo sugar B (Figure 3.2.3.35, black lines). The H8 resonance was easily assigned as this appears at a characteristic chemical shift, at 8.62ppm. The H8 resonance showed four strong crosspeaks to the H9α/β and H10α/β resonances. In turn, the H9α/β resonances showed crosspeaks to the H10α/β resonances, and the H11 resonance showed a crosspeak to the H12 resonance.

Assignment of the spin system δ

The spin system δ consists of the H12, H13, H14, H15α/β, H16 and H17 protons belonging to the azo sugar B (Figure 3.2.3.35, green lines). The identified H11 resonance, belonging to the spin system γ, helped to identify the H12 resonance and then the remaining resonances of the spin system δ. The H11 resonance showed a crosspeak to the H12 resonance, and then the H12-H13 crosspeak was identified. Subsequently, the remaining H14, H15α/β, H16 and H17 resonances were also identified. The H15α/β
resonance showed crosspeaks to H16 and H14, and then the H16-H17 and H13-H14 crosspeaks were found. Thus, H12, H13, H14, H15α/β, H16 and H17 protons were assigned to the resonances 3.52 ppm, 3.26 ppm, 1.57 ppm, 1.38 ppm, 1.88 ppm and 3.94 ppm, respectively.

**Assignment of the spin system ε**

The spin system ε is composed of the H18, H19, H20, H21, H22 and H23α/β protons belonging to sugar A (Figure 3.2.3.35, blue lines). The H18 resonance is shifted lowfield from the other resonances belonging to its spin system as the C18 carbon is bonded to two oxygen atoms rather than one. Therefore, the H18 resonance was the first to be assigned at 5.51 ppm. Subsequently, the assigned H18 helped to identify the remaining resonances within its spin system. The H18 resonance shows a clear set of three crosspeaks to the adjacent H19, H20 and H21 resonances, and then the identified H21 resonance helped to identify the remaining H22 and H23α/β resonances within the ε spin system. Subsequently, the H18, H19, H20, H21, H22 and H23α/β protons were assigned to the resonances 5.51 ppm, 3.48 ppm, 4.00 ppm, 3.90 ppm, 3.70 ppm, 1.72 ppm and 2.50 ppm, respectively. The H18-H17 crosspeak was not observed as the magnetization transfer was interrupted by the linker oxygen atom connecting the two azo sugars.
3.2.4 Assignment of the ROESY spectrum of the neamine 12

Following the resonance assignment, to elucidate through space (dipolar) interaction within the neamine 12, a $^1\text{H}-^1\text{H}$ correlated ROESY experiment in $^1\text{H}_2\text{O}$ was carried out and the measured spectrum is shown in Figure 3.2.4.36.

![ROESY spectrum of neamine 12](image)

**Figure 3.2.4.36.** 400 MHz ROESY spectrum (Spin lock mixing time $SLm = 250$ ms) of neamine 12 in $^1\text{H}_2\text{O}$ at 2°C, showing dipolar correlations between various protons of the drug molecule. For the purpose of clarity, the ROE line connectivities were traced on the upper part of the diagonal only.

Various ROEs were observed in the ROESY spectrum, a most interesting one is the distinct long range ROE shown by the amide H8 (8.62 ppm) to the H17 (3.94 ppm) proton. Similarly the resolved H18 (5.51 ppm) proton shows distinct ROE correlations to
the H22 (3.70 ppm) and H19 (3.48 ppm) protons. Subsequently, ROEs shown by H17 proton to H16 and H15α/β were observed. Similarly, ROE correlations shown by H22 proton to H21 and H23β, and by the H19 proton to H20 proton were found. Further ROE correlations were observed by the H2 proton to H3, H4, H5 and H6 and H7 protons, and by the H1 proton to H3. These ROE correlations highlight the structural significance of these protons within neamine 12 and should serve as useful distance constraints towards the determination of the NMR structure of the drug, when carried out.
3.2.5 Assignment of the HSQC spectrum of the neamine 12

The assignment of the $^1$H-$^{13}$C HSQC spectrum of neamine 12 is shown in Figure 3.2.5.37. Inspection of the spectrum shows $^1J_{\text{H,C}}$ (HSQC) correlated crosspeaks. All the protons belonging carbon nuclei of the neamine 12 showed a $^1J_{\text{H,C}}$ HSQC correlation to their directly attached proton/s. The identified $^1$H chemical shifts in the DQF-COSY spectrum were used to identify the $^{13}$C chemical shifts in the HSQC spectrum. Thus, all the $^{13}$C resonances were assigned.

![Figure 3.2.5.37. Illustration of the assignment of the $^{13}$C resonances. 400MHz $^1$H-$^{13}$C HSQC ($SL_m = 75$ ms) spectrum of neamine 12 in $^1$H$_2$O at 2°C, illustrating $^1$H-$^{13}$C crosspeaks indicated by black circles.](image-url)
3.2.6 Assignment of the HSQC-TOCSY spectrum of the neamine 12

The assignment of the HSQC-TOCSY spectrum of neamine 12 is shown in Figure 3.2.6.38. Inspection of the spectrum shows both $^1J_{\text{H,C}}$ (HSQC) and $^{n>1}J_{\text{H,C}}$ (TOCSY) correlated crosspeaks. All the carbon nuclei of the neamine 12 showed a $^1J_{\text{H,C}}$ HSQC correlation to their directly attached protons and $^{n>1}J_{\text{H,C}}$ TOCSY correlations to remote protons. In this way, all the exchangeable and non-exchangeable proton and carbon resonances of neamine 12 were unambiguously identified and assigned.

![Figure 3.2.6.38. 400 MHz $^1$H-$^{13}$C HSQC-TOCSY (Spin lock mixing time SLm = 75 ms) spectrum of neamine 12 in $^1$H$_2$O at 2°C. The spectrum shows cross peaks due to directly attached ($^1J_{\text{H,C}}$) $^1$H-$^{13}$C HSQC correlations (circled in black) together with other cross peaks due to transfer of coherence (TOCSY) to remote protons ($^{n>1}J_{\text{H,C}}$).](image)

For the purpose of clarity, the analysis of the $^1$H-$^{13}$C HSQC-TOCSY spectrum was divided into sections. In the first section (Figure 3.2.6.39), connectivities between $^{n>1}J_{\text{H,C}}$ correlated crosspeaks belonging to the α and ε spin systems are showed. In the second section (Figure 3.2.5.40), connectivities between $^{n>1}J_{\text{H,C}}$ correlated crosspeaks belonging
to the $\beta$, $\gamma$ and $\delta$ spin systems are showed. In both the spectra, the black circles represent the $^1J_{\text{H,C}}$ (HSQC) correlated crosspeaks. In the first section, the overall set of $^{n>1}J_{\text{H,C}}$ crosspeaks of the $\alpha$ spin system was highlighted by blue lines and the overall set of crosspeaks of the $\varepsilon$ spin system was highlighted by green lines.

