TARGETING A-FORM NUCLEIC ACIDS: A NEOMYCIN BASED APPROACH

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TARGETING A-FORM NUCLEIC ACIDS: A NEOMYCIN BASED APPROACH

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Chemistry

by
Nicholas N. Shaw
August 2011

Accepted by:
Dr. Dev P. Arya, Committee Chair
Dr. Ken Christensen
Dr. R. Karl Dieter
Dr. Meredith I. Newby-Spano
Dr. Bill T. Pennington
ABSTRACT

This manuscript reflects our most recent advancements in the recognition of nucleic acids. Chapter one focuses on previously established methods in nucleic acid recognition. Nucleic acid targeting by small molecules affords a unique way to potentially inhibit biological functions, which is different from targeting enzyme and protein bio-macromolecules. We seek to further develop an aminoglycoside approach to targeting nucleic acids. Since aminoglycosides were first introduced, over 60 years ago, as treatment for tuberculosis, natural product discovery and demands for therapeutically more effective aminoglycosides, with lowered toxicity, have dominated the landscape of aminoglycoside research. More recently, our group has established neomycin as a major groove binding ligand capable of binding nucleic acids outside of their traditional target, the A-site of 16S rRNA in the 20S subunit of ribosomal RNA. These non-A-site structures shared a common motif, A-form conformation within the helix.

To this end, chapter two of this manuscript focuses on advancements in targeting DNA:RNA hybrids. DNA:RNA hybrids are viable therapeutic targets as they appear in a number of biological processes: transcription, reverse transcription, DNA replication, mitochondrial DNA replication and telomerase activity. Surprisingly, the number of ligands which target DNA:RNA hybrids is fewer than ten! We report the development of a novel neomycin-methidium chloride conjugate capable of targeting DNA:RNA hybrids with selectivity as well as high affinity.
Chapters three and four introduce our most recent advancements in targeting DNA. Prior work by our group suggested conjugation of neomycin to a minor groove binding ligand can be used as a method of targeting the DNA duplex. Although these dual recognition conjugates demonstrated the ability to stabilize the duplex, improvements in affinity remained. A departure from previous work, we focus on targeting GC-rich DNA, over AT-rich DNA; as reports suggest GC-rich DNA is capable of displaying A-form characteristics. When designing our minor groove binding ligand, we utilized DNA minor groove-polyamide, pairing rules established by Dervan. Subsequent conjugation to neomycin to polyamides comprised of N-methylimidazole and N-methylpyrrole afforded us a library of polyamide conjugates. In chapter three, we focus on the development and bioanalytical studies of tetramer polyamide – neomycin conjugates. Chapter four discusses our most recent advancements in the development of hairpin polyamide - neomycin conjugates which were designed to target GC-rich DNA.
DEDICATION

This manuscript is dedicated to Leo B. Stoltaman. You are missed and loved.
ACKNOWLEDGEMENTS

I would like to thank my advisor for giving me a platform in which I could develop into scientist. At times, the amount of time, patience and understanding required to nurture a student through a Ph.D. program is more than the work being produced. I know, without question, I have tested the limitations of each of these during the time I have spent in the Arya lab and for his resolve, I am grateful to Dr. Arya. Without question, these have been the most formative years of my life.

To my wife: It is without question, I could not have completed this without you. Your love, grace and patience with such a self-centered process have been powerful tools in staving off the worst times that a Ph.D. program can bring. It’s time to do something different.

To my labmates: Labmates long gone – Liang Xue, Dr. I. Charles, Bert Willis and Hongjuan Xi. I am indebted to you for the time taken out of your schedules to teach me the tools and techniques I would need to be successful in the Arya Group. The work that was completed in this manuscript would not be possible without you. Generation India – Nihar Ranjan and Sunil Kumar, too long have I held torch for this group, it is now yours.

I would like to thank the members of my committee Dr. Ken Christensen, Dr. R. Karl Dieter, Dr. Meredith I. Newby-Spano and Dr. Bill T. Pennington for their input and suggested used to refine this dissertation. Dr. Meredith I. Newby-Spano, thank you for your kindness, generosity and friendship. It means the world to me.
Finally, to my parents, your constant and unwavering support over every single day of my life provides the fuel for accomplishments like this to be made. My success outside and inside this program is without question a reflection on my upbringing. Completion of a program such as this requires a strong belief in oneself and above all perseverance. I do not recall the period when these things crucial tools were instilled in my psyche. I can only imagine the seed was planted long before I can remember and nurtured without my notice. Without these tools, this manuscript and its author would not be where I am today.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE</td>
<td>i</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>iv</td>
</tr>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>ix</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xvii</td>
</tr>
<tr>
<td><strong>CHAPTER</strong></td>
<td></td>
</tr>
<tr>
<td>I.  NUCLEIC ACIDS AND THEIR INTERACTION WITH LIGANDS</td>
<td>1</td>
</tr>
<tr>
<td>A Historical Perspective &amp; Overview</td>
<td>1</td>
</tr>
<tr>
<td>Nucleic Acids – Presenting the Case</td>
<td>4</td>
</tr>
<tr>
<td>Targeting Nucleic Acids</td>
<td>9</td>
</tr>
<tr>
<td>References</td>
<td>22</td>
</tr>
<tr>
<td>II. TARGETING DNA:RNA HYBRIDS</td>
<td>31</td>
</tr>
<tr>
<td>Introduction</td>
<td>31</td>
</tr>
<tr>
<td>Experimental</td>
<td>44</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>55</td>
</tr>
<tr>
<td>Conclusions</td>
<td>92</td>
</tr>
<tr>
<td>References</td>
<td>94</td>
</tr>
<tr>
<td>III. ADVANCEMENTS IN DNA RECOGNITION: DEVELOPMENT OF A TETRAMER POYAMIDE – NEOMYCIN CONJUGATE LIBRARY</td>
<td>102</td>
</tr>
<tr>
<td>Introduction</td>
<td>102</td>
</tr>
</tbody>
</table>
Experimental .................................................................111
Results and Discussion ....................................................122
Conclusions .................................................................172
References .................................................................175

IV. RECOGNITION OF A-FORM DNA BY
    HAIRPIN POLYAMIDE –
    NEOMYCIN CONJUGATES ............................................181

    Introduction .............................................................181
    Experimental ...........................................................185
    Results and Discussion ..............................................200
    Conclusions ............................................................234
    References .............................................................236

APPENDICES ..................................................................241

    A: Appendix A ...........................................................241
    B: Appendix B ...........................................................278
    C: Appendix C ...........................................................359
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>UV determined thermal melting temperatures of various nucleic acids in the presence of DPA200</td>
<td>61</td>
</tr>
<tr>
<td>2.2</td>
<td>UV determined thermal denaturation temperatures of DNA:RNA hybrids with various ligands</td>
<td>65</td>
</tr>
<tr>
<td>2.3</td>
<td>CD determined thermal denaturation temperatures of DNA:RNA hybrids with various ligands</td>
<td>69</td>
</tr>
<tr>
<td>2.4</td>
<td>Thermodynamic profile of DNA:RNA hybrids interaction with neomycin, ethidium bromide and DPA200, pH 6.8</td>
<td>78</td>
</tr>
<tr>
<td>2.5</td>
<td>Thermodynamic profile of nucleic acid interactions with neomycin and ethidium bromide and DPA200, pH 5.5</td>
<td>84</td>
</tr>
<tr>
<td>3.1</td>
<td>Stoichiometry table for the synthesis of HIMImPyPyCONH(CH₂)n-tri-CH₂OCH₂-neomycin-hexahydrochloride, DPA208 – DPA213, n = 2, 3, 5, 6, 8 and 10</td>
<td>116</td>
</tr>
<tr>
<td>3.2</td>
<td>UV determined change in thermal denaturation melting temperature values for various ligands with 5’-A₂G₆C₆T₂-3’</td>
<td>145</td>
</tr>
<tr>
<td>3.3</td>
<td>UV determined thermal denaturation melting temperatures of 5’-AₘGₙCₙTₘ-3’ duplexes with tetramer polyamide – neomycin conjugates DPA214 – DPA225, neomycin and 18</td>
<td>150</td>
</tr>
<tr>
<td>3.4</td>
<td>UV determined thermal melting temperatures of target duplexes in the presence of controls and series I (DPA208 – DPA213), series II (DPA214 – DPA219), series III (DPA220 – DPA225) and series IV (DPA243 and</td>
<td></td>
</tr>
</tbody>
</table>
DPA244) tetramer polyamide – neomycin conjugates with target duplexes.................................158

3.5 UV determined change in thermal temperatures and FID assay determined change in fluorescence data.................................................................162

4.1 UV determined thermal melting, change in thermal melting, temperatures and FID assay determined change in fluorescence data.................................224
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>The ‘central dogma of molecular biology’</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>Structural elements and naming of bases, nucleosides and nucleotides</td>
<td>5</td>
</tr>
<tr>
<td>1.3</td>
<td>Schematic of structural differences between A-form and B-form nucleic acids</td>
<td>8</td>
</tr>
<tr>
<td>1.4</td>
<td>Chemical structures of representative intercalators</td>
<td>9</td>
</tr>
<tr>
<td>1.5</td>
<td>A schematic depicting DNA base pairs and the functional groups accessible through the major and minor grooves of nucleic acid duplexes</td>
<td>12</td>
</tr>
<tr>
<td>1.6</td>
<td>Chemical structures of representative minor groove binders</td>
<td>13</td>
</tr>
<tr>
<td>1.7</td>
<td>Chemical structures of representative major groove binders</td>
<td>17</td>
</tr>
<tr>
<td>1.8</td>
<td>Structures of aminoglycosides with a 2-deoxy-streptamine ring</td>
<td>19</td>
</tr>
<tr>
<td>2.1</td>
<td>Structures of compounds used in this study: DPA200 and DPA201, neomycin and ethidium bromide</td>
<td>55</td>
</tr>
<tr>
<td>2.2</td>
<td>Competition dialysis results of DPA200 with various nucleic acids</td>
<td>60</td>
</tr>
<tr>
<td>2.3</td>
<td>Mixed melting profile of various nucleic acids</td>
<td>62</td>
</tr>
</tbody>
</table>
2.4 UV thermal denaturation profiles of poly(rA):poly(dT) and poly(dA):poly(rU) with various ligands ................................................................. 66

2.5 UV thermal denaturation profiles of poly(rA):poly(dT) and poly(dA):poly(rU) with **DPA201** ................................................................. 68

2.6 CD thermal denaturation profile of poly(rA):poly(dT) and poly(dA):poly(rU) ................................................................. 71

2.7 CD scans of the titration of neomycin with poly(rA):poly(dT) and poly(dA):poly(rU) ................................................................. 73

2.8 Fluorescence emission scans of the titration of **DPA200** and ethidium bromide with poly(rA):poly(dT) and poly(dA):poly(rU) ................. 75

2.9 DSC melting profiles of poly(rA):poly(dT) and poly(dA):poly(rU). .......................................................................................... 76

2.10 ITC titration of **DPA200** into the duplex poly(dA):poly(rU). .......................................................................................... 77

2.11 Fluorescence emission scans of the titration of **DPA200** with various nucleic acids ............................................................... 85

2.12 UV thermal denaturation profiles of poly(dA):poly(rU), poly(dA-dT)2 and poly(rA):poly(rU). ............................................................................. 87

3.1 Mode for antiparallel 2:1 distamycin A binding 5’-AAATT-3’ ................................................................. 108

3.2 Mode for 1:1 HlmImPyPyDp 18 binding 5’-TGGAAA-3’ ................................................................. 109

3.3 Mode for antiparallel 2:1 HlmImPyPyDp 18 binding 5’-TGGCCA-3’ ................................................................. 110

3.4 Tetramer polyamide – neomycin conjugates used in this study: **DPA208 – DPA225, DPA243 – DPA244**, neomycin and
3.5  Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2C2AGC4-3’.................................134

3.6  Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AGC4-3’.................................135

3.7  Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-TG2C2G4-3’.................................137

3.8  Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AGC4-3’.................................138

3.9  Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2A3G4C4-3’.................................140

3.10 UV melting profiles for 5’-A2G6C6T2-3’ with controls, neomycin and HImImPyPyDp 18.................................143

3.11 UV melting profiles for 5’-A2G6C6T2-3’ with series I DPA208 – DPA213 tetramer polyamide – neomycin conjugates. ........................................144

3.12a  Plot of ∆Tm with respect to conjugate linker length upon addition of ligands to 5’-A_mG_nC_nT_m-3’.................................147

3.12b  Plot of ∆Tm with respect to conjugate linker length upon addition of ligands to 5’- A_mG_nC_nT_m -3’.................................148

3.13 CD scans for the titration of 5’-A6G2C2T6-3’ and 5’-A2G6C6T2-3’ with HImImPyPyDp 18 and CD intensity vs r_dd value plots.................................152

3.14 Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the self
complementary duplex, 5’- A\textsubscript{6}G\textsubscript{2}C\textsubscript{2}T\textsubscript{6}-3’
and 5’- A\textsubscript{2}G\textsubscript{6}C\textsubscript{2}T\textsubscript{6}-3’ by HImImPyPyDp 18

3.15 Plot of ∆Tm with respect to conjugate linker length
upon addition of ligands to 5’-G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’

3.16 Plot of ∆Tm with respect to conjugate linker
length upon addition of ligands to
5’-G\textsubscript{2}C\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’

3.17 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound
thiazole orange from the DNA duplex,
5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’, by neomycin

3.18 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound
thiazole orange from the DNA duplex,
5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’, by DPA244

3.19 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound
thiazole orange from the DNA duplex,
5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’, by DPA243

3.20 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound
thiazole orange from the DNA duplex,
5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’, by DPA208

3.21 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound
thiazole orange from the duplex,
5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’, by DPA218 and DPA224

3.22 CD scans for the titration of 5’- G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’
with DPA244

4.1 Mode for HImImPyPy\gamma\textsubscript{ImImPyPyDp 28
binding 5’-AGGCCA-3’

4.2 Hairpin polyamide – neomycin conjugates used in
this study: DPA232 – DPA242, neomycin
and HImImPyPy\gamma\textsubscript{ImImPyPyDp (28)
4.3 Retrosynthetic strategy for the convergent solution-phase synthesis of 8-ring hairpin polyamide 19

4.4 Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2A3G4C4-3’

4.5 Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2A3G4C4-3’

4.6 Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-TG2C2G4-3’

4.7 Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4-3’

4.8 Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4C4-3’

4.9 UV melting profiles for 5’-AG2C2AG4C4-3’ with hairpin polyamide – neomycin conjugates DPA232 – DPA242, neomycin and 18

4.10 Plot of ΔTm with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4C4-3’ with controls and hairpin polyamide – neomycin conjugates DPA232 – DPA242

4.11 Comparison of compounds 28 and HImImPyPyγImImPyPyβDp

4.12 Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-AG2C2AG4C4-3’, by neomycin

4.13 Fluorescence intercalator displacement titration
spectra for the displacement of pre-bound thiazole orange from the DNA duplex,
5'- AG₂C₂AG₄C₄-3’, by DPA₂³₂……………………………………227

4.14 Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex,
5’- AG₂C₂AG₄C₄-3’, by DPA₂⁴₀……………………………………228

4.15 Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex,
5’-AG₂C₂AG₄C₄-3’, by DPA₂³⁹……………………………………229

4.16 CD scans for the titration of 5’-AG₂C₂AG₄C₄-3’
with neomycin, HlmImPyPγImImPyPyDp 28, DPA₂³₂…………………………………………………………231
## LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Synthesis of DPA10 and DPA12</td>
<td>57</td>
</tr>
<tr>
<td>2.2</td>
<td>Synthesis of DPA200</td>
<td>58</td>
</tr>
<tr>
<td>2.3</td>
<td>Synthesis of DPA201</td>
<td>58</td>
</tr>
<tr>
<td>3.1</td>
<td>Synthesis of compound 6</td>
<td>125</td>
</tr>
<tr>
<td>3.2</td>
<td>Synthesis of DPA202 – DPA207</td>
<td>126</td>
</tr>
<tr>
<td>3.3</td>
<td>Synthesis of DPA213 – DPA215 and DPA216</td>
<td>127</td>
</tr>
<tr>
<td>3.4</td>
<td>Synthesis of series I tetramer polyamide – neomycin conjugates DPA208 – DPA213</td>
<td>129</td>
</tr>
<tr>
<td>3.5</td>
<td>Synthesis of series II tetramer polyamide – neomycin conjugates DPA214 – DPA219</td>
<td>130</td>
</tr>
<tr>
<td>3.6</td>
<td>Synthesis of series III tetramer polyamide – neomycin conjugates DPA220 – DPA225</td>
<td>130</td>
</tr>
<tr>
<td>3.7</td>
<td>Synthesis of series IV tetramer polyamide – neomycin conjugates DPA243 and DPA244</td>
<td>131</td>
</tr>
<tr>
<td>3.8</td>
<td>Synthesis of compound 18</td>
<td>132</td>
</tr>
<tr>
<td>4.1</td>
<td>Synthesis of compound 11</td>
<td>204</td>
</tr>
<tr>
<td>4.2</td>
<td>Synthesis of compound 20</td>
<td>205</td>
</tr>
<tr>
<td>4.3</td>
<td>Synthesis of compound 21</td>
<td>206</td>
</tr>
<tr>
<td>4.4</td>
<td>Synthesis of compound 19</td>
<td>207</td>
</tr>
<tr>
<td>4.5</td>
<td>Synthesis of DPA226 – DPA231</td>
<td>208</td>
</tr>
<tr>
<td>4.6</td>
<td>Synthesis of DPA10, DPA13 and DPA16</td>
<td>209</td>
</tr>
<tr>
<td>4.7</td>
<td>Synthesis of DPA232 – DPA237</td>
<td>210</td>
</tr>
</tbody>
</table>
4.8 Synthesis of **DPA238 – DPA240**……………………………………..211

4.9 Synthesis of **DPA241, DPA242** and compound **28**………………..212
# Glossary of Abbreviations

<table>
<thead>
<tr>
<th><strong>Abbreviation</strong></th>
<th><strong>Description</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMD</strong></td>
<td>Actinomycin D</td>
</tr>
<tr>
<td><strong>Boc</strong></td>
<td>Tert-Butyloxy carbonyl</td>
</tr>
<tr>
<td><strong>CD</strong></td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td><strong>DAPI</strong></td>
<td>4',6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td><strong>DCC</strong></td>
<td>N,N'-dicyclohexyl Carboxyimide</td>
</tr>
<tr>
<td><strong>DIPEA</strong></td>
<td>N,N-diisopropylethylamine</td>
</tr>
<tr>
<td><strong>DMAP</strong></td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td><strong>DNA</strong></td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td><strong>DOS</strong></td>
<td>2-deoxystreptamine</td>
</tr>
<tr>
<td><strong>EDC</strong></td>
<td>N,N'-diisopropylcarboxyimide</td>
</tr>
<tr>
<td><strong>Et&lt;sub&gt;3&lt;/sub&gt;N</strong></td>
<td>Triethylamine</td>
</tr>
<tr>
<td><strong>FID</strong></td>
<td>Fluorescence Intercalator Displacement</td>
</tr>
<tr>
<td><strong>HIV</strong></td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td><strong>HOBt</strong></td>
<td>1-Hydroxybenzotriazole</td>
</tr>
<tr>
<td><strong>ICB</strong></td>
<td>Inverted CCAAT boxes</td>
</tr>
<tr>
<td><strong>ITC</strong></td>
<td>Isothermal Calorimetry</td>
</tr>
<tr>
<td><strong>mtDNA</strong></td>
<td>Mitochondrial DNA</td>
</tr>
<tr>
<td><strong>NMR</strong></td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td><strong>NNRTI</strong></td>
<td>Non-Nucleoside Reverse Transcription Inhibitors</td>
</tr>
<tr>
<td><strong>NOE</strong></td>
<td>Nuclear Overhauser Effect</td>
</tr>
<tr>
<td><strong>r&lt;sub&gt;db&lt;/sub&gt;</strong></td>
<td>Ratio drug to base pairs</td>
</tr>
<tr>
<td><strong>r&lt;sub&gt;dd&lt;/sub&gt;</strong></td>
<td>Ratio drug to duplex</td>
</tr>
<tr>
<td><strong>RNA</strong></td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td><strong>rRNA</strong></td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td><strong>RT</strong></td>
<td>Reverse Transcription</td>
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<td><strong>T&lt;sub&gt;m&lt;/sub&gt;</strong></td>
<td>Melting Temperature</td>
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<td><strong>TFA</strong></td>
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<td><strong>TPS</strong></td>
<td>Triisopropylbenzenesulfonyl Chloride</td>
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<td><strong>tRNA</strong></td>
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<td><strong>UV</strong></td>
<td>Ultra Violet</td>
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NUCLEIC ACIDS AND THEIR INTERACTION WITH LIGANDS

**Nucleic Acids – A Historical Perspective & Overview.** In 1869 Miescher isolated deoxyribonucleic acid, DNA, from the nuclei of white blood cells (1). The materials extracted from the nucleus were acidic in nature and named nucleic acids. It was not long before scientists discovered nucleic acids were buried within the nuclei of all cells. In 1908 Levene and Mandel reported the isolation of nucleotides from thymus DNA (2, 3). This discovery was significant as it suggested nucleic acids were actually comprised of smaller subunits. Levene identified the four bases adenine (A), cytosine (C), guanine (G), and thymine (T) as the subunit building blocks of DNA (4).

In 1944 (5), Oswald T. Avery, Colin MacLeod and Maclyn McCarty claimed nucleic acids were the carriers of genetic information. Historically, this claim bordered on the heretical as it marked a significant departure from the biologist driven belief that proteins functioned as the carriers of genetic material. Biologists strongly believed the carriers of genetic information must be more complex than simplistic nucleic acids. The damning evidence to the biologists’ claim followed in 1949 (6).

In 1947, X-ray experiments conducted on fiber DNA revealed two notable observations. First, an interesting reflection on the X-ray pattern was observed by Astbury and interpreted as stacking of the nucleosides (7). Second, X-ray experiments
suggested significant hydrogen bonding occurred within the structure, as reported by Gulland (8). This data was eventually used to prove that nucleosides were held together by hydrogen bonds about a central helical axis.

Furberg published the first crystal structure of a nucleoside – cytidine in 1950 (9). In 1952, data suggested that the arrangement of nucleosides were not random and nucleosides were inter-stand connected by hydrogen bonds (10). Todd and co-workers confirmed, in 1953, nucleosides were intra-strand connected through 3’, 5’ phosphodiester bonds and produced a linear polymer – DNA (11). Rosalind Franklin’s work in 1953 suggested the DNA was a helix with phosphate groups occupying the outside of the helix (12). Her work marked a major leap forward in the elucidation of DNA structure.

A 1953 Nature manuscript by Watson and Crick (13) proposed DNA structure existed as a double-stranded helix, comprised of repeating, paired nucleosides, held together by phosphate groups. The pair accommodated previously reported observations, by placing the heterocyclic bases along the helical axis and the sugar-phosphate backbones in a winding anti-parallel fashion around the central axis.

![Figure 1.1](image)

**Figure 1.1.** The ‘central dogma of molecular biology’.

Crick eventually proposed the ‘central dogma of molecular biology’, **Figure 1.1**. The framework of the dogma suggests DNA acted as the carrier of genetic information. Replication of the genetic code, DNA replication, was confirmed in 1958 (14).
Beginning in 1961, Holley, Khorana and Nirenberg solved the rules by which the flow of information encoded in DNA is transcribed to RNA, which in turn is translated into proteins (15, 16, 17, 18, 19, 20, 21, 22, 23, 24). For breaking the genetic code, they were awarded the 1968 Nobel Prize in Physiology or Medicine.

The years which followed saw a number of significant advancements in nucleic acid research as recounted in the following review (1). Some advancements include methods for sequencing DNA, 1977 (25), in vitro amplification of DNA and the introduction of polymerase chain reaction, PCR, in 1983 (26). Major advancements in DNA sequencing included the complete sequencing of a free-living organism genome (27), eukaryotic organism genome (28) and human chromosome and human genome (29).

Admittedly, the birth of the nucleic acid field of study began many centuries ago when humans observed simple inheritance phenomenon in nature. In fact, the study of genetics began in 1856 with experiments in a monastery garden conducted by Gregory Mendel, far before the work of Watson and Crick (13). Nevertheless, a direct result of work conducted in the late 1940s to mid-1950s, the elucidation of DNA structure opened a floodgate of scientific investigations. Currently, our work seeks to deepen our understanding of nucleic acid recognition by small molecules. Targeting nucleic acids affords us the potential to regulate dependent biophysical functions. This is the basis for the subsequent research presented in this manuscript.
Nucleic Acids – Presenting the Case. Prior to a discussion on nucleic acid structure a case emphasizing the importance of targeting nucleic acids must be made. The transfer of genetic information follows three principles:

(i) The genetic information of an organism is encoded within its DNA. This genetic information directs most biological functions of the organism. Furthermore, genetic information can be replicated with high fidelity.

(ii) The information stored in DNA can be transcribed into ribonucleic acid, RNA, through transcription. The resulting RNA transcript of the parent DNA strand retains high fidelity as well.

(iii) RNA translates genetic information into functional proteins through translation. Proteins are very complex in nature, dependent on constituent amino acids, account for the majority of non-aqueous mass of a cell and act as the workhouse in a number of cellular functions.

These principles make up the ‘central dogma of molecular biology’, Figure 1.1 (14).

A ligand designed to bind to nucleic acids has the potential to disrupt any number of biochemical processes involved in transcription or translation. Through disruption of transcription or translation, the synthesis of dependent proteins can be artificially controlled. To this end we seek to design ligands capable of binding nucleic acids at affinities high enough to disrupt biological processes. Furthermore, these ligands can exploit the secondary structure of nucleic acids (preferential binding to a specific nucleic
acid) as well as the primary structure (specific binding to preserved sequences within nucleic acids).

*Primary Structure:* Nucleic acids provide an enticing synthetic target. Nucleic acids are comprised of repeating nucleotides, which contain three major components, **Figure 1.2** (30).

(i) Bases – Nucleic acids are planar, aromatic, heterocyclic compounds which fall into two classifications, purines and pyrimidines. Adenine and guanine populate the

<table>
<thead>
<tr>
<th>Base</th>
<th>Nucleoside</th>
<th>Deoxyribonucleoside</th>
<th>Ribonucleotide</th>
<th>Deoxyribonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>adenine</td>
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<td>2’-deoxyadenosine</td>
<td>adenosine 5’-phosphate</td>
<td>2’-deoxyadenosine 5’-phosphate</td>
</tr>
<tr>
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<td>guanosine</td>
<td>2’-deoxyguanosine</td>
<td>guanosine 5’-phosphate</td>
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<td>cytidine</td>
<td>2’-deoxycytidine</td>
<td>cytidine 5’-phosphate</td>
<td>2’-deoxycytidine 5’-phosphate</td>
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<tr>
<td>thymine</td>
<td>---------------</td>
<td>thymidine</td>
<td>---------------</td>
<td>thymidine 5’-phosphate</td>
</tr>
<tr>
<td>uracil</td>
<td>uridine</td>
<td>---------------</td>
<td>uridine 5’-phosphate</td>
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</tbody>
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**Figure 1.2.** Structural elements and naming of bases, nucleosides and nucleotides, adapted from Saenger (30).
purine class of bases while the pyrimidine class of bases consists of cytosine, thymine and uracil. DNA and RNA contain four bases, three of which, adenine, guanine and cytosine, are used in both DNA and RNA. Thymine is exclusive to DNA while uracil is exclusive to RNA. The bases are connected to the sugar at the N-9 position of purine bases and the N-1 position of pyrimidine bases.

(ii) Sugar – The sugar acts as a bridge between the DNA bases and the phosphate backbone. The ribose sugar is a furanose bound to the bases at the anomeric carbon through β-glycosidic linkages. A D-ribose or a 2-deoxy-D-ribose sugar bound to a base constitutes a nucleoside of RNA or DNA, respectively.

(iii) Phosphate Backbone – Phosphate groups join two adjacent nucleosides through an ester linkage to phosphoric acid at the 5’- or 3’- hydroxyl group. A nucleotide is a nucleoside that is phosphorylated at one of the free sugar hydroxyls. The sequence of nucleotides defines the primary structure of the nucleic acid. Furthermore, nucleoside and nucleotide function does not end with nucleic acids. Adenosine-5’-triphosphate (ATP) is a nucleotide known as the ‘energy currency in cells’ (31). Cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (GMP) (32) act as cellular process regulators (33).

Secondary Structure: Assembling the nucleotides in a sequential fashion gives rise to nucleic acid secondary structure. The assembly of two nucleotide subunits is a dinucleotide while three nucleotide subunits is a trinucleotide. In general, twenty or fewer nucleotides constitute an oligonucleotide and the term polynucleotide is used to describe a nucleic acid composed of many subunits. As nucleotide subunits are
assembled in a linear fashion the polar nature of the ribose-phosphate backbone give rise to the helical scaffold with pyrimidine and purine bases facing inward near 90° to the helical axis (30). When paired through Watson-Crick base pairing, two helical strands of nucleic acids form a double helix. The center of the helix is a spiraling cantilevered staircase of planar aromatic bases. β-glycosidic linkages between base and sugar are asymmetrical; the resulting double helix has two characteristic grooves, a major and a minor groove.

Nucleic acid secondary structure is not limited to a double helix. Nucleic acids can exist as triple helixes (34, 35, 36, 37) (association of a third strand to an existing duplex), tetraplex (38) (formed by a quartet of guanine bases), bulges (39), loops (40, 41) and stems (42) (generally found in RNA), to highlight a few. While these structures receive mention within the manuscript the focus remains on duplex nucleic acid structures.

The double helix is a dynamic molecule and exists in multiple conformations. β-glycosidic linkages are not the only contributors to overall double helix geometry. Conformational changes can be driven by changes in the duplex hydration state (43, 44), the presence of a hydroxyl group at the 2-position of the ribose sugar and base sequence. These conformational differences provide a diverse catalog of structures suitable for targeting by small binding ligands.

B-form conformation is highlighted by conformational features such as \( C_{2-\text{endo}} \) ribose sugar pucker, an axial rise between base pairs of 0.33 nm, the helical axis is not displaced and 16 bases constitute a helical turn, Figure 1.3. These features give rise to a
helix with a major groove, which is shallower and wider than the A-form counterpart, while the minor groove is deeper and narrower than the A-form counterpart. A-form nucleic acids have characteristic major (deeper and narrower) and minor (shallower and wider) grooves. Several conformational features are noteworthy: the sugar pucker is \( C_{3\text{-endo}} \), the rise between base pairs is smaller at 0.23 nm, there is a negative displacement of the helical axis and 18 base pairs constitute a helical turn.

**Figure 1.3.** Schematic of structural differences between A-form and B-form nucleic acids.
**Targeting Nucleic Acids.** Ligands designed to target duplex nucleic acids fall into two categories based on their mode of interaction. These include intercalators that insert themselves between adjacent base pairs through $\pi-\pi$ stacking interactions and ligands that bind in the grooves of the nucleic acid duplex.

*Intercalation:* Intercalation was the first proposed mechanism of ligand-nucleic acid interaction (45). The compounds in this class are planar and comprised of fused aromatic six-membered rings. The planarity of these ligands allows them to insert themselves between adjacent base pairs of a nucleic acid. Classic intercalators are highlighted by ethidium bromide (46, 47, 48), thiazole orange (49, 50), acridine orange (45, 51), actinomycin (52, 53, 54), quinacrine (55), proflavine (56, 57), coralyne chloride (58) and daunomycin (59). This mode of targeting nucleic acids does have drawbacks, Figure 1.4.

![Chemical structures of representative intercalators.](image)

**Figure 1.4.** Chemical structures of representative intercalators.
Generally these ligands do not display sequence specificity, although a slight preference for 5’-purine, 3’-pyrimidine sequences has been reported (60). However, reports suggest intercalation favors certain conformations of nucleic acids (61). Furthermore, a necessary product of intercalation is that adjacent base pairs within the duplex are forced apart and a concomitant elongation of the duplex follows.

Improvements in intercalator binding affinity have been achieved through the synthesis of intercalator-intercalator dimers. Dervan reported the synthesis of a methidium dimer which bound calf thymus DNA with $K_a$ values of $10^9$ M$^{-1}$; which is higher than the $10^5$ M$^{-1}$ value observed with the monomer (62). Utilizing an intercalator to increase binding affinity by conjugating the intercalating moiety to a major groove binding moiety has also been reported. Our group reported conjugation of a major groove binding ligand, neomycin, to a number of different intercalators (63, 64, 65). Not only was an increase in binding affinity observed, these conjugates also showed an ability to specifically target different conformations of nucleic acids as well.

Finally, a biological precedent for this method of targeting nucleic acids can be established. Topoisomerases are a class of enzymes responsible for the cutting, removal and eventual reattachment of small oligomers of DNA during DNA replication. Doxorubicin, trade name Adramycin, an intercalator, has shown the ability to halt cellular replication by preventing the reattachment of the oligomers, in turn arresting the replication process (66). Especially useful where controlling DNA replication is desired, intercalators find use in cancer chemotherapy (66, 67, 68).
The second category of ligand duplex nucleic acid interactions involves molecules which bind either the major groove or the minor groove. As previously mentioned, asymmetric $\beta$-glycosidic linkages between base and sugar give rise to the grooves found in nucleic acid duplexes. While the characteristics of these grooves can vary significantly, the grooves remain quite distinct from each other and pose unique environments for targeting by small ligands.

Watson-Crick base pair stacking presents the potential for four total base pair combinations about the helical axis, Figure 1.5. The base pairs present different functionalities to each of the grooves which are unique to each base pair. For instance, an A/T base pair presents the following: a H-bond acceptor, H-bond donor, H-bond acceptor and a methyl group to the face of the major groove. This motif is made available by the nitrogen at the 7-position and the amine at the 6-position of the purine followed by the carbonyl at the 4-position and the methyl at the 5-position of the pyrimidine, respectively. At the same time, this A/T base pair presents a palindrome motif to the minor groove face involving a H-bond acceptor, a hydrogen and a H-bond acceptor (N-3, C-2 of the purine, O-2 of the pyrimidine). Therefore, the combination of base pairs affords the major groove with four unique surfaces for approaching ligands, while the minor groove contains two unique surfaces.

Minor Groove Binding: Minor groove binding ligands are comprised of heterocyclic rings which form a crescent shaped molecule, Figure 1.6. The deeper, narrower, minor groove dictates that these molecules are relatively flat and occupy a number of base pairs. Minor groove binding ligands with optimal geometries have also
Figure 1.5. A schematic depicting DNA base pairs and the functional groups accessible through the major and minor grooves of nucleic acid duplexes, adapted from Alberts (69).
been shown to bind the minor groove with a 2:1 binding stoichiometry. The incoming second molecule occupies the same binding site with an opposite (head-to-tail) orientation with respect to the first molecule (70, 71, 72, 73). Since these molecules tend to be relatively flat, minor groove binding ligands possess a secondary intercalative binding mode as observed with 4',6-diamidino-2-phenylindol (DAPI) (74).

Figure 1.6. Chemical structures of representative minor groove binders.

Targeting the minor groove of nucleic acids has seen significant success in recent years. Minor groove binding ligands have the ability to bind DNA at high affinities, while also binding at high specificity (75, 76, 77, 78, 79, 80). Naturally occurring polyamides netropsin and distamycin A (70, 81, 82), Hoechst 33258 (83, 84, 85), Berenil (86) and DAPI (87, 88) are all examples of minor groove binding ligands. Prior to the synthesis of novel polyamides by Peter Dervan, minor groove binding ligands such as distamycin A, netropsin and Hoechst 33258, displayed preference for AT-rich DNA.
Affinities for these ligands to their target sequences were in the range of micromolar to nanomolar: netropsin \( (K_a = \sim 10^6 \text{ M}^{-1} \) to \( \sim 10^7 \text{ M}^{-1} \) \) (81), distamycin A \( (K_a = \sim 10^6 \text{ M}^{-1} \) \) (81) and Hoechst 33258 \( (K_d = 10^{-9} \text{ M}^{-1} \) \) (85).

Dervan utilized \( N \)-methylimidazole and 2-hydroxy-\( N \)-methylpyrrole to successfully bind a number of different sequences of DNA (89). Furthermore, covalent linkage of two polyamides in a head-to-tail fashion allowed Dervan to control the binding stoichiometry and increase the binding affinity of these ligands to DNA (90, 91), as discussed in chapter 4. To date, sequences as long as 16 base pairs have been bound by polyamides at very high affinities (78).

Minor groove binding ligands have found success as therapeutic agents in a number of different applications, due to their ability to bind sequence specifically (92) to the surface of the minor groove. The therapeutic potential of minor groove binding ligands have been examined for their use in cancer therapies (92, 93, 94, 95, 96, 97), and as antibacterial (98, 99, 100, 101), antiprotozal (102, 103) or antifungal agents (102, 104, 105). These ligands succeed by targeting DNA and the sequences responsible for gene expression, which in turn inhibits synthesis of the target protein.

For example, a distamycin A analog was designed to target the sequence 5’-AATTAATCAT-3’, the promoter site of the bacteria \( C. \ albicans \) with nanomolar \( K_d \) values (106). The basic leucine zipper (bZip) protein binding site, 5’-ATATAGGGTA-3’, was targeted by a novel hairpin polyamide acridine conjugate with \( K_a = 1.8 \times 10^{10} \text{ M}^{-1} \) (107). In an effort to develop small ligand transcription activators, a number of hairpin polyamide – protein conjugates were developed as
transcription factor mimics. These conjugates combined a sequence selective, 5’ – TGACCAT – 3’, component (polyamide) and protein binding component (108). The wrenchnolol component binds the Sur-2 protein, a subunit of human mediator complexes that links transcription activators to RNA polymerase II in human cells (108).

Hairpin polyamides have been shown to target the HIV-1 transcription factor IID at nanomolar affinities (106, 109, 110, 111) as well as the HIV long terminal repeat (112). The NF-κB Transcription factor, 5’-GGGACTTTCC-3’, was targeted using hairpin polyamides, achieving excellent binding affinities (113). Hairpin polyamides have successfully targeted the inverted CCAAT boxes (ICBs) of topoisomerase IIα promoter (114). Furthermore, a number of promoter sites (TATA, NRE, P53, ZIF268) were successfully targeted at nanomolar binding affinities with hairpin polyamides (115). Inhibition of protein binding to sequences preserved in the Human Estrogen-Related Receptor 2 and Estrogen Receptor α have been achieved through the binding of hairpin polyamides to DNA. Specifically, targeting the sequences 5’ – TGATCA – 3’, 5’ – AGTGAT – 3’ and 5’ – AGGTCA – 3’ with hairpin polyamides can decrease protein binding by 10 to 100 fold (116).

**Major Groove Binding:** The potential for four distinguishable surfaces per base pair greatly diversifies the surface of the major groove. The unique, sequence dependent surface of the major groove is a major contributing factor in protein – nucleic acid interactions. However, targeting the major groove of duplex nucleic acids requires ligands with surfaces of intricately distributed functional groups that complement the surface of the major groove. Furthermore, while intercalators and minor groove binding
ligands distribute their charges over a planar surface, a ligand which binds the major groove must provide a larger three-dimensional scaffold for these functional groups. This is a necessary requirement to bind the larger groove dimensions.

Due to these requirements there are very few ligands that bind the major groove of duplex nucleic acids, as shown in Figure 1.7. Synthetically functionalized ligands with metallic centers have been developed to meet these requirements and bind the major groove of DNA (117, 118). Protein mimicking ligands, based on a scaffold of amino acids, have also been synthesized to bind the major groove of DNA (119). Carbohydrates that bind in the major groove also receive attention. Carbohydrates are a relatively small class of molecules that act as DNA-cleavage agents, neocarzinostatin (120), as well as DNA alkylating agents, altromycin B (121). Carbohydrates are also employed as the major groove binding component of a dual recognition molecule, as reported for nocalamycin, respinomycin, NB-506 (intercalator-major groove binders) and neomycin-Hoechst 33258 (major-minor groove binder) (63). The binding of proteins to nucleic acid duplexes predominantly occur through the major groove. Inhibition of protein binding, through occupation of the major groove with ligands, offers a potential therapeutic approach to blocking transcription (122, 123).
Figure 1.7. Chemical structures of representative major groove binders.

The requirements necessary to bind the minor groove of duplex nucleic acids are well understood; the ability to arrest natural cellular functions by occupying the major groove remains less understood and may suffice to explain the low population of this class of ligands. Aminoglycosides represent a small subset of the class of molecules that bind the major groove of nucleic acids.

Our group has shown aminoglycoside antibiotics bind non-traditional nucleic acid targets. In fact, our group has spent considerable time understanding aminoglycoside–nucleic acid interactions. We have established neomycin, an aminoglocoside antibiotic, as an excellent molecule to probe small molecule–nucleic acid interactions.
Aminoglycosides – A Closer Examination (124). Aminoglycoside antibiotics are carbohydrates comprised of a scaffold of amino sugars joined to a central hexose ring, Figure 1.8. The aminoglycoside, streptomycin, was isolated from soil bacteria samples by Selman Waksman in 1944 (125). Fame followed when streptomycin became the first antibiotic effective against tuberculosis. Through the remaining years of the 1940s, a number of streptamine relatives were discovered giving rise to the streptomycin family. Second generation aminoglycosides followed; derived from 2-deoxystreptamine (DOS), this family of aminoglycosides includes neomycin (1949), neamine (1951), kanamycin A (1957), kanamycin B (1958) and paromomycin (1959), among others. While the number of aminoglycoside antibiotics class increased with each passing year, the mechanism of action remained a mystery. It wasn’t until the late 1950s that protein synthesis was identified as the primary target for streptomycin’s antibacterial action (102, 112, 116, 121, 124, 126, 127, 128). Spotts, in 1961 (129), proposed the ribosome as the likely target for streptomycin. In a whirlwind of research, work by Davies and others established the 30S subunit of ribosomal RNA (rRNA) as the natural target of streptomycin (127, 128).

Experiments designed to investigate the fidelity preserved during translation led to the understanding that aminoglycosides induce errors during translation. Discovery of an increasing number of aminoglycosides, highlighted by the discovery of the gentamycin class, propelled the field through the next few decades. As the years progressed, scientific interest in aminoglycosides slowly dwindled and it wasn’t until the
late 1990s that aminoglycosides saw renewed interest. Advancements in nucleic acid synthesis and combinatorial chemistry as well as a need to improve existing drugs and expand potential targets fueled the resurgence of interest in aminoglycosides.

The resurgence of interest in aminoglycoside research was amazingly one-sided. The rebirth saw a large increase in the number of aminoglycoside targets.

**Figure 1.8.** Structures of aminoglycosides with a 2-deoxystreptamine ring.
Aminoglycoside targets expanded to include the 5′-untranslated region of thymidylate synthase mRNA (130), HIV RNA Rev response (131), catalytic RNA molecules (group I introns (132), ribonuclease P RNA (133), hairpin ribozyme (134), hammerhead ribozyme (135) and the hepatitis delta virus ribozyme (136). In fact, aminoglycosides bound to HIV RNA have been shown to inhibit the binding of viral proteins to their targets, thus preventing native DNA degradation (131). Our group suggested the ability of aminoglycosides to bind to the multitude of newly discovered targets was driven by the ability to bind A-form nucleic acids, a structural motif shared by all RNA targets (124, 132, 137).

As previously discussed, RNA structures are not the only nucleic acids known to have a propensity for the A-form conformation. Based on observations made by Robinson and Wang, in 1966 (138), that suggested the binding of neomycin to DNA induced changes in the DNA generally associated with an A-form conformation; our group demonstrated the ability of neomycin to bind a number of non-traditional A-form nucleic acids. Since these discoveries, the list of A-form nucleic acids shown to bind neomycin has expanded to include the RNA triplex (139), DNA:RNA hybrid duplex (140, 141), RNA duplex (132), DNA triplex (142), A-form DNA duplex and the DNA tetraplex (143).

Since our work in the mid to late 1990’s, our group has utilized neomycin to probe the binding of a number of non-traditional nucleic acids structures. Furthermore we achieved an increased binding affinity to these nucleic acids through conjugation of neomycin to intercalators (pyrene (65), BQQ (64), ethidium bromide (140, 141)) as well
as groove binders such as Hoechst 33258 (144, 145). To this date, we continue to explore neomycin based approaches to nucleic acid binding.

Chapter two of this dissertation focuses on binding DNA:RNA hybrids, reported by our group (124, 140, 141). To this end, we report the design synthesis along with biophysical and bioanalytical studies of novel neomycin-methidium chloride conjugates. Attention will then turn to our neomycin based approach to targeting the DNA duplex.

Finally, our group had previously demonstrated the ability to bind the B-form DNA duplex through novel neomycin – Hoechst 33258 conjugates (144, 145). Expanding on this approach we report the development, synthesis and subsequent spectroscopic studies of neomycin – polyamide conjugates. Chapter three focuses on tetramer polyamide – neomycin conjugates while chapter four focuses on hairpin polyamide – neomycin conjugate, designed to target GC-rich DNA.
REFERENCES


CHAPTER II

TARGETING DNA:RNA HYBRIDS

INTRODUCTION

Six years after the report on the double-helical structure of DNA by Watson and Crick (1), the DNA:RNA hybrid was proposed to address the interaction between DNA and RNA (2). The first synthetic DNA:RNA hybrid structure was formed in 1960 (3). A year later, the first DNA:RNA hybrid helix was formed (4, 5). X-ray fiber diffraction studies (6) confirmed the conformation of the DNA:RNA hybrid was different than B-form DNA. The crystal structure of a DNA:RNA hybrid was solved in 1982 (7), while solution based conformational studies followed shortly (8). Surprisingly, a survey of articles, from the conceptual inception of the DNA:RNA hybrid to present, suggest the global helical structure of DNA:RNA hybrids has only had scholastic consent since the mid-1990’s.

In 1959, Rich published a foundational paper in which DNA:RNA hybrids were first reported (2). He proposed DNA:RNA hybrid formation occurred through the hybridization of a single-stranded DNA template strand and a newly formed RNA strand. Experimental data would support this hypothesis in 1960 (9). Temin (10) and Baltimore (11), independently, and simultaneously, reported the use of reverse transcriptase in the synthesis of a new DNA strand via a RNA template strand. This process would become

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1 As discussed in our paper (120)
known as reverse transcription affording the second example of DNA:RNA hybrid formation. In 1975, the first example of DNA:RNA hybrid formation in the replication of DNA was discovered (12).

An extensive survey of DNA:RNA hybrid stability while varying (i) the percentage of (A)<sub>n</sub>:(T or U)<sub>n</sub> content, (ii) oligomeric length and (iii) the percentage of d(Py) content in each of the strands, offers the most complete analysis of hybrid stability (31). In general: (i) an inverse relationship is displayed between the percentage of (A)<sub>n</sub>:(T or U)<sub>n</sub> content in the hybrid duplex and the thermal stability. (ii) Hybrid duplexes of equal d(Py) and d(Pu) content are thermally similar when comparing the d(Pu):r(Py) and d(Py):r(Pu) forms. (iii) Decreasing oligomeric length decreases the thermal stability. (iv) Isolation of (A)<sub>n</sub>:(T or U)<sub>n</sub> content to continuous tracts results in a lower thermally stable duplex than when the same percentage of (A)<sub>n</sub>:(T or U)<sub>n</sub> content is dispersed throughout the sequence. (v) Continuous tract (A)<sub>n</sub>:(T or U)<sub>n</sub> content results in d(Py):r(Pu) thermal stability lower than the corresponding d(Pu):r(Py) form, at physiological conditions, as expected.

It has also been suggested that DNA:RNA hybrid stability is related to the ability of the hybrid duplex to associate with a third single strand (32, 33). The d(Py):r(Pu) hybrid does not associate with a third strand of DNA, while d(Pu):r(Py) hybrids easily accommodate a third strand to form d(Pu):r(Py):d(Pu) (33). The inability to associate a third strand represents an inability to overcome a conformational energy barrier, a direct result of a more stable hybrid duplex form (32). Furthermore, with the exception of long
stretches of \((A)_n(T \text{ or } U)_n\), the stability of duplexes can also be attributed to the relative stability of the ribose chain.

The first DNA:RNA hybrid structure was solved in 1967 using X-ray fiber diffraction (6). The DNA:RNA hybrid resisted conformational changes upon relative humidity changes, a technique commonly used to drive the A-form to B-form transition in duplex DNA. This observation, coupled with solution studies of DNA:RNA hybrids (34, 35), spurred a 20 year debate on the conformation and structure of DNA:RNA hybrids.

Polymeric poly(rA):poly(dT) is a polymorphic structure. Parallel circular dichroism (CD) (36) and Raman (37) experiments refutes a complete B-form model for the DNA:RNA hybrid, evidence that poly(rA):poly(dT) is polymorphic, the conformation dependent on a number of factors. Further analysis, \(^{13}\)P solid state NMR, suggests poly(rA):poly(dT) is capable of existing in an A-like conformation at relative low humidity (38) while increasing the solvation from 87% to 92% yields a B-like conformation (39). 1D nuclear Overhauser effect (NOE), observed by NMR and empirical evidence model building, suggests an in situ model for poly(rA):poly(dT) based on B-form DNA with some exceptions (40). Currently, it is generally accepted that the conformation of poly(rA):poly(dT) is polymorphic, capable of undergoing an A- to B-type transition upon relative humidity changes. Fully hydrated this hybrid exists as a A-type duplex closer in overall conformation to B-form DNA (41, 42).

Poly(dA):poly(rU) was extensively studied using X-ray diffraction methods (43). The model proposed by Arnott and co-workers suggested a heteromorous structure for the
hybrid. This term was used to describe the overall conformation of the duplex in which one strand, the d(Pu) strand, displays B-form characteristics while the other strand, the r(Py) strand, displays A-form characteristics. The global conformation of poly(dA):poly(rU) is strongly driven by the r(Py) strand and the global conformation of the hybrid is an A-type duplex closer in conformation to the A-form of RNA (32).

Congruently, the analogous DNA:RNA hybrid, poly(dI):poly(rC) displayed similar heteromerous conformation (32, 44). However, the r(Py) has a diminutive effect on the overall conformation of the poly(rI):poly(dC) duplex, when compared to poly(dA):poly(rU), resulting in an A-type duplex closer to B-form DNA than poly(dA):poly(rU) (43). Surprisingly, the d(Pu) strands of both d(Pu):r(Py) type hybrids are conformationally similar to one another (43). Therefore differences in conformation between poly(dI):poly(rC) and poly(dA):poly(rU) are a result of the differences in conformation of the r(Py) strands.

Mixed base polymeric DNA:RNA hybrids poly(dAC):poly(rGU) and poly(dGU):poly(rAC) have also been studied (36). CD studies suggest the hybrids, poly(dAC):poly(rGU) and poly(dGU):poly(rAC), are more similar to the analogous A-form RNA duplex. Furthermore, conformational differences between the individual heteroduplexes mirror differences observed in the polymeric analogs poly(dA):poly(rU) and poly(rA):poly(dT). These differences arise from the ability of r(AC) to induce an RNA-like conformation. Therefore, poly(dGU):poly(rAC) is more A-like than poly(dAC):poly(rGU). These duplexes are susceptible to dehydration which drives the
conformation of the heteroduplexes to a complete A-form conformation, further evidence that these duplexes are A-like but retain B-form characteristics.

