ABSTRACT

BALLARD, THOMAS ERIC, JR. Small Molecule Control of Biological Function. (Under the direction of Dr. Christian Melander).

Attracted by the novel homodimeric glycosylated diazobenzo[b]fluorene natural product lomaiviticin A and its monomeric cousins the kinamycins, we investigated the ability of simplified diazofluorenes to recapitulate DNA cleaving activity of kinamycin D. Under DTT mediated conditions, we obtained high percentages of DNA nicking by kinamycin D, 1-methoxydiazofluorene and 4-aminodiazofluorene. Upon further examination, the concentrations of both DTT and kinamycin D could be lowered significantly and still retain high DNA cleavage activity. This culminated in the identification that kinamycin D effectively cleaved DNA in a concentration, temperature, and time-dependent fashion by both DTT and glutathione under mild biomimetic conditions.

The virulence and persistence of bacterial biofilm infections is responsible for many chronic illnesses as well as increased morbidity and mortality rates in a plethora of infectious diseases. Using the marine natural product oroidin as molecular inspiration, a library of reverse amide (RA) 2-aminoimidazole analogues were synthesized and assayed for anti-biofilm activity. A thorough and detailed structure-activity-relationship (SAR) culminated in the identification of a long linear aliphatic tridecyl RA analogue with sub-micromolar anti-biofilm activity against Pseudomonas aeruginosa and low micromolar activity against Acinetobacter baumannii. Additionally, a sub-class of aliphatic analogues lacking the amide moiety were synthesized that were very active at inhibiting and dispersing both P. aeruginosa and A. baumannii biofilms.

SDC-1721, a fragment of the potent HIV inhibitor TAK-779, was synthesized and conjugated to 2.0 nm diameter gold nanoparticles. Free SDC-1721 had no inhibitory effect on HIV infection; however, the (SDC-1721)−gold nanoparticle conjugates displayed activity comparable to that of TAK-779. This result suggests that multivalent presentation of small molecules on gold nanoparticle surfaces can convert inactive drugs into potent therapeutics.
Small Molecule Control of Biological Function

by

Thomas Eric Ballard, Jr.

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Chemistry

Raleigh, North Carolina

2008

APPROVED BY:

Dr. Daniel L. Comins

Dr. Bruce M. Novak

Dr. David Shultz

Dr. Christian Melander, Chair
DEDICATION

I would like to dedicate this work in the loving memory of my late grandmother Dorothy “Dot” Ballard. Her passion for life and love deeply affected me as a child and I believe she lead me down this scientific path. I miss you so very much Granny.
The author, Thomas Eric Ballard, Jr., was born in Vacaville, CA on May 20th 1982 to Eric and Kelly Ballard. He spent his early childhood growing up in Germany before moving to Kannapolis, NC where he spent the rest of his years through high school. After high school, Eric attended North Carolina State University where he graduated magna cum laude with honors, receiving his Bachelors of Science in Chemistry in 2004. As an undergraduate, Eric was a resident advisor for three years and received the Central Campus RA of the Year award in 2004. That year, Eric also received the Robert A. Osteryoung Award for Excellence in Teaching for his achievement in teaching general chemistry laboratories.

In the fall of 2004, Eric began his graduate studies at NCSU and quickly came under the guidance of the newly appointed professor, Dr. Christian Melander. In 2006, Eric was awarded the Sara and Chloe Novak Award for Teaching Excellence for teaching advanced organic chemistry laboratories. Also in 2006, he was awarded the GlaxoSmithKline Predoctoral Research Fellowship. Upon completion of his Ph.D. in 2008, he began a postdoctoral position at the University of Virginia under the direction of Professor T. L. Macdonald.
ACKNOWLEDGEMENTS

The sheer number of people who have affected my life to this point is immeasurable, but I will attempt to thank you, for you have helped achieve what I have today. First, I’d like to extend my most sincere thanks to Dr. Christian Melander. I do not believe my career as a scientist will ever be the same because of you, and I thank you for that. Your guidance, or perhaps freedom, has been decisive in my graduate career and I would not have had the same experience anywhere else. I would also like to thank Dr. Comins and Dr. Novak as they were also instructive in my scientific career. Although not nearly as productive for them when I was an undergraduate, I know that the time I spent in their labs lead me here today. Additionally, my unusual ability to deal with the NMRs in Dabney can only be attributed to Dr. Shultz. However direct or indirect, his entertaining spectroscopy class is unforgettable.