![Figure 3.2.6.39](image-url)

**Figure 3.2.6.39.** 400 MHz $^1$H-$^{13}$C HSQC-TOCSY (Spin lock mixing time $SLm = 75$ ms) spectrum of neamine in $^1$H$_2$O at 2°C, showing two out of the five spin systems within the neamine 12. The blue lines represent connectivities between crosspeaks of the spin system $\alpha$, and the green lines represent connectivities between the crosspeaks from the spin system $\varepsilon$. The H11 resonance is overlapped at 3.85 ppm.

Through analysis of the $^1$H-$^{13}$C HSQC-TOCSY spectrum, it was found that all the carbon nuclei belonging to the $\alpha$ and $\varepsilon$ spin systems showed a $^1J_{\text{H,C}}$ correlation to their directly attached proton/s and $^{n>1}J_{\text{H,C}}$ correlations to remote protons within the same spin system. For example, the C2 carbon showed a $^1J_{\text{H,C}}$ correlation to the H2 proton and one $^2J_{\text{H,C}}$ correlation to the H3 proton (Figure 3.2.6.39, blue lines). Similarly, the C18 carbon gave a $^1J_{\text{H,C}}$ correlation to H18, a $^2J_{\text{H,C}}$ correlation to H19 and a $^3J_{\text{H,C}}$ correlation to the H20
proton (Figure 3.2.6.39, green lines). A further $^2J_{H,C}$ TOCSY correlation was showed by C20 carbon to the H21 proton, and the C21 carbon gave a $^2J_{H,C}$ TOCSY correlation to H22 and a $^3J_{H,C}$ TOCSY correlation to H23α/β.

In the second section (Figure 3.2.6.40), connectivities between $^nJ_{H,C}$ correlated crosspeaks belonging to the β, γ and δ spin systems are showed. The overall set of $^nJ_{H,C}$ crosspeaks of the β spin system is highlighted by red lines, the overall set of crosspeaks of the γ spin system is highlighted by black lines and the overall set of crosspeaks of the δ spin system is highlighted by blue lines. The black circles represent the $^1J_{H,C}$ (HSQC) correlated crosspeaks.

Figure 3.2.6.40. 400 MHz $^1$H-$^13$C HSQC-TOCSY (Spin lock mixing time $SL_m = 75$ ms) spectrum of neamine 12 in $^1$H$_2$O at 2°C, showing three out of the five spin systems within the neamine 12. The red lines represent connectivities between protons belonging to the spin system β, the black lines represent connectivities among protons belonging to the spin system γ, and the blue lines represent connectivities among protons belonging to the spin system δ. The H11 resonance is overlapped at 3.85 ppm.
Through analysis of the $^1$H-$^{13}$C HSQC-TOCSY spectrum, it was found that all the carbon nuclei belonging to the β, γ and δ spin systems showed a $^1J_{H,C}$ correlation to their directly attached proton/s and $^{n>1}J_{H,C}$ correlations to remote protons within the same spin system. For example, the C4 carbon showed a $^1J_{H,C}$ crosspeak to the H4 proton, a $^2J_{H,C}$ crosspeak to the H5 proton and a $^3J_{H,C}$ crosspeak to the H6 proton (Figure 3.2.6.40, red lines). Similarly, the C9 carbon gave two $^1J_{H,C}$ crosspeaks to the H9α/β protons, a $^2J_{H,C}$ crosspeak to the H8 proton, two $^2J_{H,C}$ crosspeaks to H10α/β protons (Figure 3.2.6.40, black lines). The C15 carbon showed a $^1J_{H,C}$ crosspeak to the H15α/β protons, a $^2J_{H,C}$ crosspeak to the H14 and H16 protons and two $^3J_{H,C}$ crosspeaks to the H17 and H13 protons (Figure 3.2.6.40, blue lines).
3.2.7 \(^1\)H-NMR spectra of the neamine 12 at variable temperatures (VT series)

To probe the dynamics of the neamine 12 in solution, a series of 400MHz \(^1\)H-NMR spectra were measured in \(^1\)H\(_2\)O at temperature range of 2°C to 50°C, as shown in Figure 3.2.7.41. The temperature-induced changes in chemical shift and linewidth can be clearly observed for the exchangeable H1 and H8 and the H18 nonexchangeable proton resonances. None of the aliphatic or aromatic protons of neamine 12 produced significant changes with respect to temperature. This indicated that neamine 12 is endowed with a thermodynamically stable structure.

Figure 3.2.7.41. 400 MHz \(^1\)H-NMR VT spectra of neamine 12. Stack plot of the VT spectra (low field region) of neamine 12 in \(^1\)H\(_2\)O at (a) 2°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, (f) 50°C. The red box illustrates the progressive decrease in intensity of H1 at 9.20 ppm with increase of temperature (red box) and vice versa for H18 at 5.51 ppm as shown by the green box.
Some interesting features of the spectrum to note are that the intensities of the exchangeable H1 and H8 proton resonances and the non-exchangeable H18 proton resonances show opposite effects with progressive increase of temperature (2ºC - 50ºC). The former proton resonances undergo a steady decrease and the latter proton a steady increase on varying in temperature. The decrease in intensity of H1 and H8 resonances may be attributed to the rapid chemical exchange of the protons with water solvent at elevated temperature (e.g. > 30ºC). Furthermore, a highfield shift (Δδ≈0.4 ppm) was observed for the H8 resonance, suggesting changes in structure conformation. The increase in intensity of H18 may be associated with removal of any hindrance to the rotation of the anomeric proton i.e. increase molecular tumbling which causes longer longitudinal relaxation time ($T_1$) after increase in temperature. This retardation to exchange suggests that these protons may be involved in hydrogen bonding within the molecule. These dynamic events have implications for the NMR solution structure.
3.3 Assignment table of the neamine derivative 12

The $^1$H and $^{13}$C chemical shifts of the neamine 12 are shown in Table 3.3.4.

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*Table 3.3.4.* 400 MHz $^1$H and $^{13}$C NMR chemical shifts of neamine 12 in $^1$H$_2$O at 2°C.
3.4 Unconstrained molecular modelling of the neamine 12

The resulting structure for the neamine 12, after geometry optimization, simulated annealing and molecular dynamics, revealed a number of structurally important close vicinities (Figure 3.4.42) that act to hold the structure in its conformation. These close vicinities confirmed the retardation to exchange observed in the $^1$H-NMR VT series illustrated above.