Refinement of the secondary structure of poly(rA):poly(dT) suggests the sugar puckers, regardless of strand, were a slight variant of the C$_2$-endo conformation (38, 40). However, in order to facilitate the 2′-hydroxyl, a displacement of the RNA bases from the helical axis is observed. This displacement results in a concomitant change in the tilt and twist of these bases as well as a change in the backbone torsion angle. However, it will be shown later that there exists a high energy barrier for which the ribose sugar of RNA must pass in order to convert to C$_2$-endo, a barrier which is significantly lower to pass with the ribose of DNA (45). In fact, the existence of C$_2$-endo sugar pucker for the ribose strand in poly(rA):poly(dT) is a result of hydration and results in helical parameters (pitch, axial rise per residue and residues per turn) that mimic B-form. Subsequent dehydration converts the global ribose sugar pucker to C$_3$-endo and results in parameters closer in agreement to A-form (39). Furthermore, the analogous poly(rI):poly(dC) crystallized with helical parameters in closer agreement to A-form (44), Table 1.1.

Unlike the case of d(Py):r(Pu), in which global conformational changes can be accommodated in both strands of the duplex, d(Pu):r(Py) type polymeric duplexes are not as forgiving. Dominated by the conformational stubbornness of the r(Py) strand to remain in an A-form state with C$_3$-endo sugar pucker, global helical parameters tend to be more A-form in characteristic, whereas B-form characteristics come from the d(Pu) strand (43, 46, 47). The previously described heteromerous nature of poly(dA):poly(rU)
and poly(dI):poly(dC) arises from the overwhelming tendencies for the ribose sugars to adopt a C₃-endo sugar pucker whereas the deoxyribose sugar is found in a C₂-endo conformation. The result, these sugar puckers contribute to a DNA:RNA hybrid minor and major groove which is globally closer to A-form. Furthermore, poly(dA):poly(rU) is closer in conformation to A-form than poly(dI):poly(rC). Further analysis of helical structure reveals poly(dA):poly(rU) is less propeller twisted than poly(dI):poly(rC) ( -4° and -14°, respectively) resulting in a less rigid hybrid duplex.

DNA:RNA hybrids have been shown to be involved in transcription (13). It has been suggested that termination of the transcription process concludes with the release of the RNA strand from an unstable DNA:RNA hybrid (14), furthermore, the lability some hybrid sequences may aid the disassociation of the RNA strand from the DNA template (15, 16). DNA:RNA hybrids are formed during reverse transcription. Reverse transcription relies on the dual activity of reverse transcriptase (RT) (17, 18): (i) RT acts as a polymerase, forming DNA:RNA hybrid intermediates and (ii) as a ribonuclease H (RNase H) active enzyme, specifically degrading the RNA portions of DNA:RNA hybrid duplex domains.

The discussion of DNA replication is seemingly unbefitting when addressing DNA:RNA hybrid duplexes, however DNA:RNA hybrids play an important role in DNA replication. Okazaki fragments (19) are bridged when DNA primase synthesizes a short RNA primer of approximately ten nucleotides in length on template DNA strands. Finally DNA:RNA hybrids are also present in mitochondrial DNA. Human mitochondrial DNA (mtDNA), is present in the mitochondria of the cell; the mechanism
of replication is similar to that of nuclear DNA, however, the priming of mtDNA for DNA replication requires the use of RNA primers (20). DNA:RNA hybrids are prerequisites to the initiation of transcription and serve as primers for DNA replication of the leading strand (13, 21, 22).

There is no question that DNA:RNA hybrids are biologically significant. Even though reports have found DNA:RNA hybrids of therapeutic interest to be prevalent [reports of the persistent existence of DNA:RNA hybrids in human cytomegalovirus have been made (23)], extensive studies of DNA:RNA hybrids as potential targets for therapeutic approaches remain with the following notable exceptions.

The reverse transcription of HIV-1 has required substantial attention (24, 25). HIV-1 contains two identical polypurine tracts (PPTs). Reverse transcription (minus strand DNA synthesis) of both PPTs occurs after DNA strand transfer by reverse transcriptase, providing a unique r(Pu):d(Py) type of DNA:RNA hybrid (18). This unique PPT DNA:RNA hybrid exists until cleavage of both the RNA template strand (5’ to the PPT) and tRNA, separate events conducted by reverse transcriptase, occurs.

Telomerase activity, seemingly synonymous with G-quadruplex formation, is a well understood process, of which DNA:RNA hybrid targeting is an equally viable approach for telomerase inhibition. Telomerase activity is associated with cellular immortality (26) and its potential as a universal cancer target has been proposed (27, 28). Telomeres exist as long repeats of a simple six base sequence, d(TTAGGG)n, in all vertebrates. This non-coding extension of chromosomal DNA becomes shortened with each cellular division (29). The 3’ tail of the parent DNA telomere is longer than its
complement and forms a DNA:RNA hybrid upon binding to the active site of telomerase. Binding of small molecules to the DNA:RNA hybrid can potentially offer a therapeutic method for controlling telomerase activity, preventing telomere extension, by distorting the substrate/enzyme interaction, or preventing disassociation of the enzyme from the substrate (30).

Although polymorphic in structure, DNA:RNA hybrids offer a unique structure to target and potentially inhibit biological functions. However, a survey of literature reveals the existence of less than ten ligands which specifically bind DNA:RNA hybrids.

Actinomycin D (AMD) has demonstrated the ability to inhibit reverse transcriptase activity (48, 49) through the inhibition of double stranded DNA synthesis (49). A number of derivatives were synthesized (50), however, the preference of AMD for DNA duplexes dominates. Association constants for these conjugates to the DNA:RNA hybrids were in the $10^4$ M$^{-1}$ to $10^5$ M$^{-1}$. Surprisingly, both conjugates of AMD display a unique selectivity for leukemia cells, inhibiting cellular growth by 50% at less than 1.0 nM! Although the binding mode ultimately leading to retardation of leukemia cell growth is not known, the authors suggest the binding of these conjugates to DNA:RNA hybrids may perpetuate further development (51).

Naturally occurring polyamides, distamycin and netropsin, have been investigated for their ability to bind DNA:RNA hybrid Okazaki fragments (48, 49, 52, 53, 54). These lexitropsins are known to bind A/T stretches of DNA, displacing the spine of hydration, in the minor groove (55). A series of conformationally restrained bis-distamycin compounds, dimerized through the $ortho/para$ or meta positions of benzene and pyridine
(2,5 - and 2,4 – type, respectively), were synthesized (56). Ortho/para bis-distamycins were capable of binding both DNA duplexes as well as the Okazaki fragment at similar binding constants, \( \sim 10^8 \text{ M}^{-1} \) and \( \sim 10^7 \text{ M}^{-1} \) range, respectively. Surprisingly, mono-distamycins, linked to the benzene moiety, showed no binding to the duplexes. The meta pyridyl bis-distamycins were also studied, however, affinity for the Okazaki duplex is in the \( \sim 10^6 \text{ M}^{-1} \) range. In an effort to design a synthetic approach to DNA:RNA hybrid targeting, clear candidates, at this point, have not been found. The subsequent sections will make a case for both neomycin and ethidium bromide as moieties in which a scaffold for DNA:RNA hybrid targeting can be made.

Aminoglycosides have been recognized as A-form nucleic acid major groove binders (57, 58, 59, 60). Paramomycin, an aminoglycoside, and its complexation with DNA:RNA hybrids have been studied by Barbieri and co-workers (53). Thermal stabilization of a mixed base RNA 8-mer duplex, afforded upon paramomycin complexation, was 6.3 °C, not surprising since aminoglycosides traditionally bind RNA. However, thermal stabilization of the DNA:RNA hybrid analog was on par with the RNA duplex at 6.2 °C. Furthermore, the binding of paramomycin to the hybrid duplex was shown to induce a shift in the hybrid duplex to a more A-form like conformation. Unfortunately, the affinity of paramomycin for the RNA duplex was shown to be higher than the corresponding DNA:RNA hybrid. However, the authors suggested the binding of paramomycin to the DNA:RNA hybrid was capable of inhibiting, both, RNase H- and RNase A-mediated cleavage of the RNA strand; a strong argument for an aminoglycoside approach to targeting DNA:RNA hybrids.
Work with aminoglycosides and DNA:RNA hybrids was expanded to include aminoglycosides neomycin and ribistamycin using DNA:RNA hybrid chimeras, by Pilch and coworkers (18). These chimeras are designed as constructs of the polypurine tract (PPT) of HIV-1, found at two unique steps in the reverse transcription of HIV-1. Containing the same DNA:RNA hybrid portion, these chimeras differ in the composition, RNA (18C:18R) or DNA (18C:18D), of the complement strand. Confirming previously published results, RNase H cleavage of the DNA:RNA hybrid chimeras was inhibited by the presence of the aminoglycosides and a number of small molecule non-nucleoside reverse transcriptase inhibitors (NNRTIs) (61). In fact, of the aminoglycosides surveyed, neomycin was far superior at inhibiting RNase H cleavage, in both chimeras! Furthermore, thermal stabilization of the chimeras, afforded by the presence of aminoglycosides, reveals neomycin increases the stability of both chimeras to a larger extent than either paramomycin or ribostamycin. Isothermal titration calorimetry derived binding constants for the aminoglycosides binding the two chimeras. Neomycin displayed the highest affinity for the chimera 18C:18R at $K_a = 1.1 \times 10^6 \text{ M}^{-1}$ (18C:18D at $K_a = 5.3 \times 10^5 \text{ M}^{-1}$). In both chimeras, the association constant for neomycin was greater than the other aminoglycosides studied. Furthermore, it is clear that the addition of ring four to the 2-deoxy streptose moiety is paramount to high affinity binding of aminoglycosides to DNA:RNA hybrid structures.

Further application of neomycin based HIV-1 therapeutics has been reported by Turner and co-workers (62). Surveying a number of classical nucleic acid binding ligands, the authors identify the ability of neomycin to bind the U3 region of PPT. The
primary binding site for neomycin begins at the U3 position and extends seven base pairs into the RNase H primer grip region. The DNA:RNA hybrid binding site is primarily composed of G/C base pairs with the terminal base pair in the binding site being A/T. The binding affinity, determined by direct ITC titration, was calculated at $1.45 \times 10^7 \text{ M}^{-1}$ at 80 mM NaCl. Furthermore, a secondary binding site, located three base pairs downstream from the U3 binding site and denoted $\text{(rA)}_4: \text{(dT)}_4$ was identified. This complementary binding to the high affinity site is lower in affinity at $2.76 \times 10^5 \text{ M}^{-1}$ and only appears at lower ionic strength. The authors further suggest the A-form nature of the DNA:RNA hybrid is not the primary factor in neomycin binding, rather the binding of neomycin is complex and also driven by favorable electrostatic surfaces. Not only does this work continue to identify ligands capable of targeting DNA:RNA hybrids, it further substantiates aminoglycoside based approaches to DNA:RNA hybrid recognition and therapeutics.

Ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenathridinium bromide) belongs to a unique class of small molecules which bind double-stranded nucleic acid structures via an intercalative mechanism (63, 64). The ability of ethidium bromide to bind to nucleic acid structures and the unique fluorescent properties make ethidium bromide a laboratory standard for the staining of nucleic acids (65). The concert of ethidium bromide within biological systems has yielded potential anti-tumor (66, 67) and anti-viral (67, 68) properties. The potential of ethidium as a therapeutic agent is a double edge sword. The actual therapeutic potential of ethidium bromide remains unutilized due to its mutagenic and carcinogenic properties (69).
Ethidium bromide has been reported to interact with nucleic acid structures through a dual binding mode (65). The primary binding mode involves the interaction between the negatively charged backbone, consisting of phosphate oxygens, and the phenanthridium ring nitrogen, which is positively charged. This interaction is stabilized by the hydrophobic intercalative stacking interactions between ethidium bromide and the host double-stranded nucleic acid. A second binding mode of ethidium bromide can be observed at high ligand concentrations. In this mode, the high population of ethidium bromide molecules stack within the DNA grooves which is a direct result of the ionic interactions with the phosphate backbone (70).

In a technique developed by Muller and Crothers (71), Chaires, and co-workers identified ethidium bromide as a DNA:RNA hybrid, poly(rA):poly(dT), specific binding ligand (72). An ensuing report assayed 85 ligands against various nucleic acids and focused on binding to poly(rA):poly(dT). This assay confirmed the presence of five ligands: ellipticine, ethidium bromide, coralyne, propidium and TAS103, which uniquely recognized the poly(rA):poly(dT) structure; ethidium bromide showed the highest preference for poly(rA):poly(dT). Complete thermodynamic profiles for ellipticine, propidium and ethidium bromide binding to poly(rA):poly(dT) yield binding constants for the three ligands, all in the magnitude of $10^5 \text{ M}^{-1}$. This assay was expanded to simultaneously provide the thermal stabilization afforded by the ligand on the preferentially targeted nucleic acid structure, while in competition with other nucleic acid structures (73). Furthermore, experiments on complexes of poly(dA) and
poly(rU) suggest ethidium is capable of inducing a conversion of a 1:1 mixture of these homopolymers from a three stranded to a two stranded helix (74).

DNA:RNA hybrid targeting with ethidium bromide has been applied to targeting telomerase through its DNA:RNA hybrid duplex. Friedman and coworkers have surveyed a number of compounds in an attempt to inhibit telomerase activity through DNA:RNA hybrid binding (75). Surveying a number of intercalators, four ligands (ethidium bromide, rivanol, acridine orange and acridine yellow) were found to inhibit telomerase activity at IC$_{50}$ values in the low micromolar range. Furthermore, telomerase inhibition through the binding of these ligands to G-quadruplex structures, another substantiated method for achieving telomerase inhibition, was not observed (30). Affinity chromatography isolated the high affinity DNA:RNA binding species from a homogeneous mixture of binding ligands. It was found that ethidium bromide uniquely binds a DNA:RNA hybrid duplex, derived from the hybrid formed during the catalytic cycle of telomerase, with the highest affinity, even in the presence of other binding ligands (75). This work clearly validates ethidium bromide as another compound in the synthetic approach to targeting DNA:RNA hybrids.
EXPERIMENTAL

Materials:

**Nucleic Acids.** DNA polymers [poly(dA), lot no. GD0056; poly(dT), lot no. GC0226; poly(dC), lot no. FL0056; poly(dG);poly(dC), lot no. GB0176; poly(dA):poly(dT), lot no. 4117860021; poly(dG-dC):poly(dG-dC), lot no. 3017910011; poly(dA-dT):poly(dA-dT), lot no. 3107870011] and RNA polymers [poly(rA), lot no. 3104110011; poly(rU), lot no. GD0266; poly(rC), lot no. 8094220021] were purchased from GE Healthcare/Amersham Biosciences (Piscataway, NJ). Concentrations were determined by UV absorbance using $\varepsilon_{257} = 8600 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dA); $\varepsilon_{264} = 8520 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dT); $\varepsilon_{247} = 7400 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dC), $\varepsilon_{253} = 7400 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dG);poly(dC); $\varepsilon_{254} = 8400 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dA):poly(dT); $\varepsilon_{262} = 6600 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dG-dC):poly(dG-dC); $\varepsilon_{260} = 6000 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(dA-dT):poly(dA-dT); $\varepsilon_{258} = 9800 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(rA); $\varepsilon_{260} = 9350 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(rU) and $\varepsilon_{269} = 6200 \text{ (M}^{-1}\text{cm}^{-1})$ for poly(rC). Calf thymus DNA, lot no. GA0286, was purchased from GE Healthcare/Amersham Biosciences (Piscataway, NJ) and concentration were determined by UV absorbance using $\varepsilon_{263} = 13846 \text{ (M}^{-1}\text{cm}^{-1})$. 16S A-site rRNA (27nt) was purchased from Dharmaco Research, Inc. (Lafayette, CO). The 16S A-site rRNA (27nt) was deprotected and purity checked by HPLC using standard Dharmaco protocols. The 16S A-site RNA (27nt) was quantitated using extinction coefficients provided by the supplier. Preparation of the 16S A-site RNA was accomplished by heating to 95°C, holding for 5 minutes, followed by slow cooling to room temperature and storage at 4°C. For RNA duplex poly(rA)poly(rU), DNA:RNA hybrid duplexes poly(dA):poly(rU) and poly(rA):poly(dT), DNA triplex
poly(dA):2poly(dT), and DNA:RNA hybrid triplex poly(dA):2poly(rU), individual polymeric strands were mixed in stoichiometric ratios and formed by heating at 95 °C for 5 minutes slowly cooling to room to room temperature and storage at 4 °C between experiments. For all experiments with polymeric DNA and RNA, solutions of individual strands were dialyzed extensively (48 hrs.) against buffer using SpectraPor Float-A-Lyzer dialysis units, MW cutoff 3500 Da (Spectrum Labs, Rancho Dominguez, CA) before quantitation. Buffer conditions for all experiments are noted throughout.

**Chemicals.** Neomycin B (sulfate salt) was purchased from ICN Biomedicals and used without further purification. Sodium cacodylate, NaCl, ethidium bromide and sodium phosphate salts (Na₂HPO₄ and NaH₂PO₄) were purchased from Fisher Scientific. 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride was purchased from Sigma and used without further purification. Quantitation of ethidium bromide (ε₄₇₈ = 5680 M⁻¹cm⁻¹) and NM (ε₄₈₇ = 5811 M⁻¹cm⁻¹) in aqueous solutions were done using UV absorbance. All other reactions and solvents were purchased from Acros Organics and used without further purification. Reaction solvents were distilled over calcium hydride (pyridine, dichloromethane, DMF) or sodium metal (ethanol). Ethanol was further distilled over magnesium turnings. To ensure stability, all solutions containing ethidium bromide and NM were stored in polystyrene tubes wrapped in foil.

Methods:
**Competition Dialysis Experiments.** Competition dialysis experiments were conducted using DPA200 and a wide array of nucleic acid structures. In the assay, solutions of different nucleic acid structures (of identical concentration) were dialyzed simultaneously against a common solution of ligand using appropriately buffered conditions (58, 72, 81). The nucleic acids were allowed to equilibrate with ligand over a period of 72 hours. After equilibration, the nucleic acids were analyzed by fluorescence and the amount of bound ligand to each nucleic acid structure was determined. In these experiments, more ligand accumulated in the dialysis tube containing the structural form of highest binding affinity. Since all of the DNA samples were in equilibrium with the same free ligand concentration, the amount of ligand bound is directly proportional to the binding constant for each conformational form (58). The nucleic acid structures were chosen such that they covered a wide array of structures including quadruple helix structures – TGT tetraplex and AG$_3$T$_2$AG$_3$ tetraplex; triple helices – poly(dA):2poly(rU) and poly(dA):2poly(dT); double helices – poly(rA):poly(rU), poly(rA):poly(dT), poly(dA):poly(rU), poly(dA):poly(dT), poly(dG):poly(dC), calf thymus DNA, *Micrococcus lysodeikticus* DNA, poly(dA-dT)$_2$ and poly(dG-dC)$_2$; single stranded nucleic acids – poly(dA), poly(dG), poly(rC), poly(rU), poly(rA) poly(dC), poly(dA) and poly(dT) and the A-site RNA construct.

**Computer Modeling.** Computer modeling was used to ensure the linker length, separating the two moieties would be sufficient; however computer models were not used to optimize linker length. The DNA:RNA hybrid duplex comprised of oligomers
d(GAAGAGAAGC)and r(GCUUCUCUUC) was extracted from pdb entry 1DRR. Utilizing the backbone of the DNA:RNA hybrid, the individual bases were adjusted to d(TTTTTTTTTTT) and r(AAAAAAAAAAAAA). The adjusted DNA:RNA hybrid was subsequently minimized. Conformational optimization of neomycin, docked in the closest proximity to the hydroxyl position of 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride, was carried out prior to attachment to 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride using a Monte Carlo routine (AMBER* force field and water as solvent) in MacroModel. Five of the six amines in neomycin were protonated, in agreement with NMR studies of neomycin. Energy minimization reached a convergence threshold of 0.02 kJ/mol for all experiments.

**UV Thermal Denaturation Experiments.** All experiments were carried out using a Cary 100E UV/Vis spectrophotometer equipped with a thermoelectrically controlled 12-cell holder. All samples were analyzed in quartz cells (1 cm pathlength). Lamp stability and wavelength alignment were checked prior to each experiment. Unless otherwise noted, prior to analysis heating from 10-100 °C at a rate of 0.2 deg/min, followed by slow annealing from 95-10 °C at a rate of 0.2 deg/min prior to monitoring at 260, 264 and 280 nm performed. Nucleic acid samples were pre-formed in polystyrene tubes by heating at 95 °C for 5 minutes slowly cooling to room temperature, followed by incubation at 4 °C for 18 hours. Samples were prepared by mixing ligand and nucleic acid solutions, at appropriate ratios, followed by incubation at 4 °C for 4 hours and sample degassing. In all experiments using polymeric DNA:RNA hybrids, [nucleic acid structure] = 20 µM/base.
pair. Experiments using oligomeric DNA:RNA hybrids, [nucleic acid structure] = 20 µM/base pair. Melting temperature (Tm) assignments were aided using first derivative analysis provided by the Cary software.

**Fluorescence Titrations.** Equilibrium binding experiments were done using a Photon Technology International instrument (Lawrenceville, NJ) at ambient (22 °C) temperature. A solution of DNA:RNA hybrid (serially diluted to 1 µM) was prepared. Serial additions of ligand (EtBr or NM) were titrated into the solution followed by excitation at 525 nm (slit width = 4 nm) and resulting emission curves (from 550-700 nm) were recorded. After each addition, the solution was mixed by pipetting up and down with a micro pipette. Sample equilibrium was monitored by continually exciting/scanning the sample at different times, and was usually reached within 5 minutes. All data were normalized to account for the (small) dilution of sample upon addition of substrate.

**CD Spectropolarimetry Titrations.** Circular dichroism (CD) experiments were done at 20 °C using a Jasco J-810 spectropolarimeter. A concentrated solution of ligand was added to a solution of DNA:RNA hybrid and allowed to stir constantly before scanning from 300 - 210 nm. As with fluorescence experiments, equilibrium was determined by periodically scanning the sample over a period of time (up to 10 minutes) for the first few additions of ligand, and was reached within 5 minutes. All data were normalized to account for the (small) dilution of sample upon addition of ligand.
CD Thermal Denaturation Experiments. Circular dichroism (CD) experiments were done at 20 °C using a Jasco J-810 spectropolarimeter. All samples were analyzed in quartz cells (1 cm pathlength). Nucleic acid samples were pre-formed in polystyrene tubes by heating at 95 °C for 5 minutes slowly cooling to room temperature, followed by incubation at 4 °C for 18 hours. Samples were prepared by mixing ligand and nucleic acid solutions, at appropriate ratios, followed by incubation at 4 °C for 4 hours and sample degassing. Unless otherwise noted, prior to analysis (heating from 0-90°C at a rate of 10 deg/hr) and resulting emission curves (from 210-400 nm recorded in triplicate) samples were pre-formed in polystyrene tubes and heated at 95 °C for 5 minutes slowly cooling to room to room temperature. In all experiments, [nucleic acid structure] = 40 µM/base pair.

Isothermal Titration Calorimetry Titrations. Measurements were performed at 20 °C on a MicroCal VP-ITC (MicroCal, Inc., Northampton, MA). In a typical experiment, 8 µL aliquots of ligand (150 µM for neomycin and ethidium bromide, 50 µM for NM) were injected into an isothermal sample chamber containing 1.46 mL of nucleic acid duplex solution that was 350 µM/bp. Each experiment of this type was accompanied by the corresponding control experiment in which 8 µL aliquots of identical drug solutions were injected into a solution of buffer alone. The duration of each injection was 10 s, and the delay between injections was 300 s. The initial delay prior to the first injection was 60 s. Each injection generated a heat burst curve (microcalories per second vs. seconds). The area under each curve was determined by integration using Origin (version 7.0) software to obtain a measure of the heat associated with that injection. The heat associated with
each drug-buffer injection was subtracted from the corresponding heat associated with each drug-DNA injection to yield the heat of drug binding for that injection.

**Differential Scanning Calorimetry Experiments.** Differential scanning calorimetry (DSC) experiments were undertaken in a Microcal MC2 instrument (Microcal, Inc.). DNA:RNA hybrids at a concentration of 100 µM/bp in cacodylate buffer was used for all experiments. Primary data were corrected by subtraction of a buffer-buffer baseline and normalized to the concentration of DNA base pairs. Baseline-corrected normalized data were transferred to Origin software (Microcal, Inc.) for integration and plotting. Samples of DNA-ligand complexes (NM, ethidium bromide and neomycin) for DSC were prepared by weighing appropriate amounts of solid ligand and dissolving it directly into 1 mL of a 100 µM/bp DNA solution.

**Determination of Binding Constants by the ΔT_m Method.** The apparent ligand-DNA:RNA hybrid association constants were estimated using the following equations. Isothermal titration calorimetry (ITC) techniques were used to estimate the association constant, $K_{T_m}$, **Equation 1**.

$$\frac{1}{T_{m0}} - \frac{1}{T_m} = \frac{R}{n\Delta H_{Dup}} \ln(1 + KT \_m \ L) \quad (1)$$
In equation 1, terms $T_{m0}$ and $T_m$ are the melting temperatures of the native hybrid without and with ligand, as determined with UV thermal denaturation. $n$, the number of drug molecules bound per duplex, was determined using titrations of drug into duplex (fluorescence for ethidium bromide and 3 and CD for neomycin). $\Delta H_{Dup}$, Watson-Crick duplex enthalpy, was determined with DSC. $L$ is the free drug concentration at $T_m$, estimated at one-half the total drug concentration. The binding constant at the melting temperature, $T_m$, was extrapolated to a reference temperature ($T$) of 25 °C using the integrated van’t Hoff, Equation 2:

$$K_T = \frac{K_{T_m}}{e^{-\Delta H_T/(R(1/T_m^{-1}/T))} e^{\Delta C_p T / R(1/T_m^{-1}/T)} \left( \frac{T_m}{T} \right)^{\Delta C_p / R}} (2)$$

where, $\Delta H_T$ was determined experimentally using ITC, $R$ is the gas constant and $\Delta C_p$ was determined using Equation 3.

$$\Delta C_p = \frac{\Delta H}{\Delta T} (3)$$

Enthalpy values ($\Delta H$) were determined using binding enthalpies from excess site ITC titrations (used to identify the heat of interaction of the primary high affinity site), at various temperatures.

![Boc-protected Neomycin-Methidium Conjugate (4).](image)

**Boc-protected Neomycin-Methidium Conjugate (4).** To a solution of 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride (3) (8.6 mg, 23.1 µmol) in dry DMF (3.0 mL), dicyclohexylcarbodiimide (4.8 mg, 23.1 µmol) and dimethylaminopyridine (1.0 mg, 0.01 mmol) were added. The solution was allowed to stir under positive \( \text{N}_2 \) gas for 3 h. A solution of DPA10 (30.0 mg, 23.1 µmol) in dry DMF (3.0 mL) was added via cannula. The reaction was allowed to stir at room temperature under positive \( \text{N}_2 \) for 28 h. The volatiles were removed *in vacuo*. The dry solid was
washed with CH₂Cl₂. The resulting solid was dried in vacuo. Flash chromatography (0%-25% MeOH:CH₂Cl₂) afforded 4 (30.8 mg, 80%) as a purple solid: Rf 0.2 in 85:15 CH₂Cl₂:MeOH; UV max (95% CH₃OH) 286, 510 nm; IR (KBr) 3405, 1600 cm⁻¹; ¹H NMR (500 MHz, MeOD) δ 8.60 (d, 1H, J = 9.1), 8.56 (d, 1H, J = 9.1), 7.90 (d, 2H, J = 8.7, H18), 7.65 (d, 2H, J = 8.8), 7.56 (m, 1H, J = 9.1), 7.35-7.39 (m, 3H), 5.41 (br, 1H), 4.92 (s, 1H), 4.34 (br, 2H), 4.04 (s, 1H), 4.00-3.98 (m, 1H), 3.89-3.80 (m, 1H), 3.76-3.71 (m, 1H), 3.61-3.69 (m, 2H), 3.55-3.52 (m, 1H), 3.51 (d, 2H), 3.47 (br, 1H), 3.4-3.2 (m, 1H), 3.19 (m, 1H), 2.93-2.91 (m, 4H), 2.85-2.83 (m, 4H), 2.81 (d, 2H), 2.79-2.70 (m, 2H), 1.96-1.86 (m, 1H), 1.71 (br, 1H), 1.36-1.48 (m, 54H); ¹³C NMR (125 MHz, MeOD) δ 156.6, 167.8, 156.8, 151.5, 148.2, 136.9, 130.0, 128.8, 128.1, 127.8, 127.5, 127.4, 126.8, 124.3, 120.1, 122.0, 117.9, 108.4, 99.1, 98.9, 98.2, 81.5, 80.3, 79.3, 79.1-79.3, 77.9, 76.8, 73.1, 73.0, 71.5, 70.8, 70.2, 70.1, 66.1, 63.6, 53.3, 50.2, 43.3, 43.2, 41.7, 41.6, 33.3, 27.3; MALDI-TOF m/z (rel. intensity) calculated for C₅₁H₇₆N₁₀O₁₃SCl [M + H]⁺ 1600.09 found 1600.01.

Neomycin-Methidium Conjugate (DPA200). To a solution of 4 (30.8 mg, 18.3 µmol) in 3.0 mL dichloromethane was added trifluoroacetic acid (3.0 mL). 1,2-ethanedithiol
(1.0 mL) was added and the solution was allowed to stir for 30 min. The volatiles were removed under vacuum. The resulting oil was washed with diethylether affording a maroon solid. The solid was dissolved in nanopure water. The solution was purified with preparatory HPLC using a reverse phase column, (0%-100% H$_2$O:MeCN 0.1%TFA, 15 min). The compound eluted at 20.93 min. Fractions containing the compound were lyophilized affording **DPA200** (28.0 mg, 90%) as a maroon solid: UV max (95% H$_2$O) 288, 514 nm; IR (KBr) 3410, 1605 cm$^{-1}$; $^1$H NMR (500 MHz, D$_2$O) 8.76 (d, 1H, $J = 9.6$), 8.69 (d, 1H, $J = 9.6$), 7.86 (d, 2H, $J = 8.7$), 7.66 (d, 2H, $J = 8.8$), 7.52 (m, 1H, $J = 9.6$), 7.35-7.39 (m, 3H), 5.37 (br, 1H), 5.09 (s, 1H), 4.53 (br, 2H), 4.23 (s, 1H), 4.01-3.88 (m, 1H), 3.79-3.71 (m, 1H), 3.75-3.68 (m, 1H), 3.61-3.59 (m, 2H), 3.59-3.53 (m, 1H), 3.52 (d, 2H), 3.47 (br, 1H), 3.29-3.21 (m, 1H), 3.17-3.03 (m, 1H), 2.91-2.89 (m, 4H), 2.89-2.85 (m, 4H), 2.85 (d, 2H), 2.79 (m, 2H), 1.93 (m, 1H), 1.89 (br, 1H); $^{13}$C NMR (125 MHz, D$_2$O) δ 168.5, 155.3, 152.0, 147.3, 137.1, 131.2, 127.9, 127.8, 127.7, 127.2, 127.1, 126.7, 126.6, 123.9, 122.4, 121.3, 117.3, 110.2, 107.9, 98.9, 97.6, 95.3, 85.1, 81.2, 76.5, 74.9, 73.3, 72.8, 71.0, 70.4, 69.3, 68.4, 67.8, 67.3, 60.3, 53.9, 51.0, 50.9, 50.3, 48.7, 42.3, 40.5, 40.4, 29.3; MALDI m/z (rel intensity) calculated for C$_{46}$H$_{67}$N$_{10}$O$_{13}$SCl [M + H]$^+$ 1000.07 found [M + Na]$^+$ 1000.14.
RESULTS AND DISCUSSION

Design of Neomycin – Methidium Conjugate. We hypothesize that conjugation of neomycin with a DNA:RNA hybrid specific binding intercalator, ethidium bromide, will yield a new class of molecules capable of preferentially binding DNA:RNA hybrids. Neomycin is expected to bind in the major groove, while ethidium bromide is expected to bind via intercalation. In an effort to estimate the potential binding affinity of our conjugate, we utilized the following principle: given optimized conditions if ligand molecule A and ligand molecule B bind to a receptor, ligand A-B can be expected to bind such that the binding constant of A-B equals the product of two individual binding constant values ($K_a$). If one looks at the product of the individual $K_a$ values for ethidium bromide and neomycin, one expects an optimized $K_a$ in the $10^{12}$ M$^{-1}$ range. Even if
conditions are not optimized, the conjugate has the potential to bind our target DNA:RNA hybrids in the nanomolar range, Figure 2.1!

When choosing a suitable position on neomycin and ethidium bromide, the following assumptions were made. The amino groups on rings I, II and IV of neomycin are necessary in stabilizing and recognizing the nucleic acid grooves, aminoglycosides without any of these amino groups do not stabilize nucleic acids as efficiently (76). The 5’-OH on ring III was thus chosen to provide linkage to the intercalating unit. Two N-Boc protected neomycin derivatives were designed to accommodate variable linker lengths for conjugation.

Furthermore, when considering ethidium bromide, the electron donation afforded by the exocyclic amino groups dominate the electron withdrawing effects of the endocyclic iminium ion when examining the overall electron density of the molecule (65, 77). Therefore, these amino groups were not chosen for conjugation to neomycin. Fortunately, an ethidium bromide derivative (6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride, 6) features an accessible functional group in the form of a carboxylic acid through which conjugation can easily be achieved leaving the overall binding moiety of the molecule unperturbed.

**Synthesis.** Synthesis of compound ‘N-Boc-neomycin amine’, DPA10, has previously been reported by our group and by Tor (78, 79, 80), Scheme 2.1. Each of the six amino groups located on commercially available neomycin B sulfate were N-Boc protected. The hydroxyl at the 5’-position on ring three of neomycin was converted into a good
leaving group using 2,4,6-triisopropylbenznesulfonyl chloride (TPS) to afford compound 2. The TPS leaving group was subsequently displaced, using 2-aminoethanethiol hydrochloride in the presence of sodium metal, affording N-Boc-neomycin-amine, DPA10, in 38% overall synthetic yield. The TPS leaving group was also displaced using sodium azide to afford the intermediate N-Boc-neomycin-azide, DPA11. DPA11 was
reduced in the presence of H$_2$ (g), to afford N-Boc-neomycin-5”-amine, **DPA12, Scheme 2.1.**

![Chemical Structure](image)

**Scheme 2.2.** Reagents and conditions: (i) **DPA10**, 6-(4-carboxyphenyl)-3,8-diamino-5-methylphen-anthridinium chloride, DCC, DMAP, DMF, 28 h, 80%; (ii) 4, TFA/CH$_2$Cl$_2$, 1,2-ethanedithiol, 5 h, 90%.

6-(4-carboxyphenyl)-3,8-diamino-5-methylphen-anthridinium chloride 3 was coupled to **DPA10** in the presence of dicyclohexylcarbodiimide/dimethylaminopyridine (DCC/DMAP) to afford the N-Boc-protected conjugate 4, **Scheme 2.2.** Trifluoroacetic acid (TFA) deprotection of the N-Boc-protected conjugate 4 afforded the TFA salt of **DPA201** in good yields, **Scheme 2.2.**

![Chemical Structure](image)

**Scheme 2.3.** Reagents and conditions: (i) **DPA12**, 6-(4-carboxyphenyl)-3,8-diamino-5-methylphen-anthridinium chloride, DCC, DMAP, DMF, 28 h, 84%; (ii) 5, TFA/CH$_2$Cl$_2$, 1,2-ethanedithiol, 5 h, 94%;
Compound 3 was activated with DCC/DMAP and DPA12 was added to afford the N-Boc protected conjugate 5, Scheme 2.3. The N-Boc protected conjugate 5 was also deprotected using TFA. The resulting deprotected conjugate DPA201 was afforded in good yields, Scheme 2.3.

**Competition Dialysis Experiments.** Competition dialysis experiment allow for the screening of a compound against a number of nucleic acid structures. When the experiment was conducted at a concentration of 75 µM per base unit of nucleic acid structure, DPA200 displayed a notable preference for poly(dA):poly(rU), poly(rA):poly(dT), poly(dA):poly(rU), Figure 2.2. Little to no interaction with nucleic acid single strands (poly(dA), poly(dG), poly (rC), poly(rU), poly(rA), poly(dC), poly(dA) or poly(dT)) were detected.

In an effort to eliminate non-specific binding to various nucleic acids, a potential artifact of high nucleic acid and ligand concentrations, the experiment was conducted at 750 nM per base unit of nucleic acid, Figure 2.2. At 750 nM, DPA200 displayed a clear preference for poly(dA):poly(rU). A decrease in preference for poly(rA):poly(dT) and poly(rA):poly(U), with respect to poly(dA):poly(rU), by DPA200 was observed at 750 nM. Competition dialysis experiments were also conducted at 100 mM NaCl. At lower salt concentration, DPA200 demonstrated a clear preference of for poly(dA):poly(rU), Appendix A, Figure A8.
Figure 2.2. Competition dialysis results of DPA200 with various nucleic acids. The histograms show the amount of DPA200 bound to individual nucleic acids following dialysis. Nucleic acids were dialyzed with DPA200 in buffer solution for 72 h. In panel (a) [DPA200] = 1 µM, [nucleic acids] = 75 µM 750 nM per base unit of each polymer and panel (b) [DPA200] = 100 nM, [nucleic acids] = 750 nM 750 nM per base unit of each polymer. Buffer: 6 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 mM EDTA, pH 7.0, 185 mM NaCl.
UV Monitored Thermal Denaturation Experiments. In this experiment, pre-formed nucleic acid structures were incubated, individually, with the DPA200, Table 2.1, Appendix A, Figure A9. The samples were denatured and monitored with UV-spectroscopy. An increase in absorbance was observed upon the disassociation of the nucleic acid into single strands. The point at which the nucleic acid structure disassociates into its constituent single strands corresponds to the melting temperature, $T_m$, which was observed as the temperature in which the UV absorbance at 260 nm was halfway between the UV absorbance of the helix and single stranded forms. It is expected that nucleic acids in which DPA200 display preferential binding for will show the highest stabilization of melting temperature when complexed with DPA200 over the native

<table>
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<th>$T_m$ b ($^\circ$C)</th>
<th>$\Delta T_m$ c ($^\circ$C)</th>
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a$T_{m0}$ represents the melting temperature of the native duplex. b$T_m$ represents the melting temperature of the duplex upon addition of neomycin at a $r_{bd}$ (ratio of DNA base pairs:drug) of 2.2. c$\Delta T_m$ represents the change in melting temperature between $T_{m0}$ and $T_m$. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
unbound nucleic acid. As expected, DPA200 complexed with poly(dA):poly(rU) afforded the highest thermal stabilization of an incredible 46.9 °C increase in \( T_m \). DPA200 afforded a \( T_m \) increase of 34.3 °C for the poly(rA):poly(dT) complex, followed by the poly(rA):poly(rU) complex at \( T_m = 20.1 \) °C. Not only does DPA200 show a high preference for DNA:RNA hybrids, but the poly(dA):poly(rU) thermal stabilization afforded by DPA200 is significantly higher than any other nucleic acid structure.

Most recently, an assay using thermal denaturation of mixtures containing various nucleic acids in the presence of ligand was developed to determine structural and sequence selectivity (82). In this thermal denaturation experiment, a number of nucleic acids with different melting temperatures were combined in solution and denatured in the

\[ \text{Figure 2.3. Mixed melting profile of various nucleic acids. Panel (a) shows a melting profile for the mixed melting of various duplex nucleic acids with DPA200. Panel (b) shows a derivative plot for the mixed melting of various duplex nucleic acids with DPA200 at } r_{dd} = 20, \text{ the solid line reflects native melting and the dashed line represent the melting with DPA200. The arrows indicate the peaks that are altered by addition of the DPA200. In each panel, the peaks correspond to the following nucleic acids, poly(dG):poly(dC); peak 5, poly(dA):poly(dT); peak 4, poly(rA):poly(dT); peak 3, poly(rA):poly(rU); peak 2, poly(dA):poly(rU); peak 1. Individual polynucleotide concentration was 10 mM/bp; total polynucleotide concentration was 50 µM. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA and 46.25 mM NaCl at pH 7.0.} \]
presence of a ligand at very low ligand to nucleic acid ratios. At such low ratios, the
nucleic acid preferred by the ligand will display a thermal stabilization, while other
nucleic acids will remain unchanged. As seen in Figure 2.3, when assayed against a
solution containing poly(dA):poly(rU), poly(rA):poly(dT), poly(dA):poly(dT),
poly(rA):poly(rU) and poly(dG):poly(dC), at $r_{bd} = 20$, only the melting temperature of
poly(dA):poly(rU) is increased, while all other duplexes remain unchanged. In fact,
increasing the concentration to $r_{bd} = 10$ affords stabilization for poly(dA):poly(rU) (2.0
°C) and poly(rA):poly(dT) (0.9 °C), Appendix A, Figure A10. The smaller changes in
$T_m$, as observed in the mixed melting experiments, are consistent with the previously
reported data (82). These experiments proved irrefutable evidence that DPA200
preferentially binds to poly(dA):poly(rU). The difference in stabilization of the two
hybrids when complexed to DPA200 can be attributed to the conformational differences
adopted by the two hybrids as poly(dA):poly(rU) adopts a more A-like conformation than
poly(rA):poly(dT) (32).

**Design of Thermal Denaturation Experiments.** In an effort to further explore the
stability of DPA200 – nucleic acid complexes a number of thermal denaturation
experiments were performed. UV monitored thermal denaturation experiments were
conducted in the presence of increasing molar ratios of DPA200 and DPA201 to
poly(rA):poly(dT) and poly(dA):poly(rU). Thermal melting experiments were conducted
in the presence of neomycin, ethidium bromide and DPA200 at equivalent ratios of
ligand to DNA:RNA hybrid in an effort to compare the thermal stabilization afforded by
DPA200 to its constituent parts. As the amount of ligand required to saturate the DNA:RNA hybrids was not identical, thermal denaturation experiments were also conducted at each ligand saturation point. Finally, UV monitored thermal denaturation experiments were also expanded to include the RNA duplex poly(rA):poly(rU) and the DNA duplex poly(dA-dT)₂. These techniques have previously been used by our research group (79, 83, 84, 85, 86).

**UV Monitored Thermal Denaturation Experiments.** UV monitored thermal denaturation experiments were also conducted at pH 6.8 and 20 mM NaCl. Biphasic transitions were observed for complexes of poly(dA):poly(rU) and DPA200, where the duplex was not saturated with ligand, *Appendix A, Figure A11*. However, at r_{bd} of 4, the melting temperature of the poly(dA):poly(rU) – DPA200 complex was 31.4 °C higher than melting temperature of the duplex without ligand. The ΔTₘ of the poly(rA):poly(dT) – DPA200 complex was only 9.8 °C higher than the duplex alone. At pH 6.8 and 100 mM NaCl, UV monitored thermal denaturation of poly(dA):poly(rU) and poly(rA):poly(dT) in the presence of increasing concentration of DPA200 was conducted. The hybrid melting temperature increased with increasing amount of DPA200, *Table 2.2, Figure 2.4*. The magnitude of stabilization afforded by DPA200 varied significantly between hybrid duplexes. At a r_{bd} of 9.6, the poly(dA):poly(rU) – DPA200 complex melting temperature was 21.9 °C higher than the native duplex. However, the melting temperature of the poly(rA):poly(dT) – DPA200 complex was much lower where the ΔTₘ was only 6.1 °C.
Table 2.2. UV determined thermal denaturation temperatures of DNA:RNA hybrids with various ligands. All values are reported in °C.

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<td>3.2</td>
<td>67.7</td>
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$^aT_m$ represents the melting temperature of the native duplex. $^b\Delta T_m$ represents the change in melting temperature upon addition of ligands at a $r_{bd}$. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA (salt and pH conditions noted above).
Figure 2.4. UV thermal denaturation profiles of poly(rA):poly(dT) (a, b) and poly(dA):poly(rU) (c, d) with various ligands. Panels (a, c) represent the duplex with DPA200 at varying r_{bd} values, while panels (b, d) represent the duplex (A) was with neomycin (B), ethidium bromide (C), neomycin and ethidium bromide (D) and DPA200 (E) at 5 \( \mu \)M. Ligands were added at varying r_{bd} (ratio of drug:base pairs) values to the duplex [20 \( \mu \)M/bp]. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.

UV monitored thermal denaturation experiments were also conducted on complexes of DNA:RNA hybrid and neomycin, ethidium bromide or a non-covalent mixture of ethidium bromide and neomycin at r_{bd} of 9.6 at a pH of 6.8 and 100 mM NaCl, Table 2.2. The melting temperature of the poly(rA):poly(dT) – DPA200 complex was 6.1 °C higher than the melting temperature of the native duplex, Figure 2.4. The change
in melting temperatures of the poly(rA):poly(dT) complexes with neomycin (ΔT<sub>m</sub> = 5.3), ethidium bromide (ΔT<sub>m</sub> = 4.1), non-covalent combination of neomycin and ethidium bromide (ΔT<sub>m</sub> = 4.3) were all lower than the poly(rA):poly(dT) – DPA200 complex.  

**Appendix A, Figure A11.** The melting temperature of the poly(dA):poly(rU) – neomycin complex was only 2.8 °C higher than the duplex alone, **Figure 2.4.** The UV melting profile of the poly(dA):poly(rU) – ethidium bromide complex was biphasic which suggested simultaneous destabilization and stabilization of the duplex, ΔT<sub>m</sub> was -12.3 °C and 5.5 °C, respectively. The UV melting profile of the poly(dA):poly(rU) – non-covalent combination of neomycin and ethidium bromide complex was also biphasic, ΔT<sub>m</sub> was -12.0 ΔT<sub>m</sub> 2.6 °C, respectively.

The melting temperature poly(dA):poly(rU) – DPA200 complex was 21.9 °C higher than native duplex. Similar observations were recorded for the same complexes at a pH of 5.5 and 100 mM NaCl, **Appendix A, Figure A12.** At pH 6.8 and 100 mM NaCl, UV monitored thermal denaturation of poly(dA):poly(rU) and poly(rA):poly(dT) in the presence of increasing concentration of DPA201 was conducted, **Figure 2.5.** The stabilization afforded by DPA201 varied significantly between the duplexes, as seen with DPA200. At lower concentrations of DPA201, the UV melting profile of poly(rA):poly(dT) was biphasic, potentially due to incomplete saturation of the duplex by the conjugate. However, at r<sub>bd</sub> = 6.6 and r<sub>bd</sub> = 4, the poly(rA):poly(dT) – DPA201 complex increased in thermal stability by 4.2 °C and 10 °C. Under identical conditions and r<sub>bd</sub> = 4.0, DPA201 stabilizes the duplex by 4 °C more than DPA200.
Poly(dA):poly(rU) melting temperature increased with increasing amount of DPA201. At a $r_{bd}$ of 4.0, the poly(dA):poly(rU) – DPA200 complex melting temperature was 25.8 °C higher than the native duplex. The stabilization afforded by DPA200 on poly(dA):poly(rU) is 5.7 °C higher, at $r_{db} = 4.0$, than the stabilization afforded by DPA201 on poly(dA):poly(rU).

UV monitored thermal denaturation experiments were conducted at spectroscopically determined saturated amounts of ligand at a pH of 5.5 and 100 mM NaCl, page, Table 2.2, Appendix A, Figure A12. The poly(rA):poly(dT) – DPA200 complex was 30.5 °C higher than the melting temperature of the native duplex. While the poly(rA):poly(dT) complexes with saturated amounts of neomycin and ethidium bromide were larger than the melting temperature of the duplex alone, the stabilization afforded by neomycin was only 5.8 °C and ethidium bromide was only 8.7 °C. UV monitored melting
Table 2.3. CD determined thermal denaturation temperatures of DNA:RNA hybrids with various ligands. All values are reported in °C. $T_m^a$ values are reported below while $\Delta T_m^b$ are reported in parenthesis.

<table>
<thead>
<tr>
<th>Constant $r_{bd}$</th>
<th>poly(dA):poly(rU) $r_{bd} = 9.6$</th>
<th>poly(rA):poly(dT) $r_{bd} = 9.6$</th>
<th>poly(rA):poly(dT) $r_{bd} = 6.5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>48.9</td>
<td>63.8</td>
<td>63.8</td>
</tr>
<tr>
<td>neomycin</td>
<td>58.7 (9.8)</td>
<td>67.6 (3.8)</td>
<td>68.5 (4.7)</td>
</tr>
<tr>
<td>ethidium bromide</td>
<td>49.6 (0.7)</td>
<td>65.8 (2.0)</td>
<td>66.1 (2.3)</td>
</tr>
<tr>
<td>neomycin + ethidium bromide</td>
<td>59.2 (10.3)</td>
<td>67.8 (4.0)</td>
<td>69.8 (6.0)</td>
</tr>
<tr>
<td>DPA200</td>
<td>70.1 (21.2)</td>
<td>68.7 (4.9)</td>
<td>70.3 (6.5)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Saturation $r_{bd}$</th>
<th>poly(dA):poly(rU)</th>
<th>poly(rA):poly(dT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>48.9</td>
<td>63.8</td>
</tr>
<tr>
<td>neomycin</td>
<td>$r_{bd} = 6.0$</td>
<td>$r_{bd} = 8.0$</td>
</tr>
<tr>
<td></td>
<td>64.2 (15.3)</td>
<td>68.6 (4.8)</td>
</tr>
<tr>
<td>ethidium bromide</td>
<td>$r_{bd} = 4.4$</td>
<td>$r_{bd} = 4.2$</td>
</tr>
<tr>
<td></td>
<td>50.3 (1.4)</td>
<td>66.4 (2.6)</td>
</tr>
<tr>
<td>DPA200</td>
<td>$r_{bd} = 9.6$</td>
<td>$r_{bd} = 6.5$</td>
</tr>
<tr>
<td></td>
<td>70.1 (21.2)</td>
<td>70.3 (6.5)</td>
</tr>
</tbody>
</table>

$^aT_m$ represents the melting temperature of the native duplex. $^b\Delta T_m$ represents the change in melting temperature upon addition of ligands at various $r_{bd}$ values. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA (salt and pH conditions noted above).

Profiles of poly(dA):poly(rU) complexed with saturated amounts of neomycin and ethidium bromide were monophasic and 24.9 °C and 20.8 °C higher than the native duplex, respectively. The stabilization of the poly(dA):poly(rU) – DPA200 complex was higher, the melting temperature for the complex was 44.9 °C higher than the duplex alone.

**CD Monitored Thermal Denaturation Experiments.** Complexes of DNA:RNA hybrids and ligands were thermally denatured and monitored by circular dichorism (CD).

69
A solution containing ligand and nucleic acid is optically active, changes in the solution can be observed by following the difference in absorption for right and left polarized light (87). As in UV monitored thermal denaturation experiments, the point at which the nucleic acid – ligand complex disassociates into its constituent single strands was denoted as the melting temperature, $T_m$, Table 2.3.

CD monitored thermal denaturation of poly(rA):poly(dT) – ligand complexes were conducted at a $r_{bd}$ of 6.5. The poly(rA):poly(dT) – DPA200 complex denatured at 6.5 °C higher than the native duplex. The change in melting temperature of the poly(rA):poly(dT) – ligand complexes were 4.7 °C and 2.3 °C higher than the native duplex when complexed neomycin and ethidium bromide, respectively. The poly(rA):poly(dT) – neomycin and ethidium bromide complex denatured 6.0 °C higher than the unbound duplex, similar in magnitude to the thermal stabilization of the poly(rA):poly(dT) – DPA200 complex, Appendix A, Figure A13.