I must also thank everyone in the Melander group. We may not get along all the time, but we did what we had to do and I thank you all for helping and challenging me. I’d like to give a special acknowledgement to Robert Huigens for being the only person that I could manage to despise and then later befriend. Our insightful discussions about chemistry will surely be missed. I’d also like to thank Justin Richards who, through our discussions, helped shape the last years of my graduate career and helped me discover new chemistry to accomplish great things in our lab. There are so many others that have influenced me here at NCSU and I apologize that I cannot mention you all by name, but I would be remise not to mention: Ed D’Antonio, the best roommate and electrochemist I know; Jesse Teske, I still don’t know why you joined Dieters’ Lab; and Nick Foley, I’ll keep working long hours if
you do. Thanks to all the friends I’ve made during my time here at NCSU, you’ve all impacted my life and I’ll never forget you.

My family, I love you all and you have been my support through everything in my life. I would not be here today if it was not for all of you. To my Mom, Kelly, I don’t know what you did to raise me, but you did it right and I cannot thank you enough for all the love and support that you have given me throughout my life, and especially my scientific career. To my Dad “Papa”, Eric, your love and support have helped guide me through life as well, and although you were not always around, your support for my endeavors has never waned. Scientifically, I think you’re my biggest fan. To my sister, Melissa, you’re surely the best big sister a little brother could ever have and I hope that we’ll get to see each other more in the future. To my step-dad, Jim, although I don’t think I’ll ever be as witty as you, perhaps one day I’ll have a come-back for you. Lastly, to my girlfriend, Wendy, you are the love of my life and you have been by my side through all of my scientific pitfalls and achievements. You are my best friend and we’ve had so much fun together these last five years which I would not have ever dreamed possible (especially during graduate school). The fact that you are a chemist, and an organic chemist at that, is even more remarkable and I’ll never get tired of arguing about chemistry with you.
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<td>2-aminoimidazole</td>
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<td>biochemical induction assay</td>
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<td>bd</td>
<td>broad doublet</td>
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<td>Bn</td>
<td>benzyl</td>
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<td>Boc</td>
<td>t-butoxycarboxyl</td>
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<td>brine</td>
<td>saturated aqueous sodium chloride</td>
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<tr>
<td>&quot;Bu</td>
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<td>'Bu</td>
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<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
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</tr>
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<td>cm⁻¹</td>
<td>reciprocal centimeters</td>
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<tr>
<td>d</td>
<td>doublet</td>
</tr>
<tr>
<td>dt</td>
<td>doublet triplets</td>
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<tr>
<td>dd</td>
<td>doublet of doublets</td>
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<tr>
<td>DMAP</td>
<td>4-(dimethylamino)pyridine</td>
</tr>
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<td>DMF</td>
<td>N,N-dimethylformamide</td>
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<td>EC₅₀</td>
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<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<td>ethyl acetate</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>h</td>
<td>hour(s)</td>
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<td>HRMS</td>
<td>high resolution mass spectrometry</td>
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<td>IC₃₀</td>
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<tr>
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<td>infrared</td>
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<tr>
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<td>LBNS</td>
<td>Luria-Bertani no salt media</td>
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<td>m</td>
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<tr>
<td>MOA</td>
<td>mode-of-action</td>
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<td>MHz</td>
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<td>MIC</td>
<td>minimum inhibitory concentration</td>
</tr>
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<td>min</td>
<td>minute(s)</td>
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<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance spectrometry</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600(540)&lt;/sub&gt;</td>
<td>optical density at 600 nm (or 540 nm)</td>
</tr>
<tr>
<td>Pd/C</td>
<td>palladium on carbon</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
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<tr>
<td>q</td>
<td>quartet</td>
</tr>
<tr>
<td>QS</td>
<td>Quorum Sensing</td>
</tr>
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<td>room temperature</td>
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<td>singlet</td>
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<td>SAR</td>
<td>structure-activity-relationship</td>
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<td>TFAA</td>
<td>trifluoroacetic anhydride</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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CHAPTER 1