![Diagram of the neamine 12 structure](image)

**Figure 3.4.42.** Illustration of the unconstrained model of the neamine 12. The structure reveals four structurally significant close vicinities, including two potential hydrogen bonds. The close vicinities with the respective distances (in Å) between the involved atoms are indicated by black lines.

The most significant feature is the close vicinity between H8 and O5’ (2.99 Å), causing the H8 proton to be retarded to exchange up to 50°C and probably beyond. Other close vicinities observed in the model are between H1 and the lone pair on the azide group (2.45 Å) and between H11 and the O2 of the uracil (3.85 Å). The neamine 12 in solution has rather rigid ring conformations, but is flexible about the glycosidic linkage
connecting the rings and the linker chain. Therefore, those flexibilities allowed the linker chain to rotate to bring the H8 proton close to the H17 proton (4.89Å), justifying the ROE between H8 and H17 observed in the ROESY spectrum. The chemical structure of neamine 12 is endowed with a range of functional groups with potential to bind to the target 27mer RNA (described in Chapter 4). The interaction with the 27mer RNA would disrupt the close vicinities leading to conformational changes. The neamine 12 contains several donor atoms, such as the azide group, expected to be good nucleophile. Other donor atoms include the O5’ of the ribose group and the carbonyl groups. Some acceptor atoms include the -NH and -OH groups.
Chapter 4: NMR studies of the interaction of neamine 12 with 27mer RNA motif of the 16S rRNA

Chapter 4 will firstly describe the NMR assignment of exchangeable protons of the 27mer RNA motif derived from the A-site of 16S rRNA. Subsequently, the focus of this chapter shifts to the interaction of neamine derivative 12 (assigned in Chapter 3), with the conserved 27mer RNA motif. The NMR assignment and structure determination of the 27mer RNA has been carried out before and reported in literature.\textsuperscript{24} The 27mer RNA acts as a good model and a host target for the NMR investigation of RNA-antibiotic interaction. In this project, for sake of confirmation, the NMR assignment of the exchangeable protons of 27mer RNA and variable temperature series have been carried out independently and the results used for antibiotic binding studies are described in Section 4.6.

4.1 Predicted secondary structure of the 27mer RNA

The secondary structure (Figure 4.1.43) and empirical Gibbs energy were predicted for the 27mer RNA by the Vienna RNAfold software\textsuperscript{84} exhibiting a good thermodynamic stability of the RNA motif.

\begin{figure}[h]
\centering
\includegraphics[width=0.3\textwidth]{predicted_secondary_structure.png}
\caption{The predicted secondary structure of single stranded 27mer RNA motif, corresponding to the A-site of 16S RNA, endowed with good thermodynamic stability ($\Delta G = -10.7$ kcal/mole) and affinity for neamine 12.}
\end{figure}
The predicted secondary structure of single stranded 27mer RNA motif is composed of nine canonical base pairs (eight G•C base pairs, one A•U base pair), two A7•A21 and U5•U23 mismatches and an asymmetrical bulge A20. The 27mer RNA is G•C rich and hosts the stable UUCG tetra loop and these two features combine to confer low empirical Gibbs energy (ΔG= -10.7 kcal/mole) for the RNA.

4.2 NMR assignment of the 27mer RNA

The structure of the 27mer RNA of the A-site of the 16S RNA has been studied in solution by NMR spectroscopy. This section provides exchangeable proton assignment of a 27mer RNA. This very stable RNA oligonucleotide represents an important model system for the study of the RNA structure and its interaction using NMR spectroscopy.

4.2.1 Analysis of the ¹H-NMR spectrum of the 27mer RNA

The 1D ¹H-NMR spectrum of the 27mer RNA in ¹H₂O at 2°C showed twelve clear imino (-NH) resonances; seven imino resonances were observed in the standard canonical base pairing region (13 - 14ppm) and 5 imino resonances in the non-canonical region (9.6 - 12.4 ppm) (Figure 4.2.1.44). From the predicted secondary structure 9 imino resonances were expected in the standard canonical base pair region, indicating that two resonances could be overlapped in the 12.6-13.6 ppm region. To start the imino proton identification, at least one peak must first be identified by making certain assumptions. Chemical shifts can be used to unambiguously assign uracil and guanine imino proton resonances and most importantly distinguish between stem and loop imino proton resonances.

Therefore, to aid in the assignment of the ¹H-NMR spectrum of the 27mer RNA, the standard chemical shifts of the U12, U13 and G15 imino resonances were utilised. Firstly, it was assumed that the resonance at 9.88 ppm corresponded to the loop G15 imino resonance. This assumption was based on the fact that G15 is more likely to be stable than the remaining bases in the loop due to its ability to form a non canonical G•U sheared base pair. Next, the resonances at 11.88 ppm and 12.27 ppm were assigned to the U13 and U12 bases in the loop, respectively. The two imino resonances in the 10.0 - 11.0 ppm region were associated with U5 and U23 bases which formed a non-canonical base
pair. Furthermore, it is known that the uracil imino resonances of the standard A•U canonical base pairing are generally found lowfield from guanine imino proton resonances. Consequently, the most lowfield resonance at 13.95 ppm was assigned to the U18 imino resonance. At this stage, the $^1$H-NMR spectrum did not provide unambiguous assignment of the uracil and guanine imino protons, so the NOESY ($^1$H$_2$O/2°C) spectrum was analysed for accurate assignment.

![Diagram](image)

**Figure 4.2.1.44.** 400MHz $^1$H-NMR spectrum of the 27mer RNA in $^1$H$_2$O at 2°C, showing the imino region. Twelve imino resonances were observed corresponding to U18, G2, G22/G4, G17, G16, G1/G19, G25, U12, U13, U5, U23 and G15. **Insert:** Secondary structure of the 27mer RNA. The G15 loop imino proton resonance was clearly observed highfield of the stem imino proton peaks.
4.2.2 Imino - imino proton assignment of the 27mer RNA

The imino proton resonances were assigned by following the imino to imino sequential connectivities as illustrated in the NOESY spectrum (Figure 4.2.2.45) using established procedures. The crosspeaks observed in the NOESY (1H2O/2°C) spectrum correspond to NOEs between imino protons that were close in space. Three sets of sequential connectivity patterns were found. The first set started at G2 and stopped at G25 (Figure 4.2.2.45, red arrows). The second set of NOE connectivities connected G4/G22 overlapped imino resonances to the adjacent U5 and U23 imino resonances (Figure 4.2.2.45, blue arrows), and U5 to U23. The third set of NOE connectivities began at the U18 imino resonance and then stopped at G16 imino resonance (Figure 4.2.2.45, green arrows). Thus, imino - imino sequential assignment was obtained from G2 to G25, from G16 to U18, from G4 to U5 and from G22 to U23.