The melting temperature of poly(dA):poly(rU) – DPA200 complex was 21.2 °C, similar to the UV determined melting temperature. The poly(dA):poly(rU) complex denatured at 58.7 °C, 9.8 °C higher than the melting temperature of the native duplex. UV monitored melting profiles of poly(dA):poly(rU) complexed with ethidium bromide and the non-covalent combination of neomycin and ethidium bromide were biphasic. CD monitored melting profiles of the same complexes were monophasic and denatured at 0.7 °C and 10.3 °C higher than the unbound duplex, Appendix A, Figure A13.

CD monitored thermal denaturation of the DNA:RNA hybrid – ligand complexes were conducted at saturated amounts of neomycin, ethidium bromide and DPA200 and
pH 5.5, Table 2.3, Figure 2.6. At saturated amounts of ligand, the poly(rA):poly(dT) – neomycin complex denatured 4.8 °C higher than the melting temperature of the native duplex. The poly(rA):poly(dT) – ethidium bromide complex denatured 2.6 °C higher than the unbound duplex melting temperature while the melting temperature of the poly(rA):poly(dT) – DPA200 complex was a modest 6.5 °C higher than the native duplex melting temperature.

![Figure 2.6](image)

Figure 2.6. CD thermal denaturation profile of poly(rA):poly(dT) (left) and poly(dA):poly(rU) (right). Each panel represents the duplex (A) was with neomycin (C), ethidium bromide (B) and DPA200 (D). Ligands were added to the duplex [20 µM/bp] at saturation: poly(rA):poly(dT) neomycin ($r_{bd} = 8.0$), ethidium bromide ($r_{bd} = 4.2$) and DPA200 ($r_{bd} = 6.5$); poly(dA):poly(rU): neomycin ($r_{bd} = 6.0$), ethidium bromide ($r_{bd} = 4.4$) and DPA200 ($r_{bd} = 9.6$). The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.

The largest thermal stabilization was observed with the poly(dA):poly(rU) – DPA200 complex, a 21.2 °C increase in stabilization over the duplex alone. CD determined melting temperatures of the poly(dA):poly(rU) complex demonstrates a minimal increase to the native duplex melting temperature, only 1.5 °C. The melting
temperature of the poly(dA):poly(rU) – neomycin complex was 64.2 °C, 15.3 °C higher than the native duplex melting temperature.

**Fluorescence Monitored Thermal Denaturation Experiments.** Fluorescence techniques were conducted to further corroborate previously described results. The only drawback to this approach is the ligand of interest must contain a chromophore which can be excited and the resulting emission can be tracked, therefore complexes of the DNA:RNA hybrids and ethidium bromide or DPA200 were the only compounds studied, **Appendix A, Figure A14.** Ethidium bromide and DPA200 exhibit a strong fluorescence signal when complexed with nucleic acids. As the nucleic acid denatures the bound ligand is released into solution and a decrease in fluorescence intensity is observed. Fluorescence determined melting temperatures for the DNA:RNA hybrid – ligand complexes support previously determined values.

The following observations were made from thermal denaturation experiments. (i) The thermal stabilization of DNA:RNA hybrid duplexes increases when complexed with DPA200. The stabilization increases with increasing amounts of DPA200. Furthermore, the melting temperature increase over the native duplex melting temperature was larger when DPA200 is complexed with poly(dA):poly(rU) than when complexed with poly(rA):poly(dT). (ii) At equivalent $r_{bd}$ values, DNA:RNA hybrid complexation with DPA200 affords a larger increase in thermal stabilization than complexation with neomycin or ethidium bromide. (iii) When the DNA:RNA hybrid is
complexed with saturating amounts of ligand, the melting temperature of the DNA:RNA hybrid – **DPA200** complexes are higher than the DNA:RNA hybrid – neomycin or ethidium bromide complexes. This is observed in both poly(dA):poly(rU) and poly(rA):poly(dT). (iv) Finally, when complexed to various nucleic acids at **DPA200** saturation, the largest increase in melting temperature was observed when complexed with poly(dA):poly(rU). The poly(rA):poly(dT) – **DPA200**, poly(rA):poly(rU) – **DPA200** and poly(dA-dT)$_2$ – **DPA200** complexes were all stabilized by the conjugate,
but to a smaller magnitude than observed with the poly(dA):poly(rU) – DPA200 complex.

**DPA200 Thermodynamic Profiles with poly(rA):poly(dT) and poly(dA):poly(rU).** Ligand saturation points were defined as the number of base pairs per ligand and determined by titration of ligand into DNA:RNA hybrid. Titrations of neomycin into DNA:RNA hybrid was monitored by CD while titrations of ethidium bromide and DPA200 into DNA:RNA hybrids were monitored by fluorescence. CD titrations of ethidium bromide and DPA200 were also conducted, Appendix A, Figure A15, however binding stie size determination could not be made. A plot of CD intensity with respect to \( r_{bd} \) was made. A linear fit of pre- and post-saturation regions was conducted and the intersection of these fits defined the binding site size, Figure 2.7. Neomycin binding sites of 8.1 base pairs/ligand and 6.0 base pairs/ligand were determined for and poly(dA):poly(rU) and poly(rA):poly(dT), respectively.

Following fluorescence monitored titrations of DNA:RNA hybrids with ethidium bromide and DPA200, the fluorescence emission maxima was plotted with respect to \( r_{bd} \). Two clear inflection points were observed following analysis of the titration of ethidium bromide into poly(rA):poly(dT). A primary binding site size was observed at 4.2 base pairs/ligand and a secondary binding site was identified at 2.1 base pairs/ligand. Two binding sites were also observed following analysis of the titration of ethidium bromide into poly(dA):poly(rU). A primary binding site size of 4.4 base pairs/ligand and a
Figure 2.8. Fluorescence emission scans of the titration of DPA200 and ethidium bromide with poly(rA):poly(dT) (a, c) and poly(dA):poly(rU) (e, g) at 20°C. A plot of fluorescence intensity at 634 (b, f) and 261 (d, h) with respect to $r_{bd}$ was plotted, $r_{bd}$ is the ratio of [base pair]/[drug]. Ligands were added to the duplex [1 μM/bp] at respective $r_{bd}$ values. Excitation: 525 nm; emission: 550 – 700 nm; slits: 1.5 mm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
secondary binding site of 1.8 base pairs/ligand were identified, Figure 2.8. Analysis, following titration of DPA200 into poly(rA):poly(dT) showed only one inflection point at 6.5 base pairs/ligand. A primary binding size of 9.3 base pairs/ligand was identified upon analysis of the titration DPA200 into poly(dA):poly(rU). A secondary binding site size was also observed at high ligand concentrations; 5.7 base pairs/ligand, Figure 2.8.

Watson-Crick duplex enthalpy values, $\Delta H_{wc}$, were determined using DSC thermal denaturation experiments, conducted by Dr. Hongjuan Xi. The DSC melting profile for poly(rA):poly(dT) was exhibited one peak at 65.8 °C, Figure 2.9. The dissociation of the duplex into the single strands of RNA and DNA corresponded to a melting enthalpy of 9.21 kcal/mol. The DSC melting profile of poly(dA):poly(rU) showed one peak at 48.5 °C. The melting enthalpy of the hybrid duplex was 5.27 kcal/mol, a lower value.
Figure 2.10. ITC titration of **DPA200** into the duplex poly(dA):poly(rU) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 100 µM/bp; **DPA200** concentration was 60 µM. In the profile, the upper panels show the heat burst curves of 10 µL injection of **DPA200** into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
than observed with poly(rA):poly(dT) which correlates with previous DNA:RNA hybrid stability reports (31).

<table>
<thead>
<tr>
<th>poly(dA):poly(rU)</th>
<th>neomycin</th>
<th>ethidium bromide</th>
<th>DPA200</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H_{wc}$ (kcal/mol)$^a$</td>
<td>5.27</td>
<td>5.27</td>
<td>5.27</td>
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<tr>
<td>$T_{m0}$ (°C)$^b$</td>
<td>48.9</td>
<td>48.9</td>
<td>48.9</td>
</tr>
<tr>
<td>$T_{m}$ (°C)$^b$</td>
<td>64.2</td>
<td>50.3</td>
<td>70.9</td>
</tr>
<tr>
<td>$n$$^c$</td>
<td>6.0</td>
<td>4.4</td>
<td>9.6</td>
</tr>
<tr>
<td>$L$ (µM)</td>
<td>1.7</td>
<td>2.3</td>
<td>1.1</td>
</tr>
<tr>
<td>$\Delta H_{(10 \degree C)}$ (kcal/mol)$^d$</td>
<td>-2.14±0.07</td>
<td>-7.34±0.07</td>
<td>-11.18±0.11</td>
</tr>
<tr>
<td>$\Delta H_{(20 \degree C)}$ (kcal/mol)$^d$</td>
<td>-6.70±0.06</td>
<td>-7.69±0.03</td>
<td>-16.19±0.11</td>
</tr>
<tr>
<td>$\Delta H_{d(10 \degree C)}$ (kcal/mol)$^d$</td>
<td>-0.96±0.10</td>
<td>-1.33±0.05</td>
<td>-1.24±0.04</td>
</tr>
<tr>
<td>$\Delta H_{d(20 \degree C)}$ (kcal/mol)$^d$</td>
<td>-0.71±0.10</td>
<td>-1.11±0.07</td>
<td>-0.74±0.08</td>
</tr>
<tr>
<td>$\Delta C_p$ (cal/mol•K)$^e$</td>
<td>-427±14</td>
<td>-35±13</td>
<td>-501±22</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>poly(rA):poly(dT)</th>
<th>neomycin</th>
<th>ethidium bromide</th>
<th>DPA200</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta H_{wc}$ (kcal/mol)$^a$</td>
<td>9.21</td>
<td>9.21</td>
<td>9.21</td>
</tr>
<tr>
<td>$T_{m0}$ (°C)$^b$</td>
<td>63.8</td>
<td>63.8</td>
<td>63.8</td>
</tr>
<tr>
<td>$T_{m}$ (°C)$^b$</td>
<td>68.6</td>
<td>66.4</td>
<td>70.3</td>
</tr>
<tr>
<td>$n$$^c$</td>
<td>8.1</td>
<td>4.2</td>
<td>6.5</td>
</tr>
<tr>
<td>$L$ (µM)</td>
<td>1.2</td>
<td>2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>$\Delta H_{(10 \degree C)}$ (kcal/mol)$^d$</td>
<td>-2.48±0.02</td>
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<td>-7.76±0.10</td>
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<td>$\Delta H_{(20 \degree C)}$ (kcal/mol)$^d$</td>
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<td>-12.29±0.07</td>
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<td>$\Delta H_{d(10 \degree C)}$ (kcal/mol)$^d$</td>
<td>-0.38±0.07</td>
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<td>-1.32±0.07</td>
</tr>
<tr>
<td>$\Delta H_{d(20 \degree C)}$ (kcal/mol)$^d$</td>
<td>-0.16±0.07</td>
<td>-1.14±0.20</td>
<td>-0.80±0.05</td>
</tr>
<tr>
<td>$\Delta C_p$ (cal/mol•K)$^e$</td>
<td>-146±13</td>
<td>-97.6±4</td>
<td>-453±17</td>
</tr>
</tbody>
</table>

$^a$Data obtained from DSC melting profiles. $^b$Data obtained from CD and UV thermal denaturation profiles. $^c$Data obtained from titrations, as outlined in text. $^d$ΔH is corrected binding heat, derived by integration of heat burst curve from the sample titration, followed by subtraction of the dilution head from the control titration. $^e$ΔC$\!$$\!$p and binding affinities as defined in text. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.

Enthalpy values (ΔH) were determined using excess site ITC titrations. Excess site ITC titrations were conducted at 20 °C and 10 °C. In a typical experiment, ligands
were titrated into a DNA:RNA hybrid duplex. Experimentally binding enthalpies were
determined by the integration of the corresponding heat burst curves. The corrected
binding enthalpies, \( \Delta H_{(10 \, ^{\circ}C)} \) and \( \Delta H_{(20 \, ^{\circ}C)} \), were determined following the subtraction of
the dilution enthalpy from the binding enthalpy. Dilution enthalpies, \( \Delta H_{d(10 \, ^{\circ}C)} \) and
\( \Delta H_{d(20 \, ^{\circ}C)} \), for each ligand were determined through integration of the heat burst curves
following the titration of ligands into buffer at 20 °C and 10 °C, Figure 2.10, Appendix
A, Figure A16 – Figure A20. Corrected binding enthalpies and dilution enthalpies for
the interactions of neomycin, ethidium bromide and DPA200 with poly(rA):poly(dT) and
poly(dA):poly(rU) are located in Table 2.4.

Corrected binding enthalpies were used to calculate the observed change in heat
capacity, \( \Delta C_p \). Equation 1 (88):

\[
\Delta C_p = \frac{\Delta H}{\Delta T} \quad \text{Equation 1}
\]

The observed change in heat capacity for the interactions of neomycin and ethidium
bromide with poly(rA):poly(dT) were \(-146\pm13\) cal mol\(^{-1}\) K\(^{-1}\) and \(-97.6\pm4\) cal mol\(^{-1}\) K\(^{-1}\),
respectively. The observed heat capacity change for the interaction of DPA200 and
poly(rA):poly(dT) was \(-453\pm22\) cal mol\(^{-1}\) K\(^{-1}\). The observed change in heat capacity for
the interaction of poly(dA):poly(rU) with neomycin and ethidium bromide were \(-427\pm14\)
cal mol\(^{-1}\) K\(^{-1}\) and \(-35\pm13\) cal mol\(^{-1}\) K\(^{-1}\) while the interaction with DPA200 was \(-501\pm22\)
cal mol\(^{-1}\) K\(^{-1}\).
The design of small synthetic organic molecules is highly dependent on structure-based principles. A strong case can be made for the importance of the energetics on ligand-nucleic acid interactions (89, 90, 91). Parsing the energetics of a ligand-nucleic acid interaction provides a quantitative detail of the molecular forces that contribute to a binding interaction (92). Observed heat capacity changes, $\Delta C_p$ values, are an integral component of interaction energetics (93).

Observed heat capacity changes, for ligand-macromolecule interactions, can be impacted by the following factors. (i) The hydrophobic effect, which arises from the isolation of highly ordered pockets of water molecules surrounded by nonpolar surface area burial upon binding of ligand to nucleic acid, is a significant contributor to observed heat capacity changes (78, 94, 95, 96). (ii) Changes in electrostatic charge equilibrium have been shown to decrease $\Delta C_p$ values in aqueous media (93, 97, 98). However, it has been suggested that electrostatic charge equilibrium is not a major contributor to $\Delta C_p$ values as it is highly unlikely that ligand binding induces significant changes within the distribution of exposed electrostatic charges (99). (iii) Alterations of the internal degrees of freedom, ‘soft’ internal vibrational modes, at the polar interface between ligand and nucleic acid have been cited as potential sources for $\Delta C_p$ values (93, 100, 101). (iv) Finally, changes in equilibrium, defined as a shift between two or more states, contribute to changes in $\Delta C_p$ values. Changes in equilibrium can include conformational changes to the macromolecule host (92, 92, 93, 101, 102) as well as protonation reactions upon ligand binding (103, 104, 105).
Heat capacity changes, upon intercalative binding events, are well studied (92, 106, 107). Baldini and Varani suggest the contributions to the binding free energy arise from distortion to the DNA at the intercalation site, conformational changes to the intercalator upon complexation with the DNA, intramolecular stacking forces, long range electrostatic interactions between the intercalator and DNA and intercalator-solvent interactions (107). \( \Delta C_p \) values associated with ethidium bromide represent the delicate balance between the energetic cost of deforming the helical base pairs, necessary for forming the intercalation site, and the reduction in translational and rotational freedom of ethidium bromide upon binding (92). \( \Delta C_p \) values the binding of ethidium bromide has been reported as low as 10 cal mol\(^{-1}\) K\(^{-1}\) when complexed with calf thymus DNA (106), and -57 cal mol\(^{-1}\) K\(^{-1}\) when complexed with chromatin (108). Generally, \( \Delta C_p \) values for ethidium bromide-nucleic acid interaction have been reported around -125±40 cal mol\(^{-1}\) K\(^{-1}\) (92, 107, 109). Our observed \( \Delta C_p \) values for ethidium bromide interaction with poly(rA):poly(dT) and poly(dA):poly(rU) are consistent with previously reported values.

Aminoglycoside-nucleic acid interactions require special consideration. Groove binders exhibit negative \( \Delta C_p \) values, a result attributed to the displacement of large amounts of nonpolar surface area (95, 110). However, Barbieri and co-workers suggest aminoglycosides do not disrupt the spine of hydration (101), even at pH 5.5, and the observed negative \( \Delta C_p \) value is unrelated to change in solvent accessible areas. Perturbations to helical structure through disruption of adenine base stacking has been observed upon aminoglycoside binding to the A-site and is the main contributor to observed negative \( \Delta C_p \) values (100, 101). Experiments, to this point, have been
conducted at pH 6.8 which is advantageous since the conformations and melting enthalpy adopted by the nucleic acids as well as the aminoglycoside binding site size and stabilization reflect real biophysical conditions. Unfortunately, neomycin amino group pKa values range from 5.6 to 9.5 (105, 111). Therefore, neomycin-nucleic acid interactions studied at pH values larger than 5.6 include an inherent contribution from binding linked protonation. Binding-linked protonation leads to an increase in observed binding enthalpy (32).

While the heat capacity associated with the interaction of DPA200 and DNA:RNA hybrids, poly(rA):poly(dT) and poly(dA):poly(rU), includes a dependence on drug protonation upon binding, it is clear that the conjugate possesses contributions from both the major groove binding and intercalative moieties. The heat capacity associated with the interaction of DPA200 and poly(dA):poly(rU) was -501±14 cal mol⁻¹ K⁻¹, slightly higher in magnitude than the sum of the neomycin (427±14 cal mol⁻¹ K⁻¹) and ethidium bromide (-35±13 cal mol⁻¹ K⁻¹). The heat capacity associated with the interaction of DPA200 and poly(rA):poly(dT) (-453±17 cal mol⁻¹ K⁻¹) was significantly higher than the contributions from neomycin (-146±13 cal mol⁻¹ K⁻¹) and ethidium bromide (-97.6±4 cal mol⁻¹ K⁻¹).

While the thermodynamic profile for the interaction of ligands (neomycin, ethidium bromide and DPA200) with DNA:RNA hybrids (poly(rA):poly(dT) and poly(dA):poly(rU)), derived at pH 6.8 closely resemble physiological conditions, the intrinsic thermodynamic profile, free of the dependence on binding linked protonation remains to be determined. To this end, intrinsic thermodynamic profiles for the ligands
with the following duplexs (poly(dA-dT)$_2$, poly(rA):poly(rU) and poly(dA):poly(rU) were calculated at pH 5.5. Furthermore, as parameters calculated at pH 5.5 represent the intrinsic binding parameters, binding affinities ($K_T$) were able to be calculated.

**Binding Constant Determination – The $\Delta T_m$ Method.** The apparent ligand-DNA:RNA hybrid association constants were estimated using the following equations. The change in melting temperature of the nucleic acid in the absence and presence of drug was used to estimate the association constant, $K_{Tm}$, **Equation 2** (112):

$$\frac{1}{T_{m0}} - \frac{1}{T_m} = \frac{R}{n\Delta H_{wc}} \ln (1 + K_{Tm}L) \quad \text{Equation 2}$$

In equation 1, the terms $T_{m0}$ and $T_m$ represent the melting temperatures of the nucleic acid without ligand and at ligand saturation. The number of drug molecules bound per duplex, $n$, was determined by CD monitored and fluorescence monitored titrations of ligand into duplex. Watson-Crick duplex enthalpy, $\Delta H_{wc}$, was determined by differential scanning calorimetry, DSC. The free drug concentration at the melting temperature, $L$, was estimated at one-half the total drug concentration, **Equation 3**. The terms in equation two are defined as follow; $x$ is the concentration of nucleic acid (20 µM) and $n$ represents the number of drug molecules per duplex, as previously discussed:

$$L = \left(\frac{x}{n}\right)(0.5) \quad \text{Equation 3}$$
Table 2.5. Thermodynamic profile of nucleic acid interactions with neomycin and ethidium bromide and DPA200.

<table>
<thead>
<tr>
<th></th>
<th>poly(dA):poly(rU)</th>
<th>poly(rA):poly(rU)</th>
<th>poly(dA-dT):poly(dA-dT)</th>
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<tbody>
<tr>
<td></td>
<td>neomycin</td>
<td>ethidium bromide</td>
<td>DPA200</td>
</tr>
<tr>
<td>$\Delta H_{wc}$ (kcal/mol)$^a$</td>
<td>3.84</td>
<td>3.84</td>
<td>3.84</td>
</tr>
<tr>
<td>$T_{m0}$ (°C)$^b$</td>
<td>43.0</td>
<td>43.0</td>
<td>43.0</td>
</tr>
<tr>
<td>$T_m$ (°C)$^b$</td>
<td>65.8</td>
<td>61.8</td>
<td>90.5</td>
</tr>
<tr>
<td>$n$ (L, µM)</td>
<td>6.5 (1.5)</td>
<td>4.6 (2.2)</td>
<td>9.7 (1.0)</td>
</tr>
<tr>
<td>$\Delta H_{15°C}$ (kcal/mol)$^d$</td>
<td>3.60±0.08</td>
<td>-5.57±0.05</td>
<td>4.44±0.03</td>
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<td>$\Delta H_{20°C}$ (kcal/mol)$^d$</td>
<td>2.73±0.08</td>
<td>-6.26±0.04</td>
<td>1.90±0.05</td>
</tr>
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<td>$\Delta H_{25°C}$ (kcal/mol)$^d$</td>
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<td>-6.69±0.04</td>
<td>1.05±0.02</td>
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<tr>
<td>$\Delta C_p$ (cal/mol•K)$^e$</td>
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<td>-339±16</td>
</tr>
<tr>
<td>$K_{T(20°C)}$ (M$^{-1}$)$^f$</td>
<td>(9.93±0.10)x10$^6$</td>
<td>(9.38±0.04)x10$^6$</td>
<td>(4.77±0.13)x10$^6$</td>
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<tr>
<td></td>
<td>neomycin</td>
<td>ethidium bromide</td>
<td>DPA200</td>
</tr>
<tr>
<td>$\Delta H_{wc}$ (kcal/mol)$^a$</td>
<td>6.34</td>
<td>6.34</td>
<td>6.34</td>
</tr>
<tr>
<td>$T_{m0}$ (°C)$^b$</td>
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<td>53.4</td>
<td>53.4</td>
</tr>
<tr>
<td>$T_m$ (°C)$^b$</td>
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<td>64.2</td>
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<tr>
<td>$n$ (L, µM)</td>
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<tr>
<td>$\Delta H_{15°C}$ (kcal/mol)$^d$</td>
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<td>$\Delta H_{25°C}$ (kcal/mol)$^d$</td>
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<td>(2.14±0.15)x10$^7$</td>
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<td>9.1 (1.1)</td>
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<tr>
<td>$\Delta H_{25°C}$ (kcal/mol)$^d$</td>
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<td>0.17±0.09</td>
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<tr>
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<td>-139±6</td>
<td>-104±12</td>
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<tr>
<td>$K_{T(20°C)}$ (M$^{-1}$)$^f$</td>
<td>(7.01±0.44)x10$^4$</td>
<td>(1.97±0.05)x10$^6$</td>
<td>(1.04±0.07)x10$^6$</td>
</tr>
</tbody>
</table>

$^a$Data obtained from DSC melting profiles. $^b$Data obtained from CD and UV thermal denaturation profiles. $^c$Data obtained from titrations, as outlined in text. $^d$ΔH is corrected binding heat, derived by integration of heat burst curve from the sample titration, followed by subtraction of the dilution head from the control titration. $^e$ΔCp and binding affinities as defined in text. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure 2.11. Fluorescence emission scans of the titration of DPA200 with various nucleic acids at 20 °C. A plot of fluorescence intensity at 594 nm vs r_{bd} values for each respective nucleic acids are located in panels (b), (d) and (f). A plot of fluorescence intensity at 594 with respect to r_{bd} was plotted, r_{bd} is the ratio of [base pair]/[drug]. DPA200 was titrated into the nucleic acids [1.0 µM/bp], poly(dA):poly(rU) (a), poly(rA):poly(rU) (c) and poly(dA-dT)_{2} (e), at various r_{bd} values. Excitation: 525 nm; emission: 550 – 700 nm; slits: 1.5 mm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
The binding constant at the melting temperature, $K_{Tm}$, was calculated. The binding constant at $T_m$ was extrapolated to a reference temperature ($T$) of 20 °C using the integrated van’t Hoff equation, **Equation 4** (113):

$$K_T = \frac{K_{Tm}}{e^{\frac{-\Delta H_{obs}}{R \left( \frac{1}{T_m} - \frac{1}{T} \right)}} e^{\frac{\Delta C_p T}{R \left( \frac{1}{T_m} - \frac{1}{T} \right)}} \left( \frac{T_m}{T} \right)^{\frac{\Delta C_p}{R}}}$$  \hspace{1cm} **Equation 4**

where, $\Delta H_{obs}$ is the observed binding enthalpy, at 20 °C, of ligand to the nucleic acid which is determined experimentally using ITC excess site titrations and $R$ is the gas constant.

The saturation point, defined as the drug molecules per duplex, $n$, was determined using CD (neomycin) and fluorescence (ethidium bromide and **DPA200**), **Table 2.5**. CD titrations were successful in the determination of the $n$ value for neomycin with poly(dA):poly(rU), poly(rA):poly(dT) and poly(dA-dT)$_2$ of 6.5 base pairs/ligand, 8.0 base pairs/ligand and 7.3 base pairs/ligand, respectively, **Appendix A, Figure A21**.

Fluorescence titrations of poly(dA):poly(rU) with ethidium bromide suggest a binding site size of 4.6 base pairs/ligand and the titration of ethidium bromide into poly(rA):poly(rU) and poly(dA-dT)$_2$ were determined to be 2.5 base pairs/ligand and 5.3 base pairs/ligand, **Appendix A, Figure A22**. Finally, fluorescence determined binding site sizes for **DPA200** interaction with poly(dA):poly(rU), poly(rA):poly(rU) and poly(dA-dT)$_2$ were 9.7 base pairs/ligand, 9.6 base pairs/ligand and 9.1 base pairs/ligand,
respectively, Figure 2.11. These values were confirmed with CD titrations, Appendix A, Figure A23.

Figure 2.12. UV thermal denaturation profiles of poly(dA):poly(rU) (a), poly(dA-dT)$_2$ (b) and poly(rA):poly(rU) (c, d). The panels represent the duplex (A) with neomycin (B), ethidium bromide (C) and NM (D) at saturation. poly(dA):poly(rU): neomycin ($r_{bd} = 6.5$), ethidium bromide ($r_{bd} = 4.6$) and NM ($r_{bd} = 9.7$). poly(rA):poly(rU): neomycin ($r_{bd} = 8.0$), ethidium bromide ($r_{bd} = 2.5$) and NM ($r_{bd} = 9.6$); poly(dA):poly(dT): neomycin ($r_{bd} = 7.3$), ethidium bromide ($r_{bd} = 5.3$) and NM ($r_{bd} = 9.1$). Ligands were added to the duplexes [20 µM/bp] at varying $r_{bd}$ (ratio of drug:base pairs) values. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.

Thermal stabilization experiments, at pH 5.5 and 100 mM NaCl, were expanded to include the DNA duplex – poly(dA-dT)$_2$ and the RNA duplex – poly(rA):poly(rU), Table 2.5, Figure 2.12. The poly(rA):poly(rU) – DPA200 complex melting temperature
was 23.6 °C higher than the melting temperature of the duplex in the absence of ligand.

**Figure 2.12.** Melting temperatures of the poly(rA):poly(rU) complexes with neomycin and ethidium bromide were 11.3 °C and 10.8 °C, higher than the native duplex melting temperature, respectively. The melting profile of the poly(rA):poly(rU) – neomycin complex showed a clear decrease in UV absorbance at 280 nm followed by an increase in UV absorbance upon increasing temperatures.

An equimolar mixture of poly(rA) and poly(rU) not only has the ability to form the duplex, poly(rA):poly(rU); this mixture can also form the poly(rA):2poly(rU) triplex at highertemperatures (114) or in the presence of magnesium ions (115). In fact, it has been suggested that the formation of poly(rA):2poly(rU) in an equimolar mixture of poly(rA) and poly(rU) is an artifact of poly(rA):poly(rU) formation and disappears very slowly in ~72 hours at 200 mM sodium concentrations (116). As seen in **Figure 2.12**, panel (d), in the poly(rA):poly(rU) – neomycin complex disproportionates into a three stranded sequence poly(rA):2poly(rU) and single stranded poly(rA), evidenced by the gradual decrease in absorbance upon increasing temperature. The triplex then denatures directly from the triplex to the single stranded poly(rA) and poly(rU), as observed in the increase in absorbance beginning at 75 °C. Literature precedent establishes the midpoint of this transition as the melting temperature of poly(rA):poly(rU) (114, 117).

Melting temperatures of the poly(dA-dT)_2 – ligand complexes were not significantly higher than the native DNA duplex melting temperature in the absence of ligands. The melting temperature of the poly(dA-dT)_2 – neomycin complex was 0.5 °C higher than the native duplex melting temperature. The melting temperature of the
poly(dA-dT)$_2$ – ethidium bromide complex was 0.8 °C. However, the complex between the DNA duplex and DPA200 afforded a higher melting temperature (3.8 °C) of the native duplex, Table 2.5.

$\Delta H_{wc}$, Watson-Crick duplex enthalpy, determined using DSC thermal denaturation experiments. Watson-Crick binding enthalpy was 3.84 kcal/mol for poly(dA):poly(rU), 6.34 kcal/mol for poly(rA):poly(rU) and 3.90 kcal/mol for poly(dA-dT)$_2$, Table 2.5, Appendix A, Figure A24. $L$, the free drug concentration at $T_m$, estimated at one-half the total drug concentration, was calculated from UV thermal denaturation experiments; see Equation 4, as previously discussed, Table 2.5.

As in pH 6.8 experiments, enthalpy values ($\Delta H$) were determined using binding enthalpies from excess site ITC titrations. In an effort to attain more accurate $\Delta C_p$ values, $\Delta H$ values were calculated at three temperatures 15 °C, 20 °C and 25 °C. The corrected binding enthalpies, $\Delta H_{(15 \, \text{oC})}$, $\Delta H_{(20 \, \text{oC})}$, and $\Delta H_{(25 \, \text{oC})}$, were determined following the subtraction of the dilution enthalpy from the binding enthalpy. Dilution enthalpies were calculated as previously discussed, Appendix A, Figure A25 – Figure A27, Table 2.5. $\Delta H$ values from the excesses site ITC titrations were used to calculate $\Delta C_p$ values, Equation 1. $\Delta C_p$ values for neomycin interaction with poly(dA):poly(rU) was calculated at -168 cal/mol K$^{-1}$. $\Delta C_p$ values for the interaction of neomycin with poly(rA):poly(rU) was more negative at -224 cal/mol K$^{-1}$ while the interaction with poly(dA-dT)$_2$ was far more positive than the other duplexes at -29 cal/mol K$^{-1}$, consistent with previously reported results (53, 118). Furthermore, when comparing the $\Delta C_p$ values determined at pH 6.8 and the value determined at pH 5.5, the magnitude of heat capacity
changes are consistent as binding-linked protonation leads to an increase in observed binding enthalpy (32).

$\Delta C_p$ values for the interaction of ethidium bromide with the nucleic acid duplexes were -112 cal/mol K$^{-1}$ (poly(dA):poly(rU)), -90 cal/mol K$^{-1}$ (poly(rA):poly(rU)) and -139 cal/mol K$^{-1}$ (poly(dA-dT)$_2$). The interaction of **DPA200** with poly(dA):poly(rU) displayed the lowest $\Delta C_p$ value at -339 cal/mol K$^{-1}$. The $\Delta C_p$ values for the interaction of **DPA200** with poly(rA):poly(rU) was more positive than the DNA:RNA hybrid duplex, -256 cal/mol K$^{-1}$, while the interaction with poly(dA-dT)$_2$ was significantly more positive, -104 cal/mol K$^{-1}$. As observed at pH 6.8, $\Delta C_p$ values for the interaction between the ligands and poly(dA):poly(rU) suggest strong contributions from both the intercalative and major groove binding moieties. The $\Delta C_p$ value for the interaction of **DPA200** with poly(dA):poly(rU) and is larger than the sum of the contributions from neomycin and ethidium bromide.

$\Delta C_p$ values were used to calculate binding affinities ($K_T$) for the interactions of neomycin, ethidium bromide and **DPA200** with poly(dA):poly(rU), poly(rA):poly(rU) and poly(dA-dT)$_2$, **Table 2.5**. The binding affinity for the interaction of **DPA200** and poly(dA):poly(rU) was $4.77 \times 10^{10}$ M$^{-1}$; this was five-fold higher than observed with neomycin or ethidium bromide, $9.93 \times 10^6$ M$^{-1}$ and $9.38 \times 10^6$ M$^{-1}$, respectively. The binding affinity for the interaction of **DPA200** and poly(rA):poly(rU), was around ten-fold lower than poly(dA):poly(rU). The binding affinity for the interaction of neomycin and poly(rA):poly(rU) was higher, $2.56 \times 10^7$ M$^{-1}$, than observed for the interaction with poly(dA):poly(rU). The affinity of ethidium bromide for poly(dA):poly(rU) was similar
to the affinity of ethidium bromide for poly(dA):poly(rU). In an effort to compare the affinity of DPA200 to other nucleic acid structures, K_T values were calculated for both the RNA and DNA duplexes, Table 2.5. The conjugate binds the DNA duplex poly(dA-dT)_2 with an affinity of 1.04 x 10^6 M^{-1} and the RNA duplex poly(rA):poly(rU) with an affinity of 2.14 x 10^9 M^{-1}. The affinity for the RNA duplex, by DPA200, is higher than both neomycin (2.56 x 10^7 M^{-1}) and ethidium bromide (9.98 x 10^6 M^{-1}). The conjugate, when binding the duplex DNA, displays an increased affinity over neomycin, 7.01 x 10^4 M^{-1}, a duplex which is bound by ethidium bromide at 1.97 x 10^5 M^{-1}. Compound DPA200 clearly displays the power of conjugation and expansion of aminoglycoside based binding to non-traditional targets. In fact, not only does the conjugate bind non-traditional target, poly(dA):poly(rU), the affinity for this duplex is higher than neomycin binding the 16S A-site RNA (9.1x10^8 M^{-1}) (57).
CONCLUSIONS

The data shown in chapter two illustrates the power of conjugating two ligands that bind to a receptor at different sites. Conjugation of neomycin, a major groove binding ligand, to methidium chloride, a deriviative of ethidium bromide affords a conjugate which displays a clear preference for the DNA:RNA hybrid, poly(dA):poly(rU). Competition dialysis results were confirmed by UV thermal denaturation experiments on a representative class of nucleic acids. DPA200 preference for poly(dA):poly(rU) was corroborated by the magnitude of thermal stabilization afforded by the conjugate on poly(dA):poly(rU); larger than stabilization observed by DPA200 on other nucleic acids. In fact, a synergistic combination of the two techniques, competition melting, further illustrates the binding preference, as poly(dA):poly(rU) was the only nucleic acid stabilized by DPA200 in low micro molar amounts.

UV thermal denaturation studies of our conjugate clearly show DPA200 is a more powerful tool when binding nucleic acids than the individual constituent parts. When binding poly(dA):poly(rU), neomycin stabilizes the duplex by 24.9 °C, ethidium bromide binds the duplex by 20.8 °C, while DPA200 affords an amazing 44.9 °C, see text for buffer conditions. This observation even holds true when binding nucleic acids in which DPA200 displays a lower observed preference for, poly(rA):poly(dT) and poly(dA-dT)_2.

Finally, due to the high affinity our conjugate displays for poly(dA):poly(rU), we were unable to use traditional methods (ITC titrations or fluorescence titrations) of binding constant determination. Using the ΔT_m method for binding constant determination allowed us to compare the affinity of DPA200 to neomycin and ethidium
bromide when binding poly(dA):poly(rU) and poly(rA):poly(dT). Even though the studies, reported within, were conducted at pH 6.8 and subsequent binding affinities were intrinsically overestimated, it was clear that the affinity of DPA200 for either DNA:RNA hybrid was higher than neomycin or ethidium bromide. By parsing the binding affinity from effects of drug protonation the true power of conjugate was resolved.

When binding DNA:RNA hybrids, neomycin binds to the hybrid duplex with high $10^6$ M$^{-1}$ affinity, while ethidium bromide also binds the hybrid duplex with high $10^6$ M$^{-1}$. The conjugate, DPA200, shows a 100,000 fold improvement, with an affinity of $4.77 \times 10^{10}$ M$^{-1}$. Even though the conjugate does not display the energetic additivity estimated from the individual binding moieties, the sub-nanomolar binding affinity remains very significant and far exceeds the affinities of any known DNA:RNA binding drugs.

The conjugate reflects the first example of an aminoglycoside ligand that binds to a nucleic acid target with affinities higher than the nanomolar affinities shown for the eubacterial A-site. The work will provide a novel paradigm for aminoglycoside-based recognition of DNA:RNA hybrid structures with therapeutic applications (telomerase and RNAse H inhibitors). A recent report cites a critical need for development of such high affinity DNA:RNA binders (119).
REFERENCES


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CHAPTER III

ADVANCEMENTS IN DNA RECOGNITION: DEVELOPMENT OF A TETRAMER POLYAMIDE – NEOMYCIN CONJUGATE LIBRARY

INTRODUCTION

DNA is the carrier of genetic information and the dominant nucleic acid structure in organisms. DNA is subject to a number of biological processes such as translation and transcription. Ultimately, the genetic information stored in DNA leads to cellular growth. Friedrich Miescher isolated DNA from the nuclei of human blood cells in the late 1860s (1). Following the seminal report which proposed the double helical nature of DNA (2), reports on the polymorphic nature of DNA began appearing. Unlike DNA’s nucleic acid counterpart RNA, which exists as an A-form conformation with slight variations, DNA is capable of existing in three distinct conformational forms (3). Two of the representative forms, A-form and B-form conformation, are both right-handed helixes (4) while a third conformation, Z-form, exists as a left-handed helix (5). Our work focuses on the right-hand helical forms of DNA, A-form and B-form.

A-form and B-form DNA share conformational similarities. Both conformations feature a double helix formed by two single strands of DNA that are oriented anti-parallel to each other. Each strand features 2’-deoxy ribose sugars linked by a phosphate group which encircles the central core of hydrogen bonded base pairs. In DNA the pyrimidine bases (thymine and cytosine) and the purine bases (adenine and guanine) are hydrogen
bonded as defined by Watson and Crick (2). The topologies are both identical with each conformation featuring a major and minor groove (6).

However, differences between the two conformations are observed in the overall shape of the helix. B-form DNA is believed to be the dominant biological conformation and utilizes well order water molecules and biological cations to form a hydrated duplex. B-form DNA contains furanose conformations in a $C_2$-endo conformation and features 10 base pairs per helical turn, a major groove which is wide and shallow and a minor groove which is narrow and deep. Furthermore, B-form DNA is conformationally flexible and features numerous polymorphs defined by B-, B'-, C-, C'-, C''-, D- and E (3). In contrast, A-form DNA is conformationally rigid. Generally associated with a dehydrated form of the duplex, A-form DNA is characterized by 12 – 14 base pairs per helical turn, which is a result of the smaller axial rise per residue, 2.5 Å, than observed in B-form DNA, 3.4 Å. This, in turn, affords a helix with a major groove that is more narrow and deeper and a minor groove that is wider and shallower that the B-form counterpart. In some cases A-form DNA has been crystallized with furanose sugar puckers in a $C_3$-endo conformation (7). However, as DNA is polymorphic, it is important to note that DNA sugar pucker may exist not only in $C_3$-endo/$C_2$-endo extremes, but also anywhere within those pseudorotational extremes.

A number of literature reports suggest a strong correlation between DNA sequence and localized conformation. DNAs of like composition but vary in the sequences of bases have been shown to exhibit drastically different structural properties (8). For instance, poly(dA):poly(dT) is generally accepted as a B-form helix at high
humidity (9), while poly(dG):poly(dC) demonstrates strong A-form characteristics (10). Even when breaking homo-pyrimidine and homo-purine strands with alternating bases, poly(dA-dT)$_2$ and poly(dG-dC)$_2$, the duplexes remain conformationally distinct. Poly(dA-dT)$_2$ is highly dependent on relative humidity (9, 11) and is capable of existing between B-form and a ‘metastable A-form’ (3). Poly(dG-dC)$_2$, on the other hand, is exclusively A-form, even at high humidity (9).

Isolation of purine bases and pyrimidine bases to a single strand uniquely affects DNA conformation. Poly(dA-dG):poly(dC-dT) exists primarily in B-form unable to undergo the B-form to A-form transition, while poly(dA-dC):poly(dG-dT) is capable of existing in a number of conformations such as A-form, B-form and even the left handed Z-form (12)). Furthermore, even though mixed base sequence DNA displays predominant B-form characteristics, the ability to drive the conformation of these duplexes to A-form, through dehydration, becomes increasingly difficult as the proportion of G/C content in the helix drops below 30% (13). It is important to note, although these sequence dependent conformations are present, these conformational differences are localized and generally do little to influence the overall structure of natural occurring DNAs (14)(13)(13)(14)).

The biological significance for targeting A-form DNA is dependent on the relationship between native DNA and the basic mechanisms involved in the flow of genetic information. A strong argument has been made that suggests our current DNA based world was preceded by life based on A-form RNA (15). As such, the mechanisms required to copy genetic information most likely proceed through A-form nucleic acids.
For example, transcription of RNA to DNA occurs through DNA:RNA hybrids which are A-form (16, 17). And a case can be made that a transition from native B-form DNA to A-form DNA occurs during protein – DNA interactions. The cyclic AMP receptor protein induces a B-form to A-form transition in its DNA target (5).

Furthermore, template DNA is induced to A-form upon the binding of many polymerases (18). Proteins which induce A-form include HIV-1 reverse transcriptase (19, 20, 21), polymerase β (22), Taq polymerase (23), Bacillus polymerase I (24), T7 polymerase (25), DNase I (26, 27), I-PpoI homing endonuclease (28), PvuII restriction endonuclease (29, 30), EcoRV endonuclease (31), HhaI methyltransferase (32) and the chromosomal protein Sac7d (33). It has been suggested that induction of A-form facilitates protein binding as much the shallower minor groove of A-form exposes functional groups. These functional groups, such as the O2 of pyrimidines and the N3 of purines, are essential in DNA recognition (6). Thus, the induction of A-form is a necessary requirement influencing replication and transcription of genomic DNA.

Currently, we seek an aminoglycoside based approach to nucleic acid recognition. Implications of this approach include advancements in the area of drug development as well as a deepened understanding of the requirements for nucleic acid recognition. Our group has already identified a number of novel aminoglycoside conjugates for the recognition of A-form nucleic acids. Through the covalent attachment of neomycin to pyrene (34), BQQ (35) and methidium chloride (16, 36) we achieved increased affinity to a number of A-form nucleic acid structures.
At this time, we desire to increase the number of molecules which bind A-form DNA. Unlike B-form recognition, there are very few ligands which select for A-form characteristics (5, 18). We have previously demonstrated the ability to target B-form DNA through novel Hoechst 33258 – neomycin conjugates (37, 38). Covalent attachment of neomycin, a major groove binder, to Hoechst 33258, a minor groove binder, yielded conjugates capable of significantly enhancing the thermal stability of B-form DNA through dual recognition. Using this approach as a paradigm for A-form recognition we envision the development of a library of N-methylpyrrole and N-methylimidazole based polyamide – neomycin conjugates which target A-form DNA.

Aminoglycoside antibiotics are carbohydrate scaffolds composed of amino sugars covalently linked to a central hexose ring. Streptomycin, an aminoglycoside isolated in 1944 (39), introduced the world to aminoglycosides and was the first antibiotic effective against tuberculosis. An additional number of streptamine relatives were introduced which gave rise to the streptomycin family of antibiotics (6). Aminoglycoside development during the subsequent 20 years focused on the following two areas: increasing population of the aminoglycoside class of molecules and deciphering aminoglycoside mode of action.

Second generation aminoglycosides included the 2-deoxystreptamine (DOS) family of aminoglycosides, neomycin, neamine, kanamycin A, kanamycin B and paromomycin. Development in understanding aminoglycoside mode of action identified protein synthesis as the primary target for the antibacterial action displayed by streptomycin (40, 41, 42). Further studies demonstrated the 30S subunit of ribosomal
RNA (rRNA) was the natural target of streptomycin (42). Binding of aminoglycosides to the 30S subunit of rRNA induces a loss of fidelity during translation (40, 41) and occurs through electrostatic forces (43, 44, 45).

Since the 1990s, aminoglycoside antibiotic research has seen resurgence. A major aspect to this revival focused on exploiting the flexible scaffold of polycationic charges found on aminoglycosides to bind a number of traditional (RNA) and non-traditional nucleic acid structures. To this end, reports have shown the ability of aminoglycosides to preferentially bind a number of other RNA targets such as group I introns, a hammerhead ribozyme, the RRE transcriptional activator region from HIV, the 5’-untranslated region of thymidylate synthase a variety of RNA aptamers from in vitro selection and human mRNAs (45, 46, 47). Furthermore, neomycin binding a large number of non-traditional nucleic acid targets which include the RNA triplex (48), DNA:RNA hybrid duplex (16, 36, 49), RNA duplex (50), DNA triplex (34, 35, 48, 51), A-form DNA duplex (52) as well as the DNA tetraplex (53) have been reported. Our group suggests the ability to bind such a diverse group of nucleic acid structures lies in the ability of these nucleic acid structures to adopt A-form characteristics (3, 54, 55, 56).

Lexitropsins are a sub-class of DNA minor groove binding ligands which specifically bind DNA sequences (57). Naturally occurring lexitropsins distamycin A and netropsin consist of a polyamide scaffold of repeating pyrrole monomers (58, 59). Considerable scholastic attention has been paid to DNA binding by netropsin and distamycin A. Netropsin and distamycin A bind DNA in a sequence-specific manner (57, 60, 61). They bind through hydrogen bonds with minor groove base functionalities. For
example, when disatamycin A binds A/T rich DNA, the polyamide carboxyamides hydrogen donor groups interact directly with adenine N3 and thymidine O2 acceptor groups, Figure 3.1 (62).

**Figure 3.1.** Mode for antiparallel 2:1 distamycin A binding 5'-AAATT-3'.

Furthermore, repulsion between the amino group on the 2-position on guanine and the 3-position of the pyrrole monomers drive the sequence selectivity of netropsin and distamycin A (61). Binding affinities for netropsin and distamycin A to A/T rich DNA are moderate at ~10^6 M^{-1} (63). Furthermore, distamycin A forms a 2:1, distamycin A:DNA, complex in the minor groove where a second molecule aligns head-to-tail, antiparallel, with respect to the first distamycin A, Figure 3.1 (64).
Expanding the polyamide scaffold to include $N$-methylimidazole monomers affords differentiation of G/C base pairs from A/T base pairs (60, 65, 66, 67, 68, 69, 70). Replacement of the 3-position hydrogen with a hydrogen bond acceptor at the 3-position of imidazole overcomes repulsion between the amino group found on the 2-position of guanine and the 3-position of pyrrole (62, 71, 72, 73). $N$-methylpyrrole and $N$-methylimidazole based polyamides bind the minor groove of DNA through direct contacts with the center of each base pair, \textbf{Figure 3.2} (74).
In a 1:1 polyamide:DNA binding mode, a pyrrole monomer distinguishes A/T base pair from G/C base pairs, while an imidazole monomer distinguishes a G/C base pair from an A/T base pair, Figure 3.2 (75). In a 2:1 polyamide:DNA binding mode, a pyrrole/imidazole pairing across the floor of the minor groove distinguishes a C/G base pair and an imidazole/pyrrole pairing distinguishes a G/C base pair. A pyrrole/pyrrole pairing distinguishes and A/T or T/A base pairs from G/C or C/G base pairs, however the pyrrole/pyrrole pairing is incapable of distinguishing an A/T base pair from a T/A base pair, Figure 3.3 (62). While the binding affinity for N-methylpyrrole and N-methylimidazole based polyamides are sequence dependent, the affinity of HImImPyPyβDp for the target sequence 5’-TGGCCA-3’ was reported $K_a$ of $2.4 \times 10^7$ M$^{-1}$ (76).
EXPERIMENTAL

Materials:

**Nucleic Acids.** 16 mer DNA oligomers, 5’-AnGmCmTn-3’ (where n = 2, 3, 4, 5 and 6; m = 6, 5, 4, 3 and 2), were purchased from Integrated DNA Technologies (Coralville, IA). Concentrations were determined by UV absorbance using extinction coefficients provided by IDT. The self-complementary duplexes were pre-formed at 20 µM/duplex, by heating at 90 °C for 10 min, followed by slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h. Target DNA duplexes (target duplex 1 and target duplex 2) were purchased from Eurofins MWG Operon (Huntsville, AL). Concentrations were determined by UV absorbance using extinction coefficients provided by Operon. The duplexes were pre-formed at 20 µM/duplex, by heating at 90 °C for 10 min, followed by slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h.

**Chemicals.** Neomycin B (sulfate salt) was purchased from ICN pharmaceuticals and used without further purification (both synthesis and binding experiments); reagents were purchased from Acros organics. Solvents were purchased from VWR with the exception of pyridine, DMF and 1,4-dioxane which were purchased from Acros. Reaction solvents were distilled accordingly; dichloromethane and pyridine were distilled over calcium hydride and ethanol was distilled over sodium metal. All non-commercially available
intermediates were synthesized as previously reported: heterocyclic monomers (77) and neomycin derivatives (36, 37, 78, 79).