Diazofluorenes as Mode-of-Action Probes

1.1 Isolation, Properties and Biological Activity

The kinamycins A-D **1a-1d (Figure 1)** were first isolated in 1970 by Hata and Ohtani as bright orange crystals from the culture broth of *Streptomyces murayamaensis*.1 Within a year, the structures of kinamycins A-D **1a-1d** had been elucidated and were shown to be 6-6-5-6 ring systems possessing a unique benzo[b]tetrahydrocarbazole skeleton with an N-cyano motif never before seen in nature.2, 3

![Figure 1. Representative Kinamycins: Original (left), Revised (right).](image-url)

<table>
<thead>
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<th>Ac</th>
<th>Ac</th>
<th>Ac</th>
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<tr>
<td>1b kinamycin B</td>
<td>H</td>
<td>Ac</td>
<td>H</td>
<td>H</td>
</tr>
<tr>
<td>1c kinamycin C</td>
<td>Ac</td>
<td>H</td>
<td>Ac</td>
<td>Ac</td>
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<tr>
<td>1d kinamycin D</td>
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<table>
<thead>
<tr>
<th>2a kinamycin A</th>
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<tr>
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<td>Ac</td>
<td>H</td>
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<td>H</td>
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<td>H</td>
<td>Ac</td>
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<td>2e kinamycin E</td>
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<td>H</td>
<td>H</td>
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<tr>
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<td>H</td>
<td>H</td>
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<tr>
<td>2g kinamycin G</td>
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<td>COIPr</td>
<td>Ac</td>
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<td>2j kinamycin J</td>
<td>Ac</td>
<td>Ac</td>
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</table>

![prekinamycin (3)](image-url)

![isoprekinamycin (4)](image-url)
In addition to their intriguing structure, they were also shown to be biologically active against gram positive, and to a lesser extent, gram negative bacteria (Table 1). Even derivatives that were synthesized from kinamycin C 1c by various protections and de protections still retained biological activity (1f, 1j). In 1994, the structure of the kinamycins was revised to now contain a diazo moiety rather than a carbazole (Figure 1).

Table 1. Biological Activity of Representative Kinamycins (µM)

<table>
<thead>
<tr>
<th></th>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>R₄</th>
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<td>2e</td>
<td>Ac</td>
<td>H</td>
<td>H</td>
<td>H</td>
<td>&lt;0.01</td>
<td>0.2</td>
<td>25</td>
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</tr>
<tr>
<td>2f</td>
<td>Ac</td>
<td>H</td>
<td>H</td>
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<td>&gt;25</td>
</tr>
</tbody>
</table>

In 2001, researchers at Wyeth-Ayerst discovered Lomaiviticins A 9 and B 10, two very potent metabolites from a new species of Micromonospora, LL-37I366, later named M. lomaivitiensis (Figure 2). Both lomaiviticins exhibit potent DNA damage indicated by BIA (Biochemical Induction Assay), having minimum inhibitory concentrations of ≤ 0.1 ng/spot. Lomaivitin A 9 is the more potent metabolite and is also the more abundant of the two from the fermentation broth. Lomaivitin A 9 is a homo-dimeric diazobenzo[b]fluorene glycoside
and is extremely active against gram-positive bacteria, especially *S. aureus* and *E. Faecium* (MIC’s, 6 – 25 ng/spot), and was also shown to be very cytotoxic to a 24-cancer cell line panel with IC₅₀ values ranging from 0.01 to 98 ng/mL (Table 2). Lomaiviticin B 10 is also a homodimeric diazobenzo[b]fluorene glycoside but contains two furanol rings most likely formed by hydrolysis of the tertiary sugar with concomitant cyclization onto the proximal ketone. The cytotoxic profile for lomaiviticin A 9 in the cancer cell lines was unique compared to other DNA-damaging drugs like mitomycin C and andriamycin hinting at a different mode-of-action for the lomaiviticins. Lomaiviticin A 9 was also reported to cleave DNA under reducing conditions but no further experimental data was reported.