![Figure 4.2.2.45](image)

**Figure 4.2.2.45.** 400 MHz NOESY (τm = 250ms) spectrum (lowfield imino proton region) of 27mer RNA (0.44 mM) in 1H2O at 2°C. The arrows trace the sequential NOE connectivities between imino protons of adjacent base pairs (canonical and non-canonical) of the RNA. For the purpose of clarity, the NOE line connectivities were traced on the upper part of the diagonal only. **Insert:** Secondary structure of the 27mer RNA highlighting the imino-imino connectivities observed, represented by coloured arrows, G2 to G25 (red arrows), from G16 to U18 (green arrows), from G4 to U5 and from G22 to U23 (blue arrows).

The NOESY spectrum above conferred unambiguous assignment of the spectral imino proton base pairs. No NOEs were observed from the G16 imino resonance to the G15 imino resonance, and from the G15 imino resonance to the U12 imino resonance.
observation of imino-imino confirmed the predicted secondary structure of the 27mer RNA and also that it is endowed with a double helical A-RNA conformation. Furthermore, no imino-water crosspeaks were observed for G2, G4, G17, G22 and G25 (Figure 4.2.2.45, dotted line at 5.0 ppm), indicating the lack of imino proton exchange with water and evidence of stable base pairing. Conversely, strong cross peaks between imino protons and water were observed in the 27mer RNA for the G1, U5, U12, U13, G16, U18, G19 and U23 protons, indicating a fast exchange with water. In addition, the U12 and U18 imino-water crosspeaks were much lower in intensity, this is possibly due to their intermediate exchange with water as U18 is involved in the U•A base pair adjacent to the closing base pair and U12 is located in the loop.
4.2.3 Imino - amino proton assignment of the 27mer RNA

Once the imino proton chemical shifts were identified, the cross peaks in the imino-amino region were assigned in the next step (Figure 4.2.3.46). In the imino-amino region, connectivities can be observed from imino to the NH$_2^*$/NH$_2$ amino protons (NH$_2^*$ corresponds to the proton involved in base pair hydrogen bonding, and the H2, H5, and H1’ protons (resonances not assigned). The previously identified imino proton chemical shifts were used to assign crosspeaks in the imino-amino region. The full assignment of connectivities in the imino-amino region is shown in Figure 4.2.3.46. These connectivities provided for the evidence of base pairing between G1•C27, G2•C26, C3•G25, G4•C24, C6•G22, C8•G19, A9•U18, C10•G17 and C11•G16. The chemical shift table of the imino and amino resonances of the 27mer RNA is shown in Table 4.3.5.

Figure 4.2.3.46. 400 MHz NOESY ($\tau_m = 250$ms) spectrum of the 27mer RNA, at 2°C in $^1$H$_2$O, illustrating the imino-amino region. Connectivities from imino protons to NH$_2^*$/NH$_2$/H2/H5/H1’protons can be observed (NH$_2^*$ corresponds to the proton involved in base pair hydrogen bonding); and are marked with a black dot on the centre of the crosspeaks.
4.2.4 $^1$H-NMR spectra of the 27mer RNA at variable temperatures (VT series)

The VT $^1$H-NMR spectra of the 27mer RNA were measured over a temperature range (2ºC - 50ºC) and the results are shown in Figure 4.2.47, a-f.

![Figure 4.2.47. 400 MHz $^1$H-NMR VT spectra of 27mer RNA (0.44 mM) in $^1$H$_2$O. Stack plot of the VT spectra (imino proton region) of 27mer RNA at (a) 2ºC, (b) 10ºC, (c) 20ºC, (d) 30ºC, (e) 40ºC, (f) 50ºC.]

As expected, the 5 protons (G15, U23, U5, U13 and U12) in the non-canonical region (9.6 - 12.4 ppm) show faster chemical exchange as compared to the more stable and
hydrogen bonded (G25, G1/G19, G16, G17, G22/G4, G2 and U18) canonical protons in the 12.6 - 14.4 ppm region. The progressive decrease in intensity of the resonances may be attributed to the rapid chemical exchange of the proton with water solvent at elevated temperatures. The U13 resonance was the first to disappear, indicating the fastest imino proton exchange with water. The U12 imino resonance was observed between 2°C-40°C and then disappeared completely at 50°C. Similarly, the U13, U23 and U5 imino resonances were observed up to 30°C, but were rendered absent at 40°C. Also the G15 imino resonance disappeared at only 50°C. The largest chemical shift changes were found for U18 (highfield Δδ=0.27 ppm), U12 (highfield Δδ=0.16 ppm) and G2 (highfield Δδ=0.14 ppm), between 2°C and 50°C. This suggested that the A•U base pair, involving U18, may have a higher susceptibility to temperature-induced conformational changes. To summarise, the observation of exchange retarded base pairs even at 50°C (f) attests to the overall thermodynamic stability of the motif and justifies its selection for neamine 12 binding. These well resolved low field imino protons of the 27mer RNA thus provided an opportunity to monitor the binding of the RNA to neamine 12.
4.2.5 $^{31}$P-NMR spectra of the 27mer RNA at variable temperatures (VT series)

The $^{31}$P-NMR VT series was analyzed for the 27mer RNA in $^1$H$_2$O at different temperature points (Figure 4.2.5.48). Phosphorus chemical shifts are very sensitive to the conformation of RNA, since the phosphorus atoms are located in the RNA backbone. Therefore, the changes in phosphorus chemical shifts will signify a change in the RNA phosphate backbone. Changes in structure conformation can lead to a simultaneous highfield shift. Conversely, phosphate interactions generally produce a lowfield $^{31}$P chemical shift change due to deshielding of the phosphorus nucleus. Eleven phosphorus peaks were identified in the spectrum at 2°C. The phosphorus peaks 2-11 were observed overlapped between -2.0 and -4.4 ppm, which largely consist of stem phosphorus peaks. Peak 1 was observed significantly lowfield shifted at -1.14 ppm and was supposed to be between C14 and U13 bases in the loop. Generally, loop phosphorus peaks are observed significantly more lowfield of the stem phosphorus peaks. A decrease in phosphorus peak intensity and highfield shifts were observed in $^{31}$P-NMR VT series of the 27mer RNA. For the 27mer RNA, the largest chemical shift changes were observed for the phosphate peak 1 (highfield $\Delta \delta$=-0.5 ppm) and phosphate peak 11 (highfield $\Delta \delta$=-0.2 ppm) between 2-40°C. This strongly suggests that the elements of the phosphate backbone are susceptible to changes in conformation induced by an increase in temperature.
Figure 4.2.5.48. 161.9MHz $^{31}$P-NMR stack plot of the VT spectra of the 27mer RNA in $^1$H$_2$O at (a) 2°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C.
4.3 Assignment table of the exchangeable protons of the 27mer RNA

The NH imino, NH$_2$ and NH$_2^*$ amino chemical shifts of the 27mer RNA are shown in Table 4.3.5.