Methods:

UV Thermals Denaturation Experiments. All experiments were conducted using a Cary 100E UV/Vis spectrophotometer (Varian Inc.; Palo Alto, CA) equipped with a thermoelectrically controlled 12-cell holder. All samples were analyzed in quartz cells (1 cm pathlength). Lamp stability and wavelength alignment were checked prior to each experiment. UV melting experiments for the denaturation of the 5'-A<sub>n</sub>G<sub>m</sub>C<sub>m</sub>T<sub>n</sub>-3' duplexes, native and with neomycin, were conducted as follows. The self-complementary duplexes were pre-formed at 20 µM/duplex, by heating at 90 °C for 10 min, followed by slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h. Samples were prepared by diluting the stock solutions to 2 µM/duplex. Ligands were added and the solutions were incubated at 4 °C for 12 h. Samples were degassed prior to analysis. The samples were slowly heated from 10 °C to 90 °C at 0.2 °C/min. Buffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

CD Spectropolarimetry Titrations. Circular dichroism (CD) experiments were conducted at 20 °C using a Jasco J-810 spectropolarimeter (Jasco Inc.; Easton, MD). CD observed titration of ligands into the 5'-A<sub>6</sub>G<sub>2</sub>C<sub>2</sub>T<sub>6</sub>-3' and 5'-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3' duplexes were conducted as follows. The self-complementary duplexes were pre-formed at 20 mM/duplex by heating at 90 °C for 10 min, followed by a slow annealing at 0.2 °C/min.
and incubation at 4 °C for 12 h. The duplex was diluted to 40 mM/bp and ligands were added at varying $r_{dd}$ (ratio of drug:duplex) values. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

**Fluorescence Intercalator Displacement Assay.** 96 well equilibrium binding experiments were conducted using a Varian Eclipse Fluorescence Spectrometer (Varian Inc.; Palo Alto, CA) equipped with a 96 well plate reader. DNA duplexes were pre-formed at 5 μM/duplex by heating at 90 °C for 10 min, followed by cooling to 10 °C at 0.2 °C/min and incubation at 4 °C for 12 h. Buffer was added to each well followed by ethidium bromide (7 μM). DNA duplex was added to the individual wells such that the final concentration of DNA was 1 μM. Ligands were added to each well at a ratio of drug to duplex, $r_{dd} = 1.0$, allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. In the histogram, the values represent the percent decrease in fluorescence upon each addition of ligand. Fluorescence parameters; excitation: wavelength = 525 nm, slits = 20, filter = 335-620 nm; emission: wavelength = 550 nm; slits = 20, filter = 550-800 nm; PMTV = 610 nm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 50 mM NaCl, pH 7.0.

**Fluorescence Intercalator Displacement Titrations.** Equilibrium binding experiments were conducted using a Varian Eclipse Fluorescence Spectrometer (Varian Inc.; Palo
Alto, CA) at 20 °C. Duplexes were pre-formed at 20 mM/duplex by heating at 90 °C for 10 min, followed by a slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h. The DNA duplex was diluted to 1.0 μM/duplex, pre-bound by thiazole orange, 7 μM and allowed to equilibrate for a period of 10 min prior to addition of ligand. Ligand was added at various r_{dd} values. Fluorescence parameters; excitation: wavelength = 504, slits = 10, filter = auto; emission: wavelength = 510-610 nm; slits = 10, filter = auto; PMTV = 580 nm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 50 mM NaCl, pH 7.0.

**Determination of Binding Constants by Scatchard Analysis.** The ∆F was plotted versus molar equivalents of ligand and the ∆F_{sat} (binding stoichiometry) was determined mathematically by simultaneous solving the equations representing the pre- and post-saturation regions of the titration curve. A Scatchard plot was generated utilizing equations (1)–(3), generated where ∆F/[ligand] was plotted versus ∆F. The slope of the points representing the region immediately preceding complete saturation of the system provided -K. In these equations, [ligand] = concentration of ligand, [DNA]_{T} = total concentration of DNA, X = molar equiv of ligand versus DNA, ∆F_{x} = change in fluorescence, and ∆F_{sat} = change in fluorescence at the point where DNA is saturated with ligand.

\[
\frac{\Delta F_{x}}{\Delta F_{sat}} \frac{1}{X} = \text{Fraction of DNA - ligand complex} \quad (1)
\]
\[ 1 - \left( \frac{\Delta F_x}{\Delta F_{sat}} \right)_X = \text{Fraction of ligand} \quad (2) \]

\[ [\text{DNA}]_T \left[ X - \frac{\Delta F_x}{\Delta F_{sat}} \right] = [\text{ligand}] \quad (3) \]

Table 3.1. Stoichiometry table for the synthesis of HImImPyPyCONH(CH$_2$)$_n$-tri-CH$_2$OCH$_2$-neomycin-hexahydrochloride, DPA$_{208}$ – DPA$_{213}$, n = 2, 3, 5, 6, 8 and 10.

<table>
<thead>
<tr>
<th>Series I</th>
<th>Compound (##)</th>
<th>DPA13</th>
<th>‘HImImPy PyCONH (CH$_2$)$_n$N$_3$’</th>
<th>CuI</th>
<th>DIPEA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>amnt. (mg)</td>
<td>µmol</td>
<td>amnt. (mg)</td>
<td>µmol</td>
<td>amnt. (mg)</td>
</tr>
<tr>
<td>DPA$_{208}$</td>
<td>5.6</td>
<td>4.1</td>
<td>2.3</td>
<td>4.1</td>
<td>1.6</td>
</tr>
<tr>
<td>DPA$_{209}$</td>
<td>5.8</td>
<td>4.3</td>
<td>2.5</td>
<td>4.3</td>
<td>1.7</td>
</tr>
<tr>
<td>DPA$_{210}$</td>
<td>5.5</td>
<td>4.1</td>
<td>2.5</td>
<td>4.1</td>
<td>1.6</td>
</tr>
<tr>
<td>DPA$_{211}$</td>
<td>5.5</td>
<td>4.0</td>
<td>2.5</td>
<td>4.0</td>
<td>1.6</td>
</tr>
<tr>
<td>DPA$_{212}$</td>
<td>5.4</td>
<td>4.0</td>
<td>2.6</td>
<td>4.0</td>
<td>1.5</td>
</tr>
<tr>
<td>DPA$_{213}$</td>
<td>5.3</td>
<td>3.9</td>
<td>2.6</td>
<td>3.9</td>
<td>1.5</td>
</tr>
</tbody>
</table>

(DPA$_{208}$ – DPA$_{213}$). To a solution of N-Boc-neomycin-tri-alkyne DPA13 in toluene (1.0 mL) was added a solution consisting of CuI and DIPEA in toluene (500 µL). To this solution was a solution containing a single azide functionalized tetramer polyamides DPA$_{208}$ – DPA$_{207}$ in toluene (500 µL). The reaction mixture was allowed to stir at room temperature for 24 h, and then was concentrated in vacuo. The resulting residue was purified by flash chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugates. The solid was then suspended in 4 N HCl in dioxane (5 mL) and the reaction was allowed to stir at room
temperature for 30 min. The solution was concentrated in vacuo and the resulting solid was washed with copious amounts of CH$_2$Cl$_2$ and lyophilized to dryness to afford conjugates DPA208 – DPA213 as hydrochloride salts. Coupling and deprotection steps were near quantitative and the subsequent yields reflect the overall yield for both steps. The reported $^1$H NMR and MALDI spectra represent the deprotected compounds. Furthermore, representative $^1$H NMR and MALDI spectra of the N-Boc protected conjugate of DPA208 are also included.

(N-Boc DPA208): N-Boc DPA208 (7.9 mg, 99%) was afforded as a light brown solid: $^1$H NMR (500 MHz, CDCl$_3$, N-Boc protected) $\delta$ 8.17 (br, 2H), 7.59 (s, 1H), 7.48 (s, 1H), 7.38 (s, 2H), 7.17 (s, 1H), 7.09 (s, 1H), 6.98 (s, 1H), 6.79 (s, 1H), 6.70 (s, 1H), 6.07 (s, 1H), 5.95 (s, 1H), 5.28 (br, 1H), 5.16 (s, 1H), 4.90 (s, 1H), 4.18 (s, 2H), 4.04 (s, 6H), 3.88 (s, 3H), 3.75 (s, 3H), 3.96 (s, 1H), 3.82-3.90 (m, 3H), 3.76 (s, 1H), 3.64-3.72 (m, 4H), 3.48 (m, 6H), 3.32 (br, 2H), 3.23 (m, 2H), 3.19-3.30 (m, 5H), 2.23 (m, 1H), 1.94 (m, 1H), 1.56 (br, 4H), 1.38–1.46 (m, 54H); MALDI-TOF (N-Boc protected) $m/z$ calcd C$_{83}$H$_{126}$N$_{22}$O$_{29}$ 1896.02, found [M + Na]$^+$ 1919.22;

(DPA208): DPA208 (5.5 mg, 99%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O, deprotected) $\delta$ 8.08 (s, 1H), 7.91 (s, 1H), 7.25 (s, 1H), 7.10 (s, 1H), 7.02 (s, 1H), 6.98 (s, 1H), 6.88 (s, 1H), 6.69 (s, 1H), 6.59 (s, 1H), 6.07 (s, 1H), 5.56-5.55 (s, 1H), 5.27-5.24 (s, 1H), 5.16-5.15 (s, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.21 (m, 1H), 4.13-4.10 (m, 2H), 4.04 (s, 3H), 4.01 (s, 3H), 3.98-3.92 (m, 1H), 3.88-3.86 (m, 4H),
3.85 (s, 3H), 3.84-3.82 (m, 1H), 3.81-3.77 (m, 2H), 3.76 (s, 1H), 3.74-3.72 (m, 1H), 3.68-
3.66 (m, 1H), 3.65-3.63 (m, 4H); 3.63-3.57 (m, 1H), 3.50-3.46 (m, 1H), 3.40-3.37 (m,
1H), 3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.31 (m, 3H), 3.30-3.25 (m, 2H), 3.24-3.23
(m, 1H), 3.23-3.18 (m, 2H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.23-2.20 (m, 1H),
1.72-1.63 (m, 1H); MALDI-TOF (deprotected) m/z calcd C$_{53}$H$_{78}$N$_{22}$O$_{17}$ 1295.32, found

**DPA209**: DPA209 (5.6 mg, 94%) was afforded as a light brown solid: $^1$H NMR (500
MHz, D$_2$O) $\delta$ 8.06 (s, 1H), 7.95 (s, 1H), 7.45 (s, 1H), 7.38 (s, 1H), 7.15 (s, 1H), 7.10 (s,
1H), 7.01 (s, 1H), 6.95 (s, 1H), 6.88 (s, 1H), 6.38 (s, 1H), 5.59-5.55 (m, 1H), 5.33-5.30
(m, 1H), 5.21-5.18 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.25-4.20 (m, 1H),
4.10-4.09 (m, 2H), 3.93-3.88 (m, 7H), 3.88 (s, 2H), 3.87-3.85 (m, 7H), 3.83-3.81 (m,
1H), 3.81 (s, 3H), 3.80-3.79 (m, 2H), 3.78 (s, 1H), 3.71-3.70 (m, 1H), 3.70-3.66 (m, 4H),
3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.47-3.45 (m, 1H), 3.41-3.38 (m, 2H), 3.38-3.37
(m, 1H), 3.37-3.30 (m, 2H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.30 (m, 1H), 3.30-
3.24 (m, 2H), 3.24-3.22 (m, 1H), 3.22-3.15 (m, 3H), 3.14 3.12 (s, 2H), 2.21-2.17 (m, 1H),
1.82-1.69 (m, 2H), 1.73-1.61 (m, 1H); MALDI-TOF m/z calcd C$_{54}$H$_{80}$N$_{22}$O$_{17}$ 1309.35,
found [M + Na]$^+$ 1333.01.

**DPA210**: DPA210 (5.9 mg, 97%) was afforded as a light brown solid: $^1$H NMR (500
MHz, D$_2$O) $\delta$ 8.18 (br, 1H) 8.10 (br, 1H), 8.10 (br, 1H), 7.46 (s, 1H), 7.41 (s, 1H), 7.30 (s,
1H), 7.28 (s, 1H), 7.18 (s, 1H), 6.99 (s, 1H), 6.93 (s, 1H), 6.24 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31
(m, 1H), 5.20-5.18 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.15-4.11 (m, 2H), 4.02-3.98 (m, 2H), 3.97 (s, 6H), 3.91-3.90 (m, 1H), 3.88 (s, 3H), 3.88-3.86 (m, 1H), 3.85-3.80 (m, 2H), 3.80 (s, 3H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.60-3.53 (m, 4H), 3.46-3.45 (m, 1H), 3.40-3.38 (m, 2H), 3.37-3.30 (m, 3H), 3.34 (s, 2H), 3.33-3.31 (m, 2H), 3.30-3.29 (m, 1H), 3.27-3.25 (m, 2H), 3.25-2.23 (m, 1H), 3.21-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.27-2.21 (m, 1H), 1.70-1.61 (m, 1H), 1.58-1.53 (m, 2H), 1.52-1.47 (m, 2H), 1.32-1.28 (m, 2H); MALDI-TOF m/z calcd C_{56}H_{84}N_{22}O_{17} 1334.40, found [M + Na]^+ 1360.99.

(DPA211): DPA211 (6.0 mg, 96%) was afforded as a light brown solid: ^1^H NMR (500 MHz, D_{2}O) δ 8.11 (s, 1H), 8.07 (s, 1H), 7.48 (s, 1H), 7.41 (s, 1H), 7.32 (s, 1H), 7.22 (s, 1H), 7.07 (s, 1H), 6.95 (s, 1H), 6.90 (s, 1H), 6.04 (s, 1H), 5.56-5.55 (m, 1H), 5.27-5.24 (m, 1H), 5.16-5.15 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.21 (m, 1H), 4.13-4.10 (m, 2H), 4.01 (s, 2H), 3.98 (s, 3H), 3.98-3.92 (m, 4H), 3.88-3.86 (m, 1H), 3.85 (s, 3H), 3.84-3.82 (m, 1H), 3.82 (s, 6H), 3.81-3.77 (m, 2H), 3.76 (s, 1H), 3.74-3.72 (m, 1H), 3.68-3.66 (m, 1H), 3.65-3.60 (m, 4H), 3.63-3.57 (m, 1H), 3.50-3.46 (m, 1H), 3.40-3.37 (m, 1H), 3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 4H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 5H), 3.13-3.11 (s, 2H), 2.23-2.20 (m, 1H), 1.72-1.63 (m, 1H), 1.55-1.50 (m, 4H), 1.36-1.33 (m, 4H); MALDI-TOF m/z calcd C_{57}H_{86}N_{22}O_{17} 1351.43, found [M + Na]^+ 1375.42.
(DPA212): **DPA212** (5.9 mg, 98%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.11 (s, 1H), 7.94 (s, 1H), 7.47 (s, 1H), 7.41 (s, 1H), 7.38 (s, 1H), 7.30 (s, 1H), 7.02 (s, 1H), 6.83 (s, 1H), 6.08 (s, 1H), 5.92 (s, 1H), 5.57-5.56 (m, 1H), 5.31-5.30 (m, 1H), 5.18-5.17 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.17-4.15 (m, 2H), 4.08 (s, 3H), 4.06 (s, 3H), 4.00-3.96 (m, 1H), 3.92 (s, 1H), 3.91-3.88 (m, 1H), 3.88 (s, 3H), 3.86-3.85 (m, 4H), 3.84-3.79 (m, 8H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.73-3.70 (m, 4H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.45-3.44 (m, 1H), 3.39-3.31 (m, 3H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.28 (m, 1H), 3.27-3.24 (m, 2H), 3.24-3.20 (m, 2H), 3.23-3.22 (m, 1H), 3.21-3.17 (m, 3H), 3.15-3.10 (s, 2H), 2.25-2.22 (m, 1H), 1.75-1.64 (m, 1H), 1.57-1.50 (m, 4H), 1.31-1.25 (m, 4H), 1.28-1.20 (m, 4H); MALDI-TOF m/z calcd C$_{59}$H$_{90}$N$_{22}$O$_{17}$ 1379.48, found [M + Na]$^+$ 1403.11.

(DPA213): **DPA213** (5.9 mg, 98%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.06 (s, 2H), 7.49 (s, 1H), 7.41 (s, 1H), 7.23 (s, 2H), 7.09 (s, 1H), 6.98 (s, 1H), 6.81 (s, 1H), 6.10 (s, 2H), 5.59-5.55 (m, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.27 (s, 3H), 4.26 (s, 3H), 4.25-4.20 (m, 1H), 4.08 (s, 3H), 4.10-4.09 (m, 2H), 3.99 (s, 6H), 3.93-3.88 (m, 1H), 3.87-3.85 (m, 1H), 3.83-3.81 (m, 5H), 3.80-3.79 (m, 4H), 3.78 (s, 1H), 3.71-3.70 (m, 1H), 3.70-3.61 (m, 4H), 3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.47-3.45 (m, 1H), 3.39-3.29 (m, 2H), 3.38-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.30 (m, 1H), 3.30-3.24 (m, 4H), 3.24-3.22 (m, 1H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.21-2.17 (m, 1H), 1.73-1.61 (m, 1H),
1.60-1.47 (m, 4H), 1.31-1.20 (m, 6H); MALDI-TOF $m/z$ calcd C$_{61}$H$_{94}$N$_{22}$O$_{17}$ 1407.54, found [M + 2Na]$^+$ 1455.01.
RESULTS AND DISCUSSION

Design of Tetramer Polyamide – Neomycin Conjugates. Our research group has previously demonstrated the ability to target DNA through novel Hoechst 33258 – neomycin conjugates (37, 38). Through the conjugation of neomycin to minor groove binding Hoechst 33258, we were able to target B-form DNA. Utilizing this approach, we seek to develop a model for A-form DNA recognition through the conjugation of neomycin to a minor groove binding ligand which recognizes GC sequences. Literature suggests the propensity of DNA to adopt A-form characteristics is sequence-dependent; increased amounts of continuous guanine or cytosine content increases propensity of the duplex to adopt A-form characteristics (3, 9, 11). Polyamides based on N-methylpyrrole and N-methylimidazole have been shown to preferentially bind G/C base pairs over A/T base pairs (80, 81, 82, 83, 84).

A four-ring ‘tetramer’ polyamide will allow for the recognition of G/C minor groove DNA (71, 76). For example, the tetramer polyamide HImImPyPy- allows us to target DNA which contains the core sequence 5’-GGAA-3’ in a 1:1 binding mode (85). Furthermore, when binding head-to-tail in a 2:1 fashion, the tetramer polyamide allows us to potentially target the core sequence 5’-GGCC-3’ (71, 72, 76, 86). Therefore, conjugation of neomycin to the tetramer polyamide will allow us to potentially bind DNA which contains the sequence 5’-GGAA-3’ and 5’-GGCC-3’.

The 20 tetramer polyamide – neomycin conjugates were coupled through 1,2,3-triazoles (87, 88, 89) and amide bonds (36). The linker length separating the two moieties varied from 10 to 21 atoms, Figure 3.4. To accommodate the variation in
Figure 3.4. Tetramer polyamide – neomycin conjugates used in this study: DPA208 – DPA225, DPA243 – DPA244, neomycin and HImImPyPyDp (18).
conjugate linker length, a sub-library of tetramer polyamides DPA202 – DPA207 and a sub-library of N-Boc protected neomycin derivatives DPA13 – DPA15 and DPA17 were synthesized. Tetramer polyamide – neomycin conjugate linker length is defined as the number of atoms measured from the C-terminal carbonyl group of the tetramer polyamide to, but not counting, the 5” nitrogen on ring three of neomycin. 1,2,3-triazoles are counted through the alkene such that the lowest total number of atoms were counted. Therefore, our library of 20 tetramer polyamide- neomycin conjugates consisted of four unique series: series I tetramer polyamide – neomycin conjugates DPA208 – DPA213, series II tetramer polyamide – neomycin conjugates DPA214 – DPA219, series III tetramer polyamide – neomycin conjugates DPA220 – DPA225 and series IV conjugates DPA243 and DPA244, Figure 3.4.

Synthesis of Tetramer Polyamide Neomycin Conjugates. Solution phase synthesis of HImImPyPyOOMe 6 was achieved through the use of three monomeric building blocks 7 – 9; two imidazole based monomers 7, 8 and a single pyrrole based monomer 9, all of which were synthesized as previously reported (77, 90), Scheme 3.1.

Synthesis of tetramer polyamide 6 was conducted in solution through the sequential formation of amide bonds between the C-terminal end of the growing heterocyclic polyamide chain and the incoming monomeric building block, as reported in literature, Scheme 3.1 (84, 91). Following each addition of monomer, the desired product was recovered following column chromatography. Amide bond formation was conducted in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-
hydroxybenzotriazole (EDC/HOBt) with the exception of the first step, as described in the following paragraph.

Compound 8 was reduced to form methyl 4-amino-N-methylimidazole-2-carboxylate 8a. The amine 8 was immediately coupled to 7 through the haloform reaction (91) which afforded dimer 10, HImImCOOMe.

**Scheme 3.1.** Reagents and conditions: (i) MeOH, 10% Pd/C, H₂ (g), 23 °C; 3.5 h, CH₂Cl₂, 23 °C, 6 h, 72%; (ii) 1.5 N NaOH, MeOH, 60 °C, 4 h; (iii) MeOH, H₂, 10% Pd/C, 23 °C, 4 h; (iv) DMF, EDC, HOBt, 23 °C, 16 h, 71%; (v) 9a, DMF, EDC, HOBt, 23 °C, 16 h, 69%.

Saponification of the resulting dimer 10 was carried out in the presence of 1.5 N NaOH and was neutralized with HCl to afford intermediate 10a. Compound 10a was activated with EDC/HOBt. Concurrently, building block 9 was reduced to afford methyl
4-amino-N-methylpyrrole-2-carboxylate 9a. Compound 9a was added dropwise to the activated acid of solution 10a. The desired trimer, HImImPyCOOMe 11, was recovered as the major product following column purification. Trimer 11 was saponified in a

![Chemical Structure](image)

**Scheme 3.2.** Reagents and conditions: (i) 1.5 N NaOH:THF, MeOH, 60 °C, 5.5 h; (ii) EDC, HOBt, DMF, 23 °C, 24 h, 72% - 91%.

fashion similar to compound 10. The resulting acid 11a was activated using EDC/HOBt. Compound 9a was generated in solution and added to the activated acid. Tetramer polyamide 6 was recovered following column purification and afforded in 69% yield.

Linker length variation was afforded through α, ω–amino, azido alkanes 12 – 17 which were synthesized from corresponding dibromoalkanes using previously established procedures (92), **Scheme 3.2.** The methyl ester of tetramer polyamide 6 was saponified in the presence of 1.5 N NaOH and neutralized with HCl to afford 6a as the major
product. The resulting solid was activated using EDC/HOBt. α, ω-amino, azido alkanes 12 – 17 were dissolved in DMF and added to a solution containing 6a. The desired azide functionalized tetramer polyamides DPA202 – DPA207 were recovered following column purification in good yields.

![Diagram](image)

**Scheme 3.3.** Reagents and conditions: (i) NaN₃, DMF, 60 °C, 12 h, 89 %; (ii) CuI, DIPEA, propargyl ether, toluene, 14 h, 85%; (iii) CuI, DIPEA, 1,6-heptadiyne, toluene, 14 h, 88%; (iv) CuI, DIPEA, 1,7-octadiyne, toluene, 14 h, 86%; (v) 10% Pd/C, MeOH, H₂ (g), 7 h, 98%, (vi) propargyl chloroformate, Et₃N, CH₂Cl₂ (dry), -78 °C, 30 min, 88 %.

Linker length variation was also afforded on neomycin through the development of N-Boc protected, alkyne functionalized neomycin derivatives DPA13 – DPA15 and DPA17, Scheme 3.3. Commercially available neomycin B was prepared for conjugation as previously reported (35, 37, 79, 93). The cornerstone of our synthesis required the
preparation of an azido derivative of neomycin B, DPA11, Scheme 3.3. Beginning with natural product neomycin B, the six amino groups were N-Boc protected using di-tert-butyl dicarbonate. N-Boc neomycin-TPS 2 was prepared, as the major product, by suspending the N-Boc neomycin in anhydrous pyridine followed by the addition of 2,4,6-triisopropylbenzenesulfonyl chloride and purification by chromatography, as previously reported (36, 37, 79, 93). Compound 2 was dissolved in DMF and sodium azide was added. DPA11 was recovered as the major product following column purification, in 89% yield.

DPA11 was coupled to commercially available dialkynes using CuI/DIPEA mediated 1,2,3-triazole formation in the presence of DMF, Scheme 3.3 (87, 88, 89). DPA13 was afforded following CuI/DIPEA mediated 1,2,3-triazole formation with propargyl ether with a yield of 85%. Coupling of DPA11 to 1,6-heptadiyne in the presence of CuI/DIPEA afforded DPA14, an aliphatic analog of DPA13, in good yields, Scheme 3.3. Finally, DPA215 was synthesized following CuI/DIPEA mediated coupling of DPA211 with 1,7-octadiyne in 88% yield, Scheme 3.3. DPA11 was reduced in the presence of H₂ (g) and 10% Pd/C to afford a N-Boc protected nucleophilic neomycin derivative DPA12, Scheme 3.3. DPA12 was coupled to propargyl chloroformate to afford DPA17 as the major product following column chromatography, Scheme 3.3.

Series I tetramer polyamide – neomycin conjugates DPA208 – DPA213 were synthesized through CuI/DIPEA mediated 1,2,3-triazole formation between tetramer
polyamides, DPA202 – DPA207, and N-Boc protected neomycin DPA13, Scheme 3.4.

The N-Boc protected conjugates were purified via column chromatography followed by deprotection in the presence of 4 N HCl in 1,4-dioxane. The resulting conjugates DPA208 – DPA213 featured linkers of 12, 13, 15, 16, 18 and 20 atoms in length.

Series II tetramer polyamide – neomycin conjugates DPA214 – DPA219, Scheme 3.5 were synthesized in a similar fashion to series I conjugates. Tetramer polyamides DPA202 – DPA207 were coupled to N-Boc protected neomycin DPA14, Scheme 3.5. The purified N-Boc protected conjugates were deprotected in the presence of 4 N HCl which afforded tetramer polyamide – neomycin conjugates as hydrochloride salts. Series II conjugates featured the same linker lengths as series I (12, 13, 15, 16, 18 and 20 atoms in length) while varying the flexibility of the atoms linking the two 1,2,3-triazoles in the linker.
Scheme 3.5. Series II reagents and conditions: (i) CuI, DIPEA, \textbf{DPA202} – \textbf{DPA207}, toluene, 24 h; (ii) 4N HCl in 1,4-dioxane, 1 h. Overall yields for coupling and deprotection steps 88% - 98%.

Series III tetramer polyamide – neomycin conjugates \textbf{DPA202} – \textbf{DPA207} afforded linker lengths of 13, 14, 16, 17, 19 and 21 atoms, Scheme 3.6. Azido functionalized tetramer polyamide – neomycin conjugates \textbf{DPA202} – \textbf{DPA207} were

Scheme 3.6. Series III reagents and conditions: (i) CuI, DIPEA, \textbf{DPA202} – \textbf{DPA207}, toluene, 24 h; (ii) 4N HCl in 1,4-dioxane, 1 h. Overall yields for coupling and deprotection steps 95% - 99%.
conjugated to N-Boc protected neomycin DPA15. Following purification, the resulting N-Boc protected conjugates were deprotected in the presence of 4 N HCl in 1,4-dioxane which afforded the desired conjugates DPA220 – DPA225 as hydrochloride salts.

Series IV tetramer polyamide – neomycin conjugates DPA202 and DPA203 were synthesized to afford conjugates with shorter linker length, 10 and 11 atoms, Scheme 3.7. These conjugates featured a single 1,2,3-triazole between the two moieties. Tetramer polyamides, DPA202 and DPA203, were conjugated to DPA17. Following purification, the resulting N-Boc protected conjugates were deprotected in the presence of 4 N HCl in 1,4-dioxane which afforded the desired conjugates DPA243 and DPA244 as hydrochloride salts, Scheme 3.8.

Finally, tetramer polyamide control 18 was synthesized in a similar fashion to compounds DPA202 – DP207, Scheme 3.8. The methyl ester of tetramer polyamide 6 was saponified in the presence of 1.5 N NaOH at 60 °C to afford 6a as the major product.
The solution was neutralized with HCl and volatiles were removed *in vacuo*. The resulting solid was coupled to \( N,N \)-dimethylaminopropylamine to afford 18.

![Scheme 3.8](image)

**Scheme 3.8.** Reagents and conditions: (i) EDC, HOBt, \( N,N \)-dimethylaminopropylamine, 18 h, 55%.

**Design of DNA Duplexes to Probe Tetramer Polyamide – Neomycin Conjugate Library Binding.** Fluorescent intercalator displacement assays were conducted with a number of DNA duplex oligomers and our tetramer polyamide – neomycin conjugates, neomycin and 18. The DNA duplexes varied in length and sequence to accommodate a number of potential DNA binding sequences. DNA duplexes were designed with the two following considerations: (i) the DNA duplexes contained a potential binding site for the tetramer polyamide portion of our conjugates \( 5'-G_2A_2-3' \) or \( 5'-G_2C_2-3' \) and (ii) the DNA duplexes were lengthened to include a portion for neomycin which acted as a potential binding site \( 5'-G_4C_4-3' \) or control \( 5'-G_4-3' \) as identified by our group (94).

Applying these considerations, the following DNA duplexes were used in this study \( 5'-G_2A_3G_4C_4-3', 5'-AG_2C_2AG_4C_4-3', 5'-G_2C_2AG_4C_4-3', 5'-TG_2C_2G_4-3' \) and \( 5'-AG_2C_2AG_4-3' \). The \( 5'-G_2A_3G_4C_4-3' \) duplex contained a binding site for the tetramer polyamide portion of our conjugate when it binds in a 1:1 polyamide:DNA ratio \( 5'-G_2A_3-3' \) (74) and a portion for binding by neomycin \( 5'-G_4C_4-3' \). The \( 5'-AG_2C_2AG_4C_4-3' \) duplex contained a binding site for the tetramer polyamide neomycin portion of our conjugate...
when it binds in a 2:1 polyamide:DNA ratio (5’-AGC2A3’-3’ (62) and a portion for neomycin binding (5’-G4C4-3’). The 5’-G2C2AGC4-3’ duplex acted as a control to the 5’-G2A3G4C4-3’ DNA duplex. The duplexes are equal length and provide insight into the behavior of our tetramer polyamide – neomycin library when the duplex contains either a match and mismatch sequence for tetramer polyamide neomycin binding. Both of the remaining duplexes 5’-TG2C4-3’ and 5’-AGC2AGC4-3’ were shortened duplexes that act as controls. Neither duplex offers an optimized portion for neomycin binding or tetramer polyamide when binding in a 1:1 ligand:DNA ratio. CD scans of each of these duplexes is located in Appendix B, Figure B37.

**Tetramer Polyamide – Neomycin Conjugate Library Binding as Studied With a Fluorescent Intercalator Displacement Assay.** The DNA duplexes were pre-saturated with ethidium bromide in a 96-well plate (95, 96, 97). Neomycin, 18 and tetramer polyamide – neomycin conjugates (series I DPA208 – DPA213, series II DPA214 – DPA219, series III DPA220 – DPA225 and series IV DPA243 – DPA244) were added to the DNA at various ratios of drug to duplex ($r_{dd}$). The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. For each DNA duplex, a histogram of the normalized percent decrease in fluorescence intensity with respect to control ligands and tetramer polyamide – neomycin conjugate linker length was plotted.
When the assay was conducted using the 5'-G₂C₂AG₄C₄-3' DNA duplex, each of the tetramer polyamide – neomycin conjugates from series I DPA208 – DPA213, series II DPA214 – DPA219, series III DPA220 – DPA225 and series IV DPA243 – DPA244.

**Figure 3.5.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5'-G₂C₂AG₄C₄-3'. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 1.0$. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 conjugates are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM.duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits $ex = 20$; slits $em = 20$; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
tetramer polyamide – neomycin conjugates as well the controls, neomycin and 18, displaced pre-bound ethidium bromide, Figure 3.5. At an $r_{dd}$ of 1.0, neomycin displaced

**Figure 3.6.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5'-AG$_2$C$_2$AG$_4$-3'. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 1.0$. Controls (neomycin, HlmImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 conjugates are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 $\mu$M/duplex], pre-saturated with ethidium bromide [7 $\mu$M], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits$_{ex}$ = 20; slits$_{em}$ = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
25% of pre-bound ethidium bromide while HImImPyPyDp 18 displaced 15% of pre-bound ethidium bromide. The tetramer polyamide – neomycin conjugate that displaced the most pre-bound ethidium bromide was DPA224, linker length (L) 19 atoms, which displaced 30%. While the remaining tetramer polyamide – neomycin conjugates displaced pre-bound ethidium bromide, which suggests conjugate binding, the amount of displaced pre-bound ethidium bromide was not significantly more than neomycin. The amount of pre-bound ethidium bromide displaced by these conjugates was between 12% (DPA209, L = 13 atoms) and 26% (DPA219, L = 20 atoms) the amount displaced by neomycin and 18, Figure 3.5.

The assay was also conducted using 5’-AGC2AG4-3’, tetramer polyamide – neomycin conjugates from series I DPA208 – DPA213, series II DPA214 – DPA219, series III DPA220 – DPA225 and series IV DPA243 – DPA244 tetramer polyamide – neomycin conjugates as well the controls, neomycin and 18, Figure 3.6. Each of the compounds used in this assay displaced pre-bound ethidium bromide at $r_{dd} = 1.0$. The control compounds neomycin and 18 displaced 35% and 31% of pre-bound ethidium bromide, respectively. DPA225 (L = 21 atoms), DPA219 (L = 20 atoms) and DPA213 (L = 20 atoms) displaced significantly more pre-bound ethidium bromide than neomycin or 18, ~45%. These conjugates contain the longest linkers in our library. A number of tetramer polyamide – neomycin conjugates also displaced equivalent or slightly more pre-bound ethidium bromide than our control compounds. DPA224 (L = 19 atoms), DPA218 (L = 18 atoms) and DPA212 (L = 18 atoms) displaced ~37% while conjugates of shorter linker length, DPA215 (L = 13 atoms), DPA209 (L = 13 atoms), DPA214 (L =
12 atoms), **DPA208** (L = 12 atoms), **DPA244** (L = 11 atoms) and **DPA243** (L = 10 atoms), displaced ~40% of the pre-bound ethidium bromide. The remaining conjugates

**Figure 3.7.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-TG\_2C\_2G\_4-3’. In the histogram, the values represent the percent decrease in fluorescence at r\_dd = 1.0. Controls (neomycin, HImImPyPyDp\_18) are shown in light grey while series I **DPA208 – DPA213** conjugates are shown in blue, series II **DPA214 – DPA219** conjugates are shown in green, series III **DPA220 – DPA225** conjugates are shown in red and series IV **DPA243 – DPA244** conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 \( \mu \)M/duplex], pre-saturated with ethidium bromide [4 \( \mu \)M], at various ratios of drug to duplex (r\_dd), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits\_ex = 20; slits\_em = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na\_2HPO\_4, 0.5 mM NaH\_2PO\_4, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
of intermediate linker lengths (L = 13 to 17 atoms) displaced less pre-bound ethidium bromide than neomycin or 18.

![Figure 3.8](image-url)

**Figure 3.8.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG₂C₂AG₄C₄-3’. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 1.0$. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 conjugates are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM/duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits<sub>ex</sub> = 20; slits<sub>em</sub> = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
The assay was also conducted using a DNA duplex which eliminated a single central A/T base pair 5’-TG\textsubscript{2}C\textsubscript{2}G\textsubscript{4}-3’, Figure 3.7. Series I DPA\textsubscript{208} – DPA\textsubscript{213}, series II DPA\textsubscript{214} – DPA\textsubscript{219}, series III DPA\textsubscript{220} – DPA\textsubscript{225} and series IV DPA\textsubscript{243} – DPA\textsubscript{244} tetramer polyamide – neomycin conjugates as well the controls, neomycin and 18 displaced pre-bound ethidium bromide at $r_{dd} = 1.0$. Neomycin displaced 32% of pre-bound ethidium bromide while 18 displaced 27% of pre-bound ethidium bromide from the duplex. Generally, the tetramer polyamide – neomycin conjugates displaced equivalent amounts of pre-bound ethidium bromide as neomycin with few exceptions. Of note, DPA\textsubscript{208} (L = 12 atoms) displaced the most pre-bound ethidium bromide (47%), followed by DPA\textsubscript{209} (L = 13 atoms) and DPA\textsubscript{214} (L = 12 atoms), 42% and 33%, respectively.

The FID titration was also conducted using the 5’-AG\textsubscript{2}C\textsubscript{2}AG\textsubscript{4}C\textsubscript{4}-3’ duplex, Figure 3.8. As observed with previously discussed duplexes, series I DPA\textsubscript{208} – DPA\textsubscript{213}, series II DPA\textsubscript{214} – DPA\textsubscript{219}, series III DPA\textsubscript{220} – DPA\textsubscript{225} and series IV DPA\textsubscript{243} – DPA\textsubscript{244} tetramer polyamide – neomycin conjugates as well the controls, neomycin and 18 displaced pre-bound ethidium bromide at $r_{dd} = 1.0$. The controls displaced 27% and 16% (neomycin and 18, respectively) of the pre-bound ethidium bromide from the duplex. The conjugate with the longest linker length DPA\textsubscript{225} (L = 21 atoms) displaced the most pre-bound ethidium bromide, 40%. As the linker length decreased, the conjugates DPA\textsubscript{214} (L = 12 atoms), DPA\textsubscript{208} (L = 12 atoms), DPA\textsubscript{244} (L = 11 atoms) and DPA\textsubscript{243} (L = 10 atoms), 33% on average.

Results from the assay of 5’-G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3’ with series I DPA\textsubscript{208} – DPA\textsubscript{213}, series II DPA\textsubscript{214} – DPA\textsubscript{219}, series III DPA\textsubscript{220} – DPA\textsubscript{225} and series IV DPA\textsubscript{243} –
DPA244 tetramer polyamide – neomycin conjugates as well the controls, neomycin and 18 is shown in Figure 3.9. Neomycin displaced 28% of the pre-bound ethidium bromide.
upon binding the DNA duplex at $r_{dd} = 1.0$. The control 18 displaced 20% of the pre-bound ethidium bromide. Each of the tetramer polyamide – neomycin conjugates displaced pre-bound ethidium bromide upon addition to the duplex. Tetramer polyamide – neomycin conjugates containing linker lengths of 10 to 12 atoms, DPA243 (L = 10 atoms), DPA244 (L = 11 atoms), DPA208 (L = 12 atoms) and DPA214 (L = 12 atoms), displaced the largest amount (~50%) of pre-ethidium bromide. A second set of tetramer polyamide – neomycin conjugates displaced a high amount of pre-bound ethidium bromide. The conjugates DPA212 (L = 18 atoms), DPA218 (L = 18 atoms), DPA224 (L = 19), DPA213 (L = 20 atoms) and DPA219 (L = 20 atoms) displaced ~42% of pre-bound ethidium bromide.

A strong dependence on conjugate linker length and the amount of displaced pre-bound ethidium bromide was observed at other $r_{dd}$ values. At a $r_{dd}$ of 0.5, the tetramer polyamide – neomycin conjugates of longer linker length, DPA212 (L = 18 atoms), DPA218 (L = 18 atoms), DPA224 (L = 19), DPA213 (L = 20 atoms) and DPA219 (L = 20 atoms) and DPA225 (L = 20 atoms) displaced significantly more pre-bound ethidium bromide than other tetramer polyamide – neomycin conjugates, neomycin or 18, Appendix B, Figure B38. At a $r_{dd}$ of 2.0, tetramer polyamide – neomycin conjugates of longer linker length DPA212 – DPA219 (L = 18-20 atoms) and shorter linker length DPA243 – DPA214 (L = 10-12 atoms) clearly displace more pre-bound ethidium bromide than any other ligands used in the study, Appendix B, Figure B39.
Tetramer Polyamide – Neomycin Conjugate Library Binding as Studied With UV Thermal Denaturation Experiments. UV thermal denaturation concentration dependence experiments were conducted using a G/C rich 16 mer duplex 5’-A₂G₆C₆T₂-3’ and the following ligands: series I DPA208 – DPA213, series II DPA214 – DPA219 and series III DPA220 – DPA225 tetramer polyamide – neomycin conjugates as well as neomycin and HImImPyPyDp 18, Figure 3.10, Figure 3.11, Table 3.2 and Appendix B, Figure B40 – Figure B41.

Addition of the control HImImPyPyDp 18 stabilized the duplex by 2.7 °C at r_{dd} = 0.25, Table 3.2, Figure 3.10. The thermal stabilization at r_{dd} = 1.0 was 9.1 °C and 10.6 °C at r_{dd} = 2.0. The thermal stabilization afforded by 18 on the duplex increased with increasing concentration such that the stabilization afforded at r_{dd} = 2.5 was the highest at 12.1 °C. UV thermal denaturation of the duplex in the presence of neomycin suggests neomycin is slightly better at stabilizing the duplex than 18. At r_{dd} = 1.0, neomycin stabilized the duplex by 10.9 °C, 1.8 °C higher than 18 at the same r_{dd}, Figure 3.10. Addition of a second equivalent of neomycin increased the thermal stabilization by 1.7 °C. The largest increase in thermal stabilization was observed at r_{dd} = 2.0 (12.6 °C).

UV thermal denaturation of 5’-A₂G₆C₆T₂-3’ was conducted in the presence of series I tetramer polyamide – neomycin conjugates, DPA208 – DPA213, Figure 3.11. In this series, the highest stabilization afforded by a conjugate on, at r_{dd} = 1.0, was observed with DPA210 (L = 15 atoms) and DPA211 (L = 16 atoms), Table 3.2. DPA210 and DPA211 stabilized the duplex by 22.2 °C and 21.6 °C, respectively. These values were significantly higher than those observed for either neomycin or 18 alone. At r_{dd} values
greater than 2.0, **DPA210** and **DPA211** saw little to no increase in thermal stabilization. The magnitude of thermal stabilization afforded by shorter linkers, **DPA208** (L = 12 atoms) and **DPA209** (L = 13 atoms) on 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ was very close to the stabilization afforded by neomycin and 18.

UV thermal denaturation of 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ was conducted in the presence of series II tetramer polyamide – neomycin conjugates, **DPA214 – DPA219**, Appendix B.

**Figure 3.10.** UV melting profiles for 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ with controls, neomycin (a) and HImImPyPyDp 18 (b). The histograms on the right are a plot of ΔT<sub>m</sub> with respect to the ratio of drug:duplex (r<sub>dd</sub>). Ligands were added to the duplex [2 μM/duplex] at varying r<sub>dd</sub> values. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Each panel represents the duplex with a conjugate at varying r<sub>dd</sub> values. Buffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0.
Figure 3.11. UV melting profiles for 5′-A₂G₆C₆T₂-3′ with series I DPA208 – DPA213 tetramer polyamide – neomycin conjugates. The histograms on the right are a plot of $\Delta T_m$ with respect to the ratio of drug:duplex ($r_{dd}$). Ligands were added to the duplex [2 µM/duplex] at varying $r_{dd}$ values. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Each panel represents the duplex with a conjugate at varying $r_{dd}$ values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0.
Table 3.2. UV determined change in thermal denaturation melting temperature (ΔT_{m}^{a}) values for various ligands with 5'-A2G6C6T2-3'.

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^aΔT_{m} represents the change in melting temperature upon addition of various ligands. ^bN represents tetramer polyamide – neomycin conjugate linker length in atoms. Buffer: 1.5 mM Na_{2}HPO_{4}, 0.5 mM NaH_{2}PO_{4}, 0.25 mM EDTA, pH 7.0.

Figure B40. The highest thermal stabilization afforded on the duplex at r_{dd} value of 1.0 was observed with DPA216 (L = 15 atoms), 17.5 °C, Table 3.2. Shorter linkers, DPA214 (L = 12 atoms) and DPA215 (L = 13 atoms), as well as longer linkers, DPA217 – DPA219 (L = 16, 18 and 20 atoms), all stabilized the duplex. However, the magnitude of stabilization was lower than observed with DPA216 (L = 15 atoms). As observed with series I tetramer polyamide – neomycin conjugates, the magnitude of stabilization afforded by shorter conjugate linkers DPA214 (L = 12 atoms) and DPA215 (L = 13...
atoms) upon complexation with 5’-A₂G₆C₆T₂-3’ was closer to neomycin and 18. Furthermore, these conjugates do not substantially increase the thermal stability of the 5’-A₂G₆C₆T₂-3’ duplex at \( r_{dd} \) values larger than 1.0.

UV thermal denaturation of 5’-A₂G₆C₆T₂-3’ in the presence of series III tetramer polyamide – neomycin conjugates, DPA220 – DPA225, Appendix B, Figure B41. The highest UV observed thermal stabilization at \( r_{dd} = 1.0 \) was afforded by DPA222 (L = 16 atoms) and DPA223 (L = 17 atoms), 20.3 °C and 19.0 °C, respectively, Table 3.2. The thermal stabilization afforded by the shorter linkers, DPA220 (L = 13 atoms) and DPA221 (L = 14 atoms) were significantly lower at 9.6 °C and 11.4 °C, respectively; closer to the values observed for neomycin and 18. Furthermore, these conjugates do not significantly improve the thermal stabilization on the duplex upon increasing \( r_{dd} \) values.

Plots of ΔTm with respect to linker length at \( r_{dd} = 0.5, 1.0 \) and 2.0 were plotted and the following generalizations were made, Appendix B, Figure B42. Generally speaking, in each series, the linkers that afforded the duplex with the largest thermal stabilization were between 15 – 17 atoms in length, consistent with previous polyamide – major groove binding conjugates (98). Tetramer polyamide – neomycin conjugates of 15 – 17 atom linkers did not show increased thermal stabilization as the \( r_{dd} \) values increased past \( r_{dd} = 1.0 \). Finally, the magnitude of stabilization afforded by conjugates with linkers shorter than 15 atoms were closer in magnitude to those observed with neomycin and 18.

Expanding UV Monitored Thermal Denaturation Experiments with a Shrinking GC Pocket. UV thermal denaturation experiments were conducted on a number of 16 mer
Figure 3.12a. Plot of $\Delta T_m$ with respect to conjugate linker length upon addition of ligands to $5'$-A$_m$G$_n$C$_n$T$_m$-3'. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series II DPA214 – DPA219 conjugates are shown in green and series III DPA220 – DPA225 conjugates are shown in red. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 $\mu$M/duplex] at a ratio of drug to duplex 1.0. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. $^\Delta$T$_m$ values for the duplex with no additional NaCl.
Figure 3.12b. Plot of $\Delta T_m$ with respect to conjugate linker length upon addition of ligands to 5'-$A_mG_nC_nT_m$-3'. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series II DPA214 – DPA219 conjugates are shown in green and series III DPA220 – DPA225 conjugates are shown in red. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 µM/duplex] at a ratio of drug to duplex 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. $^a$$\Delta T_m$ values for the duplex with no additional NaCl.
DNA duplexes and series II DPA214 – DPA219 and series III DPA220 – DPA225 tetramer polyamide – neomycin conjugates. In an effort to focus solely on the primary binding site, these studies were conducted at $r_{dd} = 1.0$ in the presence of 50 mM NaCl. Where duplex formation and stability allowed; the duplexes 5'-A$_2$G$_6$C$_6$T$_2$-3', as previously discussed, and 5'-A$_3$G$_5$C$_5$T$_3$-3' were also conducted in the absence of additional salt to the buffer. Experimental data for these experiments are located in Table 3.3 and Figure 3.12a and Figure 3.9b. Normalized UV thermal denaturation curves are located in Appendix B, Figure B43 – Figure B44.

In an effort to expand our studies, thermal denaturation experiments with the 5'-A$_2$G$_6$C$_6$T$_2$-3’ duplex were conducted in the presence of 10 mM additional NaCl. The stabilization afforded by neomycin on the 5'-A$_2$G$_6$C$_6$T$_2$-3’ duplex was 8.6 °C while the control polyamide 18 only afforded a 4.2 °C increase in thermal stability. The largest thermal stabilization afforded on the 5'-A$_2$G$_6$C$_6$T$_2$-3’ duplex was observed when complexed with conjugates DPA218 (L = 18 atoms), DPA219 (L = 20 atoms), and DPA220 (L = 13 atoms), 6.8 °C, 4.2 °C and 4.7 °C, respectively.

The G/C pocket was decreased by two G/C base pairs and UV thermal denaturation experiments were conducted on the 5'-A$_3$G$_5$C$_5$T$_3$-3’ duplex. In the absence of NaCl, the largest stabilization afforded by tetramer polyamide – neomycin conjugates was 8.1 °C and 7.5 °C observed with DPA216 (L = 15 atoms) and DPA217 (L = 16 atoms). However, these conjugates were better at stabilizing the duplex than 18, 4.9 °C, but did not stabilize the duplex as well as neomycin alone, 4.4 °C. In the presence of 50 mM NaCl, the largest thermal stabilization afforded by the conjugates on 5’-A$_3$G$_5$C$_5$T$_3$-3’
Table 3.3. UV determined thermal denaturation melting temperatures ($T_m$) of 5’-AmG₅C₅T₃-3’ duplexes with tetramer polyamide – neomycin conjugates DPA214 – DPA225, neomycin and 18. All values are reported in °C.

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</table>

aN represents conjugate linker length in atoms. bTₘ represents the melting temperature of the native duplex. cΔTₘ represents the change in melting temperature upon addition of neomycin at a tₐₜ. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. dBuffer used in these melts do not include 50 mM NaCl.
was observed with **DPA215** (L = 13 atoms) and **DPA216** (L = 15 atoms), 3.2 °C. These conjugates were effective in stabilizing the duplex by ~4 °C less than neomycin, but ~1.5 °C more **18**. As the size of the GC pocket is decreased to 5’-A₄G₄C₄T₄-3’, 5’-A₅G₃C₃T₃-3’ and 5’-A₆G₂C₂T₆-3’ the magnitude of stabilization afforded by series II and series III tetramer polyamide – neomycin conjugates increase. The average magnitude of stabilization afforded by series II and series III conjugates on 5’-A₄G₄C₄T₄-3’ was only 0.4 °C. The largest stabilization was afforded by **DPA225** (L = 21 atoms) at 1.7 °C, which was 1 °C higher than the stabilization afforded by **18** and 2.3 °C less than the stabilization afforded by neomycin, 4.0 °C. As the G/C pocket was decreased by a G/C base pair, 5’-A₅G₃C₃T₅-3’, the average magnitude of stabilization afforded by series II and series III conjugates was 3.4 °C. Neomycin stabilized the duplex by 5.5 °C while **18** stabilized the duplex by 5.2 °C. The largest conjugate stabilization was 6.3 °C as observed with **DPA214** (L = 12 atoms). As the central pocket was decreased by another G/C base pair, 5’-A₆G₂C₂T₆-3’, average magnitude of stabilization increased to 5.4 °C. The largest magnitude of stabilization afforded on the duplex was observed with **DPA215** (L = 13 atoms), 5.5 °C and **DPA218** (L = 20), 5.7 °C. The average magnitude of stabilization by tetramer polyamide – neomycin conjugates was 2.8 °C. Neomycin stabilized the 5’-A₆G₂C₂T₆-3’ duplex by 4.3 °C while compound **18** stabilized the duplex by 1.8 °C.

**Binding of HImImPyPyDp 18 to 5’-A₂G₆C₆T₂-3’ and 5’-A₆G₂C₂T₆-3’ duplexes, Implications of Target Sequence.** CD monitored titrations of HImImPyPyDp **18** into
both 5'-A₂G₆C₆T₂-3' and 5'-A₆G₂C₂T₆-3' duplexes were conducted, Figure 3.13. The CD spectra of native 5'-A₆G₂C₂T₆-3' consists of a positive couplet at 270 nm and negative couplet at 210 nm which suggests the duplex retains A-form characteristics. However the strong negative band at 240 nm and the shoulder at 280 nm suggest the duplex also retains B-form characteristics. The binding of HImImPyPyDp 18 to 5'-A₆G₂C₂T₆-3' is clearly illustrated by the increase in CD intensity at 300 nm – 350 nm, the

![CD scans for the titration of 5'-A₆G₂C₂T₆-3' (a) and 5'-A₂G₆C₆T₂-3' (c) with HImImPyPyDp 18 and CD intensity vs r₆d value plots, 246 nm (b) and 240 nm (d) at 20 °C. 18 was added to the duplex [40 µM/bp] at varying r₆d values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.](image)
polyamide absorption region. Analysis of the DNA region at 246 nm shows distinct inflection points at 1:1 and 2:1, ligand:DNA, ratios as expected (99).