![Lomaiviticins](image)

**Table 2. Cytotoxicity Data for Lomaiviticin A.**

<table>
<thead>
<tr>
<th>CELL LINE</th>
<th>IC₅₀ (ng/mL)</th>
<th>CELL LINE</th>
<th>IC₅₀ (ng/mL)</th>
<th>CELL LINE</th>
<th>IC₅₀ (ng/mL)</th>
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<tbody>
<tr>
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<td>Leukemia</td>
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<td>MCF7</td>
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<td>HL60</td>
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<tr>
<td>SKBR3</td>
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<td>SW948</td>
<td>62</td>
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<tr>
<td>Ovarian Cancer</td>
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<td></td>
<td></td>
<td>LNCAP</td>
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</tr>
<tr>
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<td>HCT15</td>
<td>8.2</td>
<td>RC3</td>
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<tr>
<td>A2780</td>
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</table>
1.2 DNA/Mode-of-Action Studies

The kinamycins and lomaiviticins have attracted a great deal of interest from the synthetic and biosynthetic community. However, little is known about their mode-of-action (MOA). The diazo moiety, as it is so rare in nature, is the potential driving force for the cytotoxic, antibacterial and antitumor properties. Several diazo containing compounds have been synthesized in the past twenty years and have been reported to cleave DNA (Figure 3).\textsuperscript{6-11} However, these compounds are activated toward DNA cleavage either photochemically, oxidatively, or with heat, while it has been established that lomaiviticin A cleaved DNA under reducing conditions.

1.2.1 Jebaratnam’s Oxidation Hypothesis

Assuming that the kinamycins work by DNA damage via reaction with the diazo group, Jebaratnam tested this hypothesis using diazofluorene 7 and β-naphthyl phenyl diazomethane 8 against plasmid DNA (pBR322) (Scheme 1).\textsuperscript{12} Jebaratnam was already interested in activating diazonium compounds with copper salts,\textsuperscript{13} and he hypothesized that the kinamycins were simply the deprotonated forms of diazonium compounds and protonation at physiological pH might generate an active and unstable diazonium ion.\textsuperscript{14} To
this end, he submitted pBR322 to DNA cleavage conditions that consisted of 7 with 500 or 1000 µM of the following salts: AgOAc, Tl(OAc)$_3$, Cu(OAc)$_2$, or Hg(OAc)$_2$. Varying the metal salt and the salt concentration, he obtained single strand nicking of the plasmid DNA with diazofluorene 7 and β-naphthyl phenyl diazomethane 8 under Cu(OAc)$_2$ conditions.

![Scheme 1](image)

**Scheme 1.** Oxidative Activation.

Jebaratnam proposed that the cupric acetate oxidized the diazo moiety, then with loss of N$_2$, yielded the one-electron radical that could then cleave DNA by known radical pathways.$^{15-18}$ β-naphthyl phenyl diazomethane 8 also cleaved the plasmid DNA in the presence of cupric acetate while diazofluorene 7 or cupric acetate alone (as controls) did not lead to DNA cleavage.

### 1.2.2 Dmitrienko’s Electrophilicity Hypothesis

The next MOA proposal for the kinamycins and lomaiviticins was brought forth by Dmitrienko. Dmitrienko believed that the increased diazonium ion character played a large part in the biological activity of these natural products.$^{19}$ To test his hypothesis he synthesized dideoxyisoprekinamycin derivative 9 (Scheme 2).

Suzuki cross-coupling of the boronate ester of 10 with bromobenzoate 11 afforded biaryl 12. Bromination of biaryl 12 followed by heating in methanesulfonic acid gave the
desired cyclized product 13. Palladium-catalyzed amination with benzylamine followed by
hydrogenation and diazotization/demethylation yielded dideoxyisoprekinamycin 9.

Dmitrienko’s mechanistic rationale was based on end product analysis of the reaction
of β-naphthol with either isoprekinamycin 4 or isoprekinamycin analogue 9 (Scheme 3). This
observation was rationalized by invoking a key hydrogen bond between the phenolic
hydroxyl and the ketone alpha to the diazo moiety which produced a more electrophilic
diazonium ion. Biologically, this data presents the hypothesis that DNA amino groups
(Guanine and Adenine) would add to the diazo group, forming labile triazenes. Subsequent
disassociation with concomitant loss of N₂, would generate a radical intermediate on the
DNA base and the aryl ring of the natural product. The resulting radicals could then alkylate
the DNA base or act independently (i.e. reaction with O₂) to cause DNA damage.¹⁶,¹⁷
1.2.3 Feldman’s Reductive Activation Hypothesis