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Table 4.3.5. 400 MHz NH imino, NH$_2$ and NH$_2^*$ amino chemical shifts of the 27mer RNA in $^1$H$_2$O at 2°C.
4.4 Unconstrained molecular modelling: simulated annealing and molecular dynamics of the 27mer RNA

After geometry optimization, simulated annealing and molecular dynamics, the unconstrained model of the 27mer RNA showed an A-form helix with all the hydrogen bonded base pairs within standard distances and a hairpin loop corresponding to the UUCG tetraloop (Figure 4.4.49, a). The loop was zoomed to show some interactions between base pairing (Figure 4.4.49, b).

![Figure 4.4.49](image)

**Figure 4.4.49.** Illustration of (a) the unconstrained modelling of the 27mer RNA. (b) Zoom of the 5’-ACCUUCGGGU-3’ sequence of the 27mer RNA, showing the G•U mismatch and base-phosphate interaction.62

The N3 and O4 of U5 made hydrogen bonds with the O2 and N3 of U23 to form the U5•U23 mismatch base pair (Figure 4.4.50, a). The N6 and N7 of A21 made hydrogen bonds with the N7 and N6 of A6 to form the A7•A21 mismatch37 (Figure 4.4.50, b).
The unconstrained model (Figure 4.4.49, b) reveals that the U13 base is flipped out of the loop. This confirms the strong U13 imino-water crosspeaks observed in the NOESY (\(^1\)H\(_2\)O/2°C) spectrum of the 27mer RNA, indicating a fast exchange with water. Further, the analysis of the model, revealed the main characteristics of the loop structure which is the U•G wobble base pair that contains two hydrogen bonds between the 2'-OH group and O2 of U12 and N1 and O7 of G15 to form the U12•G15 mismatch. These interactions are regarded as the main determinants for the thermal stability. Additional, stabilizing effects arise from extensive base stacking interactions and specific base-phosphate interactions. A specific base-phosphate interaction is one hydrogen bond between the C14 amino proton and one phosphate oxygen between the U12 and U13 bases. The hairpin has a loop of only two nucleotides and both adopt C2'-endo sugar pucker.
4.5 Discussion about the NMR assignment and model of 27mer RNA

Nearly complete assignments of the exchangeable protons have been achieved by the NOESY ($\tau_m = 250\text{ms}$) experiment in $^1\text{H}_2\text{O}$ at 2°C. Several specific interactions in the loop were expected to contribute to the hairpin thermodynamic stability. The presence of imino-imino connectivities indicated a stable, double-helical RNA. Furthermore, the imino-imino sequential assignment observed in Figure 4.2.2.45 confirmed the predicted secondary structure of the 27mer RNA. The motif is composed of nine stable canonical base pairs, two non-canonical A7•A21 and U5•U23 mismatches and the asymmetrical bulge A20. The imino-amino connectivities observed in the NOESY spectrum of the 27mer RNA, provided strong evidence of base pairing between C6•G22, C8•G19 and A9•U18, in the absence of antibiotic. Consequently, the existence of the imino-imino and imino-amino NOEs from G4/G22 to U5/U23 could support also that the U5•U23 mismatch is closed. Similarly, weak imino-amino C8•G19 crosspeaks and no imino-imino connectivities from G19 to U18 were observed in the NOESY spectrum, suggesting a decreased stability of the C8•G19 base pair due to proximity to the A7•A21 mismatch. No NOEs were observed from the G16 imino resonance to the G15 imino resonance, and from the G15 imino resonance to the U12 imino resonance, indicating that imino-imino distances could be more than 5.0Å. Furthermore, strong U12, U13, G15 and G19 imino-water crosspeaks were observed in the NOESY ($^1\text{H}_2\text{O}/2^\circ\text{C}$) spectrum, indicating a greater exposure to water. Consequently, the C6•G22, C8•G19, the A9•U18 base pairs, the A7•A21 mismatch, the asymmetrical bulge A20 and the U12, U13 bases in the loop are predicted to be the likely site(s) of interaction with neamine 12.
4.6 NMR studies of the interaction of neamine 12 with 27mer RNA

A systematic NMR study of the interaction of the neamine 12 with the conserved and highly stable 27mer RNA motif of the A-site 16S rRNA was carried out. As described in Chapter 3, the antibiotic showed well-resolved and dispersed resonances including exchange retarded amide protons suggesting a stable conformation of the drug. Similarly, the 27mer RNA exhibited well-resolved and stable exchangeable imino protons in the lowfield region of the NMR spectrum. The imino-imino and imino-amino regions of the NMR spectrum of the neamine 12-RNA complex were analyzed to discover any differences in NOE patterns and intensity after the addition of the neamine 12. Changes in these two parameters would be indicative of conformational change in the tertiary RNA structure. ³¹P- and ¹H-NMR VT series were performed to attest the stability of the complex at higher temperatures.

4.6.1 Comparison of the ¹H-NMR spectra (imino proton region) of 27mer RNA and its neamine 12 complex

No detectable chemical shift changes to either the RNA or neamine 12 protons were observed in the 1D ¹H-NMR spectrum of the RNA-neamine 12 complex in ¹H₂O (Figure 4.6.1.51, b), suggesting a weak binding of neamine 12 to the 27mer RNA motif. A comparison of the ¹H-NMR spectra (imino proton region) of 27mer RNA (Figure 4.6.1.51, a) and its neamine 12 complex (Figure 4.6.1.51, b) in ¹H₂O at 2°C showed considerable line broadening induced by the canonical U18 (13.95 ppm), G22 (13.29 ppm) and G19 (12.81 ppm) protons and to the non canonical mismatch U5 (10.71 ppm) and U23 (10.14 ppm) protons due to complex formation. There is also broadening of non-canonical U12 (12.27 ppm) and U13 (11.84 ppm) imino protons of the tetraloop which may arise due to non-specific interaction between the two moieties.
A comparison of the $^1$H-NMR spectra of neamine 12 (Figure 4.6.1.51, c) and RNA-neamine 12 complex (Figure 4.6.1.51, d) in $^1$H$_2$O at 2°C shows the total disappearance of H1 (9.2 ppm) (green box) and the cluster of high field protons (0.5 - 2.5 ppm) of the cyclohexyl rings A and B (green box) of the neamine 12. This suggests that both the uracil and the two cyclohexyl rings of neamine 12 are involved in binding to the 27mer RNA.
4.6.2 Comparison of the NOESY spectra (imino - imino proton region) of 27mer RNA and its neamine 12 complex

A comparison of the NOESY spectra of 27mer RNA and its complex with neamine 12 (Figure 4.6.2.52) was carried out in order to probe the effect of interaction between the two components by monitoring the assigned imino proton resonances of the RNA. These changes in peak intensity appear to be due to an increase in line broadening of the resonances in the 1D spectrum of the complex, indicating an intermediate rate of chemical exchange of neamine 12 between the bound and free states of 27mer RNA. The two symmetrical A7•A21 and U5•U23 mismatches, the lone asymmetrical bulge A20 and the U12 and U13 bases of the loop are predicted to be the likely site(s) of interaction with neamine 12.