The 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ duplex also displays a positive and negative couplet at 270 nm and 210 nm in the CD scan of the native duplex. The CD scan clearly shows a negative band at 240 which suggests the duplex still retains B-form characteristics, the decreased intensity of this band and the lack of a clear shoulder at 280 nm suggests this duplex, when compared to the 5’-A<sub>6</sub>G<sub>2</sub>C<sub>2</sub>T<sub>6</sub>-3', retains less B-form characteristics. However, when 18 is titrated into the 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3', no clear ligand absorption bands resolve; analysis of the DNA region at 240 nm displays no inflection points upon ligand addition. While this data alone could suggest 18 does not bind the duplex, thermal denaturation experiments have clearly suggested 18 is capable of thermally stabilizing the 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ duplex.

In an effort to support the results obtained from the CD monitored titration of 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ and 5’-A<sub>6</sub>G<sub>2</sub>C<sub>2</sub>T<sub>6</sub>-3’ by 18, the titration was repeated using a fluorescence displacement titration (FID). In this experiment, the 16 mer DNA duplexes were pre-saturated with thiazole orange. Compound 18 was added at varying r<sub>dd</sub> values. The results from the FID displacement of pre-bound thiazole orange from the 5’-A<sub>6</sub>G<sub>2</sub>C<sub>2</sub>T<sub>6</sub>-3’ duplex by 18 results in a decrease in fluorescence intensity indicative of ligand binding, Figure 3.14. Plotting the change in fluorescence intensity at 526 nm with respect to the r<sub>dd</sub> clearly shows binding sites at 1:1 and 2:1 ligand:DNA ratios as observed with CD.

Upon titration of 18 into the 5’-A<sub>2</sub>G<sub>6</sub>C<sub>6</sub>T<sub>2</sub>-3’ duplex pre-bound with thiazole orange a clear decrease in fluorescence intensity is observed. This supports our claim
that 18 is capable of binding the 5’-A₂G₆C₆T₂-3’ duplex. However, when the change in fluorescence intensity at 526 nm is plotted against the r₃d value no clear inflection points are observed. Binding of linear polyamides comprised N-methylimidazole and N-methylpyrrole monomers have shown the ability to bind target sequences in a 1:1 and 2:1

![Diagram](image)

**Figure 3.14.** Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the self complementary duplex, 5’-A₆G₂C₂T₆-3’, (a) and (b), and 5’-A₂G₆C₆T₂-3’ by HImImPyPyDp 18 at 20 °C. A plot of fluorescence intensity vs r₃d is located in panel (b), (d). The duplexes, 1.0 µM/duplex, 16 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min, prior to addition of 18. 18 was added to the duplexes at various r₃d values. Excitation: 504 nm; emission: 510-610 nm; slits: 1.0 nm. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 100 mM NaCl.
ligand:DNA binding stoichiometry (99). Furthermore, these polyamides are also capable of targeting non-specific sequences as well. The trimer polyamide H1mPyPyDp binds duplex DNA with 2:1 ligand:DNA binding stoichiometry and has been reported to bind the target sequence 5’-GTAC-3’ with an association constant $K_a$ of $8.6 \times 10^6 \text{M}^{-1}$ while it binding the non-target sequence 5’-GACA-3’ with a $K_a$ of $1.3 \times 10^5 \text{M}^{-1}$ (99). Similar conclusions have been reported (100, 101).

Experimental evidence suggests tetramer polyamide – neomycin conjugates increase the thermal stability of a number of DNA duplexes. However, it is clear from CD and FID titrations 5’-A$_m$G$_n$C$_n$T$_m$-3’ type duplexes with 18 the binding of 18 to DNA is strongly sequence dependent. Optimized 2:1 stoichiometry binding of 18 to DNA occurs when DNA contains the sequence 5’-AG$_2$C$_2$T-3’ (76). In our current design of 5’-A$_m$G$_n$C$_n$T$_m$-3’ type duplexes, the only duplex which contains our target sequence is 5’-A$_6$G$_2$C$_2$T$_6$-3’. Unfortunately, the 5’-A$_6$G$_2$C$_2$T$_6$-3’ duplex contains a large percentage of A/T content suggesting a conformation closer to B-form (13) and weakly bound by neomycin (37). Data collected by our group suggests duplexes containing of large percentage of G/C content are bound with higher affinities by neomycin (94). Unfortunately, the duplex used in this study 5’-A$_2$G$_6$C$_6$T$_2$-3’ does not contain the optimized target tetramer polyamide sequence. Therefore, in order to receive synergistic contributions from both binding moieties a target DNA duplex must be identified.

**Identifying Potential Target Duplexes.** The following two target DNA duplexes were used in subsequent studies: target duplex 1 5’-G$_2$A$_3$G$_4$C$_4$-3’ and target duplex 2 5’-
G₂C₂AG₄C₄-3’. These duplexes each contained a neomycin binding site 5’-G₄C₄-3’, as identified by our group (94). Target duplex 1 contains the target polyamide sequence for the 1:1 binding mode, 5’-G₂A₃-3’, within the following duplex 5’-G₂A₃G₄C₄-3’. Target duplex 2 contains the target polyamide sequence for the 2:1 binding mode, 5’-G₂C₂A-3’, within the following duplex 5’-G₂C₂AG₄C₄-3’.

**UV Monitored Thermal Denaturation of Target Duplexes.** UV thermal denaturation experiments were conducted using both target duplexes. In an effort to focus solely on the primary binding site, these studies were conducted at r_{dd} = 1.0 in the presence of 50 mM NaCl. Target duplex 1, 5’-G₂A₃G₄C₄-3’, was denatured in the presence of series I (DPA208 – DPA213), series II (DPA214 – DPA219), series III (DPA220 – DPA225) and series IV (DPA243 and DPA244) tetramer polyamide – neomycin conjugates as well as neomycin and 18. A histogram which plots the ΔT_m with respect to conjugate linker length is found in Figure 3.15 while UV thermal denaturation profiles are located in Appendix B, Figure B46. Experimentally determined values are located in Table 3.5.

Each of the tetramer polyamide – neomycin conjugates, neomycin and 18 increased the melting temperature of 5’-G₂A₃G₄C₄-3’, target duplex 1. Neomycin increased the melting temperature of the duplex by 6.2 °C and the tetramer polyamide control 18 increased the melting temperature by a similar magnitude of 6.3 °C. The largest increase in melting temperature was afforded by tetramer polyamide – neomycin conjugates which featured the shortest linker lengths between the two moieties.
Conjugates DPA243 (L = 10 atoms) and DPA244 (L = 11 atoms) afford a 21.5 °C and 24.7 °C increase in thermal stability, respectively. In each series the two conjugates with the shortest linker length increase the thermal stability by a larger magnitude than the remaining conjugates in the series. Furthermore, as the linker length increases, in

![Figure 3.15](image-url). Plot of ΔTₘ with respect to conjugate linker length upon addition of ligands to 5’-G₂A₃G₄C₄-3’. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA23 and DPA244 are shown in dark grey. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 µM/duplex] at a ratio of drug to duplex = 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Table 3.4. UV determined thermal melting temperatures of target duplexes in the presence of controls and series I (DPA208 – DPA213), series II (DPA214 – DPA219), series III (DPA220 – DPA225) and series IV (DPA243 and DPA244) tetramer polyamide – neomycin conjugates with target duplexes duplexes. All values are reported in °C.

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*^N* represents tetramer polyamide – neomycin conjugate linker length in atoms. *^T_m* represents the melting temperature of the native duplex. *^ΔT_m* represents the change in melting temperature upon addition of ligands at a ratio 1:1. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
each series, the magnitude of thermal stabilization decreases. Examination of series I tetramer polyamide – neomycin conjugates shows DPA208 (L = 12 atoms) and DPA209 (L = 13 atoms) increase the thermal stability of target duplex 1 by 20.9 °C and 15.2 °C, Table 3.5. As linker length is increased to 15 atoms, the stabilization afforded by DPA210 (L = 15 atoms) is 10.9 °C. DPA211 (L = 16 atoms), DPA212 (L = 18 atoms) and DPA213 (L = 20 atoms) stabilize the duplex by 5.5 °C, 6.7 °C and 6.3 °C, respectively. Series II tetramer polyamide – neomycin conjugates DPA214 (L = 12 atoms) and DPA215 (L = 13 atoms) stabilize target duplex 1 by 22.5 °C and 16.1 °C, similar in magnitude to the values obtained with conjugates of the same linker length from series I. As observed with series I conjugates, thermal stabilization decreases with increasing linker length such that DPA219 (L = 20 atoms) only stabilizes target duplex 1 by 6.4 °C.

Series III tetramer polyamide – neomycin conjugates feature some conjugates with intermediate linker lengths, not found in series I or series II. DPA220 (L = 13 atoms) is the conjugate with the shortest linker length in the series and increases the thermal stabilization of the duplex by 18.5 °C. The magnitude of stabilization afforded by this conjugate is nominally larger than observed with tetramer polyamide – neomycin conjugates DPA209 (L = 13 atoms) and DPA215 (L = 13 atoms). DPA221 (L = 14 atoms) stabilizes the duplex by 17.1 °C. DPA222 (L = 16 atoms) features the same linker length as DPA217 (L = 16 atoms) and DPA211 (L = 16 atoms) and stabilizes target duplex 1 by 9.0 °C. As linker length increases in series III, the magnitude of stabilization decreases such that DPA225 (L = 12 atoms) affords 6.2 °C of stabilization.
Target duplex 2, 5’-G₂C₂AG₄C₄-3’, was denatured in the presence of series II (DPA214 – DPA219) and series III (DPA220 – DPA225) tetramer polyamide – neomycin conjugates as well as neomycin and 18. A histogram which plots the $\Delta T_m$ with

![Figure 3.16](image-url)

**Figure 3.16.** Plot of $\Delta T_m$ with respect to conjugate linker length upon addition of ligands to 5’-G₂C₂AG₄C₄-3’. Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 are shown in dark grey. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 µM/duplex] at a ratio of drug to duplex 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
respect to conjugate linker length is found in Figure 3.16 while UV thermal denaturation profiles are located in Appendix B, Figure B47. Experimentally determined values are located in Table 3.5.

The magnitude of stabilization afforded by neomycin on target duplex 2, 5’-G_2C_2AG_4C_4-3’ is 5.5 °C, which is similar to the magnitude afforded by neomycin on target duplex 1, 5’-G_2A_3G_4C_4-3’. The magnitude of stabilization afforded by the tetramer polyamide control 18 on target duplex 2 was only 1.9 °C; much lower in magnitude than observed with target duplex 1 (6.2 °C). Furthermore, of the tetramer polyamide – neomycin conjugates studied with target duplex 2, the magnitude of stabilization is lower than observed with target duplex 1. Since UV thermal denaturation experiments focused on the primary binding site, r_{dd} = 1.0, the 4.3 °C difference in stabilization afforded by 18 on target duplex 1 and target duplex two is not unexpected. When binding the duplex in a 1:1 stoichiometry, 18 prefers the sequence 5’-G_2A_3-3’ over 5’-G_2C_2A-3’.

As observed with target duplex 1, tetramer polyamide – neomycin conjugates DPA243 (L = 10 atoms) and DPA244 (L = 11 atoms) stabilize the duplex higher than any other conjugates studied. DPA243 (L = 10 atoms) increased the thermal stability of target duplex 2 by 11.4 °C while DPA244 (L = 11 atoms) increased the thermal stabilization of target duplex 2 by 13.2 °C. However, the magnitude of stabilization afforded by these two conjugates on target duplex 2 were ~10 °C lower than observed than observed with target duplex 1.

When examining the thermal stability afforded by series II tetramer polyamide – neomycin conjugates on target duplex 2, the largest stabilization was afforded by
Table 3.5. UV determined change in thermal melting (ΔTₘ) temperatures and FID assay determined change in fluorescence (ΔF) data. Data for the addition of controls and series I (DPA208 – DPA213), series II (DPA214 – DPA219), series III (DPA220 – DPA225) and series IV (DPA243 and DPA244) tetramer polyamide – neomycin conjugates with target duplexes at rₐd = 1.0.

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aN represents tetramer polyamide – neomycin conjugate linker length in atoms.

bΔF represents the change in fluorescence intensity upon addition of ligand at rₐd = 1.0.  cΔTₘ represents the change in melting temperature upon addition of ligands at rₐd = 1.0. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

DPA214 (L = 12 atoms), 9.3 °C. Unlike the trend observed with series II conjugates and target duplex 1, the magnitude of stabilization did not decrease with increasing linker length. In fact, the second largest magnitude of stabilization was observed with DPA219.
(L = 20 atoms). **DPA215** (L = 13 atoms) stabilized target duplex 2 by 5.7 °C while **DPA218** (L = 18 atoms) stabilized the duplex by 3.9 °C. Intermediate linker lengths of 15 and 16 atoms, **DPA216** (L = 15 atoms) and **DPA217** (L = 16 atoms), did not change the melting temperature of the duplex by any appreciable amount.

Finally, for ease of comparison, a table comparing ΔTₘ values (obtained from UV thermal denaturation experiments) and ΔF values (obtained from FID assays) with respect to linker length for tetramer polyamide – neomycin conjugates and controls with target duplex 1 and target duplex two is located in Table 3.5.

The largest magnitude of stabilization afforded by series III conjugates on target duplex 2 was observed with **DPA225** (L = 21 atoms), 7.0 °C. Tetramer polyamide – neomycin conjugates **DPA224** (L = 19 atoms) stabilized the duplex by 4.9 °C. Surprisingly, **DPA220** (L = 13 atoms) only stabilized the melting temperature of target duplex 2 by 2.2 °C, while the remaining tetramer polyamide – neomycin conjugates did not significantly change the melting temperature of the duplex.

**Fluorescence Intercalator Displacement Titrations and Binding Affinity.** In an effort to obtain quantitative binding affinities Kₐ and relate them to the results obtained from our assay, full FID titrations were conducted. Target duplex 1 was pre-saturated with thiazole orange. A departure from ethidium bromide was made due to the large difference in fluorescence intensity observed between the bound and unbound states of thiazole orange (102). Ligand was added at varying r_dd values and the decrease in fluorescence intensity was recorded.
Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-G2A3G4C4-3’, by neomycin at 20 °C (a). A plot of fluorescence intensity vs r_{dd} is located in panel (b). A related plot of ΔF vs r_{dd} is located in panel (c). Scatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 μM/duplex, 14 μM/base pair, was pre-bound by thiazole orange, 7 μM and allowed to equilibrate for a period of 10 min prior to addition of neomycin, the fluorescence intensity is denoted as F_{max}. Neomycin was added to the duplex at various r_{dd} values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-G₂A₃G₄C₄-3’, by DPA244 at 20 °C (a). A plot of fluorescence intensity vs r_{dd} is located in panel (b). A related plot of ∆F vs r_{dd} is located in panel (c). Scatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of DPA244, the fluorescence intensity is denoted as F_{max}. DPA244 was added at various r_{dd} values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure 3.19. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5'-G\textsubscript{2}A\textsubscript{3}G\textsubscript{4}C\textsubscript{4}-3', by DPA243 at 20 °C (a). A plot of fluorescence intensity vs \( r_{dd} \) is located in panel (b). A related plot of \( \Delta F \) vs \( r_{dd} \) is located in panel (c). Schatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of DPA243, the fluorescence intensity is denoted as \( F_{\text{max}} \). DPA243 was added at various \( r_{dd} \) value. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure 3.20. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-G₂A₃G₄C₄-3’, by DPA208 at 20 °C (a). A plot of fluorescence intensity vs $r_{dd}$ is located in panel (b). A related plot of $\Delta F$ vs $r_{dd}$ is located in panel (c). Schatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of DPA208, the fluorescence intensity is denoted as $F_{\text{max}}$. DPA208 was added at various $r_{dd}$ values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the duplex, 5'-G₂A₃G₄C₄-3', by DPA218 (a) and DPA224 (c) at 20 °C. A related plot of ΔF vs r_{dd} is located in panel (b and d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of ligand, the fluorescence intensity is denoted as F_{max}. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Titration of neomycin into the duplex pre-bound with thiazole orange resulted in a decrease in fluorescence intensity, Figure 3.17. The decrease in fluorescence intensity was plotted against $r_{dd}$ and a clear 1:1 binding stoichiometry was observed. Scatchard analysis was performed on the pre-saturation region of the titration curve and provided an association constant $K_a$ of $1.1 \times 10^6 \text{ M}^{-1}$. Titration of the target duplex 1 by potential high affinity tetramer polyamide – neomycin conjugates DPA244 (L = 11 atoms), DPA243 (L = 10 atoms) and DPA208 (L = 12 atoms) were conducted, Figure 3.18, Figure 3.19 and Figure 3.20, respectively. The following association constants for DPA244 (L = 11 atoms), DPA43 (L = 10 atoms) and DPA208 (L = 12 atoms) were obtained following Scatchard analysis: $K_a$ of $1.9 \times 10^7 \text{ M}^{-1}$, $8.6 \times 10^6 \text{ M}^{-1}$ and $6.6 \times 10^6 \text{ M}^{-1}$, respectively.

In an effort to extend this approach to include other tetramer polyamide – neomycin conjugates, DPA218 (L = 18 atoms) and DPA214 were titrated into target duplex 1 at various $r_{dd}$ values, Figure 3.21. A decrease in fluorescence intensity was observed upon titration of the tetramer polyamide – neomycin conjugates into target duplex 1 pre-saturated with thiazole orange. Surprisingly, we could not extend Scatchard analysis to the titration of the tetramer polyamide – neomycin conjugates DPA218 (L = 18 atoms) and DPA214 (L = 12 atoms). The lack of a clear saturation point precluded the use of Scatchard analysis. Whether the high amount of displaced ethidium bromide observed in our single point FID assay is a result of a secondary binding mode or non-optimized linker length between neomycin and the tetramer polyamide remains to be seen. While association constants could not be derived for all tetramer polyamide – neomycin conjugates the association constants for DPA208 (L = 12 atoms), DPA243 (L
= 10 atoms) and DPA244 (L = 11 atoms) are larger than observed with neomycin and significantly higher than previously reported association constants for N-methylimidazole and N-methylpyrrole polyamides and their target sequences.

Figure 3.22. CD scans for the titration of 5’-G₂A₃G₄C₄-3’ with DPA244 (a, b). A plot of CD intensity at 265 nm vs r_dd value (c). Titrations were conducted at 20 °C. In plot (b) the open circle represents the CD scan of the duplex in the absence of neomycin while the closed circle represents the CD scan of the duplex in the presence of neomycin r_dd = 2.0. The conjugate was added to the duplex [40 μM/bp] was added at varying r_dd values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
**CD Monitored Titrations of Target Duplexes.** In effort to further characterize the binding of tetramer polyamide – conjugates, CD monitored titration of target duplex 1 (5’-G₂A₃G₄C₄-3’) with **DPA244** (L = 11atoms) was conducted, **Figure 3.22.** The CD spectra of native 5’-G₂A₃G₄C₄-3’ consists of a positive peak at 270 nm and negative peak at 210 nm which suggests the duplex retains A-form characteristics. However the strong negative band at 240 nm and the shoulder at 280 nm suggest the duplex also retains B-form characteristics. The binding of **DPA244** (L = 11atoms) to 5’-G₂A₃G₄C₄-3’ is clearly illustrated by the increase in CD intensity at 300 nm – 350 nm, the polyamide absorption region. Furthermore, the CD spectra display an increase in CD intensity at 270 nm and 210 nm upon addition of **DPA244** (L = 11atoms) which suggests an increase in A-form characteristics of the duplex occurs upon conjugate addition.
CONCLUSIONS

A library consisting of 20 tetramer polyamide – neomycin conjugates DPA208 – DPA219, DPA243 and DPA244 was designed and synthesized. The tetramer polyamide 1 consists of N-methylimidazole and N-methylpyrrole monomeric units which were coupled through EDC/HOBt mediated amide bond coupling in solution. Tetramer polyamide 1 was functionalized with the azide functional group through amide bond coupling with a number of α,ω-amino, azido alkanes to afford DPA202 – DPA207. Tetramer polyamide – neomycin conjugates were synthesized through 1,2,3-trirole formation between DPA202 – DPA207 and neomycin derivatives DPA13, DPA14, DPA15 and DPA17.

Spectroscopic techniques were used to characterize the binding of neomycin, 18 and tetramer polyamide – neomycin conjugates DPA208 – DPA219, DPA243 and DPA244 and the following conclusions were drawn: (i) Tetramer polyamide – neomycin conjugates increase the thermal stability of DNA. UV thermal denaturation experiments increased the melting temperature of 5’-A₂G₆C₆T₂-3’. The increase in thermal stability was dependant on linker length. The melting temperature of the duplex was stabilized by ~20 °C with conjugates of intermediate linker lengths of 16 and 17 atoms, which was double the increase in melting temperature afforded by neomycin or 18. (ii) Thermal stabilization afforded by tetramer polyamide – neomycin conjugates suggests strong sequence dependence. Series II and series III tetramer polyamide – neomycin conjugates afforded the largest increase in melting temperature when complexed with 5’-A₆G₂C₂T₆-3’ at 50 mM NaCl. Generally, as the size of the G/C pocket increased the magnitude of
the thermal stabilization decreased. (iii) *Compound 18 offers insight into tetramer polyamide – neomycin conjugate binding.* Our polyamide control 18 was titrated into 5’-A₆G₂C₂T₆-3’ and 5’-A₂G₆C₆T₂-3’ was monitored by CD and fluorescence. Upon titration of 18 in 5’-A₆G₂C₂T₆-3’ clear binding stoichiometries are observed at 1:1 and 2:1 polyamide:DNA. However, no clear changes in the CD signal for 5’-A₂G₆C₆T₂-3’ when titrated by 18. In fact binding was only confirmed by the decrease in fluorescence intensity upon addition of 18 to the 5’-A₂G₆C₆T₂-3’ duplex. (iv) *A target duplex for quantitative binding studies is paramount.* CD and FID titrations suggested optimal binding to DNA by the polyamide occurred complexed with the DNA at 2:1 and contained the sequence 5’-AG₂C₂A-3’. Unfortunately, the duplex that met the criteria, 5’-A₆G₂C₂T₆-3’, featured two A/T rich tails which are not suitable for neomycin binding. Therefore we designed target duplex 1, 5’-G₂A₃G₄C₄-3’, which featured the target DNA sequence for 18 when bound 1:1 and 5’-G₂C₂AG₄C₄-3’, which feature the target DNA sequence for 18 when bound 2:1. (v) *FID assay identified potential high affinity binding tetramer polyamide – neomycin conjugates.* We assayed each of the conjugates with target duplex 1 and target duplex 2. A clear preference for target duplex 1 was displayed by tetramer polyamide – neomycin conjugates. Target duplex 1 was the only duplex in the conjugates displaced more pre-bound ethidium bromide than neomycin. In fact, the assay identified two separate groups of conjugates which potentially bind target duplex 1 with affinities larger than neomycin or 18. (vi) *UV thermal denaturation experiments aids in the identification of potential high affinity binding tetramer polyamide – neomycin conjugates.* Tetramer polyamide – neomycin conjugates increase the thermal
stabilization of target duplex 1 better than target duplex 2. The largest magnitude of thermal stabilization afforded by the conjugates on 5'-G₂A₃G₄C₄-3' occurs with linker lengths between 10 and 14 atoms. (vii) **CD Titration of 5'-G₂A₃G₄C₄-3' by DPA244 suggests binding.** Binding of DPA244 (L = 11 atoms) to 5'-G₂A₃G₄C₄-3' is clearly illustrated by the increase in CD intensity at 300 nm – 350 nm. Furthermore, CD suggests addition of DPA244 (L = 11 atoms) to 5'-G₂A₃G₄C₄-3' increase A-form conformation in the duplex. (viii) **Full FID titrations suggest tetramer polyamide – neomycin conjugates bind the sequence with Kₐ of 10⁶ - 10⁷ M⁻¹.** Full FID titrations were conducted with tetramer polyamide – neomycin conjugates identified in the assay. The highest Scatchard derived association constant for 5'-G₂A₃G₄C₄-3' was observed with DPA244 (L = 11 atoms) Kₐ of 1.9 x 10⁷ M⁻¹. Binding constants were determined for the target duplex and DPA243 (L = 10 atoms), Kₐ of 8.6 x 10⁶ M⁻¹, and DPA208 (L = 8 atoms) Kₐ of 6.6 x 10⁶ M⁻¹.
REFERENCES


94. manuscript in press.


CHAPTER IV

RECOGNITION OF A-FORM DNA BY HAIRPIN POLYAMIDE – NEOMYCIN CONJUGATES

INTRODUCTION

In our ongoing effort to target DNA we report continued advancements in A-form DNA recognition. A-form DNA is an enticing nucleic acid target since it differs in conformation from biologically dominant B-form DNA (1). The A-form DNA duplex features an axial rise per residue of 2.5 Å and 12 – 14 base pairs per helical turn. The major groove of A-form DNA is narrow and deep, while the minor groove is wide and shallow. Some A-form DNA duplexes have even been crystallized with furanose sugar puckers in a $C_3$-endo conformation (2).

Changes in the relative humidity can induce A-form conformation. For example, a decrease in humidity drives to conformation of poly(dA-dT)$_2$ to A-form (3, 4). The ability of a duplex to adopt A-from conformation is sequence dependent. While changes in humidity drives the B to A transition in poly(dA-dT)$_2$, poly(dG-dC)$_2$ is conformationally rigid and exists exclusively in A-form (3). Furthermore, several self-complementary DNA oligomers d(CCGG)$_2$, d(GGCCG GCC)$_2$ and d(GGTAT ACC)$_2$ crystallized as A-form duplexes (5, 6, 7). Since numerous other oligomers, in conjunction with the self-complementary DNA oligomers, were crystallized as B-form
duplexes in similar conditions, a strong emphasis was placed on A-form sequence
dependence. And while it is accepted that mixed base sequence adopts B-form, the
ability of a DNA duplex to adopt A-form decrease when the percentage of G/C content
drops below 30% (8).

A transition from B-form to A-form DNA is also observed upon protein and small
molecule binding. Proteins which induce A-form upon binding include HIV-1 reverse
transcriptase (9, 10, 11), polymerase β (12), Taq polymerase (13), *Bacillus* polymerase I
(14), T7 polymerase (15) and DNAs I (16, 17), to name a few. Furthermore, small
molecules neomycin, spermine and hexaamminecobalt (III) induce A-form upon binding
(18).

Recent advancements by our group and others suggest aminoglycoside
(neomycin) is capable of binding a number of non-traditional A-form nucleic acid
structures: RNA triplex (19), DNA:RNA hybrid duplex (20, 21, 22), RNA duplex (23),
DNA triplex (19, 24, 25, 26), A-form DNA duplex (18) as well as the DNA tetraplex
(27). We currently desire to expand our polyamide – neomycin conjugates approach to
DNA recognition by targeting DNA sequences with a high propensity to adopt the A-
form conformation. To this end we desire to target DNA sequences of high G/C content.

Aminoglycosides feature polycationic charges about a flexible carbohydrate
scaffold which allows them to preferentially bind prokaryotic rRNA as well as a number
of other RNA targets such as group I introns, a hammerhead ribozyme, the RRE
transcriptional activator region from HIV, the 5'-untranslated region of thymidylate
synthase a variety of RNA aptamers from in vitro selection and human mRNAs (28, 29,
Since their discovery by Selman Waksman (31), considerable attention has been paid to understanding aminoglycoside mode of action. Previous work by our group suggests conjugation of neomycin to a minor groove binding ligand can act as a paradigm for an aminoglycoside based approach to DNA recognition (32, 33).

Significant work has been conducted on the lexitropsin sub-class of DNA minor groove binding ligands (34). Natural products netropsin and disamycin A are di-N-
methylpyrrole and tri-N-methylpyrrole polyamides, respectively, which bind A/T rich DNA sequence (35, 36, 37). Advancements in polyamide design allow polyamides to read G/C sequences (38, 39, 40, 41, 42, 43, 44). Substitution of N-methylpyrrole with N-methylimidazole offers a solution to overcome the repulsion between the amino group found on the 2-position of guanine and the 3-position of pyrrole (45, 46, 47, 48). However, true recognition of DNA base pairs requires linear polyamide to bind in a 2:1 head-to-tail fashion in the minor groove. Pairing heterocyclic monomers across the floor of the minor groove in a 2:1 binding stoichiometry, a pyrrole/imidazole pairing recognizes a C/G base pair while an imidazole/pyrrole pairing recognizes a G/C base pair. A pyrrole/pyrrole pairing recognizes A/T containing base pairs from G/C containing base pairs, but does not recognize A/T base pairs from T/A base pairs (49).

Hairpin polyamides offer a unique method for preserving minor groove specificity/pairing rules by convalently linking two linear polyamides (50), Figure 4.1. Hairpin polyamides are advantageous as the use of a γ-aminobutyrate linker when covalently linking linear polyamides allows the polyamides align staggered, hear-to-tail, in the minor groove (51, 52). Second, compared to linear homodimers, the hairpin motif displays ~100 fold higher affinity (53). Six-ring hairpin polyamides bind their target sequences at low $10^7$ M$^{-1}$ affinity (54). Eight-ring hairpin polyamides have been successfully used to target the sequence 5’-TGGCCA-3’ with an association constant $K_a = 9.7 \times 10^9$ M$^{-1}$, significantly higher than the affinity observed for the linear tetramer polyamide $< 2 \times 10^5$ M$^{-1}$ (55).
EXPERIMENTAL

Materials:

**Nucleic Acids.** DNA oligomers were purchased from Eurofins MWG Operon (Huntsville, AL). Concentrations were determined by UV absorbance using extinction coefficients provided by Operon. The duplexes were pre-formed at 20 µM/duplex, by heating at 90 °C for 10 min, followed by slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h.

**Chemicals.** Neomycin B (sulfate salt) was purchased from ICN pharmaceuticals and used without further purification (both synthesis and binding experiments); reagents were purchased from Acros organics. Solvents were purchased from VWR with the exception of pyridine, DMF and 1,4-dioxane which were purchased from Acros. Reaction solvents were distilled accordingly; dichloromethane and pyridine were distilled over calcium hydride and ethanol was distilled over sodium metal. All non-commercially available intermediates were synthesized as previously reported: heterocyclic monomers (56) and neomycin derivatives (33, 57, 58, 59).

Methods:

**Fluorescence Intercalator Displacement Assay.** 96 well equilibrium binding experiments were conducted using a Varian Eclipse Fluorescence Spectrometer (Varian Inc.; Palo Alto, CA) equipped with a 96 well plate reader. DNA duplexes were pre-formed at 5 µM/duplex by heating at 90 °C for 10 min, followed by cooling to 10 °C at
0.2 °C/min and incubation at 4 °C for 12 h. Buffer was added to each well followed by ethidium bromide (7 μM). DNA duplex was added to the individual wells such that the final concentration of DNA was 1 μM. Ligands were added to each well at a ratio of drug to duplex, \( r_{dd} = 1.0 \), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. In the histogram, the values represent the percent decrease in fluorescence upon each addition of ligand. Fluorescence parameters; excitation: wavelength = 525 nm, slits = 20, filter = 335-620 nm; emission: wavelength = 550 nm; slits = 20, filter = 550-800 nm; PMTV = 610 nm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 50 mM NaCl, pH 7.0.

**Fluorescence Intercalator Displacement Titrations.** Equilibrium binding experiments were conducted using a Varian Eclipse Fluorescence Spectrometer (Varian Inc.; Palo Alto, CA) at 20 °C. Duplexes were pre-formed at 20 mM/duplex by heating at 90 °C for 10 min, followed by a slow annealing at 0.2 °C/min and incubation at 4 °C for 12 h. The DNA duplex was diluted to 1.0 μM/duplex, pre-bound by thiazole orange, 7 μM and allowed to equilibrate for a period of 10 min prior to addition of ligand. Ligand was added at various \( r_{dd} \) values. Fluorescence parameters; excitation: wavelength = 504, slits = 10, filter = auto; emission: wavelength = 510-610 nm; slits = 10, filter = auto; PMTV = 580 nm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, 50 mM NaCl, pH 7.0.
Determination of Binding Constants by Scatchard Analysis. The $\Delta F$ was plotted versus molar equivalents of ligand and the $\Delta F_{\text{sat}}$ (binding stoichiometry) was determined mathematically by simultaneous solving the equations representing the pre- and post-saturation regions of the titration curve. A Scatchard plot was generated utilizing equations 1 – 3, generated where $\Delta F/[\text{ligand}]$ was plotted versus $\Delta F$. The slope of the points representing the region immediately preceding complete saturation of the system provided $-K_a$. In these equations, $[\text{ligand}] = \text{concentration of ligand}$, $[\text{DNA}]_T = \text{total concentration of DNA}$, $X = \text{molar equiv of ligand versus DNA}$, $\Delta F_x = \text{change in fluorescence}$, and $\Delta F_{\text{sat}} = \text{change in fluorescence at the point where DNA is saturated with ligand}$.

\[
\left(\frac{\Delta F_x}{\Delta F_{\text{sat}}} \right) \frac{1}{X} = \text{Fraction of DNA – ligand complex} \quad (1)
\]

\[
\left[ 1 - \left(\frac{\Delta F_x}{\Delta F_{\text{sat}}} \right) \frac{1}{X} \right] = \text{Fraction of ligand} \quad (2)
\]

\[
[\text{DNA}]_T \left[ X - \frac{\Delta F_x}{\Delta F_{\text{sat}}} \right] = [\text{ligand}] \quad (3)
\]

Synthesis. Synthesis of intermediate compounds is located in the Appendix C. Listed in the order of appearance: HImImCOOMe (10), Appendix B p. 278; HImImPyCOOMe (11) Appendix B p. 280; BocNHPyγCOOMe (25), p. 360; BocNHPyγImCOOMe (20), p. 361; BocNHlImPyCOOMe (26), p. 362; BocNHlImPyPyCOOMe (21), p. 363; HImlMpyPyImCOOMe (27), p. 364; HImlMpyPyImImPyPyCOOMe (19), p. 366;
azide functionalized tetramer polyamides (DPA226 – DPA231), p. 367 – 370; N-Boc
neomycin-NHC(O)CCH (DPA16), p. 372; and HImImPyPyγImImPyPyDp (28), p. 373.

(DPA232 – DPA237). To a solution of DPA13 (10.0 mg, 7.3 µmol) in DMF (1.0 mL)
was added a homogeneous solution consisting of CuI (2.8 mg, 14.6 µmol) and DIPEA
(47.4 mg, 366.4 µmol) in DMF (500 µL). To this solution was added a solution
containing a single azide functionalized hairpin polyamides DPA226 – DPA231 (7.3
µmol) suspended in DMF. The solution was allowed to stir at 80 °C for 24 h. The
solution was concentrated *in vacuo*. The resulting residue was purified by flash
chromatography (silica gel, CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield
the N-Boc protected conjugate which was suspended in 1,4-dioxane (2.0 mL). 4N HCl in
1,4-dioxane (4.0 mL) was added and the solution was stirred for 1 h. The solid was
decanted, and washed with copious amounts of CH₂Cl₂ and lyophilized to afford
DPA232 – DPA237 as hydrochloride salts. The reported data reduction of ¹H NMR and
MALDI spectra reflect the deprotected compounds. Furthermore, representative ¹H
NMR and MALDI spectra of the N-Boc protected conjugate of **DPA232** are also included.

**N-Boc DPA232**. **N-Boc DPA232** (3.0 mg, 15%) was afforded as a light brown solid: TLC (protected, 90:10 CH₂Cl₂:MeOH v/v) Rₜ 0.48; **¹H NMR** (500 MHz, (CD₃)₂CO, N-Boc protected) δ 8.11 (br, 2H), 7.51 (s, 1H), 7.26 (s, 2H), 7.18 (s, 1H), 7.07 (s, 1H), 6.91 (s, 1H), 6.80 (s, 1H), 6.74 (s, 1H), 6.67 (s, 1H), 6.61 (s, 2H), 6.55 (s, 2H), 6.10 (br, 1H), 5.95 (br, 1H), 5.28 (br, 1H), 5.16 (s, 1H), 4.90 (s, 1H), 4.18 (s, 2H), 4.04 (s, 6H), 3.88 (s, 12H), 3.75 (s, 3H), 3.96 (s, 1H), 3.82-3.90 (m, 4H), 3.76 (s, 1H), 3.64-3.72 (m, 4H), 3.48 (m, 6H), 3.32 (br, 2H), 3.23 (m, 2H), 3.19-3.30 (m, 5H), 2.23 (m, 1H), 1.94 (m, 1H), 1.56 (br, 4H), 1.38–1.46 (m, 54H); MALDI-TOF (N-Boc protected) m/z calcd C₁₀₉H₁₅₅N₃₃O₃₄ 2471.60, found [M + Na]+ 2495.22;

**DPA232**. **DPA232** (2.3 mg, 98%) was afforded as a light brown solid: **¹H NMR** (500 MHz, D₂O) δ 8.09 (s, 1H), 9.01 (s, 1H), 7.94 (s, 1H), 7.78 (s, 1H), 7.61 (s, 2H), 7.51 (s, 1H), 7.20 (s, 1H), 7.16 (s, 1H), 6.95 (s, 1H), 6.88 (s, 1H), 6.56 (s, 2H), 6.50 (s, 2H), 6.48 (s, 1H), 5.67-5.66 (s, 2H), 5.31-5.30 (m, 1H), 5.18 (br, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.22 (s, 6H), 4.17-4.15 (m, 2H), 4.15 (s, 6H), 4.00 (s, 6H), 4.00-3.96 (m, 1H), 3.96 (s, 3H), 3.90 (s, 4H), 3.86-3.85 (m, 1H), 3.84 (s, 3H), 3.84-3.79 (m, 2H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.73-3.59 (m, 4H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.45 (br, 1H), 3.39-3.36 (m, 1H), 3.35 (s, 4H), 3.35-3.33 (m, 4H), 3.32-3.28 (m, 1H), 3.27 (br, 2H), 3.23-3.22 (m, 1H), 2.81-2.78 (m, 3H), 3.15-3.10 (s, 2H), 2.66-
2.55 (t, $J = 5.1$, 2H), 2.25-2.22 (m, 1H), 2.18-1.95 (t, $J = 6.4$, 2H), 1.75-1.64 (m, 1H), 1.55-1.41 (m, $J_1 = 6.4, J_2 = 5.2$, 2H); MALDI-TOF (deprotected) $m/z$ calcd C$_{79}$H$_{107}$N$_{33}$O$_{22}$ 1870.90, found [M + Na]$^+$ 1896.19.

(DPA233). DPA233 (3.3 mg, 22%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.06 (s, 2H), 7.71 (s, 1H), 7.52 (s, 2H), 7.50 (s, 1H), 7.46 (s, 1H), 7.09 (s, 1H), 7.00 (s, 1H), 6.75 (s, 2H), 6.68 (s, 1H), 6.60 (s, 1H), 6.55 (s, 1H), 6.48 (s, 1H), 6.03 (s, 2H), 5.69-5.65 (m, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.25-4.20 (m, 1H), 4.10-4.09 (m, 2H), 3.93-3.88 (m, 1H), 3.78 (s, 6H), 3.70 (s, 6H), 3.60 (s, 6H), 3.54 (s, 3H), 3.50 (s, 3H), 3.87-3.85 (m, 1H), 3.83-3.81 (m, 4H), 3.80-3.79 (m, 2H), 3.78 (s, 1H), 3.71 (br, 1H), 3.70-3.65 (m, 4H), 3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.47-3.45 (m, 1H), 3.38-3.36 (m, 1H), 3.35 (s, 2H), 3.35 (br, 2H), 3.33-3.25 (m, 7H), 3.24-3.22 (m, 1H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.67-2.54 (t, $J = 5.1$, 2H), 2.21-2.17 (m, 1H), 2.18-1.90 (t, $J = 6.4$, 2H), 1.86 (m, 2H), 1.73-1.61 (m, 1H); MALDI-TOF $m/z$ C$_{80}$H$_{109}$N$_{33}$O$_{22}$ 1884.93, found [M + Na]$^+$ 1907.22.

(DPA234). DPA234 (3.5 mg, 23%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.01 (br, 2H), 7.53 (s, 1H), 7.18 (s, 1H), 7.12 (s, 2H), 6.89 (s, 1H), 6.85 (s, 1H), 6.79 (s, 1H), 6.75 (s, 1H), 6.68 (s, 2H), 6.60 (s, 1H), 6.56 (s, 1H), 6.49 (s, 1H), 6.11 (s, 1H), 5.36-5.35 (m, 1H), 5.27-5.24 (m, 1H), 5.16 (br, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.27 (s, 3H), 4.23-4.21 (m, 1H), 4.18 (s, 3H), 4.13-4.10 (m, 2H), 3.99 (s, 6H), 3.96 (s, 6H), 3.94 (s, 7H), 3.87 (m, 4H), 3.84-3.82 (m, 4H), 3.81-3.77 (m, 4H), 3.76
(s, 1H), 3.74-3.72 (m, 1H), 3.68-3.66 (m, 1H), 3.63-3.57 (m, 5H), 3.50-3.46 (m, 1H), 3.41-3.31 (m, 5H), 3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 2H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.66-2.54 (t, J = 5.1, 2H), 2.23-2.20 (m, 1H), 2.18-1.90 (t, J = 6.3, 2H), 1.72-1.63 (m, 1H), 1.55-1.52 (m, 6H); MALDI-TOF m/z calcd C_{82}H_{113}N_{33}O_{22} 1912.98, found [M + Na]^+ 1934.71.

(DPA235). **DPA235** (4.2 mg, 27%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.09 (br, 2H), 7.53 (s, 2H), 7.47 (s, 2H), 7.18 (s, 2H), 7.12 (s, 1H), 6.89 (s, 2H), 6.85 (s, 1H), 6.79 (s, 1H), 6.75 (s, 1H), 6.68 (s, 1H), 6.60 (s, 1H), 6.81 (s, 1H), 6.56 (s, 1H), 6.49 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20 (br, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.22 (s, 3H), 4.15-4.11 (m, 2H), 4.00 (s, 7H), 3.96 (s, 6H), 3.90 (s, 6H), 3.91-3.90 (m, 1H), 3.88-3.86 (m, 4H), 3.85-3.80 (m, 5H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.65-3.61 (m, 1H), 3.60-3.53 (m, 4H), 3.46 (br, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H), 3.33-3.32 (m, 2H), 3.30-3.29 (m, 3H), 3.27-3.25 (m, 4H), 3.25-2.23 (m, 1H), 3.21-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.27-2.21 (m, 1H), 2.63-2.54 (t, J = 5.1, 2H), 2.18-1.95 (t, J = 6.4, 2H), 1.70-1.61 (m, 1H), 1.55-1.41 (m, 8H); MALDI-TOF m/z calcd C_{83}H_{115}N_{33}O_{22} 1927.01, found [M + Na]^+ 1951.33.

(DPA236). **DPA236** (2.8 mg, 18%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.11 (s, 1H), 8.06 (s, 1H), 7.86 (s, 1H), 7.55 (s, 2H), 7.50 (s, 1H), 7.46 (s, 1H), 7.40 (s, 2H), 7.18 (s, 1H), 7.10 (s, 1H), 6.79 (s, 2H), 6.71 (s, 1H), 6.64 (s, 1H), 6.09 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.51-4.43 (m, 1H),
4.37-4.33 (m, 1H), 4.30 (s, 3H), 4.28-4.22 (m, 1H), 4.20 (m, 1H), 4.15-4.11 (m, 2H), 4.00 (s, 1H), 4.02-3.98 (m, 6H), 3.93 (s, 6H), 3.91-3.90 (m, 1H), 3.90 (s, 6H), 3.88-3.86 (m, 1H), 3.85-3.80 (m, 4H), 3.79 (s, 4H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.57-3.53 (m, 4H), 3.46-3.45 (m, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H), 3.33-3.31 (m, 2H), 3.30-3.29 (m, 1H), 3.27-3.25 (m, 2H), 3.25-2.23 (m, 3H), 3.21-3.16 (m, 5H), 3.13-3.10 (s, 2H), 2.67-2.54 (t, J = 5.2, 2H), 2.27-2.21 (m, 1H), 2.18-1.90 (t, J = 6.4, 2H), 1.70-1.61 (m, 1H), 1.59 (m, 4H) 1.31 (m, 4H), 1.28 (m, 4H); MALDI-TOF m/z calcd C_{85}H_{119}N_{33}O_{22} 1955.06, found [M + Na]^+ 1978.01.

**DPA237.** DPA237 (3.3 mg, 21%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.01 (s, 2H), 7.71 (s, 1H), 7.50 (s, 2H), 7.45 (s, 1H), 7.41 (s, 1H), 7.13 (s, 1H), 7.00 (s, 1H), 6.98 (s, 1H), 6.75 (s, 2H), 6.68 (s, 1H), 6.60 (s, 1H), 6.55 (s, 1H), 6.18 (s, 1H), 5.57-5.56 (m, 1H), 5.31 (br, 1H), 5.18-5.17 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.27 (s, 3H), 4.25-4.22 (m, 1H), 4.17-4.15 (m, 2H), 4.18 (s, 3H), 4.00-3.96 (m, 1H), 3.97 (s, 12H), 3.90 (s, 9H), 3.87 (m, 4H), 3.84-3.79 (m, 2H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.70-3.64 (m, 5H), 3.65-3.55 (m, 1H), 3.45-3.44 (m, 1H), 3.39-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.36-3.23 (m, 9H), 3.23-3.22 (m, 1H), 3.21-3.17 (m, 3H), 3.15-3.10 (s, 2H), 2.67-2.54 (t, J = 5.2, 2H), 2.25-2.22 (m, 1H), 2.18-1.90 (t, J = 6.4, 2H), 1.75-1.64 (m, 1H), 1.66 (m, 4H), 1.38 (m, 8H); MALDI-TOF m/z calcd C_{87}H_{123}N_{33}O_{22} 1983.12, found [M + Na]^+ 2007.19.
(DPA238 – DPA240). To a solution of DPA16 (9.2 mg, 7.3 µmol) in DMF (1.0 mL) was added a solution of the azide-terminal hairpin polyamides (7.3 µmol) suspended in DMF. To this solution was added a homogeneous solution consisting of CuI (2.8 mg, 14.6 µmol) and DIPEA (47.4 mg, 366.4 µmol) in DMF (500 µL). The reaction was allowed to stir for 18 h at 80 °C. The solution was concentrated in vacuo. The resulting residue was purified by flash chromatography (silica gel, CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugate which was suspended in 1,4-dioxane (2.0 mL). 4N HCl in 1,4-dioxane (4.0 mL) was added and the solution was stirred for 1 h. The solid was decanted, and washed with copious amounts of CH₂Cl₂ and lyophilized to afford DPA238 – DPA240 as a hydrochloride salts. The reported data reduction reflects the deprotected compounds.

(DPA238). DPA238 (3.8 mg, 26%) was afforded as a light brown solid: ¹H NMR (500 MHz, D₂O) δ 7.91 (s, 1H), 7.73 (s, 1H), 7.63 (s, 1H), 7.60 (s, 2H), 7.56 (s, 1H), 7.43 (s,
1H, 7.26 (s, 1H), 7.17 (s, 1H), 7.07 (s, 1H), 6.93 (s, 1H), 6.77 (s, 2H), 6.69 (s, 1H), 5.97 (s, 2H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.22 (m, 1H), 4.22 (s, 1H), 4.15 (s, 6H), 4.13-4.10 (m, 6H), 4.02-3.98 (m, 7H), 3.91 (s, 3H), 3.91-3.90 (m, 1H), 3.90 (s, 3H), 3.88-3.86 (m, 1H), 3.84 (s, 3H), 3.83-3.80 (m, 2H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.50-3.46 (m, 1H), 3.40-3.37 (m, 1H), 3.36 (s, 2H), 3.36-3.32 (m, 6H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 2H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.66-2.55 (t, J = 5.1, 2H), 2.25-2.22 (m, 1H), 2.18-1.95 (t, J = 6.4, 2H), 1.75-1.64 (m, 1H), 1.55-1.41 (m, J1=6.4, J2=5.2, 2H); MALDI-TOF m/z calcd C_{77}H_{107}N_{31}O_{22} 1818.87, found [M + Na]^+ 1843.62.