In 2005, Feldman proposed a third MOA that was underpinned by the reductive activation by a one-electron reduction of the quinone to generate reactive aryl radical 14. Once generated this radical could abstract a hydrogen atom from DNA inducing DNA strand scission (Scheme 4). In addition, once 14 abstracts a hydrogen an orthoquinonemethide is generated. Orthoquinonemethides are known DNA damaging agents (i.e. mitomycin C, hydroxymethylacylfulvene).21-25

Feldman tested the putative e⁻/H⁺ reduction with the use of Bu₃SnH/AIBN in refluxing benzene. In a more recent account, Feldman admits to the limitations of the
chosen abiological conditions, but holds fast that the work helps to explore the chemistry of the diazoparaquinone moiety irrespective of the environment. However, there is no biological equivalent for Bu3SnH/AIBN and refluxing benzene is also not biomimetic. It is important to note the lack of reactivity in non-aromatic solvents (THF, 1,4-dioxane, CCl4, CH3CN, EtOH, or CHCl3). Feldman attributes this to the stabilization or promoting effect that aromatic solvents may give to the intermediate radical which, we believe, presents even more evidence that this is not the appropriate means of activation for this class of natural products. The kinamycins and lomaiviticins will not have the benefit of being stabilized by aromatic solvents in vitro or in vivo.

Feldman also describes several reaction sets that, overall, suggest the intermittency of a radical and an orthoquinonemethide under his reaction conditions. The orthoquinonemethide was inferred by the isolation of addition products of the solvent or selenium/sulfur nucleophiles (Scheme 5).
1.3 Diazofluorene Analogue Design and Synthesis

Unable to accept the previous MOA studies as indicative of biomimetic activation, we sought to delineate an appropriate means of testing our own activation hypothesis for the diazofluorene class of compounds. To this end, we synthesized a small library of diazofluorene analogues with varying electronic properties to probe the MOA and assayed against the ability of the analogue library to recapitulate the DNA cleavage activity of kinamycin D.\textsuperscript{27}

It has already been established that the activity of the kinamycins and lomaiviticins is directly related to the presence of the diazo moiety. The hydroxylated naphthoquinone subunit has also been identified as possibly playing an assisting role in the MOA for these
natural products. With these two ideas in mind, the synthetic library would consist of substituted diazofluorenes to explore how the differences in ring electronics would affect the diazo moiety.

![Scheme 6](image_url)


1-Aminofluorenone 16 and 4-aminofluorenone 18 were chosen as convenient starting materials to access most of the library through various functionalizations of the amino group (Schemes 6 & 7). The parent diazo compound, 9-diazofluorene 7, was synthesized as reported from fluorenone. The methoxyfluorenones were synthesized by diazotization of the aminofluorenone (16/18) at 0 °C with NaNO₂/H₂SO₄ followed by the addition of the diazonium salt to a solution of refluxing 50% H₂SO₄. The isolated hydroxyfluorenones were then methylated with NaH/MeI or Me₂SO₄/NaOH to give the methoxyfluorenones in high yields. Oxidation of the aminofluorenones (16/18) with TFAA/H₂O₂ afforded the nitrofluorenones in moderate yields. Finally, installation of the diazo group was
accomplished by either: hydrazinolysis followed by mercuric oxide oxidation or by condensation with tosyl hydrazine followed by base induced elimination of the tosyl group. The chlorodiazofluorenes were accessed from the known chlorofluorenones under standard diazo installation conditions. Additionally, the aminodiazofluorenes were accessed directly from the aminofluorenones through tosyl hydrazine condensation followed by base induced oxidation (Scheme 7).

Scheme 7. Synthesis of Chlorodiazofluorenes and Aminodiazofluorenes.