![Figure 4.6.2.52. 400 MHz NOESY (τ_m = 250ms) overlaid spectra of 27mer RNA and its neamine 12 complex in H2O at 2°C, showing a comparison of the imino-imino region of the 27mer RNA and the 27mer RNA-neamine 12 spectra. The red cross peaks belong to the spectrum of the 27mer RNA and the green cross peaks belong to the spectrum of the 27mer RNA-neamine 12 complex. The black boxes indicate the detectable changes in imino diagonal peak intensity. The largest changes in imino diagonal peak intensities were observed for U5 and U23, belonging to the U•U mismatch. Other changes in diagonal peak intensity were found for the U12 imino proton, located in the loop. These results are consistent with the changes observed in the 1D 1H-NMR spectrum. When two molecules interact, the chemical environment of the nuclei is altered, leading to changes in chemical shift. In addition, a](image-url)

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very small lowfield shift observed for the U18 and U12 imino diagonal peaks, was likely due to a deshielding of the imino and amino proton nuclei.

4.6.3 Comparison of the NOESY spectra (imino - amino proton region) of 27mer RNA and its neamine 12 complex

A comparison of the imino-amino region NOESY (\(\text{H}_2\text{O}/ 2^\circ\text{C}\)) spectra of the 27mer RNA and its neamine 12 complex is shown in the Figure 4.6.3.53.

Through comparison of the NOESY (\(\text{H}_2\text{O}/2^\circ\text{C}\)) spectra of the 27mer RNA and its neamine complex, differences were observed in the NOE crosspeaks corresponding to imino-amino and imino-water connectivities. In the spectrum of the 27mer RNA-neamine 12 complex the imino-amino crosspeaks had either significantly reduced in intensity (G1•C27, G19•C8 base pairs) or were undetectable (U12 base, U18•A9 base pair). A
small lowfield shift was observed for the imino-amino U18•A9 crosspeaks, due to a deshielding of the imino and amino nuclei, suggesting interactions with the neamine 12. Small highfield shift was observed for the imino-amino G19•C8 and G1•C27 crosspeaks, suggesting changes in structure conformation, such as increased base stacking interactions. Furthermore, the intensities of the imino proton (F2)→water proton (F1) exchange cross peaks (Figure 4.6.3.53, dotted line along 5 ppm) are much reduced in the NOESY spectrum of the 27mer RNA-neamine 12 complex indicating retardation to exchange caused to the RNA by bound neamine 12. The U12 and U23 imino-water crosspeaks have reduced intensity, indicating that the neamine 12 was having the effect of reducing imino proton exchange. Furthermore the U12 imino-water crosspeak was slightly lowfield shifted. The imino-amino crosspeaks of the G2•C26, G4•C24, G22•C6, G17•C10, G16•C11 and G25•C3 base pairs were retained in the presence of the neamine 12. The G19•C8 base pair is close to the bulge and mismatch regions of the 27mer RNA motif and the observed line broadening and loss of NOE correlations may be a consequence of the binding of neamine 12 to this unstructured region of the RNA.
4.6.4 Comparison of the NOESY spectrum of the 27mer RNA - neamine 12 complex and the ROESY spectrum of the neamine 12

Through comparison of the NOESY spectrum of the 27mer RNA-neamine 12 complex and the ROESY spectrum of the neamine 12 (Figure 4.6.4.54), it was found that the long-range ROEs shown by the amide H8 proton to H17 proton was absent in the NOESY spectrum of the 27mer RNA-neamine 12 complex.

**Figure 4.6.4.54.** Illustration of the 400 MHz NOESY and ROESY ($^1$H$_2$O/2°C) spectra (overlapped) of the 27mer RNA-neamine 12 complex and the neamine 12 uncomplexed, showing a comparison of the imino-amino region. The black boxes indicate the detectable changes in imino diagonal peak intensity. The ROEs arising from H1, H3, H8 and H18 to H3, H4, H17, H19 and H22 of neamine 12 were rendered absent after complex formation to the RNA (black square boxes).

Furthermore, it was also found that the ROEs due to H1→H3, H3→H4, H18→H19, H22 correlations are absent in the NOESY spectrum of the 27mer RNA – neamine 12 complex. This comparison of the ROESY spectrum of the neamine 12 with the NOESY spectrum of the RNA-neamine 12 complex gave an opportunity to monitor the effect of interaction between the two components on the resolved exchangeable proton resonances H1 and H8 of neamine 12.
4.6.5 $^1$H-NMR spectra of the 27mer RNA-neamine 12 complex at variable temperatures (VT series)

Analogous to the $^1$H-NMR spectra of the variable temperature series of neamine 12 and 27mer RNA, the $^1$H-NMR spectra of the variable temperature series of 27mer RNA-neamine 12 complex (Figure 4.6.5.55) was also measured over the same temperature range (2ºC-50ºC) to monitor changes in chemical shift and linewidth with temperature.

![400 MHz $^1$H-NMR stack plot of the VT spectra (imino proton region) of 27mer RNA-neamine 12 complex (1:1) in $^1$H$_2$O at (a) 2°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, (f) 50°C.](image)

**Figure 4.6.5.55.** 400 MHz $^1$H-NMR stack plot of the VT spectra (imino proton region) of 27mer RNA-neamine 12 complex (1:1) in $^1$H$_2$O at (a) 2°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, (f) 50°C.