(DPA239). DPA239 (3.9 mg, 27%) was afforded as a light brown solid: ¹H NMR (500 MHz, CDCl₃) δ 8.20 (s, 1H), 7.57 (s, 1H), 7.33 (s, 1H), 7.21 (s, 2H), 7.06 (s, 1H), 7.00 (s, 1H), 6.90 (s, 1H), 6.84 (s, 1H), 6.61 (s, 2H), 6.58 (s, 1H), 6.50 (s, 2H), 6.11 (s, 2H), 5.56-5.55 (m, 1H), 5.27-5.24 (m, 1H), 5.16-5.15 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.27 (s, 3H), 4.25-4.20 (m, 1H), 4.18 (s, 3H), 4.10-4.09 (m, 2H), 4.07 (s, 6H), 4.02-3.98 (m, 7H), 3.96 (s, 6H), 3.94 (m, 2H), 3.91-3.90 (m, 1H), 3.88-3.86 (m, 4H), 3.85-3.80 (m, 5H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.45-3.44 (m, 1H), 3.39-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.28 (m, 5H), 3.27-3.24 (m, 2H), 3.23-3.22 (m, 1H), 3.21-3.17 (m, 3H), 3.15-3.10 (s, 2H), 2.66-2.54 (t, J = 5.4, 2H), 2.21-2.18 (m, 1H), 2.18-1.90 (t, J = 6.2, 2H), 1.73-1.61 (m, 1H), 1.55-1.52 (m, 2H); MALDI-TOF m/z calcd for C_{78}H_{109}N_{31}O_{22} 1832.89, found [M + Na]^+ 1854.99.
**DPA240.** DPA240 (4.4 mg, 30%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.11 (s, 1H), 7.34 (s, 2H), 7.32 (s, 2H), 7.19 (s, 2H), 7.09 (s, 1H), 6.94 (s, 3H), 6.81 (s, 1H), 6.61 (s, 1H), 6.60 (s, 1H), 5.59-5.55 (m, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.30 (s, 3H), 4.28-4.22 (m, 1H), 4.20 (s, 3H), 4.15-4.11 (m, 2H), 4.10 (s, 6H), 4.00-3.96 (m, 1H), 3.93 (s, 6H), 3.91-3.88 (m, 7H), 3.86-3.85 (m, 1H), 3.84-3.79 (m, 5H), 3.78 (s, 1H), 3.77-3.74 (m, 4H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.50-3.46 (m, 1H), 3.40-3.37 (m, 1H), 3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.25 (m, 7H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.67-2.54 (t, $J = 5.1$, 2H), 2.27-2.21 (m, 1H), 2.18-1.90 (t, $J = 6.2$, 2H), 1.70-1.61 (m, 1H), 1.59 (m, 2H); MALDI-TOF $m/z$ calcd for C$_{80}$H$_{113}$N$_{31}$O$_{22}$ 1860.95, found [M + Na]$^+$ 1883.00.
(DPA241). To a solution of compound 19 (10.0 mg, 9.2 µmol) in methanol (10 mL) was added 1.5 M NaOH (1 mL) and the solution was allowed to stir for 3.0 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF (10 mL). EDC (2.2 mg, 11.5 µmol) and HOBT (1.6 mg, 11.5 µmol) were added and the reaction was allowed to stir for 30 min. Compound DPA10 (11.8 mg, 9.2 µmol) was added and reaction was allowed to stir at room temperature for 12 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, inc. CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugate which was suspended in 1,4-dioxane (2.0 mL). 4N HCl in 1,4-dioxane (4.0 mL) was added and the solution was stirred for 1 h. The solid was decanted, and washed with copious amounts of CH₂Cl₂ and lyophilized to afford
DPA241 (13.9 mg, 88%) as a light yellow hydrochloride salt: $^1$H NMR (500 MHz, D$_2$O) δ 7.61 (s, 1H), 7.48 (s, 2H), 7.41 (s, 1H), 7.20 (s, 1H), 7.17 (s, 1H), 7.03 (s, 1H), 7.01 (s, 1H), 6.78 (s, 2H), 6.70 (s, 1H), 6.64 (s, 2H), 6.10 (s, 2H), 5.57-5.56 (m, 1H), 5.31-5.30 (m, 1H), 5.18-5.17 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.29 (s, 1H), 4.23-4.21 (m, 2H), 4.19 (s, 1H), 4.13-4.10 (m, 8H), 4.00 (s, 6H), 3.90 (s, 6H), 3.93-3.88 (s, 7H), 3.87-3.85 (m, 4H), 3.83-3.81 (m, 4H), 3.80-3.79 (m, 2H), 3.78 (s, 1H), 3.71-3.70 (m, 1H), 3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.46-3.45 (m, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H), 3.33-3.31 (m, 2H), 3.30-3.29 (m, 1H), 3.27-3.25 (m, 2H), 3.25-2.23 (m, 1H), 3.21-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.68-2.55 (m, 4H), 2.60-2.54 (t, $J = 5.3$, 2H), 2.23-2.20 (m, 1H), 2.18-1.95 (t, $J = 6.1$, 2H); MALDI-TOF m/z calcd for C$_{73}$H$_{101}$N$_{27}$O$_{21}$S 1724.82, found [M + Na]$^+$ 1747.65.
(DPA242). To a solution of compound 19 (10.0 mg, 9.2 µmol) in methanol (10 mL) was added 1.5 M NaOH (1 mL) and the solution was allowed to stir for 3.0 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF (10 mL). EDC (2.2 mg, 11.5 µmol) and HOBt (1.6 mg, 11.5 µmol) were added and the reaction was allowed to stir for 30 min. Compound DPA11 (11.1 mg, 9.2 µmol) was added and reaction was allowed to stir at room temperature for 18 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, inc. CH₂Cl₂:MeOH, 5% incremental increases in MeOH) to yield the N-Boc protected conjugate which was suspended in 1,4-dioxane (2.0 mL). 4N HCl in 1,4-dioxane (4.0 mL) was added and the solution was stirred for 1 h. The solid was decanted, and washed with copious amounts of CH₂Cl₂ and lyophilized to afford
**DPA242** (14.3 mg, 81%) as a light yellow hydrochloride salt: $^1$H NMR (500 MHz, D$_2$O-) $\delta$ 7.65 (s, 1H), 7.45 (s, 2H), 7.41 (s, 1H), 7.23 (s, 1H), 7.19 (s, 1H), 7.00 (s, 1H), 6.95 (s, 1H), 6.78 (s, 2H), 6.70 (s, 1H), 6.65 (s, 2H), 6.10 (s, 2H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.18 (s, 1H), 4.17-4.15 (m, 2H), 4.07 (s, 1H), 3.98-3.96 (m, 2H), 3.94 (s, 6H), 3.88-3.86 (m, 6H), 3.84-3.82 (m, 6H), 3.81-3.77 (m, 2H), 3.76 (s, 4H), 3.74-3.72 (m, 3H), 3.68-3.66 (m, 1H), 3.63-3.57 (m, 1H), 3.47-3.45 (m, 1H), 3.38-3.36 (m, 1H), 3.36-3.23 (m, 4H), 3.35-3.33 (m, 2H), 3.32-3.30 (m, 1H), 3.30-3.24 (m, 2H) 3.24-3.22 (m, 1H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.67-2.54 (t, $J = 6.2$, 2H), 2.25-2.22 (m, 1H), 2.18-1.90 (t, $J = 5.1$, 2H), 1.75-1.64 (m, 2H); MALDI-TOF $m/z$ calcd for C$_{71}$H$_{97}$N$_{27}$O$_{21}$ 1664.70, found [M + Na]$^+$ 1687.13.
RESULTS AND DISCUSSION

Design of Hairpin Polyamide – Neomycin Conjugates. Advancements by our research group have led to the development of a model for aminoglycoside, neomycin recognition of DNA. Conjugation of neomycin to minor groove binding ligands have allowed us to target B-form DNA (32, 33) as well as DNA containing GC sequences, Chapter 4. We seek to expand the scope of our model to include the ability to target DNA which contains a core sequence of four GC base pairs. Literature suggests an increase in guanine or cytosine content in DNA increases the ability of the duplex to adopt A-form characteristics (1, 3, 4). Chapter 4 utilized the ability of N-methylpyrrole and N-methylimidazole to preferentially bind G/C base pairs over A/T base pairs (60, 61, 62, 63, 64). In this chapter, N-methylpyrrole and N-methylimidazole heterocycles are paired across the floor of the minor groove to preferentially bind stretches of G/C base pairs, a technique previously established (60, 61, 62, 63, 64).

An eight-ring hairpin polyamide will allow for the recognition of a core sequence of four G/C base pairs (55, 60). While the tetramer polyamide HImImPyPy- allowed us to target DNA which contained the core sequence 5’-GGAA-3’ in a 1:1 binding mode, the pairing of this tetramer polyamide, head-to-tail, in the floor of the minor groove allows us to potentially target the core sequence 5’-GGCC-3’ in the 2:1 fashion (55). Hairpin polyamides afford a method of controlling the binding stoichiometry, in turn allow for the recognition of a core sequence of four G/C base pairs (54, 55, 64, 65, 66, 67).
Figure 4.2. Hairpin polyamide – neomycin conjugates used in this study: DPA232 – DPA242, neomycin and HImImPyPyImImPyPyDp (28).
The 11 hairpin polyamide – neomycin conjugates were coupled through 1,2,3-triazoles, ‘single- and double-Click’ (68, 69, 70) and amide bonds (21). The linker length separating the two moieties varied from one to 20 atoms, Figure 4.2. To accommodate the variation in conjugate linker length, a sub-library of hairpin polyamides 18, DPA226 – DPA231 and a sub-library of N-Boc protected neomycin derivatives DPA10, DPA12, DPA13 and DPA16 were synthesized. Hairpin polyamide – neomycin conjugate linker length is defined as the number of atoms measured from the C-terminal carbonyl group of the hairpin polyamide to, but not including, the 5’ nitrogen on ring three of neomycin. Conjugates containing 1,2,3-triazoles are counted through the alkene bond such that the lowest total number of atoms were counted. Therefore, our library of 11 hairpin polyamide – neomycin conjugates ‘single-Click’ hairpin polyamide – neomycin conjugates DPA238 – DPA240, ‘double-Click’ hairpin polyamide – neomycin conjugates DPA232 – DPA237, and amide bond conjugates DPA241 and DPA242, Figure 4.2.

**Synthesis of Hairpin Polyamide – Neomycin Conjugates.** In the design of our hairpin polyamide – neomycin conjugates DPA232 – DPA242 we focused on the following retrosynthetic approach, Figure 4.3. Each of our hairpin polyamide – neomycin conjugates DPA232 – DPA242 contained the same 8-ring hairpin polyamide HImImPyPyγImImPyPyCOOMe 19 as shown in Figure 4.3. We envisioned this parent hairpin polyamide, 19, as a product of sequential couplings of three intermediate
**Figure 4.3.** Retrosynthetic strategy for the convergent solution-phase synthesis of 8-ring hairpin polyamide 1.
polyamide trimers. The three trimers consisted of an ImImPy containing trimer 11, PyγIm 20 a γ-turn moiety containing trimer and ImPyPy containing trimer 21, Figure 4.3. Furthermore, we envisioned the preparation of intermediate trimers 11, 20 and 21 as coupling products of monomeric building blocks. For example, the ImImPy containing trimer could be constructed from three monomeric building blocks: 2-trichloroacetyl-1-methylimidazole 7, methyl 4-nitro-1-methylimidazole-2-carboxylate 8 and methyl 4-nitro-1-methylpyrrole-2-carboxylate 9, Figure 4.3. In a similar fashion, the γ-turn moiety containing PyγIm 20 could be assembled from commercially available methyl 4-aminobutyrate 24, and two monomeric building blocks: methyl 4-[(tert-butoxy carbonyl)amino]-1-methylpyrrole-2-carboxylate 23 and methyl 4-nitro-1-methylimidazole-2-carboxylate 8, Figure 4.3. Finally, the ImPyPy containing trimer 21 was easily prepared using two monomeric building blocks: methyl 4-[(tert-butoxy

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\text{Scheme 4.1. Reagents and conditions: (i) dry MeOH, H}_2\text{(g), 10% Pd/C, 23 °C, 3.5 h, 99%}; (ii) CH}_2\text{Cl}_2, 23 \text{ °C, 6 h, 72%}; (iii) MeOH, 1.5 N NaOH, 23 \text{ °C, 4 h}; (iv) MeOH, H}_2, 10\% \text{ Pd/C, 23 °C, 4 h, 97%}; (v) DMF, EDC, HOBt, 23 \text{ °C, 16 h, 71%}.}
\]
carbonyl)amino]-1-methylimidazole-2-carboxylate 22 and methyl 4-nitro-1-methylpyrrole-2-carboxylate 9, **Figure 4.3**.

Compound 19, and the subsequent trimers were conducted in solution in a modification of Dervan’s method (64). The intermediate trimers were prepared from building blocks 7 – 9 and 22 - 24, which were synthesized as previously reported (56, 71). Synthesis of trimer 11 was conducted as previously discussed in chapter 4 (**Scheme 3.1**, page 155) and shown in **Scheme 4.1** (72).

The γ-turn trimer 20 was synthesized in two coupling steps from methyl 4-[(tert-butoxy carbonyl)amino]-1-methylpyrrole-2-carboxylate 23, **Scheme 4.2**. Compound 23 was saponified with 1.5 N NaOH followed by neutralization with HCl to afford 23a. Compound 23a was activated with benzotriazol-1-yl-oxytripyrrolidinophosphonium

![Scheme 4.2: Reagents and conditions:](image)

**Scheme 4.2.** Reagents and conditions: (i) MeOH, 1.5 N NaOH, 60 °C, 4 h; (ii) DMF, 24, PyBOP, DIPEA, 23 °C, 15 h, 89%; (iii) MeOH, 1.5 N NaOH, 60 °C, 3.5 h; (iv) DMF, EDC, HOBT, 23 °C, 16 h, 89%.
hexafluorophosphate/\(N,N\)-diisopropylethylamine (PyBOP/DIPEA). Commercially available methyl 4-aminobutyrate 24 was added to the solution containing the activated acid of 23a. The desired dimer, BocNHPy\(\gamma\)COOMe 25, was recovered as major product. Dimer 25 was saponified in a fashion similar to compound 23. The resulting acid 25a was activated using EDC/HOBt. Compound 8a was added dropwise to the activated acid solution of 25a. The \(\gamma\)-turn moiety BocNHPy\(\gamma\)ImCOOMe 20, was recovered in 58% yield.

![Scheme 4.3](image)

**Scheme 4.3.** Reagents and conditions: (i) MeOH, 1.5 N NaOH, 60 °C, 4 h; (ii) DMF, 9a, EDC, HOBt, 23 °C, 15 h, 71%; (iii) MeOH, 1.5 N NaOH, 60 °C, 6 h; (iv) DMF, 9a, EDC, HOBt, 23 °C, 15 h, 71%.

Trimer 21 was synthesized in two steps from methyl 4-[(\textit{tert}-butoxy carbonyl)amino]-1-methylimidazole-2-carboxylate 22, **Scheme 4.3**. The monomer 22 was saponified in the presence of 1.5 N NaOH at neutralized with HCl to afford 22a. Compound 22a activated with EDC/HOBt. Compound 8a was added dropwise to the activated acid solution of 22a. The desired dimer, BocNHImPyCOOMe 26, was recovered as the major product. Dimer 26 was saponified in a fashion similar to compound 22. The resulting acid 26a was activated using EDC/HOBt followed by the dropwise addition of compound 9a. Trimer 21, BocNHImPyPyCOOMe, was afforded in 63% yield.
Parent hairpin polyamide 19 was synthesized as follows, Scheme 4.4.

Compound 11 was saponified with NaOH and neutralized with HCl to afford 11a. Compound 11a activated using PyBOP/DIPEA. Congruently, compound 20 was deprotected using 4 N HCl. Compound 20a was coupled 11a to afford 27. Compound 27 was recovered following precipitation and subsequent filtration in 88% yield. Compound 27 was saponified to afford the corresponding acid 27a in a method similar to compound 11. The resulting acid 27a was activated using PyBOP/DIPEA. At the same time, trimer 21 was deprotected in the presence of 4 N HCl. Compound 21a was added to a solution containing 27a to afford the parent hairpin polyamide 19, as the major product, in 89% yield.
Linker length variation was afforded through \( \alpha, \omega - \text{amino, azido alkanes} \) \( 12 \) – \( 17 \) which were synthesized from corresponding dibromoalkanes using previously established procedures (73), Scheme 4.5. Parent hairpin polyamide was saponified in the presence of 1.5 N NaOH and neutralized with HCl to afford \( 19a \). Compound \( 19a \) was activated using EDC/HOBt. In individual reactions, \( \alpha, \omega - \text{amino, azido alkanes} \) \( 12 \) – \( 17 \) were added to the solution containing \( 19a \). The desired azide functionalized tetramer polyamides \( \text{DPA226} \) – \( \text{DPA231} \) were recovered following column purification in good yields.

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\text{Scheme 4.5. Reagents and conditions: (i) MeOH, 1.5 N NaOH, 60 \degree C, 5.5 h; (ii) EDC, HOBt, DMF, 23 \degree C, 24 h, 77\% - 91\%}
\]

Commercially available neomycin B was prepared for conjugation as previously reported (25, 32, 59, 74), Scheme 4.6. The six amino groups on neomycin B were \( N \)-Boc protected using di-\( \text{tert} \)-butyl dicarbonate. \( N \)-Boc neomycin-TPS 2 was prepared as the
major product following the suspension of N-Boc neomycin in anhydrous pyridine and addition of 2,4,6-triisopropylbenzenesulfonyl chloride followed purification via chromatography, as previously reported (21, 32, 59, 74). Synthesis of DPA10 was

**Scheme 4.6.** Reagents and conditions: (i) NaN$_3$, DMF, 60 °C, 12 h, 89 %; (ii) 10% Pd/C, MeOH, H$_2$ (g), 7 h, 98%, (iii) propiolic acid, DCC, CH$_2$Cl$_2$ (dry), 0 °C, 30 min, DPA12, rt, 18 h, 86 %; (iv) CuI, DIPEA, propargyl ether, toluene, 14 h, 85%.

conducted as previously discussed in chapter 2 (Scheme 2.1, page 57) and shown in Scheme 4.1. Following addition of sodium azide to compound 2, DPA11 was recovered as the major product following purification, in 89% yield, Scheme 4.6. DPA11 was reduced in the presence of H$_2$ (g) and 10% Pd/C to afford DPA12, Scheme 4.6. DPA12 was coupled to propiolic acid to afford DPA16 as the major product following column chromatography (75), Scheme 4.6. Finally, DPA13 was synthesized following the coupling of DPA11 to commercially available propargyl ether using CuI/DIPEA mediated 1,2,3-triazole formation with a yield of 85%, Scheme 4.6 (68, 69, 70).
Scheme 4.7. Reagents and conditions: (i) CuI, DIPEA, DPA13, DPA226 – DPA231, DMF, 36 h; (ii) 4N HCl in 1,4-dioxane, 1 h. Overall yields for coupling and deprotection steps 15% – 27%.

‘Double-Click’ hairpin polyamide – neomycin conjugates DPA232 – DPA237 were synthesized through CuI/DIPEA mediated 1,2,3-triazole formation between hairpin polyamides, DPA226 – DPA231, and N-Boc protected neomycin DPA1, Scheme 4.7. The N-Boc protected conjugates were purified with flash column chromatography followed by deprotection in the presence of 4 N HCl to afford DPA232 – DPA237, Scheme 4.7. These conjugates represent the longest linkers in our library where the linker length was 12, 13, 15, 16, 18 and 20 atoms in length DPA232 – DPA237, respectively.

‘Single-Click’ hairpin polyamide – neomycin conjugates DPA238 – DPA240, Scheme 4.8 were synthesized in a similar fashion to ‘Double-Click’ hairpin polyamide – neomycin conjugates. Hairpin polyamides (DPA226 – DPA231) were coupled to
**DPA16** through Cul/DIPEA mediated 1,2,3-triazole formation., **Scheme 4.8.** The desired conjugates **DPA238 – DPA240** were afforded following flash column chromatography purification of the N-Boc protected conjugates followed by deprotection in the presence of 4 N HCl. The resulting conjugates feature intermediate linker lengths of 8, 9 and 11 atoms.

**Scheme 4.8.** Reagents and conditions: (i) Cul, DIPEA, **DPA16**, **DPA226 – DPA228**, DMF, 36 h; (ii) 4N HCl in 1,4-dioxane, 1 h. Overall yields for coupling and deprotection steps 26% - 30%.

Amide bond coupled hairpin polyamide – neomycin conjugates **DPA241** and **DPA242** were afforded by EDC/HOBt mediated amide bond formation, **Scheme 4.9.** Parent hairpin polyamide was saponified in the presence of 1.5 N NaOH to afford to 19a, as previously discussed in **Scheme 4.4.** N-Boc protected neomycin **DPA10** was dissolved added to 19a. Following column purification of the N-Boc protected conjugate
and deprotection in the presence of 4 N HCl, the desired azide hairpin polyamide – neomycin conjugate DPA241 was recovered in 88% yield, Scheme 4.9. DPA241

Scheme 4.9. Reagents and conditions: (i) EDC, HOBr, DMF, DPA10, 12 h; (ii) 4N HCl in 1,4-dioxane, 1 h, 88%; (iii) EDC, HOBr, DMF, DPA12, 18 h; (iv) 4N HCl in 1,4-dioxane, 1 h, 81%; (v) EDC, HOBr, N,N-dimethylaminopropylamine, 12 h, 94%. Yields for the synthesis of DPA241 and DPA242 are reported as overall yields for coupling and deprotection steps.

featured a linker length of four atoms. Analogous to the synthesis of DPA241, compound DPA12 was added the activated acid solution of 19a. The desired azide hairpin polyamide – neomycin conjugate DPA242 was recovered following column
purification of the N-Boc protected conjugate and deprotection in the presence of 4 N HCl. **DPA242** was recovered in 81% yield and featured a single atom linker length between the two moieties, Scheme 4.9.

Finally, the synthesis of the hairpin polyamide, HIImPyPyγImImPyPyDp 28, was conducted using EDC/HOBt mediated coupling between 19a and N,N-dimethylaminopropylamine, Scheme 4.9; similar to the synthesis of **DPA241** and **DPA242**. The major product 28 was recovered, with 94% yield, following purification.

**Design of DNA Duplexes to Probe Hairpin Polyamide – Neomycin Conjugate Library Binding.** Fluorescent intercalator displacement assays were conducted with a number of DNA duplex oligomers and our hairpin polyamide – neomycin conjugates, neomycin and 28. The DNA duplexes varied in length and sequence to accommodate a number of potential DNA binding sequences. DNA duplexes were designed with the two following considerations: (i) the DNA duplexes contained a potential binding site for the tetramer polyamide portion of our conjugates (5’-AG₂C₂A-3’), mismatch site (5’-G₂A₃-3’) or control (5’-G₂C₂A-3’ or 5’-G₂C₂A-3’). (ii) The DNA duplexes were lengthened to include a portion for neomycin which acted as a potential binding site (5’-G₄C₄-3’) or control (5’-G₄-3’) as identified by our group (76). Applying these considerations, the following DNA duplexes were used in this study 5’-AG₂C₂AG₄C₄-3’, 5’-G₂A₃G₄C₄-3’, 5’-G₂C₂AG₄C₄-3’, 5’-TG₂C₂G₄-3’ and 5’-AG₂C₂AG₄C₄-3’. The 5’-AG₂C₂AG₄C₄-3’ was the target duplex for our studies and featured a binding site for the hairpin polyamide portion of our conjugate (5’-AG₂C₂A-3’) (55) and a portion for binding by neomycin (5’-
G₄C₄-3’). The 5’-G₂A₃G₄C₄-3’ duplex acted as a control in our study as it contained a mismatch site (5’-G₂A₃-3’) which does not bind the hairpin polyamide portion of our conjugates but does include a portion for binding by neomycin (5’-G₄C₄-3’). In an effect to see if loss of a the 5’ terminal A/T base pair effects the binding of our tetramer polyamide – neomycin conjugate, the 5’-G₂C₂AG₄C₄-3’ duplex was designed. Both of the remaining duplexes 5’-TG₂C₂G₄-3’ and 5’-AG₂C₂AG₄-3’ were shortened duplexes that act as controls. CD scans of each of these duplexes is located in Appendix B, Figure B37.

**Hairpin Polyamide – Neomycin Conjugate Library Binding as Studied With a Fluorescent Intercalator Displacement Assay.** An assay was conducted in which neomycin, 28 and hairpin polyamide – neomycin conjugates DPA232 – DPA242 were added to individual wells that contained the target duplex, pre-saturated with ethidium bromide, at various drug:duplex ratio (rₜd) in a 96-well plate (77, 78, 79). A histogram of the normalized percent decrease in fluorescence intensity with respect to control ligands and hairpin polyamide – neomycin conjugate linker length was plotted.

Each of the hairpin polyamide – neomycin conjugates as well as neomycin and 28 displaced pre-bound ethidium bromide DPA232 – DPA242 upon addition to wells containing the 5’-G₂A₃G₄C₄-3’ duplex at rₜd = 1.0, Figure 4.4. The 5’-G₂A₃G₄C₄-3’ duplex acted as a control in our study as it contained a mismatch site (5’-G₂A₃-3’) which does not bind the hairpin polyamide portion of our conjugates but does include a portion for binding by neomycin (5’-G₄C₄-3’). As the neomycin binding portion of this DNA
Figure 4.4. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5'-G₂A₃G₄C₄-3’. In the histogram, the values represent the percent decrease in fluorescence at \( r_{dd} = 1.0 \). Data was obtained from a FID assay. Ligands were added to the DNA [1 \( \mu \)M/duplex], pre-saturated with ethidium bromide [7 \( \mu \)M], at various ratios of drug to duplex \( (r_{dd}) \), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits\(_{ex} = 20\); slits\(_{em} = 20\); Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT \( V = 810 \) and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

duplex (5’-G₄C₄-3’) was identical to our target duplex, 5’-AG₂C₂AG₄C₄-3’, the amount of pre-bound ethidium bromide, 26%, was nearly identical. However, as the 5’-G₂A₃G₄C₄-3’ duplex contains a mismatch site, the decreased amount of displaced pre-bound ethidium bromide upon addition of 28 to the duplex (only 7%) was expected. Each of the hairpin polyamide – neomycin conjugates DPA232 – DPA242 used in our study displaced pre-bound ethidium bromide upon addition to the 5’-G₂A₃G₄C₄-3’.
duplex. However, none of these conjugates displaced more pre-bound ethidium bromide than neomycin.

![Figure 4.5](image)

**Figure 4.5.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2C2AG4C4-3’. In the histogram, the values represent the percent decrease in fluorescence at r_{dd} = 1.0. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM/duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex (r_{dd}), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits_{ex} = 20; slits_{em} = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na_{2}HPO_{4}, 0.5 mM NaH_{2}PO_{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

An FID assay was used to study the effect of the loss of the 5’ terminal A/T base pair (5’-G2C2AG4C4-3’) on the binding of our tetramer polyamide – neomycin conjugates, DPA232 – DPA242. **Figure 4.5.** Neomycin displaced 24% of pre-bound ethidium bromide while compound 18 displaced 18% at r_{dd} = 1.0. The assay identified four hairpin polyamide – neomycin conjugates which displaced more pre-bound ethidium bromide.
that neomycin and 28. **DPA237** (L = 20 atoms) displaced 31% of pre-bound ethidium bromide while hairpin polyamide – neomycin conjugates of intermediate linker lengths **DPA232**, **DPA239** and **DPA240** (L = 12, 9, 11 atoms, respectively) displaced ~29%.

The control ligands neomycin and HImImPyPyDp 28 displaced pre-bound ethidium bromide from the remaining duplexes 5’-TG₂C₂G₄-3’ and 5’-AG₂C₂AG₄-3’.

**Figure 4.6.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-TG₂C₂G₄-3’. In the histogram, the values represent the percent decrease in fluorescence at r̄ₜd = 1.0. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM/duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex (r̄ₜd), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slitsₑₓ = 20; slitsₑₘ = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
shortened duplexes. The same was true for our hairpin polyamide – neomycin conjugates DPA232 – DPA242 in our library.

Addition of neomycin to wells containing the 5’-TG2C2G4-3’ duplex pre-bound with ethidium bromide resulted in a 36% decrease in fluorescence intensity at r_{dd} = 1.0, Figure 4.6. The hairpin polyamide control 28 only displaced 17% or pre-bound ethidium bromide upon addition to the duplex. The only hairpin polyamide – neomycin conjugate that displaced more pre-bound ethidium bromide was DPA237 (L = 20 atoms) at 45%. All remaining hairpin polyamide – neomycin conjugates of shorter linker length did not displace as much pre-bound ethidium bromide as neomycin. Hairpin polyamide – neomycin conjugates of intermediate linker length DPA232 (L = 12 atoms), DPA239 (L = 9 atoms) and DPA240 (L = 11 atoms) all displaced approximately equivalent amounts of pre-bound ethidium bromide from the 5’-TG2C2G4-3’ duplex, ~35%.

Addition of neomycin (at r_{dd} = 1.0) to the 5’-AG2C2AG4-3’ duplex pre-bound with ethidium bromide displaced 21% of the pre-bound intercalator, while 28 (at r_{dd} = 1.0) displaced 29%, Figure 4.7. Each of the tetramer polyamide – neomycin conjugates DPA232 – DPA242 displaced pre-bound ethidium bromide at r_{dd} = 1.0. However, none of these conjugates displaced appreciably more ethidium bromide than neomycin or 28. The only hairpin polyamide – neomycin conjugates which displace more pre-bound ethidium bromide were DPA232 (L = 12 atoms), DPA233 (L = 13 atoms) and DPA238 – DPA240 (L = 8, 9, 11 atoms, respectively). As conjugate linker length increase the amount of stabilization was DPA238 (L = 8 atoms) 30%, DPA239 (L = 9 atoms) 32%,
DPA240 (L = 11 atoms) 31%, DPA232 (L = 12 atoms) 30% and DPA233 (L = 13 atoms) 31%.

Figure 4.7. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4-3’. In the histogram, the values represent the percent decrease in fluorescence at r_{dd} = 1.0. Data was obtained from a FID assay. Ligands were added to the DNA [1 μM/duplex], pre-saturated with ethidium bromide [7 μM], at various ratios of drug to duplex (r_{dd}), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits_{ex} = 20; slits_{em} = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na_{2}HPO_{4}, 0.5 mM NaH_{2}PO_{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

Neomycin displaced 30% of the pre-bound ethidium bromide upon binding the DNA duplex, Figure 4.8. The control hairpin polyamide 28 displaced 20% of the pre-bound ethidium bromide, not surprising with respect to neomycin, since the binding site for the hairpin polyamide is smaller than the binding site for neomycin. Each of the hairpin polyamide – neomycin conjugates DPA232 – DPA242 displaced pre-bound
ethidium bromide upon addition to wells containing the pre-saturated target duplex at \( r_{dd} = 1.0 \). The assay identified five hairpin neomycin conjugates that displaced more pre-

![Bar chart showing normalized decrease in fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG\(_2\)C\(_2\)AG\(_4\)C\(_4\)-3’](image)

**Figure 4.8.** Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG\(_2\)C\(_2\)AG\(_4\)C\(_4\)-3’. In the histogram, the values represent the percent decrease in fluorescence at \( r_{dd} = 1.0 \). Data was obtained from a FID assay. Ligands were added to the DNA [1 \( \mu \)M/duplex], pre-saturated with ethidium bromide [7 \( \mu \)M], at various ratios of drug to duplex (\( r_{dd} \)), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits\(_{ex} = 20\); slits\(_{em} = 20\); Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na\(_2\)HPO\(_4\), 0.5 mM NaH\(_2\)PO\(_4\), 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

bound ethidium bromide than neomycin or 28. **DPA237**, which featured the longest linker (L = ) of 20 atoms, displaced the most pre-bound ethidium bromide, 41%.

Conjugates **DPA236** (L = 18 atoms) and **DPA235** (L = 16 atoms) displaced 38% and 35% of pre-bound ethidium bromide, respectively. There were also two more hairpin polyamide – neomycin conjugates which displaced more pre-bound ethidium bromide
than neomycin or 28. These conjugates DPA232 (L = 12 atoms) and DPA240 (L = 11 atoms) displaced 34% and 36% of pre-bound ethidium bromide.

Furthermore, a plot of linker length with respect to percent decrease in fluorescence intensity was plotted at $r_{dd} = 0.5$ and $r_{dd} = 2.0$, Appendix C, Figure C26 and Figure C27. A $r_{dd} = 0.5$, neomycin displaced 19% and 28 displaced 14% of pre-bound ethidium bromide. However, conjugates of linker length of 11 and 12 atoms (DPA240 and DPA232) displaced significantly more pre-bound ethidium bromide at 26% and 30%, respectively, Appendix C, Figure C26. Increasing the $r_{dd}$ value to 2.0, DPA232 (L = 12 atoms) and DPA240 (L = 11 atoms) both displace significantly more amounts of pre-bound ethidium bromide than neomycin or 28. Furthermore, the ability of hairpin polyamide – neomycin conjugates of longer linker length DPA235 (L = 16 atoms), DPA236 (L = 18 atoms) DPA237 (L = 20 atoms) to displace more pre-bound ethidium bromide than neomycin or 28, previously observed at $r_{dd} = 1.0$ was also observed at $r_{dd} = 2.0$, Appendix C, Figure C27.

**UV Monitored Thermal Denaturation of Target Duplex.** UV thermal denaturation experiments were conducted using the target duplex 5’-AG₂C₂AG₄C₄-3’. The target duplex was denatured in the presence of hairpin polyamide – neomycin conjugates, neomycin and the hairpin polyamide control 28, Figure 4.9 and Table 4.1. In an effort to focus solely on the primary binding site, these studies were conducted at $r_{dd} = 1.0$ in the presence of 50 mM NaCl. Each of the compounds studied increased the melting temperature of the duplex as seen in the histogram, Figure 4.10.
Neomycin increased the melting temperature of the duplex by 7.1 °C and the hairpin polyamide control 28 increased the melting temperature of the duplex by 5.4 °C. The largest increase in thermal stabilization was afforded by DPA233 (L = 13 atoms) at 22.8 °C. As the linker length decreased, the thermal stabilization afforded by the conjugates on the duplex decreased in magnitude. DPA232 (L = 12 atoms) increased the melting temperature by 21.9 °C. Decreasing the linker length to 11 atoms (DPA240) and 9 atoms (DPA239) stabilized the duplex by 20.8 °C and 18.0 °C, respectively. The lowest increase in thermal stabilization afforded by hairpin polyamide – neomycin conjugates on the duplex 5’-AG₂C₂AG₄C₄-3’occurred with DPA241 (L = 4 atoms), which increased the thermal stability of the duplex by 12.6 °C. DPA234 (L = 15 atoms) increases the melting temperature of the duplex by 16.3 °C. As linker length increases beyond 15 atoms, the increase in melting temperature afforded by hairpin polyamide – neomycin conjugates
remains constant. **DPA235** (L = 16 atoms), **DPA236** (L = 18 atoms) and **DPA237** (L = 20 atoms) which feature linker lengths of 16, 18 and 20 atoms all increase the melting temperature of the duplex by ~19 °C.

**Figure 4.10.** Plot of ΔTm with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4C4-3’ with controls and hairpin polyamide – neomycin conjugates **DPA232 – DPA242**. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 µM/duplex] at a ratio of drug to duplex 1.0. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na2HPO4, 0.5 mM NaH2PO4, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

Finally, for ease of comparison, a table comparing ΔTm values (obtained from UV thermal denaturation experiments) and ΔF values (obtained from FID assays) with respect to linker length for hairpin polyamide – neomycin conjugates **DPA32 – DPA242** and controls with 5’-AG2C2AG4C4-3’ is located in **Table 4.1**.
**Table 4.1.** UV determined thermal melting ($T_m$) change in thermal melting ($\Delta T_m$) temperatures and FID assay determined change in fluorescence ($\Delta F$) data. Data for the addition of hairpin polyamide – neomycin conjugates DPA232 – DPA242, neomycin and 28 to 5'-AG$_2$C$_2$AG$_4$C$_4$-3' at $r_{dd} = 1.0$.

<table>
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<th>$T_m$</th>
<th>$\Delta T_m$</th>
<th>$\Delta F$</th>
<th>$T_m$</th>
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<td>-</td>
<td>-</td>
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<td>12.6</td>
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<td>75.3</td>
<td>19.2</td>
<td>DPA241</td>
<td>46.8</td>
<td>12.6</td>
</tr>
</tbody>
</table>

$^a$ $T_m$ represents the melting temperature of the native duplex, values are reported in °C. $^b$ $\Delta T_m$ represents the change in melting temperature upon addition of neomycin at a $r_{dd}$, values are reported in °C. $^c$ $\Delta F$ represents the change in fluorescence intensity upon addition of ligand at $r_{dd} = 1.0$. $^d$ N represents tetramer polyamide – neomycin conjugate linker length in atoms. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

**Fluorescence Intercalator Displacement Titrations and Binding Affinity.** In an effort to obtain quantitative binding affinities $K_a$ and relate them to the results obtained from our assay and thermal denaturation experiments, full FID titrations were conducted. The target duplex was pre-saturated with thiazole orange. A departure from ethidium bromide was made due to the large difference in fluorescence intensity observed between the bound and unbound states of thiazole orange (80). Ligand was added at varying $r_{dd}$ values and the decrease in fluorescence intensity was recorded.
Dervan has established the affinity of hairpin polyamides to a number of target sequences (55, 67). The hairpin polyamide HImImPyPyγImImPyPyβDp analogous to our control 28, Figure 4.11, was reported to recognize the target sequence 5'-TGGCCA-3’ with an equilibrium constant $K_a$ of $9.7 \times 10^9$ M$^{-1}$ as determined by DNase I footprint titration experiments (55). A decrease in fluorescence intensity upon the titration of 28 into the target duplex was observed. A clear inflection point was observed at $r_{dd} = 1.0$, Appendix C, Figure C28. However, addition of 28 beyond $r_{dd} = 1.0$ resulted in a continued decrease in fluorescence intensity. We believe the continued decrease in fluorescence intensity is a result of 28 binding the lower affinity mismatch site found the target duplex as previously reported (54, 55, 63, 67). As a result, we were unable to perform Scatchard analysis on the pre-saturation region of the titration curve, observed at $r_{dd} = 1.0$ and an association constant $K_a$ for our control 28 with 5'-AG$_2$C$_2$AG$_4$C$_4$-3’ was not determined.

Titration of neomycin into the 5’-AG$_2$C$_2$AG$_4$C$_4$-3’ duplex pre-bound with thiazole orange resulted in a decrease in fluorescence intensity, Figure 4.12. The decrease in fluorescence intensity was plotted against $r_{dd}$ and a clear 1:1 binding stoichiometry was observed. Scatchard analysis was performed on the pre-saturation region of the titration curve and provided an association constant $K_a$ of $3.3 \times 10^6$ M$^{-1}$. Fluorescence intensity
Figure 4.12. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-AGCAGC4-3’, by neomycin at 20 °C (a). A plot of fluorescence intensity vs $r_{dd}$ is located in panel (b). A related plot of $\Delta F$ vs $r_{dd}$ is located in panel (c). Scatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of neomycin, the fluorescence intensity is denoted as $F_{max}$. Neomycin was added at various $r_{dd}$ values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-AG\textsubscript{2}C\textsubscript{2}AG\textsubscript{4}C\textsubscript{4}-3’, by \textbf{DPA232} at 20 °C (a). A plot of fluorescence intensity vs \(r_{dd}\) is located in panel (b). A related plot of \(\Delta F\) vs \(r_{dd}\) is located in panel (c). Scatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of \textbf{DPA232}, the fluorescence intensity is denoted as \(F_{\text{max}}\). \textbf{DPA232} was added at various \(r_{dd}\) values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

**Figure 4.13.** Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-AG\textsubscript{2}C\textsubscript{2}AG\textsubscript{4}C\textsubscript{4}-3’, by \textbf{DPA232} at 20 °C (a). A plot of fluorescence intensity vs \(r_{dd}\) is located in panel (b). A related plot of \(\Delta F\) vs \(r_{dd}\) is located in panel (c). Scatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of \textbf{DPA232}, the fluorescence intensity is denoted as \(F_{\text{max}}\). \textbf{DPA232} was added at various \(r_{dd}\) values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure 4.14. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5’-AG₂C₂AG₄C₄-3’, by DPA₂₄₀ at 20 °C (a). A plot of fluorescence intensity vs r₆₆ is located in panel (b). A related plot of ∆F vs r₆₆ is located in panel (c). Schatchard analysis of the pre-saturation region is located in panel (d). The DNA duplex, 1.0 μM/duplex, 14 μM/base pair, was pre-bound by thiazole orange, 7 μM and allowed to equilibrate for a period of 10 min prior to addition of DPA₂₄₀, the fluorescence intensity is denoted as Fₘₐₓ. DPA₂₄₀ was added at various r₆₆ values. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5'-AGCAGCAGC-3', by DPA239 (a) at 20 °C. A related plot of ΔF vs r_{dd} is located in panel (b). The DNA duplex, 1.0 μM/duplex, 14 μM/base pair, was pre-bound by thiazole orange, 7 μM and allowed to equilibrate for a period of 10 min prior to addition of ligand, the fluorescence intensity is denoted as F_{max}. Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.

decreased upon titration of DPA232 (L = 12 atoms) and DPA240 (L = 11 atoms) into the duplex pre-bound with thiazole orange. Both hairpin polyamide – neomycin conjugates clearly demonstrate 1:1 binding with the target duplex, Figure 4.13 and Figure 4.14, respectively. Scatchard analysis of the pre-saturation region of the titration cure afforded association constants for the conjugates with the target duplex; DPA232 (L = 12 atoms), K_a = 3.8 x 10⁹ M⁻¹, and DPA240 (L = 11 atoms), K_a = 7.8 x 10⁸ M⁻¹. Surprisingly, we could not extend Scatchard analysis to the titration of the remaining hairpin polyamide – neomycin conjugates.
Titration of **DPA239** into the target duplex resulted in a decrease in fluorescence intensity, *Figure 4.15*, as observed with previously discussed hairpin polyamide – neomycin conjugates. Unfortunately, the lack of a clear saturation point precluded the use of Scatchard analysis.

Furthermore, similar observations were made upon the titration of hairpin polyamide – neomycin conjugates of longer linker length **DPA236** (*L* = 18 atoms) and **DPA237** (*L* = 20 atoms) into the target duplex, *Appendix C, Figure C29*.

While the decrease in fluorescence intensity suggests conjugate binding, the inability to fit this data may be a result of the insufficient linker length. Although we still observe a decrease in fluorescence intensity, the linker separating the two binding moieties may not allow the conjugate to bind in its preferred orientation. Assuming the hairpin polyamide portion of our conjugates binds at a similar magnitude to analogous hairpin polyamides developed by Dervan $10^8 – 10^9$ M$^{-1}$ (55), the hairpin polyamide portion drives the binding of our conjugates as neomycin (from our Scatchard analysis binds 5'-AG$_2$C$_2$AG$_4$C$_4$-3' with $K_a$ of $3.3 \times 10^6$ M$^{-1}$). It is possible that if linker length is insufficient the hairpin portion binds 5'-AG$_2$C$_2$AG$_4$C$_4$-3’ leaving neomycin to bind in a less preferential orientation. However, this leaves space for additional amounts of conjugate to bind the duplex thus resulting in the continuing decrease in fluorescence intensity as observed with **DPA236** (*L* = 18 atoms), **DPA232** (*L* = 12 atoms) and **DPA240** (*L* = 11 atoms).

Our group has previously shown the ability of neomycin minor groove binding conjugates of longer linker length to bind DNA (32). Unfortunately, we were unable to
Figure 4.16. CD scans for the titration of 5'-AG₂C₂AG₄C₄-3' with neomycin (a, d), HImImPyPyγImImPyPyDp 28 (b, e), DPA232 (c, f) at 20 °C. In plot (d, e, f) the open circle represents the CD scan of the duplex in the absence of neomycin while the closed circle represents the CD scan of the duplex in the presence of ligand r_{dd} = 2.0. The conjugate was added to the duplex [40 μM/bp] was added at varying r_{dd} values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
derive quantitative association constants for hairpin polyamide – neomycin conjugates of longer linker lengths. Whether the high amount of displaced ethidium bromide observed in our single point FID assay is a result of a secondary binding mode or non-optimized linker length remains to be seen. While association constants could not be derived for all hairpin polyamide – neomycin conjugates the association constants for **DPA232** (L = 12 atoms) and **DPA240** (L = 11 atoms) to the target duplex are the highest observed association constants for neomycin binding DNA.

**CD Monitored Titrations of Target Duplexes.** In effort to further characterize the binding of hairpin polyamide – conjugates, CD monitored titration of the target duplex 5’-AG₂C₂AG₄C₄-3’ with neomycin, **18** and **DPA232** (L = 12 atoms), Figure 4.16. The CD spectra of native 5’-AG₂C₂AG₄C₄-3’ consists of a positive band at 270 nm which suggests the duplex retains A-form characteristics. However the negative band at 240 nm and the slight shoulder at 280 nm suggest the duplex also retains some B-form characteristics. Addition of neomycin to the duplex results in an increase in CD intensity at 270 nm and the appearance of a negative peak at 210 nm which suggests an increase in A-form characteristics upon binding. The addition of **18** to the duplex does little to change the overall shape of the CD spectra between 200 nm – 300 nm. Binding of **18** is illustrated by the increase in CD intensity between 300 nm – 400 nm. The positive band at 270 nm and a negative band decreases in intensity upon addition of **18**. The negative band at 240 nm and the slight shoulder at 280 nm suggest addition of **18** results in an increase in B-form characteristics. The binding of **DPA232** (L = 12 atoms) to 5’-5’-
AG₂C₂AG₆C₄-3’ is clearly illustrated by the increase in CD intensity at 300 nm – 350 nm, the polyamide absorption region. Furthermore, the CD spectra displays an increase in CD intensity at 270 nm and 210 nm upon addition of DPA₂₃₂ (L = 12 atoms) which suggests an increase in A-form characteristics of the duplex occurs upon conjugate addition.
CONCLUSIONS

This chapter reflects our current advancements in an aminoglycoside based approach to DNA recognition. A library of hairpin polyamide – neomycin conjugates DPA232 – DPA240 which differ in linker length between the two binding moieties was synthesized. The hairpin polyamide was designed to target the sequence 5’-AG₂C₂A-3’ and synthesized in solution through the sequential coupling of polyamide trimers. The hairpin polyamide was functionalized with the azide functional group through the coupling with a number of α,ω-amino, azido alkanes with our parent polyamide 19 to afford DPA226 – DPA231. Hairpin polyamide – neomycin conjugates DPA232 – DPA240 were synthesized through 1,2,3,-triole formation between DPA226 – DPA231 and neomycin derivatives DPA13 and DPA16. Shorter hairpin polyamide – neomycin conjugates were afforded through amide bond formation between 19 and neomycin derivatives DPA10 and DPA12.

Spectroscopic techniques were used to characterize the binding of neomycin, HlmImPyPyImlmPyDp 28, and hairpin polyamide – neomycin conjugates DPA232 – DPA240 and the following conclusions were drawn. (i) FID assay identified potential high affinity binding tetramer polyamide – neomycin conjugates. Each of the conjugates was assayed against a DNA duplex containing the hairpin target sequence as well as a GC rich pocket for neomycin. The assay clearly illustrated the binding of each hairpin polyamide – neomycin conjugate to the target duplex. Furthermore, results from the FID single point assay identified five conjugates which displaced more pre-bound ethidium bromide than neomycin or the control polyamide. (ii) UV thermal denaturation
experiments aids in the identification of potential high affinity binding tetramer polyamide – neomycin conjugates. Each of the compounds used in our study stabilized the target duplex. In fact, each of the hairpin polyamide – neomycin conjugates DPA232 – DPA242 stabilized the thermal stability of the target duplex better than neomycin of 28. Furthermore, hairpin polyamide – neomycin conjugates of linker length 11, 12 and 13 atoms in length (DPA240 (L = 11 atoms), DPA232 (L = 12 atoms) and DPA233 (L = 13 atoms), respectively) stabilized the duplex by the largest magnitude. (iii) CD Titration of 5’-AG2C2AG4C4-3’ by DPA232 suggests binding. Binding of DPA232 (L = 12 atoms) to 5’-AG2C2AG4C4-3’ is clearly illustrated by the increase in CD intensity at 300 nm – 350 nm. Furthermore, CD suggests addition of DPA232 (L = 12 atoms) to 5’-AG2C2AG4C4-3’ increase A-form conformation in the duplex. (iv) Full FID titrations suggest tetramer polyamide – neomycin conjugates bind the sequence with $K_a$ of $10^8 – 10^9$ M$^{-1}$. In an effort to obtain quantitative association constants, full FID titrations were conducted with the hairpin polyamide – neomycin conjugates and the target duplex. While each of these conjugates displaced pre-bound thiazole orange, indicative of conjugate binding, the lack of a clear saturation point was not observed for a number of these conjugates. When saturation was observed upon titration of DPA232 (L = 12 atoms) and DPA240 (L = 11 atoms) into the target duplex, Scatchard analysis was performed to determine quantitative association constants $K_a$. Scatchard derived association constants for DPA232 (L = 12 atoms) and DPA240 (L = 11 atoms) with the target duplex were $K_a = 3.8 \times 10^9$ M$^{-1}$ for $K_a = 7.8 \times 10^8$ M$^{-1}$, respectively.
REFERENCES


76. manuscript in press.


APPENDIX A

**N-Boc neomycin-S(CH$_2$)$_2$NH$_2$ (DPA10).** Synthesized as previously described: $^1$H NMR (300 MHz, CDCl$_3$) δ 6.28 (s, 1H), 6.22 (s, 1H), 5.25 (s, 1H), 5.05 (s, 1H), 4.88 (s, 2H), 4.80 (s, 1H), 4.61 (s, 1H), 4.30-4.00 (m, 5H), 3.89 (s, 2H), 3.80 (s, 1H), 3.74 (s, 2H), 3.70 (s, 2H), 3.65 (s, 2H), 3.60 (s, 3H), 3.40 (s, 1H), 3.31 (s, 2H), 3.14 (s, 2H), 1.57-1.34 (m, 54 H); MALDI-TOF m/z calcd for C$_{55}$H$_{99}$N$_7$O$_{24}$S 1274.47, found [M + Na]$^+$ 1298.36.

**N-Boc neomycin-N$_3$ (DPA11).** To a solution of 2 (100.0 mg, 67.5 mmol) in DMF (10 mL) was added sodium azide (21.9 mg, 337.4 mmol). The solution was allowed to stir at 60 °C for 12 h. The solution was suspended in brine (250 mL) and washed with CH$_2$Cl$_2$ (150 mL). The organic layer was dried over Na$_2$SO$_4$ and concentrated in vacuo. The residue was purified by flash chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 0.5% incremental increases in MeOH) to yield DPA11 (74.5 mg, 89%) as a white solid: TLC
(92:8 CH₂Cl₂:MeOH v/v) Rₙ 0.68; ¹H NMR (500 MHz, CDCl₃) δ 6.21 (s, 1H), 6.15 (s, 1H), 5.22 (s, 1H), 4.97 (s, 1H), 4.89 (s, 2H), 4.74 (s, 1H), 4.60 (s, 1H), 4.29-4.00 (m, 5H), 3.86 (s, 2H), 3.76 (s, 1H), 3.70 (s, 2H), 3.50 (s, 3H), 3.45 (s, 1H), 3.25 (s, 2H), 3.00 (s, 2H), 1.55-1.36 (m, 54H); MALDI-TOF m/z calcd for C₅₃H₉₅N₇O₂₄Na 1240.35, found [M + Na]⁺ 1264.71.