The attenuation in intensity of the imino protons of the RNA complex with progressive increase of temperature follows a trend that is similar to the decrease observed before with the uncomplexed 27mer RNA (Figure 4.2.4.47, a-f). Changes in chemical shift and linewidth were observed in this VT series, the U18, U13, U12, U23 and U5 resonances
were clearly exchange retarded at 2°C after the addition of the neamine 12. The U13, U5 and U23 imino resonances are clearly visible up to 20°C and the U12 and U18 resonances disappear above 40°C. At 30°C, the resonances of the U12, U23 and U5 were absent in the presence of the neamine 12 but were still observable in the spectrum at 40°C of the 27mer RNA uncomplexed. The largest chemical shift changes in chemical shift were observed for U18 (highfield, Δδ=0.13 ppm) and G2 (highfield, Δδ=0.07 ppm). Both the U18 and G2 resonances were absent in the 1H-NMR spectrum at 50°C but were still observable in the spectrum at 50°C of the VT series of the 27mer RNA uncomplexed. No chemical shift changes were observed for the other imino resonances. Interestingly, no temperature-induced chemical shift changes were observed for the U12 imino proton, but were observed (highfield Δδ=0.16 ppm) in the case of the 1H-NMR VT series of the 27mer RNA. Additionally, for both the 27mer RNA and the 27mer RNA-neamine 12 complex, the imino resonances of guanine, located in the stem, appear to be broader at a lower temperature than at higher temperatures. The U18 imino resonance remains broad even at a higher temperature. No temperature-induced chemical shift and linewidth changes were observed for the aromatic protons of the 27mer RNA-neamine 12 complex spectrum (data not shown). The retention of the line broadening effect observed for the above noted imino protons of the RNA-neamine12 complex at higher temperatures attests to the stability of the complex.
4.6.6 $^{31}$P-NMR spectra of the 27mer RNA-neamine 12 at variable temperatures (VT series)

The $^{31}$P-NMR VT series was analyzed for the 27mer RNA-neamine 12 complex in $^{1}$H$_2$O at different temperature points (Figure 4.6.6.56). Eleven phosphorus peaks were identified in the $^{31}$P-NMR spectrum.

Figure 4.6.6.56. 161.9MHz $^{31}$P-NMR stack plot of the VT spectra of the 27mer RNA-neamine 12 complex in $^{1}$H$_2$O at (a) 2°C, (b) 10°C, (c) 20°C, (d) 30°C, (e) 40°C, (f) 50°C.
Through comparison of the $^{31}$P-NMR spectra of the 27mer RNA-neamine 12 complex and the 27mer RNA in $^1$H$_2$O at 2°C (Figure 4.6.6.57), it was found that small but detectable changes in line broadening and chemical shift were observed for all the phosphorus peaks. Lowfield shifts were observed in the $^{31}$P-NMR spectrum of the 27mer RNA-neamine 12 complex in $^1$H$_2$O at 2°C. Generally, phosphate interactions produce a lowfield $^{31}$P chemical shift change due to deshielding of the phosphorus nucleus, suggesting binding.

**Figure 4.6.6.57.** Illustration of the comparison of the $^{31}$P-NMR spectra of (a) the 27mer RNA and (b) the 27mer RNA-neamine complex in $^1$H$_2$O at 2°C.
4.7 Discussion of the comparison between the complexed and uncomplexed 27mer RNA

In summary, the main spectral effect observed due to the binding of a molar equivalent of neamine 12 to the 27mer RNA is considerable line broadening in the $^1$H-NMR spectra of both the molecules. This suggests that the affinity between neamine 12 and 27mer RNA belongs to the weak category and the observed line broadening may be due to an intermediate rate of chemical exchange, on the NMR chemical shift time scale, of neamine 12 between the bound and free states of 27mer RNA. Small but detectable chemical shift changes were observed in the NOESY ($^1$H$_2$O/2°C) spectrum of the 27mer RNA-neamine 12 complex. The largest changes in imino diagonal peak intensities were observed for U5, U12, U13 and U23 with increasing retardation to exchange after complex formation.

Another interesting feature of the NOESY spectrum of the 27mer RNA-neamine 12 complex is that the U23 imino-water crosspeak had reduced in intensity. The line broadening, the loss of NOE correlation between U5•U23 and the reduce intensity of the U23 imino-water crosspeak may be a consequence of interactions of neamine 12 to this unstructured region of the RNA. A lowfield shift was observed for the U18 and U12 imino diagonal peaks that could be attributed to changes in base stacking or to direct binding of the neamine 12. The imino-amino crosspeaks of the G2•C26, G4•C24, G22•C6, G17•C10, G16•C11 and G25•C3 base pairs were retained in presence of neamine 12. The imino-amino crosspeaks had significantly reduced in intensity in the case of the G19•C8 base pair, and were undetectable in the case of the U12 base and the U•A base pair. Furthermore, a lowfield shift was observed for the imino-amino U•A crosspeaks and a highfield shift was observed for the imino-amino G19•C8 crosspeaks. These base pairs occur close to the bulge and mismatch regions of the 27 RNA motif and the observed line broadening and loss of NOE correlations may be a consequence of the binding of neamine 12 to this unstructured region of the RNA. Although the binding is weak, the absence of the H1, H8 and sugar protons of neamine 12 and selective line broadening of the U18, U5, U23, U12 and U13 imino proton of the RNA host suggests
that the site of interaction within the complex may involve these proton groups of the ligand and host.

4.8 Unconstrained model of the 27mer RNA – neamine 12 complex

The unconstrained model of the 27mer RNA-neamine 12 complex, after geometry optimization, simulated annealing and molecular dynamics, revealed intermolecular interaction between the 27mer RNA and the neamine 12 (Figure 4.8.58). As mentioned before, critical nucleotides for neamine 12 binding to the A-site include the C8•G19 and A9•U18 base pairs, the two symmetrical A7•A21 and U•U mismatches, the lone asymmetrical bulge A and the loop (U12, U13).

![Diagram of the unconstrained model of the 27mer RNA-neamine 12 complex. The green molecule represents the neamine 12 and the blue structure represents the 27mer RNA.](image)

**Figure 4.8.58.** Illustration of the unconstrained modelling of the 27mer RNA-neamine 12 complex. The green molecule represents the neamine 12 and the blue structure represents the 27mer RNA.
The RNA backbone is distorted by the presence of the bulged nucleotide A20 and noncanonical A7•A21 pair. This distortion arises from non-standard values of backbone dihedral torsion angles and leads to formation of a distinct binding pocket for neamine 12. The bulge A20 of the RNA is well poised to undergo an interaction with the uracil moiety of neamine 12 as shown by the unconstrained molecular model shown in Figure 4.8.59, a. The uracil moiety of the neamine 12 might interact with the A20 base through two hydrogen bonds, the N6 and N1 of A20 forming hydrogen bonds with O4 and H1 of uracil. The neamine 12 may have formed a linear array from the A20 base to the UUCG loop, the rings A and B were positioned close to the loop, near the U12 and U13 bases (Figure 4.8.59, b). Both A and B rings might adopt chair conformations, with the amino and hydroxyl substituents in equatorial positions, making specific interactions to stabilize the neamine12-RNA complex. The azide and hydroxyl groups of A and B rings suggest probable electrostatic contacts with the phosphate backbone.