![Diagram of DPA11 and DPA12](image)

N-Boc neomycin-NH₂ (DPA12). To a solution of DPA11 (100.0 mg, 80.6 mmol) in MeOH (20 mL) was added 10% Pd/C (15.0 mg, 140.9 mmol). The reaction was allowed to stir for 7 h under slightly positive H₂ (g), afforded via submerged syringe needle. The catalyst filtered and the solvent was removed in vacuo to afford DPA12 (95.8 mg, 98%), sufficiently pure, as a yellowish oil: TLC (90:10 CH₂Cl₂ v/v) Rₙ 0.25; ¹H NMR (500 MHz, CDCl₃) δ 7.84 (br, 2H), 6.41 (s, 1H), 6.33 (s, 1H), 5.28 (s, 1H), 5.16 (s, 1H), 4.80 (s, 2H), 4.72 (s, 1H), 4.60(s, 1H), 4.30-4.00 (m, 5H), 3.95 (s, 2H), 3.87 (s, 1H), 3.76 (s, 2H), 3.71 (s, 2H), 3.60 (s, 2H), 3.55 (s, 3H), 3.54 (s, 2H), 3.47 (s, 1H), 3.30 (s, 2H), 1.66-1.38 ( m, 54 H); MALDI-TOF m/z calcd for C₅₃H₉₅N₇O₂₄ 1214.35, found [M + Na]⁺ 1238.58.
Boc-protected Neomycin-Methidium Conjugate (5). To a solution of 6-(4-carboxyphenyl)-3,8-diamino-5-methylphenanthridinium chloride (3) (10.0 mg, 26.7 µmol) in dry DMF (5.5 mL), dicyclohexylcarbodiimide (5.5 mg, 26.7 µmol) and dimethylaminopyridine (1.5 mg, 15.0 µmol) were added. The solution was allowed to stir under positive N₂ gas for 3 h. A solution of DPA12 (34.8 mg, 26.7 µmol) in dry DMF (4.0 mL) was added via cannula. The reaction was allowed to stir at room temperature under positive N₂ for 28 h. The volatiles were removed in vacuo. The dry solid was washed with CH₂Cl₂. The resulting solid was dried in vacuo. Flash chromatography (0%-25% MeOH:CH₂Cl₂) afforded 5 (33.8 mg, 83%) as a purple solid: \( R_f \) 0.2 in 85:15 CH₂Cl₂:MeOH; \(^1\)H NMR (500 MHz, methanol-d₄, 25 °C) \( \delta \) 8.52 (d, 1H, \( J = 9.0 \)), 8.48 (d, 1H, \( J = 9.0 \)), 7.80 (d, 2H, \( J = 8.8 \)), 7.58 (d, 2H, \( J = 8.8 \)), 7.47 (m, 1H, \( J = 9.1 \)), 7.30-7.25 (m, 3H), 5.31 (br, 1H), 5.22 (s, 1H), 4.64 (br, 2H), 4.54 (s, 1H), 4.40-3.98 (m, 1H), 3.89-3.80 (m, 1H), 3.77-3.70 (m, 1H), 3.69-3.60 (m, 2H), 3.59-3.54 (m, 4H), 3.50 (d, 2H), 3.41 (br, 4H), 3.40-3.20 (m, 1H), 3.19 (m, 1H), 3.13-1.01 (m, 4H), 2.95-2.93 (m, 4H), 2.91 (d, 2H), 1.96-1.86 (m, 2H), 1.71 (br, 1H), 1.36-1.48 (m, 54H); MALDI-TOF \( m/z \) (rel. intensity) calculated for \( C_{73}H_{108}N_{10}O_{25} \) [M + Na]⁺ 1525.69 found 1549.16.
Neomycin-Methidium Conjugate (DPA201). To a solution of 5 (33.8 mg, 22.2 µmol) in 3.0 mL dichloromethane was added trifluoroacetic acid (3.0 mL). 1,2-ethanediithiol (100.0 µL) was added and the solution was allowed to stir for 30 min. The volatiles were removed under vacuum. The resulting oil was washed with diethylether affording a maroon solid. The solid was dissolved in nanopure water. The solution was purified with preparatory HPLC using a reverse phase column, (0%-100% H₂O:MeCN 0.1%TFA, 15 min). The compound eluted at 20.93 min. Fractions containing the compound were lyophilized affording DPA201 (34.1 mg, 95%) as a maroon solid: ^1^H NMR (500 MHz, MEOD) 8.56 (d, 1H, J = 9.5 Hz), 8.71 (d, 1H, J = 9.4), 7.56 (d, 2H, J = 8.7), 7.50 (d, 2H, J = 8.8), 7.32 (m, 1H, J = 9.6), 7.31-7.30 (m, 3H), 6.27 (br, 1H), 4.33-4.23 (s, 3H), 4.01-3.88 (m, 2H), 3.99-3.81 (m, 1H), 3.75-3.71 (m, 1H), 3.61-3.59 (m, 2H), 3.29-3.21 (m, 4H), 3.17-3.03 (m, 4H), 2.91-2.89 (m, 2H), 2.89-2.85 (m, 2H), 2.85 (m, 2H), 2.77 (m, 2H), 1.93-1.70 (m, 4H), 1.89 (m, 2H); MALDI m/z (rel intensity) calculated for C₄₄H₆₃N₁₀O₁₃ [M + H]⁺ 940.03 found [M + Na]⁺ 961.60.
Figure A1. Characterization of compound DPA10.
Figure A2. Characterization of compound DPA11.
Figure A3. Characterization of compound DPA12.
**Figure A4.** Characterization of compound 4.
(i) MALDI-TOF

(j) Reverse phase HPLC chromatograph of purified NM. Column and conditions: Phenomenex Luna Su-C18(2) reverse phase column, 250 x 10.00 mm 5 u micro, (0%-100% H₂O:MeCN 0.1%TFA, 30 min). Y-axis units AU₄₈₈.

Figure A5. Characterization of compound DPA200.
Figure A6. Characterization of compound 5.
Figure A7. Characterization of compound DPA201.
Figure A8. Competition dialysis results of DPA200 with various nucleic acids. The histograms show the amount of DPA200 bound to individual nucleic acids following dialysis. Nucleic acids were dialyzed with DPA200 in buffer solution for 72 h. In the panel [DPA200] = 100 nM, [nucleic acids] = 750 nM per base unit of each polymer. Buffer: 6 mM Na$_2$HPO$_4$, 2 mM NaH$_2$PO$_4$, 1 mM EDTA, pH 7.0, 100 mM NaCl.
Figure A9. UV thermal denaturation profiles of various nucleic acids. In each panel the native nucleic acids, dotted red line, **DPA200**, solid lie. **DPA200** was added to the nucleic acids [20 μM/bp] at $r_{bd} = 2.2$ μM. The samples were incubated at 4 °C for 6 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure A10. Mixed melting profile of various nucleic acids. Each panel shows the melting mixture of poly(dG):poly(dC); peak 5, poly(dA):poly(dT); peak 4, poly(rA):poly(dT); peak 3, poly(rA):poly(rU); peak 2, poly(dA):poly(rU); peak 1. Panel (a) is the melting of the mixed polynucleotide solution without \textbf{DPA200}. Panels (b), (c) and (d) are the melting of the mixed polynucleotide solution in the presence of \textbf{DPA200} (r_{bd} = 20, 10, and 8, respectively). Panel (e) is the melting profiles of all mixed melting experiments. Panels (f), (g) and (h) are the first derivative plots melting of the mixed polynucleotide solution in the presence of \textbf{DPA200} (r_{bd} = 20, 10, and 8, respectively). In each panel, the solid line reflects the melting in the absence of ligand, while the dashed line represents the mixed melting in the presence of \textbf{DPA200}. The concentration of each polynucleotide was 10 µM/bp, the total concentration of polynucleotide was 50 µM/bp. Data was normalized for ease of comparison. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.50 mM EDTA, pH 7.0, 46.25 mM NaCl.
Figure A11. UV thermal denaturation profiles of poly(rA):poly(dT) (a, b) and poly(dA):poly(rU) (c, d). Panels (a, c) represent the duplex with DPA200 at varying r_{bd} values, while panels (b, d) represent the duplex (A) was with neomycin (B), ethidium bromide (C), neomycin and ethidium bromide (D) and DPA200 (E) at 5 µM. The ligands were added to the duplex [20 µM/bp] at varying r_{bd} (ratio of drug:base pairs) values. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 20 mM NaCl.
Figure A12. UV thermal denaturation profiles of poly(rA):poly(dT) (a, b) and poly(dA):poly(rU) (c, d). Panels (a, c) represent the duplex (A) was with neomycin (B), ethidium bromide (C), neomycin and ethidium bromide (D) and DPA200 (E) at 2.1 µM, while panels ((b, d) represent the duplex (A) with neomycin (B), ethidium bromide (C) and DPA200 (D) at saturation. poly(rA):poly(dT): neomycin ($r_{bd} = 8.0$), ethidium bromide ($r_{bd} = 4.2$) and DPA200 ($r_{bd} = 6.5$); poly(dA):poly(rU): neomycin ($r_{bd} = 6.5$), ethidium bromide ($r_{bd} = 4.6$) and DPA200 ($r_{bd} = 9.7$). The ligands were added to the duplex [20 µM/bp] at varying $r_{bd}$ (ratio of drug:base pairs) values. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure A13. CD thermal denaturation profiles of poly(rA):poly(dT) (a, b) and poly(dA):poly(rU) (c) in the presence of various ligands. Panels (a, c) represent the duplex with ligands at $r_{bd} = 9.6$, while panel (b) represents the duplex with ligands at $r_{bd} = 6.5$. Ligands, neomycin (C), ethidium bromide (B), DPA200 (D), neomycin and ethidium bromide (E) were added to the duplex (A) [20 µM/bp] and the added at varying $r_{bd}$ (ratio of drug:base pairs) values. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A14. Fluorescence melting profiles of poly(rA):poly(dT) (a), poly(dA):poly(rU) (b) and poly(rA):poly(dT) (c) with DPA200 and ethidium bromide (EtBr). The ligands were added to the duplex [20 µM/bp]. DNA:RNA hybrid (20.0 µM/bp) was mixed with ligands at r_{bd} values of 9.6, 9.6 and 6.5 (a, b, c, respectively). The samples were incubated at 4°C for 12 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Excitation, 525 nm – emission \lambda_{max}, 595 nm (ethidium bromide), 614 nm (DPA200) ; slits: 1.5 mm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A15. CD scans of the titration of ethidium bromide and DPA200 with poly(rA):poly(dT) and poly(dA):poly(rU) at 20 °C, (a, b; c, d; respectively). In the expanded chromophore regions the open circles denote the CD signal of native hybrid without drug, while closed circles denote the CD signal after max addition of the ligand. Ligands were added to the duplex [20 µM/bp] at respective r_{bd} values. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A16. ITC titration of neomycin into the duplex poly(rA):poly(dT) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 200 µM/bp; neomycin concentration was 150 µM. In the profile, the upper panels show the heat burst curves of 8 µL injection of neomycin into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A17. ITC titration of neomycin into the duplex poly(dA):poly(rU) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 100 µM/bp; neomycin concentration was 100 µM. In the profile, the upper panels show the heat burst curves of 10 µL injection of neomycin into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A18. ITC titration of ethidium bromide into the duplex poly(rA):poly(dT) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 75 µM/bp; ethidium bromide concentration was 80 µM. In the profile, the upper panels show the heat burst curves of 10 µL injection of ethidium bromide into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
**Figure A19.** ITC titration of ethidium bromide into the duplex poly(dA):poly(rU) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 100 µM/bp; ethidium bromide concentration was 80 µM. In the profile, the upper panels show the heat burst curves of 10 µL injection of ethidium bromide into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A20. ITC titration of DPA200 into the duplex poly(rA):poly(dT) 20 °C (a) and 10 °C (b). Control titration was carried out with drug into buffer solution alone (c) and (d). The sample titration was conducted at a concentration of 75 µM/bp; DPA200 concentration was 60 µM. In the profile, the upper panels show the heat burst curves of 10 µL injection of DPA200 into a sample containing the DNA:RNA hybrid. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 6.8, 100 mM NaCl.
Figure A21. CD scans of the titration of neomycin with poly(dA):poly(rU) (a, b), poly(rA):poly(rU) (c, d) and poly(dA-dT)₂ (e, f) at 20 °C. A plot of CD intensity at 243 (b), 244 (d) and 246 (f) with respect to \( r_{bd} \) was plotted, \( r_{bd} \) is the ratio of [base pair]/[drug]. In the expanded chromophore regions the open circles denote the CD signal of native hybrid without drug, while closed circles denote the CD signal after max addition of the ligand. Ligands were added to the duplex [20 μM/bp] at respective \( r_{bd} \) values. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure A22. Fluorescence emission scans of the titration of ethidium bromide with various nucleic acids at 20 °C. Ethidium bromide was titrated into poly(dA):poly(rU) (a), poly(rA):poly(rU) (c) and poly(dA-dT)\textsubscript{2} (e) at various $r_{bd}$ values. A plot of fluorescence intensity at 594 nm vs $r_{bd}$ values for each respective nucleic acids are located in panels (b), (d) and (f). A plot of fluorescence intensity at 594 with respect to $r_{bd}$ was plotted, $r_{bd}$ is the ratio of [base pair]/[drug]. Ligand was added to the duplex [1 µM/bp] at respective $r_{bd}$ values. Excitation: 525 nm; emission: 550 – 700 nm; slits: 1.5 mm. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure A23. CD scans of the titration of DPA200 with poly(rA):poly(rU) (a, b) and poly(dA-dT)$_2$ (c, d) at 20 °C. A plot of CD intensity at 264 (b) and 277 (d) with respect to $r_{bd}$ was plotted, $r_{bd}$ is the ratio of [base pair]/[drug]. In the expanded chromophore regions the open circles denote the CD signal of native hybrid without drug, while closed circles denote the CD signal after max addition of the ligand. Ligands were added to the duplex [20 µM/bp] at respective $r_{bd}$ values. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Figure A24. DSC melting profiles of poly(dA):poly(rU) (a), poly(rA):poly(rU) (b) and poly(dA-dT)\(_2\) (c). Integration of the melting peak yielded \(\Delta H_{wc}\). The duplex was pre-formed at 100 µM/bp prior to analysis, slow heating from 25 ºC to 90 ºC at 0.75 ºC/min. Buffer: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl. Data collected by Dr. Hongjuan Xi.
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Figure A25. ITC titration of 50 µM neomycin into various solutions. Control titrations were conducted of neomycin into buffer alone at 15 °C (a), 20 °C (e) and 25 °C (i). Sample titrations of neomycin into poly(dA):poly(rU) [(b), (f) and (j)], poly(rA):poly(rU) [(c), (g) and (k)] and poly(dA-dT)2 [(d), (h) and (l)] were conducted at 15 °C, 20 °C and 25 °C, respectively. Sample cell titrations were conducted at a concentration of 350 µM/bp nucleic acids. In each profile, the upper panels show the heat burst curves of 8 µL injection of neomycin into sample. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. ∆C_p plots were plotted according to Equation 3. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
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Figure A26. ITC titration of 50 µM ethidium bromide into various solutions. Control titrations were conducted of ethidium bromide into buffer alone at 15 °C (a), 20 °C (e) and 25 °C (i). Sample titrations of ethidium bromide into poly(dA):poly(rU) [(b), (f) and (j)], poly(rA):poly(rU) [(c), (g) and (k)] and poly(dA-dT)₂ [(d), (h) and (l)] were conducted at 15 °C, 20 °C and 25 °C, respectively. Sample cell titrations were conducted at a concentration of 350 µM/bp nucleic acids. In each profile, the upper panels show the heat burst curves of 8 µL injection of ethidium bromide into sample. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. \( \Delta C_p \) plots were plotted according to Equation 3. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Blank Titrations | poly(dA):poly(rU) | poly(rA):poly(rU) | poly(dA-dT)$_2$
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(a) | (b) | (c) | (d)
Figure A27. ITC titration of 50 µM DPA200 into various solutions. Control titrations were conducted of DPA200 into buffer alone at 15 °C (a), 20 °C (e) and 25 °C (i). Sample titrations of DPA200 into poly(dA):poly(rU) [(b), (f) and (j)], poly(rA):poly(rU) [(c), (g) and (k)] and poly(dA-dT)\textsubscript{2} [(d), (h) and (l)] were conducted at 15 °C, 20 °C and 25 °C, respectively. Sample cell titrations were conducted at a concentration of 350 µM/bp nucleic acids. In each profile, the upper panels show the heat burst curves of 8 µL injection of DPA200 into sample. The corrected injection heats shown in the sample titrations were derived by integration of heat burst curve from sample titration, followed by subtraction of the dilution heat from control titration. The data points reflect the experimental injection heats. The average value was calculated as the binding enthalpy. ΔC\textsubscript{p} plots were plotted according to Equation 3. Buffer conditions: 10 mM sodium cacodylate, 0.5 mM EDTA, pH 5.5, 100 mM NaCl.
Methyl 4-amino-1-methylimidazole-2-carboxylate (8a). To a solution of compound 8 (3.0 g, 16.2 mmol) in dry MeOH (80.0 mL) was added 10% Pd/C (0.3 g). The mixture was stirred under positive hydrogen atmosphere, afforded by slow bubbling of H\(_2\) (g) via submerged syringe, for 3.5 h. The catalyst was filtered over Celite and the solvent was removed in vacuo to afford 8a (2.5 mg, 99%) as a yellow oil: TLC (70:30 CH\(_2\)Cl\(_2\):Acetone v/v) R\(_f\) 0.25; \(^1\)H NMR (500 MHz, CDCl\(_3\)) \(\delta\) 7.28 (s, 2H), 6.30 (s, 1H), 3.84 (s, 3H), 3.78 (s, 1H); \(^13\)C NMR (125 MHz, CDCl\(_3\)) \(\delta\) 158.9, 145.5, 131.6, 109.4, 53.5, 35.7; MALDI-TOF m/z calcd for C\(_6\)H\(_9\)N\(_3\)O\(_2\) 155.15, found [M + H]+ 156.55.

HImImCOOMe (10). To a solution of compound 8 (925.9 mg, 5 mmol) in dry MeOH (80.0 mL) was added 10% Pd/C (92.5 mg). The solution was stirred under positive hydrogen atmosphere, afforded by slow bubbling of H\(_2\) (g) via submerged syringe, for 3.5 h. The catalyst was filtered over Celite and the solvent was removed in vacuo to afford 8a as yellow oil. To a solution of 7 (1.4 g, 4.0 mmol) in CH\(_2\)Cl\(_2\) (100 mL) was added 8a,
suspended in CH$_2$Cl$_2$ (10 mL), and the solution was stirred for 6 h. The solution was concentrated *in vacuo*. The residue was purified by flash chromatography (silica gel, hexane:acetone 0.5% incremental increases in acetone) to yield 10 (758.2 mg, 72%) as a light yellow solid: TLC (1:1 hexane:acetone v/v) $R_f$ 0.23; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.60 (s, 1H), 7.49 (s, 1H), 6.96 (s, 1H), 6.92 (s, 1H), 3.99 (s, 3H), 3.94 (s, 3H), 3.86 (s, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 159.3, 156.5, 138.2, 136.6, 131.6, 128.4, 126.2, 114.9, 52.4, 36.0, 35.6; MALDI-TOF $m/z$ calcd for C$_{11}$H$_{13}$N$_5$O$_3$ 263.26, found [M + H]$^+$ 263.37.

Methyl 4-amino-1-methylpyrrole-2-carboxylate (9a). To a solution of compound 9 (3.0 g, 16.2 mmol) in dry MeOH (80.0 mL) was added 10% Pd/C (0.3 g). The mixture was stirred under positive hydrogen atmosphere, afforded by slow bubbling of H$_2$ (g) via submerged syringe, for 4 h. The catalyst was filtered over Celite and the solvent was removed *in vacuo* to afford 9a (2.3 mg, 97%) as a yellow oil: TLC (70:30 CH$_2$Cl$_2$:Acetone v/v) $R_f$ 0.30; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.91 (s, 2H), 7.23 (s, 1H), 6.78 (s, 1H), 3.85 (s, 3H), 3.74 (s, 1H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 160.8, 124.2, 121.6, 111.9, 51.9, 37.2; MALDI-TOF $m/z$ calcd for C$_7$H$_{10}$N$_3$O$_2$ 154.17, found [M - H]$^+$ 153.55.
HIImPyCOOMe (11). To a solution of compound 9 (194.4 mg, 1.1 mmol) in dry MeOH (80.0 mL) was added 10% Pd/C (19.4 mg). The solution was stirred under positive hydrogen atmosphere, afforded by slow bubbling of H₂ (g) via submerged syringe, for 4 h. The catalyst was filtered over Celite and the solvent was removed in vacuo to afford 9a as a yellow oil. To a solution of 10 (200.0 mg, 0.7 mol) in methanol (10 mL) was added 1.5 M NaOH (2 mL) and the solution was allowed to stir for 4 h. The solution was neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF (30 mL), EDC (172.1 mg, 0.9 mmol) and HOBt (121.6 mg, 0.9 mmol), were added and the reaction was allowed to stir for 1 h. A solution of 9a (139.7 mg, 0.9 mmol) in DMF, 10 mL, was added dropwise over the period of 10 min and the reaction was allowed to stir for 16 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, acetone:CH₂Cl₂, 5.0 % incremental increases of acetone) to yield pure 11 (164.2 mg, 71%) as a dark brown solid: TLC (50:50 CH₂Cl₂:acetone v/v) Rₖ 0.80; ¹H NMR (500 MHz, CDCl₃) δ 9.51 (s, 1H), 8.88 (s, 1H), 7.49 (s, 1H), 7.44 (d, 1H, J = 3.0), 7.09 (s, 1H), 7.04 (s, 1H), 6.84 (d, 1H J = 3.0), 4.12, (s, 3H), 4.11 (s, 3H), 3.94 (s, 3H), 3.84 (s, 3H); ¹³C NMR (125 MHz, CDCl₃) δ 161.6, 156.3, 155.9, 138.5, 135.5, 130.9, 128.9, 128.3, 126.2, 121.2, 120.1,
114.1, 108.4, 51.2, 36.8, 35.8, 35.7; MALDI-TOF m/z calcd for C\textsubscript{17}H\textsubscript{19}N\textsubscript{7}O\textsubscript{4} 385.38, found [M]\textsuperscript{+} 385.22.

HImImPyPyCOOMe (6). To a solution of 11 (150.0 mg, 0.4 mol) in methanol:tetrahydrofuran (1:3, v:v) was added 1.5 M NaOH (5 mL) and the solution was allowed to stir at 60 °C for 3 h. The solution was neutralized with HCl. Volatiles were removed \textit{in vacuo}. The resulting solid was dissolved in DMF (30 mL), EDC (114.7 mg, 0.6 mmol) and HOBt (81.1 mg, 0.6 mmol) were added and the reaction was allowed to stir for 1 h. A solution of 9a (93.1 mg, 0.6 mmol) in DMF, 10 mL, was added dropwise over the period of 10 min and the reaction was allowed to stir for 16 h. DMF was evaporated \textit{in vacuo} and the resulting solid was purified via flash chromatography (silica gel, acetone:CH\textsubscript{2}Cl\textsubscript{2}, 5.0 % incremental increases of acetone) to yield pure 6 (102.5 mg, 69%) as a dark brown solid: TLC (50:50 CH\textsubscript{2}Cl\textsubscript{2}:acetone v/v) R\textsubscript{f} 0.81; \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}) \(\delta\) 9.47 (s, 1H), 8.87 (s, 1H), 7.57 (s, 1H), 7.41 (s, 1H), 7.35 (s, 1H), 7.13 (s, 1H), 6.96 (s, 1H), 6.70 (s, 1H), 6.69 (s, 1H), 4.03, (s, 3H), 4.02 (s, 3H), 3.89 (s, 3H), 3.84 (s, 3H), 3.71 (s, 3H); \textsuperscript{13}C NMR (125 MHz, CDCl\textsubscript{3}) \(\delta\) 161.5, 158.8, 156.2, 156.0, 138.3, 135.4, 134.1, 128.2, 126.2, 123.3, 121.7, 121.0, 120.9, 119.9, 119.1, 114.15, 108.2, 103.4,
51.1, 36.8, 36.7, 35.7, 35.6; MALDI-TOF m/z calcd for C_{23}H_{25}N_{9}O_{5} 507.50, found [M - H]^+ 505.56.

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HImImPyPyCONH(CH$_2$)$_n$N$_3$ (DPA 202 – DPA207). Compound 6 was dissolved in MeOH:THF (1:3; v:v; 10 mL). 1.5 N NaOH (2.0 mL) was added and the reaction was stirred at 60 °C and followed by TLC. When TLC showed the starting material was no longer present, 4 – 6 h, the reaction was neutralized with HCl. The solution was evaporated to dryness. The resulting acid was suspended in DMF (20 mL), EDC and HOBt were added and the solution was stirred for 1 h. The amino-azido alkane (12 - 17) was added and the solution was allowed to stir overnight. DMF was removed in vacuo and the resulting solid was purified by flash chromatography (silica gel, CH$_2$Cl$_2$:acetone 25% incremental increases in acetone) to yield DPA202 – DPA207.
**HImImPyPyCONH(CH$_2$)$_2$N$_3$ (DPA202):** DPA202 was afforded (109.8 mg, 84%) as a light yellow solid: TLC (70:30, hexane:ethyl acetate, v/v) $R_f$ 0.60. $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.54 (br, 1H), 8.97 (s, 1H), 7.91 (s, 1H), 7.41 (s, 1H), 7.15 (s, 1H), 7.10 (s, 1H), 7.02 (s, 1H), 6.98 (s, 1H), 6.69 (s, 1H), 6.59 (s, 1H), 6.07 (s, 1H), 4.04 (s, 3H), 4.01 (s, 3H), 3.88 (s, 3H), 3.85 (s, 3H), 3.32 (t, $J$ = 6.5, 2H), 3.23 (t, $J$ = 6.3, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 162.1, 159.1, 156.1, 155.9, 138.3, 135.4, 134.3, 126.2, 126.1, 123.3, 122.8, 121.7, 120.9, 119.3, 119.1, 114.4, 103.8, 103.4, 51.5, 41.2, 36.9, 36.4, 35.7, 35.6; MALDI-TOF $m/z$ calcd C$_{23}$H$_{27}$N$_{13}$O$_4$ 561.57, found [M – 2H]$^+$ 559.85.

**HImImPyPyCONH(CH$_2$)$_3$N$_3$ (DPA203):** DPA203 was afforded (86.0 mg, 76%) as an off white solid: TLC (70:30, hexane:ethyl acetate, v/v) $R_f$ 0.60. IR (neat) 2933, 2867, 2053, 1667, 1648 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 9.59 (s, 1H), 9.03 (s, 1H), 8.09 (s, 1H), 7.39 (s, 1H), 7.15 (s, 1H), 7.14 (s, 1H), 7.00 (s, 1H), 6.97 (s, 1H), 6.66 (s, 1H), 6.58 (s, 1H), 6.38 (s, 1H), 4.02 (s, 3H), 4.00 (s, 3H), 3.88 (s, 3H), 3.80 (s, 3H), 3.41 (t, $J_1 = 6.5$, 2H), 3.37 (t, $J_2 = 6.4$, 2H), 1.82 (p, $J_1 = 6.4$, $J_2 = 6.5$, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 162.1, 159.1, 156.3, 155.9, 138.4, 135.5, 134.3, 128.2, 126.3, 123.4, 123.3, 121.3, 120.9, 119.2, 119.0, 114.4, 103.6, 103.4, 49.5, 39.9, 36.8, 36.7, 36.6, 35.8, 29.0; MALDI-TOF $m/z$ calcd C$_{25}$H$_{29}$N$_{13}$O$_4$ 575.59, found [M – 2H]$^+$ 572.97.

**HImImPyPyCONH(CH$_2$)$_5$N$_3$ (DPA204):** DPA204 was afforded (104.3 mg, 91%) as a yellow solid: TLC (70:30, hexane:ethyl acetate, v/v) $R_f$ 0.58. IR (neat) 2923, 2853,
HImImPyPyCONH(CH$_2$)$_8$N$_3$ (DPA205): DPA205 was afforded (71.1 mg, 72%) as a light yellow powder: TLC (70:30, hexane:ethyl acetate, v/v) R$_f$ 0.53. IR (neat) 2922, 2841, 2075, 1671, 1663 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) δ 9.44 (br, 1H), 8.94 (s, 1H), 7.88 (s, 1H), 7.38 (s, 1H), 7.13 (s, 1H), 7.07 (s, 1H), 6.99 (s, 1H), 6.95 (s, 1H), 6.66 (s, 1H), 6.56 (s, 1H), 6.04 (s, 1H), 4.01 (s, 3H), 3.98 (s, 3H), 3.85 (s, 3H), 3.82 (s, 3H), 3.29 (t, $J_1 = 5.7$, 2H), 3.20 (t, $J_2 = 6.1$, 2H), 1.55 (m, 4H), 1.36 (m, 4H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 161.8, 159.0, 156.3, 156.2, 138.4, 135.5, 134.2, 128.2, 126.3, 123.6, 123.4, 121.5, 121.0, 119.2, 118.7, 114.3, 103.6, 103.3, 51.3, 39.1, 36.8, 36.7, 35.8, 35.7, 29.8, 29.5, 28.6, 24.2; MALDI-TOF m/z calcd C$_{27}$H$_{33}$N$_{13}$O$_4$ 603.65, found [M – 2H]$^+$ 601.21.

HImImPyPyCONH(CH$_2$)$_8$N$_3$ (DPA206): DPA206 was afforded (72.6 mg, 75%) as a yellow solid: TLC (70:30, hexane:ethyl acetate, v/v) R$_f$ 0.50. IR (neat) 2928, 2841, 2065, 1675, 1665, 1531 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) δ 9.53 (s, 1H), 8.94 (s, 1H), 8.04 (s, 1H), 7.39 (s, 1H), 7.16 (s, 1H), 7.11 (s, 1H), 7.03 (s, 1H), 6.997 (s, 1H), 6.68 (s, 1H), 6.63 (s, 1H), 6.44 (s, 1H), 4.00 (s, 3H), 3.97 (s, 3H), 3.88 (s, 3H), 3.80 (s, 3H), 3.40 (t, $J_1 = 6.1$, 2H), 3.37 (t, $J_2 = 6.7$, 2H), 1.55 (p, $J_1 = 6.0$, 2H), 1.52 (p, $J_2 = 6.7$, 2H), 1.82 (m, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 162.0, 159.6, 156.3, 156.2, 138.2, 135.6, 134.3, 128.2, 126.7, 123.5, 123.2, 121.2, 121.1, 119.3, 119.1, 114.4, 103.3, 103.1, 49.5, 39.3, 36.9, 36.8, 36.7, 36.6, 31.8, 28.7, 23.0; MALDI-TOF m/z calcd C$_{28}$H$_{35}$N$_{13}$O$_4$ 617.68, found [M]$^+$ 617.54.
7.71 (s, 1H), 7.45 (s, 1H), 7.17 (s, 1H), 7.11 (s, 1H), 7.08 (s, 1H), 7.00 (s, 1H), 6.73 (s, 1H), 6.58 (s, 1H), 5.92 (s, 1H), 4.08 (s, 3H), 4.06 (s, 3H), 3.92 (s, 3H), 3.88 (s, 3H), 3.38 (m, 2H), 3.21 (m, 2H), 1.55 (m, 4H), 1.31 (m, 4H), 1.28 (m, 4H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 161.8, 159.0, 156.3, 156.0, 138.4, 135.5, 134.2, 128.2, 126.3, 123.8, 123.4, 121.3, 121.1, 119.1, 118.5, 114.2, 103.4, 103.2, 51.5, 39.7, 36.8, 36.7, 35.8, 35.7, 29.8, 29.5, 28.6, 27.9, 26.7, 26.2; MALDI-TOF m/z calcd C$_{30}$H$_{37}$N$_{13}$O$_4$ 645.73, found [M – 3H]$^+$ 642.00.

HImImPyPyCONH(CH$_2$)$_{10}$N$_3$ (DPA207): DPA207 was afforded (88.9 mg, 88%) as an off white crystalline solid: TLC (70:30, hexane:ethyl acetate, v/v) R$_f$ 0.50. IR (neat) 2911, 2831, 2054, 1661, 1653, 1581 cm$^{-1}$; $^1$H NMR (300 MHz, CDCl$_3$) δ 9.61 (br, 1H), 9.06 (s, 1H), 7.80 (s, 1H), 7.49 (s, 1H), 7.23 (s, 1H), 7.11 (s, 1H), 7.06 (s, 1H), 7.05 (s, 1H), 6.81 (s, 1H), 6.64 (s, 1H), 6.00 (s, 1H), 4.27 (s, 3H), 4.26 (s, 3H), 4.08 (s, 3H), 3.91 (s, 3H), 3.39 (m, 2H), 3.27 (m, 2H), 1.60 (m, 4H), 1.31 (m, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$) δ 161.9, 159.1, 156.1, 156.0, 135.5, 135.2, 132.1, 130.2, 126.3, 123.8, 123.4, 121.4, 121.2, 119.2, 118.7, 114.9, 103.5, 103.1, 51.7, 39.4, 37.1, 36.9, 36.8, 35.9, 29.8, 29.5, 29.3, 29.2, 28.9, 27.1, 26.8; MALDI-TOF m/z calcd C$_{32}$H$_{43}$N$_{15}$O$_4$ 673.78, found [M – H]$^+$ 672.97.
**N-Boc neomycin-N₃ (DPA11).** See Appendix A for synthetic procedure, compound data reduction and characterization.

**Table B2.** Stoichiometry table for the synthesis of N-Boc-neomycin alkynes, DPA13, DPA14 and DPA15.

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**N-Boc neomycin-'tri'-alkynes (DPA13 – DPA15).** To a solution of dialkyne in toluene (2 mL) was added copper iodide and DIPEA. A solution of DPA11, in toluene (2 mL), was added and the reaction was allowed to stir at 60 °C for 12 h. Volatiles were removed *in vacuo* and the resulting solid was purified by chromatography (silica gel, CH₂Cl₂:2-propanol, 1.0 % incremental increases of 2-propanol) to afford the desired N-Boc neomycin-alkynes, DPA25, DPA26 and DPA27.
**$N$-Boc neomycin-‘tri’-$\text{CH}_2\text{OCH}_2\text{CCH}$ (DPA13).** DPA13 was afforded (155.2 mg, 85%) as a white solid TLC (90:10 CH$_2$Cl$_2$:EtOH $v/v$) $R_f$ 0.60; FT-IR (neat) 3383, 2180, 1686, 1523, 1366, 1169 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.61 (br, 1H), 6.12 (s, 1H), 6.00 (s, 1H), 5.27 (s, 1H), 4.97 (s, 1H), 4.88 (s, 2H), 4.76 (s, 1H), 4.61 (s, 1H), 4.21-4.05 (m, 5H), 3.87 (s, 2H), 3.76 (s, 1H), 3.68 (s, 2H), 3.51 (s, 3H), 3.44 (s, 1H), 3.25 (s, 2H), 3.02 (s, 2H), 2.79 (s, 2H), 2.61-2.59 (t, 2H), 1.56-1.36 (m, 54 H); MALDI-TOF $m/z$ calcd for C$_{59}$H$_{99}$N$_9$O$_{25}$ 1334.46, found [M + Na]$^+$ 1356.14.

**$N$-Boc neomycin-‘tri’-(CH$_2$)$_3$CCH (DPA14).** DPA14 was afforded (166.3 mg, 88%) as a white solid TLC (90:10 CH$_2$Cl$_2$:EtOH $v/v$) $R_f$ 0.55; FT-IR (neat) 3376, 2185, 1677, 1520, 1300, 1100 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.73 (br, 1H), 6.10 (s, 1H), 5.87 (s, 1H), 5.23 (s, 1H), 4.90 (s, 1H), 4.89 (s, 2H), 4.77 (s, 1H), 4.63 (s, 1H), 4.21-4.05 (m, 5H), 3.91 (s, 2H), 3.73 (s, 1H), 3.64 (s, 2H), 3.50 (s, 3H), 3.37 (s, 1H), 3.22 (s, 2H), 3.00 (s, 2H), 2.68-2.59 (m, 6H), 1.59-1.32 (m, 54 H); MALDI-TOF $m/z$ calcd for C$_{60}$H$_{101}$N$_9$O$_{24}$ 1332.49, found [M + Na]$^+$ 1355.29.

**$N$-Boc neomycin-‘tri’-(CH$_2$)$_4$CCH (DPA15).** DPA15 was afforded (680.2 mg, 86%) as a white solid TLC (90:10 CH$_2$Cl$_2$:EtOH $v/v$) $R_f$ 0.55; FT-IR (neat) 3386, 2100, 1674, 1520, 1333, 1115 cm$^{-1}$; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 7.76 (br, 1H), 6.21 (s, 1H), 5.91 (s, 1H), 5.24 (s, 1H), 4.99 (s, 1H), 4.84 (s, 2H), 4.77 (s, 1H), 4.60 (s, 1H), 4.31-4.15 (m, 5H), 3.90 (s, 2H), 3.75 (s, 1H), 3.61 (s, 2H), 3.59 (s, 3H), 3.32 (s, 1H), 3.20 (s, 2H), 3.15 (s,
2H), 2.90-2.41 (t, 8H), 1.55-1.33 (m, 54 H); MALDI-TOF m/z calcd for C_{61}H_{103}N_{9}O_{24} 1346.52, found [M + Na]^+ 1367.49.

N-Boc neomycin-NH₂ (DPA12). See Appendix A for synthetic procedure, compound data reduction and characterization.

N-Boc neomycin-NHCH₂(O)OCH₂CCH (DPA17). A solution of DPA12 (78.3 mg, 64.5 µmol) in dry CH₂Cl₂ (20 mL) was cooled to -78 °C under inert atmosphere afforded by Ar (g). Triethylamine (13.0 mg, 129.0 µmol) was added via syringe and the solution was stirred for 10 min. Propargyl chloroformate (764.5 mg, 6.5 mmol) was added dropwise via syringe and the solution was stirred for 30 min. The solution was allowed to come to room temperature. Volatiles were removed in vacuo and the resulting solid was purified by chromatography (silica gel, CH₂Cl₂:2-propanol, 1.0 % incremental increases of 2-propanol) to afford DPA17 (73.5 mg, 88%) as a white solid TLC (90:10
CH$_2$Cl$_2$:EtOH $\nu/\nu$) $R_f$ 0.71; $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.12 (s, 1H), 6.00 (s, 1H), 5.27 (s, 1H), 4.97 (s, 1H), 4.88 (s, 2H), 4.76 (s, 1H), 4.61 (s, 1H), 4.21-4.05 (m, 5H), 3.87 (s, 2H), 3.76 (s, 1H), 3.68 (s, 2H), 3.51 (s, 3H), 3.44 (s, 1H), 3.25 (s, 2H), 3.02 (s, 2H), 2.79 (s, 2H), 2.61-2.59 (t, 2H), 1.56-1.36 (m, 5H); MALDI-TOF $m/z$ calcd for C$_{57}$H$_{98}$N$_8$O$_{26}$ 1295.28.43, found [M + Na]$^+$ 1317.29.

**Table B3.** Stoichiometry table for the synthesis of HImImPyPyCONH(CH$_2$)$_n$-tri-(CH$_2$)$_3$-neomycin-hexahydrochloride, DPA214 – DPA219, $n$ = 2, 3, 5, 6, 8 and 10.

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(DPA214 – DPA219). To a solution of $N$-Boc neomycin-tri-alkyne (DPA14) in toluene (1.0 mL) was added a solution consisting of CuI and DIPEA in toluene (500 $\mu$L). To this
solution was added a suspension of the azide functionalized tetramer polyamides in toluene (500 µL). The reaction mixture was allowed to stir at room temperature for 24 h, and then was concentrated in vacuo. The resulting residue was purified by flash chromatography (silica gel, CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugates. The solid was then suspended in 4 N HCl in dioxane (5 mL) and the reaction was allowed to stir at room temperature for 30 min. The solution was concentrated in vacuo and the resulting solid was washed with copious amounts of CH₂Cl₂ and lyophilized to dryness to afford conjugates DPA214 – DPA219 as hydrochloride salts. Coupling and deprotection steps were near quantitative and the subsequent yields reflect the overall yield for both steps. The reported ¹H NMR and MALDI spectra represent the deprotected compounds.

(DPA214): DPA214 (2.8 mg, 95%) was afforded as a light brown solid: ¹H NMR (500 MHz, D₂O) δ 8.10 (s, 2H), 7.48 (s, 1H), 7.42 (s, 1H), 7.35 (s, 1H), 7.25 (s, 1H), 7.08 (s, 1H), 6.89 (s, 1H), 6.79 (s, 1H), 6.12 (s, 2H), 5.57-5.56 (m, 1H), 5.31-5.30 (m, 1H), 5.18-5.17 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.21 (m, 1H), 4.13-4.10 (m, 2H), 4.10 (s, 1H), 3.93-3.89 (m, 1H), 3.89 (s, 6H), 3.88 (s, 3H), 3.87-3.85 (m, 1H), 3.83-3.81 (m, 1H), 3.80-3.79 (m, 6H), 3.78 (s, 1H), 3.71-3.70 (m, 1H), 3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.46-3.45 (m, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H), 3.33-3.31 (m, 2H), 3.30-3.29 (m, 1H), 3.30-3.28 (m, 2H), 3.27-3.20 (m, 5H), 3.20-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.23-2.20 (m, 1H), 1.72-1.63 (m, 1H), 7.98 (s, 2H) 2.68-2.55 (m, 4H) 1.78-1.70 (m, 2H); MALDI-TOF m/z calcd C₅₄H₈₀N₂₂O₁₆ 1293.35, found [M + Na]⁺ 1316.00.
**DPA215:** DPA215 (3.2 mg, 97%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.15 (s, 2H) 7.45 (s, 1H), 7.40 (s, 1H), 7.31 (s, 1H), 7.23 (s, 1H), 7.02 (s, 1H), 6.97 (s, 1H), 6.76 (s, 1H), 6.05 (s, 1H), 6.303 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.17-4.15 (m, 2H), 4.09 (s, 1H), 4.00 (s, 3H), 3.98-3.92 (m, 1H), 3.89 (s, 3H), 3.88-3.86 (m, 4H), 3.84-3.82 (m, 4H), 3.81-3.77 (m, 5H), 3.76 (s, 1H), 3.74-3.72 (m, 1H), 3.68-3.66 (m, 1H), 3.63-3.57 (m, 1H), 3.47-3.45 (m, 1H), 3.43-3.36 (m, 2H), 3.37-3.36 (m, 1H), 3.36-3.27 (m, 7H), 3.24-3.22 (m, 1H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.74-2.62 (m, 4H), 2.25-2.22 (m, 1H), 1.88-1.77 (m, 4H), 1.75-1.64 (m, 1H); MALDI-TOF $m/z$ calcd C$_{55}$H$_{82}$N$_{22}$O$_{16}$ 1307.38, found [M + Na]$^+$ 1331.71.

**DPA216:** DPA216 (3.8 mg, 98%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) $\delta$ 8.10 (s, 2H), 7.48 (s, 1H), 7.43 (s, 1H), 7.36 (s, 1H), 7.29 (s, 1H), 7.01 (s, 1H), 6.91 (s, 1H), 6.79 (s, 1H), 6.03 (s, 1H), 5.88 (s, 1H), 5.59-5.55 (m, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.15-4.11 (m, 2H), 4.00 (s, 1H), 4.00-3.96 (m, 1H), 3.98 (s, 6H), 3.91-3.88 (m, 1H), 3.87 (s, 3H), 3.86-3.85 (m, 1H), 3.85 (s, 3H), 3.84-3.79 (m, 2H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.50-3.46 (m, 1H), 3.44-3.42 (m, 2H), 3.41-3.36 (m, 3H), 3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 2H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.27-2.21 (m, 5H), 1.77-1.59
(m, 2H), 1.70-1.61 (m, 1H), 1.58-1.56 (m, 2H), 1.55-1.50 (m, 2H), 1.38-1.29 (m, 2H); MALDI-TOF m/z calcd C$_{57}$H$_{86}$N$_{22}$O$_{16}$ 1335.43, found [M]$^+$ 1357.44.

**DPA217**: DPA217 (3.0 mg, 98%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.15 (s, 1H), 8.10 (s, 1H), 7.45 (s, 1H), 7.40 (s, 1H), 7.31 (s, 1H), 7.23 (s, 1H), 7.02 (s, 1H), 6.97 (s, 1H), 6.76 (s, 1H), 6.05 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.17-4.15 (m, 2H), 4.08 (s, 1H), 3.99 (s, 2H), 3.98-3.92 (m, 1H), 3.91 (s, 1H), 3.88 (s, 6H), 3.88-3.86 (m, 1H), 3.84-3.82 (m, 1H), 3.81-3.77 (m, 5H), 3.76 (s, 4H), 3.74-3.72 (m, 1H), 3.68-3.66 (m, 1H), 3.63-3.57 (m, 1H), 3.47-3.45 (m, 1H), 3.38-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.30 (m, 1H), 3.30-3.24 (m, 3H), 3.24-3.22 (m, 1H), 3.22-3.16 (m, 5H), 3.14-3.12 (s, 2H), 2.74-2.62 (m, 4H), 2.25-2.22 (m, 1H), 1.82-1.66 (m, 3H), 1.57-1.47 (m, 4H), 1.36-1.29 (m, 4H); MALDI-TOF m/z calcd C$_{58}$H$_{88}$N$_{22}$O$_{16}$ 1349.46, found [M + Na]$^+$ 1373.90.

**DPA218**: DPA218 (3.5 mg, 96%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.17 (s, 1H), 8.03 (s, 1H), 7.35 (s, 1H), 7.30 (s, 1H), 7.26 (s, 1H), 7.21 (s, 1H), 7.06 (s, 1H), 6.98 (s, 1H), 6.88 (s, 1H), 5.94 (s, 1H), 5.59-5.55 (m, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.15-4.11 (m, 2H), 4.07 (s, 3H), 4.00 (s, 1H), 4.00-3.96 (m, 1H), 3.91 (s, 6H), 3.91-3.88 (m, 1H), 3.86 (s, 2H), 3.86-3.85 (m, 1H), 3.84-3.79 (m, 5H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.50-3.46 (m, 1H), 3.40-3.37 (m, 1H), 3.36
(s, 2H), 3.36-3.33 (m, 2H), 3.32-3.31 (m, 3H), 3.30-3.25 (m, 2H), 3.24-3.23 m, 3H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.66-2.61 (m, 4H), 2.27-2.21 (m, 1H), 1.77-1.59 (m, 2H), 1.70-1.61 (m, 1H), 1.57-1.48 (m, 4H), 1.35-1.31 (m, 4H), 1.31-1.27 (m, 4H); MALDI-TOF m/z calcd C_{60}H_{92}N_{22}O_{16} 1377.51, found [M + H + Na]^+ 1402.47.

(DPA219): DPA219 (3.1 mg, 88%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.09 (s, 2H), 7.47 (s, 1H), 7.40 (s, 1H), 7.32 (s, 1H), 7.22 (s, 1H), 7.02 (s, 1H), 6.98 (s, 1H), 6.76 (s, 1H), 5.92 (s, 1H), 5.56-5.55 (m, 1H), 5.27-5.24 (m, 1H), 5.16-5.15 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.25-4.20 (m, 1H), 4.10-4.09 (m, 2H), 4.08 (s, 2H), 4.06 (s, 1H), 4.02-3.98 (m, 1H), 3.92 (s, 3H), 3.91-3.90 (m, 4H), 3.88 (s, 3H), 3.88-3.86 (m, 1H), 3.85-3.80 (m, 5H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.45-3.44 (m, 1H), 3.39-3.36 (m, 1H), 3.36-3.35 (m, 2H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.28 (m, 1H), 3.27-3.24 (m, 2H), 3.24-3.20 (m, 2H), 3.23-3.22 (m, 1H), 3.21-3.17 (m, 3H), 3.15-3.10 (s, 2H), 2.70-2.63 (m, 4H), 2.21-2.17 (m, 1H), 1.80-1.69 (m, 2H), 1.73-1.61 (m, 1H), 1.57-1.50 (m, 4H), 1.31-1.25 (m, 6H); MALDI-TOF m/z calcd C_{62}H_{96}N_{22}O_{16} 1405.56, found [M + Na]^+ 1427.72.
Table B4. Stoichiometry table for the synthesis of HlmImPyPyCONH(CH₂)n-tri-(CH₂)₄-neomycin-hexahydrochloride, DPA220 – DPA225, n = 2, 3, 5, 6, 8 and 10.

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(DPA220 – DPA225). To a solution of N-Boc-neomycin-tri-alkyne DPA15 in toluene (1.0 mL) was added a solution consisting of CuI and DIPEA in toluene (500 µL). To this solution was added a suspension of the azide functionalized tetramer polyamides in toluene (500 µL). The reaction mixture was allowed to stir at room temperature for 24 h, and then was concentrated in vacuo. The resulting residue was purified by flash...
chromatography (silica gel, CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugates. The solid was then suspended in 4 N HCl in dioxane (5 mL) and the reaction was allowed to stir at room temperature for 30 min. The solution was concentrated in vacuo and the resulting solid was washed with copious amounts of CH₂Cl₂ and lyophilized to dryness to afford conjugates DPA220 – DPA225 as hydrochloride salts. Coupling and deprotection steps were near quantitative and the subsequent yields reflect the overall yield for both steps. The reported ¹H NMR and MALDI spectra represent the deprotected compounds.

(DPA220): DPA220 (4.9 mg, 98%) was afforded as a light brown solid: ¹H NMR (500 MHz, D₂O) δ 7.88 (s, 2H), 7.48 (s, 1H), 7.42 (s, 1H), 7.35 (s, 1H), 7.25 (s, 1H), 7.08 (s, 1H), 6.89 (s, 1H), 6.79 (s, 1H), 6.05 (s, 2H), 5.57-5.56 (m, 1H), 5.31-5.30 (m, 1H), 5.18-5.17 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.25-4.20 (m, 1H), 4.10-4.09 (m, 2H), 4.09 (s, 1H), 4.08 (s, 2H), 4.00-3.96 (m, 1H), 3.91 (s, 6H), 3.91-3.88 (m, 1H), 3.88 (s, 3H), 3.86-3.85 (m, 1H), 3.84-3.79 (m, 2H), 3.78 (s, 4H), 3.77-3.74 (m, 1H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.47-3.45 (m, 1H), 3.38-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.33-3.28 (m, 2H), 3.32-3.30 (m, 1H), 3.30-3.24 (m, 2H), 3.24-3.21 (m, 3H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.65-2.59 (m, 4H), 2.23-2.20 (m, 1H), 1.77-1.58 (m, 4H), 1.72-1.63 (m, 1H); MALDI-TOF m/z calcd C₅₅H₈₂N₂₂O₁₆ 1307.38, found [M + Na]⁺ 1330.73.
(DPA221): **DPA221** (5.0 mg, 97%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.16 (s, 2H), 7.38 (s, 1H), 7.32 (s, 1H), 7.31 (s, 1H), 7.26 (s, 1H), 7.08 (s, 1H), 6.99 (s, 1H), 6.89 (s, 1H), 6.17 (s, 1H), 5.59-5.56 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.21 (m, 1H), 4.13-4.10 (m, 2H), 4.09 (s, 1H), 4.06-4.01 (m, 1H), 4.00 (s, 1H), 3.91-3.90 (m, 1H), 3.88-3.86 (m, 4H), 3.85-3.80 (m, 2H), 3.80 (s, 3H), 3.79 (s, 4H), 3.79-3.75 (m, 4H), 3.74 (s, 3H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.50-3.46 (m, 1H), 3.44-3.39 (m, 2H), 3.40-3.37 (m, 1H), 3.36 (s, 2H), 3.36-3.32 (m, 3H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 2H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.70-2.55 (m, 4H), 2.25-2.22 (m, 1H), 1.93-1.89 (m, 2H), 1.75-1.44 (m, 5H); MALDI-TOF m/z calcd C$_{56}$H$_{84}$N$_{22}$O$_{16}$ 1321.40, found [M + Na]$^+$ 1344.56.

(DPA222): **DPA222** (5.2 mg, 97%) was afforded as a light brown solid: $^1$H NMR (500 MHz, D$_2$O) δ 8.11(s, 2H), 7.35 (s, 1H), 7.27 (s, 1H), 7.20 (s, 1H), 7.17 (s, 1H), 7.00 (s, 1H), 6.95 (s, 1H), 6.82 (s, 1H), 6.09 (s, 1H), 5.59 (s, 1H), 5.33-5.30 (m, 1H), 5.21-5.18 (m, 1H), 4.50-4.55 (m, 1H), 4.36-4.34 (m, 1H), 4.25-4.22 (m, 1H), 4.17-4.15 (m, 2H), 4.10 (s, 1H), 3.97 (s, 1H), 3.93-3.88 (m, 1H), 3.88 (s, 3H), 3.87-3.85 (m, 7H), 3.83-3.81 (m, 1H), 3.81 (s, 3H), 3.80-3.79 (m, 2H), 3.78 (s, 4H), 3.71-3.70 (m, 1H), 3.66-3.63 (m, 1H), 3.61-3.56 (m, 1H), 3.45-3.40 (m, 2H), 3.45-3.44 (m, 1H), 3.41-3.31 (m, 3H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.28 (m, 1H), 3.27-3.24 (m, 2H), 3.23-3.22 (m, 1H), 3.21-3.17 (m, 3H), 3.15 3.10 (s, 2H), 2.60-2.51 (m, 4H), 2.27-2.21 (m, 1H), 1.70-1.61 (m,
5H), 1.56-1.50 (m, 2H), 1.50-1.47 (m, 2H), 1.33-1.27 (m, 2H); C_{58}H_{88}N_{22}O_{16} 1349.46, found [M + Na]^+ 1374.10.

**DPA223**: DPA223 (4.8 mg, 96%) was afforded as a light brown solid: \(^1\)H NMR (500 MHz, D\textsubscript{2}O) \(\delta\) 8.11 (s, 1H), 8.04 (s, 1H), 7.47 (s, 1H), 7.40 (s, 1H), 7.32 (s, 1H), 7.22 (s, 1H), 7.02 (s, 1H), 6.98 (s, 1H), 6.76 (s, 1H), 6.02 (m, 2H), 5.56-5.55 (m, 1H), 5.37-5.34 (m, 1H), 5.16-5.15 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.15-4.11 (m, 2H), 4.07 (s, 1H), 3.98-3.92 (m, 1H), 3.92 (s, 1H), 3.88 (s, 6H), 3.86 (m, 1H), 3.86 (s, 3H), 3.84-3.82 (m, 4H), 3.81-3.77 (m, 5H), 3.76 (s, 1H), 3.74-3.72 (m, 1H), 3.68-3.66 (m, 1H), 3.63-3.57 (m, 1H), 3.46-3.45 (m, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H), 3.33-3.31 (m, 2H), 3.30-3.30 (m, 1H), 3.30-3.27 (m, 2H), 3.27-3.25 (m, 2H), 3.26-2.23 (m, 3H), 3.21-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.73-2.66 (m, 4H), 2.21-2.17 (m, 1H), 1.83-1.71 (m, 4H), 1.73-1.61 (m, 1H), 1.55-1.51 (m, 2H), 1.51-1.44 (m, 2H), 1.36-1.30 (m, 4H); MALDI-TOF m/z calcd C_{59}H_{90}N_{22}O_{16} 1363.48, found [M + Na]^+ 1386.47.

**DPA224**: DPA224 (5.3 mg, 99%) was afforded as a light brown solid: \(^1\)H NMR (500 MHz, D\textsubscript{2}O) \(\delta\) 8.11 (br, 2H), 7.45 (s, 1H), 7.40 (s, 1H), 7.31 (s, 1H), 7.25 (s, 1H), 7.08 (s, 1H), 6.98 (s, 1H), 6.76 (s, 1H), 6.14 (s, 1H), 5.65-5.64 (m, 1H), 5.27-5.24 (m, 1H), 5.16-5.15 (m, 1H), 4.51-4.43 (m, 1H), 4.37-4.33 (m, 1H), 4.28-4.22 (m, 1H), 4.15-4.11 (m, 2H), 4.07 (s, 1H), 4.00 (s, 1H), 3.98-3.92 (m, 1H), 3.91 (s, 3H), 3.89 (s, 3H), 3.88-3.86 (m, 1H), 3.84-3.82 (m, 4H), 3.81-3.77 (m, 5H), 3.76 (s, 1H), 3.74-3.72 (m, 1H), 3.68-
3.66 (m, 1H), 3.63-3.57 (m, 1H), 3.46-3.45 (m, 1H), 3.37-3.34 (m, 1H), 3.34 (s, 2H),
3.33-3.29 (m, 4H), 3.29-3.28 (m, 2H), 3.28-3.27 (m, 1H), 3.27-3.25 (m, 2H), 3.25-2.23
(m, 1H), 3.21-3.16 (m, 3H), 3.13-3.10 (s, 2H), 2.73-2.66 (m, 4H), 2.21-2.17 (m, 1H),
1.83-1.74 (m, 4H), 1.73-1.61 (m, 1H), 1.57-1.48 (m, 4H), 1.35-1.31 (m, 4H), 1.31-1.27
(m, 4H); MALDI-TOF m/z calcd C₆₁H₉₄N₂₂O₁₆ 1391.54, found [M + Na]⁺ 1414.55.

(DPA225):  DPA225 (4.3 mg, 95%) was afforded as a light brown solid: ¹H NMR (500
MHz, D₂O) δ 8.06 (s, 2H), 7.35 (s, 1H), 7.28 (s, 1H), 7.21 (s, 1H), 7.14 (s, 1H), 7.03 (s,
1H), 6.81 (s, 1H), 6.07 (s, 1H), 5.51 (s, 1H), 5.49-5.46 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.28 (s, 3H), 4.23-4.21 (m, 1H), 4.17
(s, 3H), 4.13-4.10 (m, 2H), 4.07 (s, 1H), 4.02-3.98 (m, 1H), 3.96 (s, 3H), 3.91-3.90 (m,
1H), 3.88-3.86 (m, 4H), 3.85-3.80 (m, 2H), 3.79 (s, 1H), 3.79-3.75 (m, 4H), 3.72-3.66
(m, 4H), 3.66-3.59 (m, 1H), 3.50-3.46 (m, 1H), 3.46-3.41 (m, 2H), 3.40-3.37 (m, 1H),
3.36 (s, 2H), 3.36-3.32 (m, 2H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 4H), 3.24-3.23 (m,
1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.70-2.55 (m, 4H), 2.25-2.22 (m, 1H), 1.80-
1.49 (m, 5H), 1.61-1.55 (m, 4H), 1.31-1.22 (m, 6H); MALDI-TOF m/z calcd
C₆₃H₉₈N₂₂O₁₆ 1419.59, found [M + Na]⁺ 1442.25.
(DPA243). To a solution of DPA17 (15 mg, 11.6 µmol) suspended in toluene (1.0 mL) was added a homogeneous solution consisting of CuI and DIPEA in toluene (500 µL). To this solution was added a solution of DPA202 (6.5 mg, 11.6 µmol) suspended in toluene (500 µL). The reaction was allowed to stir at room temperature for 24 h. The solution was concentrated in vacuo. The resulting residue was purified by flash chromatography (silica gel, CH₂Cl₂:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugate. The solid was then suspended in 4 N HCl in dioxane (5 mL) and the reaction was allowed to stir at room temperature for 30 min. The solution was concentrated in vacuo and the resulting solid was washed with copious amounts of CH₂Cl₂ and lypholized to dryness to afford DPA243 as a hydrochloride salt. ¹H NMR (500 MHz, D₂O) δ 7.41 (s, 1H), 7.39 (s, 1H), 7.28 (s, 1H), 7.21 (s, 1H), 7.03 (s, 1H), 6.97 (s, 1H), 6.87 (s, 1H), 6.05 (s, 1H), 5.57-5.56 (m, 1H), 5.31-5.30 (m, 1H), 5.18-5.17 (m, 1H), 4.47-4.39 (m, 1H), 4.36-4.33 (m, 1H), 4.25-4.20 (m, 1H), 4.10-4.09 (m, 2H), 4.09
(s, 3H), 4.08 (s, 3H), 4.00-3.96 (m, 1H), 3.91 (s, 3H), 3.91-3.88 (m, 1H), 3.88 (s, 3H), 3.86-3.85 (m, 1H), 3.84-3.79 (m, 2H), 3.78 (s, 1H), 3.77-3.74 (m, 1H), 3.70-3.64 (m, 1H), 3.65-3.55 (m, 1H), 3.47-3.45 (m, 1H), 3.38-3.36 (m, 1H), 3.35 (s, 2H), 3.35-3.33 (m, 2H), 3.32-3.30 (m, 1H), 3.30-3.24 (m, 2H), 3.24-3.21 (m, 3H), 3.22-3.15 (m, 3H), 3.14-3.12 (s, 2H), 2.65-2.59 (m, 4H), 2.23-2.20 (m, 1H), 1.77-1.58 (m, 4H), 1.72-1.63 (m, 1H); MALDI-TOF m/z calcd C$_{51}$H$_{76}$N$_{20}$O$_{18}$ 1257.27, found [M + H$_2$O]$^+$ 1276.11.

(DPA244). To a solution of DPA17 (15 mg, 11.6 µmol) suspended in toluene (1.0 mL) was added a homogeneous solution consisting of CuI and DIPEA in toluene (500 µL). To this solution was added a solution of DPA203 (6.5 mg, 11.6 µmol) suspended in toluene (500 µL). The reaction was allowed to stir at room temperature for 24 h. The solution was concentrated in vacuo. The resulting residue was purified by flash
chromatography (silica gel, CH$_2$Cl$_2$:MeOH, 2% incremental increases in MeOH) to yield the N-Boc protected conjugate. The solid was then suspended in 4 N HCl in dioxane (5 mL) and the reaction was allowed to stir at room temperature for 30 min. The solution was concentrated in vacuo and the resulting solid was washed with copious amounts of CH$_2$Cl$_2$ and lypholized to dryness to afford DPA244 as a hydrochloride salt. $^1$H NMR (500 MHz, D$_2$O) $\delta$ 7.38 (s, 1H), 7.32 (s, 1H), 7.31 (s, 1H), 7.26 (s, 1H), 7.08 (s, 1H), 6.99 (s, 1H), 6.89 (s, 1H), 6.57 (s, 1H), 6.37 (s, 1H), 5.89-5.86 (m, 1H), 5.33-5.31 (m, 1H), 5.20-5.18 (m, 1H), 4.47-4.40 (m, 1H), 4.35-4.34 (m, 1H), 4.23-4.21 (m, 1H), 4.13-4.10 (m, 2H), 4.09 (s, 3H), 4.06-4.01 (m, 1H), 4.00 (s, 3H), 3.91-3.90 (m, 1H), 3.88-3.86 (m, 1H), 3.85-3.80 (m, 2H), 3.80 (s, 3H), 3.79 (s, 1H), 3.79-3.75 (m, 1H), 3.74 (s, 3H), 3.72-3.66 (m, 1H), 3.66-3.59 (m, 1H), 3.50-3.46 (m, 1H), 3.44-3.39 (m, 2H), 3.40-3.37 (m, 1H), 3.36 (s, 2H), 3.36-3.32 (m, 3H), 3.32-3.31 (m, 1H), 3.30-3.25 (m, 2H), 3.24-3.23 (m, 1H), 3.22-3.14 (m, 3H), 3.13-3.11 (s, 2H), 2.70-2.55 (m, 4H), 2.25-2.22 (m, 1H), 1.93-1.89 (m, 2H), 1.75-1.44 (m, 5H); MALDI-TOF $m/z$ calcd C$_{52}$H$_{78}$N$_{20}$O$_{18}$ 1271.30, found [M + H$_2$O]$^+$ 1289.71.

HlmImPyPyDp (18). Compound 6 (50 mg, 0.09 mmol) was dissolved in MeOH:THF (1:3; v:v; 10 mL). 1.5 N NaOH (2.0 mL) was added and the reaction was stirred at 60 °C
and followed by TLC. When TLC showed the starting material was no longer present, 4 – 6 h, the reaction was neutralized with HCl. The solution was evaporated to dryness. The resulting acid was suspended in DMF (20 mL), EDC and HOBr were added and the solution was stirred for 1 h. N,N-dimethylanopropylamine (19.4 mg, 0.19 mmol) was added and the solution was allowed to stir for 18 h. DMF was removed in vacuo and the resulting solid was suspended in CH$_2$Cl$_2$ (25 mL). The solution was decanted and the resulting solid was suspended in MeOH (15 mL). The solution filtered and dried to afford 18 as an off white solid (28.6 mg, 55%): $^1$H NMR (500 MHz, D$_2$O) $\delta$ 9.53 (s, 1H), 8.88 (s, 1H), 7.66 (s, 1H), 7.44 (s, 1H), 7.36 (s, 1H), 7.24 (s, 1H), 7.00 (s, 1H), 6.77 (s, 1H), 6.63 (s, 1H), 4.03 (s, 6H), 4.02 (s, 3H), 3.91 (s, 3H), 3.89 (s, 3H), 3.80 (s, 3H), 3.22 (t, $J_1$ = 4.2, 2H), 2.75 (t, $J_2$ = 6.1, 2H), 2.63 (q, $J_1$ = 4.3, $J_2$ = 6.1, 2H); $^{13}$C NMR (125 MHz, CDCl$_3$) $\delta$ 162.5, 158.9, 154.2, 139.6, 138.7, 138.0, 1.37.0, 136.6, 133.1, 127.6, 126.3, 125.8, 122.1, 121.4, 120.3, 119.9, 119.0, 114.18, 108.9, 105.9, 66.4, 63.8, 50.1, 36.3, 36.4, 34.2, 32.6; MALDI-TOF m/z calcd for C$_{27}$H$_{35}$N$_{11}$O$_4$ 577.64, found [M + Na]$^{+}$ 599.64.
Figure B1. Characterization of compound 8a.
Figure B2. Characterization of compound 10.
(a) $^1$H NMR

(b) $^{13}$C NMR
Figure B3. Characterization of compound 9a.

(a) $^1$H NMR
Figure B4. Characterization of compound 11.
Figure B5. Characterization of compound 6.
Figure B6. Characterization of compound DPA202.
Figure B7. Characterization of compound DPA203.

(a) $^1$H NMR
Figure B8. Characterization of compound DPA204.
(a) $^1$H NMR

(b) $^{13}$C NMR
Figure B9. Characterization of compound DPA205.

(a) $^1$H NMR
Figure B10. Characterization of compound DPA206.
(a) $^1$H NMR

(b) $^{13}$C NMR
Figure B11. Characterization of compound DPA207.

(a) $^1$H NMR
Figure B12. Characterization of compound DPA13.

(b) MALDI-TOF

(a) $^1$H NMR
Figure B13. Characterization of compound DPA14.
Figure B14. Characterization of compound DPA12.
Figure B15. Characterization of compound DPA17.

(a) $^1$H NMR (N-Boc protected)
(b) MALDI-TOF (N-Boc protected)

(a) $^1$H NMR (deprotected)
Figure B16. Characterization of compound DPA208.
(b) MALDI-TOF

Figure B17. Characterization of compound DPA209.

(a) $^1$H NMR
Figure B18. Characterization of compound DPA210.
Figure B19. Characterization of compound DPA211.
Figure B20. Characterization of compound DPA212.
(b) MALDI-TOF

Figure B21. Characterization of compound DPA213.

(a) $^1$H NMR
(b) MALDI-TOF

Figure B22. Characterization of compound DPA214.

(a) $^1$H NMR
Figure B23. Characterization of compound DPA215.

Figure B23. Characterization of compound DPA215.
Figure B24. Characterization of compound DPA216.
Figure B25. Characterization of compound DPA217.
Figure B26. Characterization of compound DPA218.

(b) MALDI-TOF

(a) $^1$H NMR
Figure B27. Characterization of compound DPA219.

(a) $^1$H NMR

(b) MALDI-TOF
Figure B28. Characterization of compound DPA220.
Figure B29. Characterization of compound DPA221.

(a) $^1$H NMR

(b) MALDI-TOF
Figure B30. Characterization of compound DPA222.

(a) $^1$H NMR

(b) MALDI-TOF
Figure B31. Characterization of compound DPA223.
Figure B32. Characterization of compound DPA224.

(a) $^1$H NMR

(b) MALDI-TOF
Figure B33. Characterization of compound DPA225.

(b) MALDI-TOF

(a) $^1$H NMR
Figure B34. Characterization of compound DPA243.
Figure B35. Characterization of compound DPA244.

(a) $^1$H NMR

(b) MALDI-TOF
Figure B36. Characterization of compound 18.
Figure B37. CD scans for the following duplexes: 5’-G₂A₃G₄C₄-3’ (a), 5’-AG₂C₂AG₄C₄-3’ (b), 5’-G₂C₂AG₄C₄-3’ (c), 5’-TG₂C₂G₄-3’ (d) and 5’-AG₂C₂AG₄-3’ (e). Scans were conducted at 20 °C. The concentration of the duplexes was 40 µM/bp. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure B38. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G_2A_3G_4C_4-3’. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 1.0$. Controls (neomycin, HlmImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 conjugates are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 μM/duplex], pre-saturated with ethidium bromide [7 μM], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits_{ex} = 20; slits_{em} = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure B39. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-G2A3G4C4-3’. In the histogram, the values represent the percent decrease in fluorescence at \( r_{dd} = 1.0 \). Controls (neomycin, HelimImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 conjugates are shown in blue, series II DPA214 – DPA219 conjugates are shown in green, series III DPA220 – DPA225 conjugates are shown in red and series IV DPA243 – DPA244 conjugates are shown in dark grey. Data was obtained from a FID assay. Ligands were added to the DNA [1 \( \mu \)M/duplex], pre-saturated with ethidium bromide [7 \( \mu \)M], at various ratios of drug to duplex (\( r_{dd} \)), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits\(_{ex} = 20\); slits\(_{em} = 20\); Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na2HPO4, 0.5 mM NaH2PO4, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure B40. UV melting profiles for 5'-A₂G₆C₆T₂-3' with series II DPA214 – DPA219 tetramer polyamide – neomycin conjugates. The histograms on the right are a plot of $\Delta T_m$ with respect to the ratio of drug:duplex ($r_{dd}$). Ligands were added to the duplex [2 $\mu$M/duplex] at varying $r_{dd}$ values. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Each panel represents the duplex with a conjugate at varying $r_{dd}$ values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0.
Figure B41. UV melting profiles for 5'-A₂G₆C₆T₂-3' with series III DPA220 – DPA225 tetramer polyamide – neomycin conjugates. The histograms on the right are a plot of $\Delta T_m$ with respect to the ratio of drug:duplex ($r_{dd}$). Ligands were added to the duplex [2 µM/duplex] at varying $r_{dd}$ values. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Each panel represents the duplex with a conjugate at varying $r_{dd}$ values. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0.
Figure B42. Plot of $\Delta T_m$ with respect to conjugate linker length upon addition of ligands to 5'-A$_2$G$_6$C$_2$-3' at $r_{dd} = 0.5$ (a), $r_{dd} = 1.0$ (b) and $r_{dd} = 2.0$ (c). Controls (neomycin, HImImPyPyDp 18) are shown in light grey while series I DPA208 – DPA213 are shown in blue, series II DPA214 – DPA219 conjugates are shown in green and series III DPA220 – DPA225 conjugates are shown in red. Data was obtained from UV thermal denaturation experiments. Ligands were added to the DNA [2.0 µM/duplex] at varying ratios of drug to duplex. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. $^a\Delta T_m$ values for the duplex with no additional NaCl.
Figure B43. UV melting profiles for 5’-A\textsubscript{m}G\textsubscript{n}C\textsubscript{n}T\textsubscript{m}-3’ type duplexes with controls, neomycin and HiImPyPyDp 18 (a – f). Controls were added to the duplex [2.0 \mu M/duplex] at \textit{r}_{dd} = 1.0. The samples were incubated at 4 \degree C for 12 h prior to analysis, slow heating from 10 \degree C to 90 \degree C at 0.2 \degree C/min. Buffer: 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. \textsuperscript{a}Buffer: 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0.
Figure B44a. UV melting profiles for 5’-A<sub>m</sub>G<sub>n</sub>C<sub>n</sub>T<sub>m</sub>-3’ type duplexes with series II DPA214 – DPA219 tetramer polyamide-neomycin conjugates. Ligands were added to the duplex [2.0 µM/duplex] at r<sub>dd</sub> = 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. *Buffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0.
Figure B44b. UV melting profiles for 5'-A<sub>m</sub>G<sub>n</sub>C<sub>n</sub>T<sub>m</sub>-3' type duplexes with series II DPA214 – DPA219 tetramer polyamide - neomycin conjugates. Ligands were added to the duplex [2.0 μM/duplex] at r<sub>dd</sub> = 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. aBuffer: 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.25 mM EDTA, pH 7.0.
Figure B45a. UV melting profiles for 5'-A<sub>m</sub>G<sub>n</sub>C<sub>n</sub>T<sub>m</sub>-3' type duplexes with series III DPA220 – DPA225 tetramer polyamide - neomycin conjugates. Ligands were added to the duplex [2.0 µM/duplex] at r<sub>ld</sub> = 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 1.5 mM Na₃HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0. *Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0.
Figure B44b. UV melting profiles for 5’-AₙGₖCₖTₙ-3’ type duplexes with series III DPA220 – DPA225 tetramer polyamide - neomycin conjugates. Ligands were added to the duplex [2.0 µM/duplex] at r_{ld} = 1.0. The samples were incubated at 4 °C for 12 h prior to analysis, slow heating from 10 °C to 90 °C at 0.2 °C/min. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl. "a"Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0.
Figure B46. UV melting profiles for 5’-G₂A₃G₄C₄-3’ with series I DPA208 – DPA213 (a), series II DPA214 – DPA219 (b), series III DPA220 – DPA225 (c) and series IV DPA243 and DPA244 (d) tetramer polyamide - neomycin conjugates. UV melting profiles for the controls are also plotted in panel (d). Ligands were added to the duplex [2.0 µM/duplex] at r_{dd} (ratio of drug:duplex) = 1.0. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. The panels above correspond to the melts. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure B47. UV melting profiles for 5'-G₂C₂AG₄C₄-3' with series II DPA214 – DPA219 (a), series III DPA220 – DPA225 (b) and series IV DPA243 and DPA244 (c) tetramer polyamide - neomycin conjugates. UV melting profiles for the controls are also plotted in panel (c). Ligands were added to the duplex [2.0 µM/duplex] at $r_{dd}$ (ratio of drug:duplex) = 1.0. The samples were incubated at 4 °C for 12 h. prior to analysis, slow heating from 10 °C to 100 °C at 0.2 °C/min. The panels above correspond to the melts. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
APPENDIX C

Methyl 4-amino-1-methylimidazole-2-carboxylate (8a). See Appendix B for synthetic procedure, compound data reduction and characterization.

HImImCOOMe (10). See Appendix B for synthetic procedure, compound data reduction and characterization.

Methyl 4-amino-1-methylpyrrole-2-carboxylate (9a). See Appendix B for synthetic procedure, compound data reduction and characterization.
HIImPyCOOMe (11). See Appendix B for synthetic procedure, compound data reduction and characterization.

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23  25

BocNHPγCOOMe (25). To a solution of 23 (313.0 mg, 1.2 mmol) in methanol (10 mL) was added 1.5 M NaOH (2 mL) and the solution was allowed to stir for 4 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed \textit{in vacuo}. The resulting solid was dissolved in DMF (10 mL). PyBOP (768.7 mg, 1.5 mmol) and DIPEA (1.072 mL, 6.1 mmol) were added and the reaction was allowed to stir for 30 min. Methyl-4-aminobutyrate hydrochloride, 24, (207.4 mg, 1.4 mmol) was added and the reaction was allowed to stir for 15 h. DMF was evaporated \textit{in vacuo} and the resulting solid was purified via flash chromatography (silica gel, 75:25 hexane:EtOAc \(\nu/\nu\)) to yield pure 25 (371.5 mg, 89%) as a light brown solid: TLC (EtOAc) \(R_f\) 0.80; \(^1\)H NMR (300 MHz, CDCl\(_3\)) \(\delta\) 6.84 (s, 1H), 6.64 (br, 1H), 6.45 (s, 1H), 6.23 (br, 1H), 3.85 (s, 3H), 3.69 (s, 3H), 3.88-3.66 (m, 2H), 2.41-2.37 (d, \(J = 9.3, 2\)H), 2.02-1.79 (m, \(J_1 = 6.1, J_2 = 9.2, 2\)H), 1.47 (s, 9H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \(\delta\) 162.8, 159.3, 154.5, 123.2, 120.9, 119.7, 102.7, 80.8, 51.1, 38.9, 36.7, 35.7, 33.8, 28.4; MALDI-TOF \(m/z\) calcd for C\(_{16}\)H\(_{25}\)N\(_3\)O\(_5\) 339.39, found [M + H]\(^+\) 340.11.


**BocNHPyγImCOOMe (20).** Compound 8 (204.1 mg, 1.1 mmol) was dissolved in MeOH (20 mL). 10% Pd/C (20.4 mg) was added and the solution was stirred under positive H₂ atmosphere, supplied by submerged syringe needle in the solution. The solution was stirred for 3.5 h. The catalyst was filtered over celite and the filtrate was concentrated *in vacuo*, followed by co-evaporation with copious amounts of CH₂Cl₂. The resulting oil was suspended in DMF and placed under vacuum for addition to the activated acid solution. To a solution of 25 (371.5 mg, 1.1 mmol) in methanol (10 mL) was added 1.5 M NaOH (2 mL) and the solution was allowed to stir for 4 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed *in vacuo*. The resulting solid was dissolved in DMF (10 mL). EDC (229.9 mg, 1.2 mmol) and HOBt (162.0 mg, 1.2 mmol) were added and the reaction was allowed to stir for 30 min. The amine solution, in 10 mL of DMF, was added dropwise to the activated acid solution over the period of 10 min and the reaction was allowed to stir for 16 h. DMF was evaporated *in vacuo* and the resulting solid was purified via flash chromatography (silica gel, 50:50 CH₂Cl₂:Acetone v/v) to yield pure 20 (511.7 mg, 89%) as a light brown solid: TLC (50:50 CH₂Cl₂:Acetone v/v) Rₗ 0.71; ¹H NMR (300 MHz, CDCl₃) δ 9.08 (s, 1H), 7.46 (s, 1H), 6.83 (s, 1H), 6.51 (s, 1H), 6.49 (s, 1H), 6.39 (s, 1H), 3.96 (s, 3H), 3.88 (s, 3H), 3.83 (s, 3H), 3.42-3.40 (m, 2H), 2.46-2.42 (d, J = 6.1, 2H), 1.96-1.93 (m, J₁ = 6.1, J₂ = 5.7,
$^{13}$C NMR (75 MHz, CDCl$_3$) δ 170.6, 161.9, 159.0, 153.3, 137.1, 131.3, 123.2, 121.7, 117.6, 115.5, 103.6, 80.0, 52.2, 38.6, 36.5, 35.9, 33.8, 30.9, 29.3; MALDI-TOF m/z calcd for C$_{21}$H$_{30}$N$_6$O$_6$ 462.50, found [M + Na$^+$] 485.49.

**BocNHImPyCOOMe (26).** Compound 9 (152.2 mg, 0.8 mmol) was dissolved in MeOH (20 mL). 10% Pd/C (15.2 mg) was added and the solution was stirred under positive H$_2$ atmosphere, supplied by submerged syringe needle in the solution. The solution was stirred for 3 h. The catalyst was filtered over celite and the filtrate was concentrated in vacuo, followed by co-evaporation with copious amounts of CH$_2$Cl$_2$. The resulting oil was suspended in DMF and placed under vacuum for addition to the activated acid solution. Compound 22 (211.1 mg, 0.8 mmol) was dissolved in MeOH (20 mL) and 1.5 M NaOH (2 mL) was added. The solution was allowed to stir for 4 h at 60 °C. The ester solution was quenched by neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF (10 mL). EDC (158.5 mg, 0.8 mmol) and HOBt (111.7 mg, 0.8 mmol) were added and the reaction was allowed to stir for 30 min. The amine solution, in 8 mL of DMF, was added dropwise to the activated acid solution over the period of 10 min and the reaction was allowed to stir for 15 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, acetone:CH$_2$Cl$_2$, 5.0 % incremental increases of acetone) to yield pure 26 (218.4 mg,
71%) as a white solid: TLC (50:50 CH₂Cl₂:Acetone v/v) Rf 0.71; ¹H NMR (500 MHz, CDCl₃) δ 8.98 (s, 1H), 7.92 (s, 1H), 7.39 (s, 1H), 7.17 (s, 1H), 6.73 (s, 1H), 4.03 (s, 3H), 3.87 (s, 3H), 3.78 (s, 3H), 1.47 (s, 9H); ¹³C NMR (75 MHz, CDCl₃) δ 161.5, 155.9, 152.6, 136.7, 133.8, 121.2, 120.8, 119.8, 112.5, 108.4, 80.8, 51.1, 36.7, 35.7, 28.2; MALDI-TOF m/z calcd for C₁₇H₂₃N₅O₅ 377.40 found [M + H]⁺ 377.37.

**BocNHImPyPyCOOME (21).** Compound 9 (106.6 mg, 0.6 mmol) was dissolved in MeOH (20 mL). 10% Pd/C (10.6 mg) was added and the solution was stirred under positive H₂ atmosphere, supplied by submerged syringe needle in the solution. The solution was stirred for 3 h. The catalyst was filtered over celite and the filtrate was concentrated *in vacuo*, followed by co-evaporation with copious amounts of CH₂Cl₂. The resulting oil was suspended in DMF and placed under vacuum for addition to the activated acid solution. Compound 26 (218.4 mg, 0.6 mmol) was dissolved in MeOH (20 mL) and 1.5 M NaOH (2 mL) was added. The solution was allowed to stir for 6 h at 60 °C. The ester solution was quenched by neutralized with HCl. Volatiles were removed *in vacuo*. The resulting solid was dissolved in DMF (10 mL). EDC (110.9 mg, 0.6 mmol) and HOBt (78.2 mg, 0.6 mmol) were added and the reaction was allowed to stir for 30 min. The amine solution, in 8 mL of DMF, was added dropwise to the activated acid
solution over the period of 10 min and the reaction was allowed to stir for 15 h. DMF was evaporated *in vacuo* and the resulting solid was purified via flash chromatography (silica gel, acetone:CH$_2$Cl$_2$, 5.0 % incremental increases of acetone) to yield pure 21 (218.4 mg, 71%) as a white solid: TLC (50:50 CH$_2$Cl$_2$:Acetone *v/v*) R$_f$ 0.71; $^1$H NMR (500 MHz, CDCl$_3$) $\delta$ 8.86 (s, 1H), 8.04 (s, 1H), 7.68 (s, 1H), 7.44 (s, 1H), 7.28 (s, 1H), 7.18 (s, 1H), 6.78 (s, 1H), 6.66 (s, 1H), 4.05 (s, 3H), 3.94 (s, 3H), 3.92 (s, 3H), 3.83 (s, 3H), 1.53 (s, 9H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 170.8, 161.3, 156.8, 153.3, 138.2, 134.1, 123.5, 121.8, 120.9, 120.0, 119.4, 118.3, 112.5, 107.2, 106.3, 80.9, 51.0, 37.2, 36.7, 35.0, 27.9; MALDI-TOF m/z calcd for C$_{23}$H$_{29}$N$_7$O$_6$ 499.52, found [M + H]$^+$ 500.01.

**HImImPyPyγImCOOMe (27).** Compound 20 (69.2 mg, 149.6 µmol) was suspended in diethyl ether (5 mL) and 4 N HCl in 1,4-dioxane was added (5 mL) and the reaction was allowed to stir for 5.5 h. The solvent was removed *in vacuo* and the resulting solid was washed multiple times with excessive amounts of diethyl ether to afford a dry white solid, sufficiently pure for addition to the activated acid. To a solution of compound 11 (57.6 mg, 149.6 µmol) in methanol (10 mL) was added 1.5 M NaOH (2 mL) and the solution was allowed to stir for 5.5 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed *in vacuo*. The resulting solid was dissolved in DMF (10
PyBOP (77.9 mg, 149.6 µmol) and DIPEA (19.3 mg, 149.6 µmol) were added and the reaction was allowed to stir for 30 min. The amine hydrochloride solid was added to the activated acid solution over the period of 10 min and the reaction was allowed to stir for 16 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, Acetone v/v) to yield pure 27 (94.3 mg, 88%) as a light brown solid: TLC (Acetone) R_f 0.48; ^1H NMR (500 MHz, MeOD) δ 9.28 (br, 1H), 9.01 (br, 1H), 8.73 (br, 1H), 8.26 (br, 1H), 8.19 (br, 1H), 7.72 (s, 1H), 7.70 (s, 1H), 7.62 (s, 1H), 7.58 (s, 1H), 7.31 (s, 1H), 7.18 (s, 1H), 7.00 (s, 1H), 6.79 (s, 1H), 4.22 (s, 3H), 4.10 (s, 3H), 4.05 (s, 3H), 3.94 (s, 6H), 3.87 (s, 3H), 2.53-2.51 (t, J = 5.7, 2H), 2.03-1.99 (t, J = 6.3, 2H), 1.52-1.42 (m, J_1=6.3, J_2=5.7, 2H); ^13C NMR (75 MHz, MeOD) δ 172.6, 161.9, 161.6, 159.0, 156.3, 155.9, 153.3, 138.5, 137.1, 135.5, 131.3, 130.9, 128.9, 128.3, 126.2, 123.2, 121.7, 121.2, 120.1, 117.6, 115.5, 114.1, 108.4, 103.6, 80.0, 52.2, 51.2, 38.6, 36.8, 36.5, 35.9, 35.8, 35.7, 33.8, 30.9, 29.3; MALDI-TOF m/z calcd for C_{32}H_{37}N_{13}O_{7} 715.72, found [M + Na]^+ 739.21.
HIImPyPyγImPyPyCOOMe (19). Compound 21 (69.2 mg, 149.6 μmol) was suspended in diethyl ether (5 mL) and 4 N HCl in 1,4-dioxane was added (5 mL) and the reaction was allowed to stir for 6 h. The solvent was removed in vacuo and the resulting solid was washed multiple times with excessive amounts of diethyl ether to afford a dry white solid, sufficiently pure for addition to the activated acid. To a solution of compound 27 (90.0 mg, 125.7 μmol) in methanol (10 mL) was added 1.5 M NaOH (3 mL) and the solution was allowed to stir for 4.5 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF (10 mL). PyBOP (81.8 mg, 157.2 μmol) and DIPEA (20.4 mg, 157.2 μmol) were added and the reaction was allowed to stir for 30 min. The amine hydrochloride solid was added to the activated acid solution over the period of 10 min and the reaction was allowed to stir for 24 h. DMF was evaporated in vacuo and the
resulting solid was purified via flash chromatography (silica gel, acetone) to yield pure 19 (110.6 mg, 89%) as a light brown solid: TLC (95:5 CH$_2$Cl$_2$:MeOH v/v) R$_f$ 0.48; $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ 7.75 (s, 1H), 7.70 (s, 1H), 7.68 (s, 1H), 7.66 (s, 1H), 7.60 (s, 1H), 7.49 (s, 1H), 7.33 (s, 1H), 7.31 (s, 1H), 7.18 (s, 2H), 7.05 (s, 1H), 6.85 (s, 1H), 6.78 (s, 1H), 6.66 (s, 1H), 4.22 (s, 3H), 4.10 (s, 3H), 4.05 (s, 6H), 3.94 (s, 6H), 3.90 (s, 6H), 3.81 (s, 3H), 2.60-2.54 (t, J = 5.1, 2H), 2.18-1.95 (t, J = 6.4, 2H), 1.55-1.41 (m, J$_1$=6.4, J$_2$=5.2, 2H); $^{13}$C NMR (75 MHz, MeOD) δ 174.6, 171.3, 162.5, 161.6, 161.0, 159.9, 156.9, 156.1, 155.7, 154.1, 153.8, 138.6, 138.1, 137.5, 136.1, 133.7, 131.4, 130.1, 128.5, 128.0, 126.1, 123.5, 123.0, 121.9, 121.1, 121.0, 120.7, 120.4, 120.2, 119.7, 118.0, 117.6, 115.5, 114.1, 112.8, 108.7, 107.6, 106.9, 103.2, 80.8, 80.0, 52.7, 51.1, 51.0, 38.6, 37.2, 36.5, 36.3, 36.0, 35.8, 35.4, 35.7, 34.4, 33.7, 30.1; MALDI-TOF m/z calcd for C$_{49}$H$_{54}$N$_{20}$O$_{10}$ 1083.08, found [M + Na]$^+$ 1106.07.

![Diagram](image)

HImImPyPyγImImPyCONH(CH$_2$)$_n$N$_3$ (DPA226 – DPA231). To a solution of compound 19 (10.0 mg, 9.2 µmol) in methanol (10 mL) was added 1.5 M NaOH (1 mL) and the solution was allowed to stir for 5.5 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed in vacuo. The resulting solid was dissolved in DMF.
(10 mL). EDC (2.2 mg, 11.5 μmol) and HOBT (1.6 mg, 11.5 μmol) were added and the reaction was allowed to stir for 30 min. The α,ω-amino, azido alkanes (11.5 μmol) were added to the activated acid solution over the period of 10 min and the reaction was allowed to stir for 24 h. DMF was evaporated in vacuo and the resulting solid was purified via flash chromatography (silica gel, inc. % of MeOH in CH₂Cl₂) to yield the desired pure azide-terminal hairpin polyamides, DPA226 – DPA231.

**HImImPyPyγImImPyCONH(CH₂)₂N₃ (DPA226).** DPA226 (9.2 mg, 88%) was afforded as a light brown solid: TLC (85:15 CH₂Cl₂:MeOH v/v) Rₓ 0.50; ¹H NMR (500 MHz, (CD₃)₂CO) δ 7.81 (s, 1H), 7.77 (s, 1H), 7.71 (s, 1H), 7.68 (s, 1H), 7.61 (s, 1H), 7.43 (s, 1H), 7.33 (s, 1H), 7.30 (s, 1H), 7.26 (s, 2H), 7.15 (s, 1H), 6.94 (s, 1H), 6.88 (s, 1H), 6.61 (s, 1H), 4.22 (s, 6H), 4.15 (s, 6H), 4.00 (s, 6H), 3.96 (s, 3H), 3.90 (s, 3H), 3.36-3.23 (m, 4H), 2.66-2.55 (t, J = 5.1, 2H), 2.18-1.95 (t, J = 6.4, 2H), 1.55-1.41 (m, J₁=6.4, J₂=5.2, 2H); MALDI-TOF m/z calcd C₅₀H₅₆N₂₄O₁₁ 1137.14, found [M + Na]⁺ 1161.07.

**HImImPyPyγImImPyCONH(CH₂)₃N₃ (DPA227).** DPA227 (9.7 mg, 91%) was afforded as a light brown solid: TLC (85:15 CH₂Cl₂:MeOH v/v) Rₓ 0.50; ¹H NMR (500 MHz, (CD₃)₂CO) δ 7.81 (s, 1H), 7.72 (s, 1H), 7.69 (s, 1H), 7.64 (s, 1H), 7.55 (s, 1H), 7.43 (s, 1H), 7.35 (s, 1H), 7.28 (s, 1H), 7.20 (s, 2H), 7.17 (s, 1H), 6.94 (s, 1H), 6.89 (s, 1H), 6.63 (s, 1H), 4.30 (s, 6H), 4.20 (s, 6H), 4.00 (s, 6H), 3.94 (s, 3H), 3.90 (s, 3H), 3.33-3.25 (m, 4H), 2.67-2.54 (m, 2H), 2.18-1.90 (m, 4H), 1.86 (m, 2H); MALDI-TOF m/z calcd C₅₁H₅₈N₂₄O₉ 1151.16, found [M + Na + H₂O]⁺ 1176.67.
**HImImPyPyγImImPyCONH(CH$_2$)$_6$N$_3$ (DPA228).** DPA228 (9.9 mg, 91%) was afforded as a light brown solid: TLC (85:15 CH$_2$Cl$_2$:MeOH v/v) $R_f$ 0.50; $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ 7.84 (s, 1H), 7.73 (s, 1H), 7.71 (s, 1H), 7.66 (s, 1H), 7.63 (s, 1H), 7.40 (s, 1H), 7.35 (s, 1H), 7.31 (s, 1H), 7.22 (s, 2H), 7.18 (s, 1H), 7.14 (s, 1H), 6.96 (s, 1H), 6.89 (s, 1H), 6.61 (s, 1H), 4.27 (s, 6H), 4.18 (s, 6H), 4.07 (s, 6H), 3.96 (s, 3H), 3.94 (s, 3H), 3.41-3.31 (m, 4H), 2.66-2.54 (m, 2H), 2.18-1.90 (m, 4H), 1.55-1.52 (m, 6H); MALDI-TOF $m/z$ calcd C$_{53}$H$_{62}$N$_{24}$O$_9$ 1179.21, found [M + Na]$^+$ 1202.21.

**HImImPyPyγImImPyCONH(CH$_2$)$_6$N$_3$ (DPA229).** DPA229 (8.5 mg, 77%) was afforded as a light brown solid: TLC (85:15 CH$_2$Cl$_2$:MeOH v/v) $R_f$ 0.50; $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ 7.84 (s, 1H), 7.73 (s, 1H), 7.70 (s, 1H), 7.65 (s, 1H), 7.60 (s, 1H), 7.40 (s, 1H), 7.33 (s, 1H), 7.30 (s, 1H), 7.26 (s, 2H), 7.20 (s, 1H), 6.94 (s, 1H), 6.88 (s, 1H), 6.61 (s, 1H), 4.22 (s, 6H), 4.15 (s, 6H), 4.00 (s, 6H), 3.96 (s, 3H), 3.90 (s, 3H), 3.32-3.21 (m, 4H), 2.63-2.54 (m, 2H), 2.18-1.95 (m, 4H), 1.55-1.41 (m, 8H); MALDI-TOF $m/z$ calcd C$_{54}$H$_{64}$N$_{24}$O$_9$ 1193.24, found [M + Na]$^+$ 1215.99.

**HImImPyPyγImImPyCONH(CH$_2$)$_8$N$_3$ (DPA230).** DPA230 (9.6 mg, 85%) was afforded as a light brown solid: TLC (85:11 CH$_2$Cl$_2$:MeOH v/v) $R_f$ 0.45; $^1$H NMR (500 MHz, (CD$_3$)$_2$CO) δ 7.84 (s, 1H), 7.72 (s, 1H), 7.69 (s, 1H), 7.61 (s, 1H), 7.55 (s, 1H), 7.43 (s, 1H), 7.36 (s, 1H), 7.28 (s, 1H), 7.22 (s, 2H), 7.20 (s, 1H), 6.94 (s, 1H), 6.89 (s, 1H), 6.61 (s, 1H), 4.30 (s, 6H), 4.20 (s, 6H), 4.10 (s, 6H), 3.93 (s, 3H), 3.90 (s, 3H), 3.38-
3.21 (m, 4H), 2.67-2.54 (m, 2H), 2.18-1.90 (m, 4H), 1.59 (m, 4H) 1.31 (m, 4H), 1.28 (m, 4H); MALDI-TOF m/z calcd C_{56}H_{68}N_{24}O_{9} 1221.29, found [M + Na]^+ 1245.22.

HImImPyPyImImPyCONH(CH_2)_10N_3 (DPA231). DPA231 (10.1 mg, 91%) was afforded as a light brown solid: TLC (85:15 CH_2Cl_2:MeOH v/v) R_f 0.40; ¹H NMR (500 MHz, (CD_3)_2CO) δ ¹H NMR (500 MHz, (CD_3)_2CO) δ 7.84 (s, 1H), 7.73 (s, 1H), 7.70 (s, 1H), 7.66 (s, 1H), 7.63 (s, 1H), 7.40 (s, 1H), 7.35 (s, 1H), 7.31 (s, 1H), 7.22 (s, 2H), 7.18 (s, 1H), 6.96 (s, 1H), 6.89 (s, 1H), 6.61 (s, 1H), 4.27 (s, 6H), 4.18 (s, 6H), 4.07 (s, 6H), 3.96 (s, 3H), 3.94 (s, 3H), 3.36-3.23 (m, 4H), 2.67-2.54 (t, J = 5.2, 2H), 2.18-1.90 (t, J = 6.4, 2H), 1.66 (m, 4H), 1.38 (m, 8H); MALDI-TOF m/z calcd C_{58}H_{72}N_{24}O_{9} 1249.35, found [M + Na]^+ 1273.01.

N-Boc neomycin-S(CH_2)_2NH_2 (DPA10). See Appendix A for compound data reduction and characterization.
**N-Boc neomycin-N$_3$ (DPA11).** See Appendix A for synthetic procedure, compound data reduction and characterization.

**N-Boc neomycin-NH$_2$ (DPA12).** See Appendix A for synthetic procedure, compound data reduction and characterization.

**N-Boc neomycin-‘tri’-CH$_2$OCH$_2$CCH (DPA13).** See Appendix B for compound data reduction and characterization.
N-Boc neomycin-NHC(O)CCH (DPA16). To a flame dried round bottom flask under inert atmosphere, afforded by argon gas, was added DCC (12.9 mg, 62.6 µmol) and anhydrous CH₂Cl₂ (10 mL). The solution was cooled to 0 °C and propiolic acid (3.7 mg, 52.7 µmol) was added via syringe. The solution was allowed to stir at 0 °C for 30 min. Compound DAP12 (32.0 mg, 26.3 µmol) was dissolved in anhydrous CH₂Cl₂ and added dropwise to the activated acid solution, via syringe, under positive Ar (g), over a period of 15 min. The solution was allowed to warm to room temperature and was stirred for 18 h. Volatiles were removed in vacuo and the resulting solid was purified by chromatography (silica gel, CH₂Cl₂:2-propanol, 1.0 % incremental increases of 2-propanol) to afford DPA16 (28.7 mg, 86%) as an off white solid TLC (90:10 CH₂Cl₂:EtOH v/v) Rf 0.55; ¹H NMR (500 MHz, CDCl₃) δ 7.61 (br, 1H), 6.12 (s, 1H), 6.00 (s, 1H), 5.27 (s, 1H), 4.97 (s, 1H), 4.88 (s, 2H), 4.76 (s, 1H), 4.61 (s, 1H), 4.21-4.05 (m, 5H), 3.87 (s, 2H), 3.76 (s, 1H), 3.68 (s, 2H), 3.51 (s, 3H), 3.44 (s, 1H), 3.25 (s, 2H), 3.02 (s, 2H), 2.79 (s, 2H), 2.61-2.59 (m, 2H), 1.56-1.36 (m, 54 H); MALDI-TOF m/z calcd for C₅₆H₉₃N₇O₂₅ 1266.38, found [M + H₂O]⁺ 1277.91
**HImImPyPyγImImPyPyDp (28).** To a solution of compound 19 (10.0 mg, 9.2 µmol) in methanol (10 mL) was added 1.5 M NaOH (1 mL) and the solution was allowed to stir for 3.0 h at 60 °C. The ester solution was neutralized with HCl. Volatiles were removed *in vacuo*. The resulting solid was dissolved in DMF (10 mL). EDC (2.2 mg, 11.5 µmol) and HOBt (1.6 mg, 11.5 µmol) were added and the reaction was allowed to stir for 30 min. *N,N*-dimethylaminopropylamine (9.4 mg, 92.0 µmol) was added and the solution was allowed to stir for 18 h. DMF was removed *in vacuo* and the resulting solid was suspended in CH$_2$Cl$_2$ (25 mL). The solution was decanted and the resulting solid was suspended in MeOH (15 mL). The solution was decanted and the resulting solid was suspended in water (15 mL). The solution was decanted and the solid was dried to afford 28 as an off white solid (8.9 mg, 84%): $^1$H NMR (500 MHz, acetone-d$_6$) δ 7.81 (s, 1H), 7.78 (s, 1H), 7.71 (s, 1H), 7.70 (s, 1H), 7.67 (s, 1H), 7.53 (s, 1H), 7.41 (s, 1H), 7.37 (s, 1H), 7.22 (s, 2H), 7.00 (s, 1H), 6.91 (s, 1H), 6.84 (s, 1H), 6.74 (s, 1H), 4.29 (s, 3H), 4.20 (s, 3H), 4.19 (s, 3H), 4.12 (s, 6H), 4.00 (s, 6H), 3.90 (s, 6H), 3.88 (s, 6H), 2.60-2.54 (t, J = 5.6, 2H), 2.18-1.95 (t, J = 6.6, 2H), 1.55-1.41 (m, J$_1$=6.6, J$_2$=5.5, 2H); MALDI-TOF m/z calcd for C$_{53}$H$_{64}$N$_{22}$O$_9$ 1153.22, found [M + Cl]$^+$ 1188.54.
Figure C1. Characterization of compound 25.

(a) $^1$H NMR

Figure C1. Characterization of compound 25.

(a) $^1$H NMR
Figure C2. Characterization of compound 20.
Figure C3. Characterization of compound 26.
Figure C4. Characterization of compound 21.
**Figure C5.** Characterization of compound 27.
Figure C6. Characterization of compound 19.
Figure C7. Characterization of compound DPA226.
Figure C8. Characterization of compound DPA227.
Figure C9. Characterization of compound DPA228.
Figure C10. Characterization of compound DPA229.
Figure C11. Characterization of compound DPA230.
Figure C12. Characterization of compound DPA231.
Figure C13. Characterization of compound DPA16.
(a) $^1$H NMR (N-Boc protected)

(b) MALDI-TOF (N-Boc protected)
Figure C14. Characterization of compound DPA232.
Figure C15. Characterization of compound DPA233.
Figure C16. Characterization of compound DPA234.
Figure C17. Characterization of compound DPA235.
Figure C18. Characterization of compound DPA236.
Figure C19. Characterization of compound DPA237.
Figure C20. Characterization of compound DPA238.
Figure C21. Characterization of compound DPA239.
Figure C22. Characterization of compound DPA240.
Figure C23. Characterization of compound DPA241.
Figure C24. Characterization of compound DPA242.
Figure C25. Characterization of compound 28.
Figure C26. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG\textsubscript{2}C\textsubscript{2}AG\textsubscript{4}C\textsubscript{4}-3’. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 0.5$. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM/duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits\textsubscript{ex} = 20; slits\textsubscript{em} = 20; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na\textsubscript{3}HPO\textsubscript{4}, 0.5 mM NaH\textsubscript{2}PO\textsubscript{4}, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure C27. Plot of normalized fluorescence intensity with respect to conjugate linker length upon addition of ligands to 5’-AG2C2AG4C4-3’. In the histogram, the values represent the percent decrease in fluorescence at $r_{dd} = 2.0$. Data was obtained from a FID assay. Ligands were added to the DNA [1 µM/duplex], pre-saturated with ethidium bromide [7 µM], at various ratios of drug to duplex ($r_{dd}$), allowed to equilibrate for 5 min and the subsequent decrease in fluorescence was recorded. The data was normalized such that the well containing ethidium bromide and buffer was denoted as 0% fluorescence and the well containing buffer, DNA and ethidium bromide was denoted as 100% fluorescence. Fluorescence parameters: Excitation = 525 nm; Emission = 608 nm; slits$_{ex} = 20$; slits$_{em} = 20$; Excitation filter = 335 – 620 nm; Emission filter = 550 – 1100 nm; PMT V = 810 and triplicate measurements were averaged. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure C28. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5'-AG₂C₂AG₄C₄-3', by hairpin polyamide – neomycin control 28 at 20 °C (a). The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of 28, the fluorescence intensity is denoted as Fₘₐₓ. Compound 28 was added at various r_dd values, a plot of fluorescence intensity vs r_dd is located in panel (b). A related plot of ΔF vs r_dd is located in panel (c). Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.
Figure C29. Fluorescence intercalator displacement titration spectra for the displacement of pre-bound thiazole orange from the DNA duplex, 5'-AG2C2AG4C4-3, by DPA237 (a) and DPA236 (c) at 20 °C. The DNA duplex, 1.0 µM/duplex, 14 µM/base pair, was pre-bound by thiazole orange, 7 µM and allowed to equilibrate for a period of 10 min prior to addition of ligand, the fluorescence intensity is denoted as $F_{\text{max}}$. A related plot of $\Delta F$ vs $r_{dd}$ is located in panel (b and d). Fluorescence parameters: Excitation = 504 nm; Emission = 510-610 nm; Excitation filter = open; Emission filter = open; Slits = 20; PMTV = 550 and triplicate measurements were averaged. Buffer: 1.5 mM Na$_2$HPO$_4$, 0.5 mM NaH$_2$PO$_4$, 0.25 mM EDTA, pH 7.0, 50 mM NaCl.