**Figure 4.8.59.** Unconstrained molecular model of the 27mer RNA-neamine 12 complex, showing (a) interactions between the uracil (U) of neamine 12 and the bulge A20 of the 27mer RNA. The dotted lines represent hydrogen bonding between the H1 of neamine 12 and the N1 of A20 of the 27mer RNA (2.89Å), and O4 of the neamine 12 and NH2 of A20 of the 27mer RNA (2.51Å). (b) Interactions between one azide group and the H8 proton of neamine 12, and the N3 (3.57Å) and O2 (4.19Å) of the U12 of the 27mer RNA. The dotted lines represent hydrogen bonding between the azide group of neamine 12 and the N3 of U12 (27mer RNA), and H8 of the neamine 12 and O2 of U12 (27mer RNA).
Chapter 5: Conclusion and suggestions for future work

5.1 Conclusion

The project provided an opportunity to learn and apply a range of 1D and 2D NMR (homo and heteronuclear) methods to characterize biologically important molecules, neamine 12 and 27mer RNA. A systematic characterisation of the \(^1\)H-NMR spectra of the novel neamine 12 and its binding target, a stable 27 RNA, in native aqueous conditions was carried out. The full assignment of the \(^1\)H and \(^{13}\)C resonances of the neamine 12, and the exchangeable protons of the 27mer RNA were obtained. Furthermore, the \(^1\)H-NMR spectra of the RNA-neamine 12 complex (1:1), provided direct experimental evidence for interaction between the two components, although the binding was weak.

The addition of a molar equivalent of neamine to 27mer RNA caused considerable line broadening to both the components, especially to the A9•U18 base pair, the unstructured mismatch U5•U23 and the loop (U12, U13), providing evidence for their affinity. Specific interactions occur between the chemical groups of neamine 12 and conserved nucleotides in the RNA and were observed. The antibiotic likely binds in the minor groove of the model A-site RNA within a pocket created by an A7•A21 base pair and a single bulged adenine, since chemical shift changes where observed for C8•G19 base pair that is close to these.

In addition to the NMR data, an unconstrained model of the neamine 12 and RNA-neamine 12 complex was generated using molecular dynamics methods. The model clearly showed a folded structure for the antibiotic. In view of the disposition of various functional groups endowed by neamine, the results of this work provide a good opportunity to modify selected groups and render stronger affinity to the RNA.
5.2 Suggestions for future work

The NMR results of this project showed that although neamine 12 (azidodicaccharide nucleoside conjugate) has weak affinity for A-site 27mer RNA of the 12S rRNA, there is considerable potential to use the compound to generate other derivatives with enhanced affinity for the RNA. For example, the four azido groups can be converted to four amino groups (Figure 5.2.60) to facilitate greater electrostatic and hydrogen bond interactions with the RNA.

![Chemical structure of the modified neamine 12 showing the substitution of the four azido groups with amino functionalities (in red).](image)

Figure 5.2.60. Chemical structure of the modified neamine 12 showing the substitution of the four azido groups with amino functionalities (in red).

When the above has been achieved, it should be possible to determine proper NMR structure of the optimised neamine derivative using experimental geometrical constraints derived from NOE and scalar coupling data. The unconstrained model of the neamine 12 determined in this project shows that the molecule has a natural ability to adopt a folded conformation. Thus, the model provided a framework to generate a proper NMR structure of the optimized neamine derivative.

The NMR methods used to characterize RNA-neamine 12 complex in this project provide a basis and can be successfully applied to determine the NMR structure of the RNA-
optimized neamine 12 complex. When completed, the results should enable a better understanding of the molecular basis of antibiotic action.
References

42. The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.


77. HyperChem (TM) Professional 7.51, Hyperform, Inc., 1115 NW 4th Street, Gainesville, Florida 32601, USA.


84. Vienna RNAfold software.


Appendices

Appendix I: NMRPipe script

source /home/chemistry/Desktop/NMRpipe/com/nmrInit.linux9.com
endif
if (-e /home/chemistry/Documents/NMRpipe/dynamo/com/nmrInit.linux9.com)
then
source /home/chemistry/Documents/NMRpipe/dynamo/com/nmrInit.linux9.com
endif
#!/bin/csh

bruk2pipe -in /media/DATAPART1/Grace/12-mer/15/ser \
   -bad 0.0 -aswap -DMX -decim 4544 -dspfvs 20 -grpdlY 67.9842987060547 \
   -xN 4096 -yN 512 \
   -xT 2048 -yT 256 \
   -xMODE DQD -yMODE States-TPPI \
   -xSW 4401.408 -ySW 4402.552 \
   -xOBS 400.232 -yOBS 400.232 \
   -xCAR 4.774 -yCAR 4.774 \
   -xLAB 1Hx -yLAB 1Hy \
   -ndim 2 -aq2D States \ 
   -out /media/DATAPART1/Grace/12-mer/15.fid -verb -ov

sleep 5

nmrPipe -verb -in /media/DATAPART1/Grace/12-mer/15.fid \ 
   | nmrPipe -fn GMB -1b -5.0 -gb 0.08 -c 0.5 \ 
   | nmrPipe -fn ZF -auto \ 
   | nmrPipe -fn FT -auto \ 
   | nmrPipe -fn PS -p0 -103.5 -p1 -4 -di \ 
   | nmrPipe -fn TP \ 
   | nmrPipe -fn SP -off 0.5 -end 0.98 -c 1 \ 
   | nmrPipe -fn ZF -auto \ 
   | nmrPipe -fn FT -auto \ 
   | nmrPipe -fn PS -p0 -92.4 -p1 191 \ 
   | nmrPipe -fn TP \ 
   | nmrPipe -ov -verb -out /media/DATAPART1/Grace/12-mer/15.ft2

/home/chemistry/Documents/sparky/bin/.pipe2ucsf
/media/DATAPART1/Grace/12-mer/15.ft2 /media/DATAPART1/Grace/12-mer/15.ucsf
Appendix II: Training and Development

Poster related to the project:


Award:

I was awarded a competitive student bursary for the meeting: Structure2013, on 26th – 27th Feb 2013, Holywell Park, Loughborough University.

Publications:


Teaching experiences:

I undertook demonstration duties to the School of Chemistry, University of Manchester:

1) Demonstration assignment 1: MSc workshop and lab demonstration about NMR methods (16 hours).
2) Demonstration assignment 2: NMR workshop, “Intensive 2-day workshop in NMR 10th and 11th January 2013” directed by Dr. Vasudevan Ramesh at the School of Chemistry, University of Manchester.

My Training and Development:

1) Studying Large-Scale Brain Networks: Electrical Stimulation & Neural-Event-Triggered fMRI. Speaker: Nikos Logothetis, Max-Planck-Institute for Biological Cybernetics, Tübingen. On 5th June, 2013, at the Stopford Building, University of Manchester.
2) NMR and EPR UK users’ meeting, on 13th -14th November, 2012. Coventry Hilton hotel.

3) Academic Tutorial Writing, Sackville building, University of Manchester.

4) Introduction to Research-Essentials. On 09/11/2012, University Place 1.219, University of Manchester.