The synthesis of two 1,4-substituted fluorenones was also envisioned to more closely mimic the substitution of the natural products. Following the work of Jones, 1,4-dimethoxybenzene 26 was acylated with 2-iodobenzoic acid 27 using TFAA/TFA in 97% yield (Scheme 8). Palladium catalyzed cyclization afforded 1,4-dimethoxyfluorenone 28 in 50% yield. Condensation of 1,4-dimethoxyfluorenone 28 with tosyl hydrazine followed by elimination with sodium methoxide yielded the target analogue 1,4-dimethoxydiazofluorene 29 in 15% yield over two steps. The 1,4-diazoquinofluorene 30 was synthesized by quantitative demethylation of 1,4-dimethoxyfluorenone 29 with BBr₃, then hydrazinolysis and Fetizon’s reagent affected the tandem oxidation of the hydroquinone and hydrazone in 42% yield.³¹
1.4 DNA Cleavage with Diazofluorene Analogues

With the synthesis of the diazofluorene library complete, it was then necessary to assay them for their ability to recapitulate the DNA cleaving ability of the natural products. Since lomaiviticin A was reported to cleave double stranded DNA under reducing conditions,\(^5\) we sought to mimic the reducing conditions of the cell *in vitro*. To this end, we explored the DNA cleaving activity of kinamycin D (2d) under a variety of preliminary activating conditions (Figure 4).\(^3\) First we incubated plasmid pBR322 DNA with kinamycin D but no cleavage occurred even after 3 days. Kinamycin D was then incubated with pBR322 in the presence of either dithiothreitol (DTT), nicotinamide adenine dinucleotide phosphate (NADPH), or sodium cyanide (NaCN). Appreciable DNA damage (single strand scission) was observed for the DNA/kinamycin D solution incubated with DTT, while no other significant DNA damage was observed for the other additives. A preliminary time course for optimal cleavage was also conducted for kinamycin D in the presence of DTT and maximal cleavage was obtained after 2 days with 1 mM of kinamycin D and 1 M DTT. It is worth noting that DTT is a free-radical scavenger so the intermediacy of a diffusible free-radical as the active intermediate is not very likely.

![Scheme 8. Synthesis of 1,4-substituted Diazo Analogues.](image)
Using these optimized conditions for the natural product, we assayed the synthetic diazofluorene analogues for their ability to cleave DNA (Figure 5). The methoxy group (17, 19, 29) was chosen specifically because it mimics the reduced form of the paraquinone natural products, which had already been hypothesized by Feldman as being an active intermediate.

Two compounds assayed, 1-methoxydiazofluorene 17 and 4-aminodiazofluorene 25, cleaved DNA in an efficient manner comparable to kinamycin D 2d under DTT conditions (Figure 6). A control experiment was also conducted; 1-methoxyfluorenone and 4-aminofluorenone were incubated with DNA/DTT as before and showed no cleavage of the DNA clearly indicating that the diazo group is essential for DNA cleavage. To further
explore these as kinamycin mimics, 1-methoxydiazofluorene 17 and 4-aminodiazofluorene 25 were assayed for their ability to cleave DNA in the absence of DTT. Under these conditions, 4-aminodiazofluorene 25 retained DNA cleavage activity, while 1-methoxydiazofluorene 17 displayed minimal activity.

Although strange, these results are thought to show the increased activity of the 4-aminodiazofluorene 25, indicating that it does not need to be activated. A clear analogy would be that the 1-aminodiazofluorene 24 should then be very active and indeed it is so active that it could not be accurately assayed due to its instability even at -80 °C. We also assayed 1-methoxydiazofluorene 17 for its ability to cleave DNA in the presence of NADPH or NaCN but no appreciable amount of cleavage was detected (Figure 7).

We quantitated the extent of DNA cleavage from supercoiled DNA (type I) to nicked DNA (type II) and these results are summarized in Table 3. Most notably, in the presence of DTT, 1-methoxydiazofluorene 17 outperforms kinamycin D 2d, exhibiting 150% of the cleavage activity of the natural product. In the presence of DTT, 4-aminodiazofluorene 25
only exhibits 64% activity in comparison with kinamycin D, but in the absence of DTT, it exhibits 160% of the cleavage activity that kinamycin D exhibited in DTT (or 900% as compared to kinamycin D in the absence of DTT).

In addition to the DNA cleavage assay, we also explored the antiproliferative activity against HeLa cells for 1-methoxydiazofluorene 17, 4-aminodiazofluorene 25 and the diazo parent compound 7. Earlier, diazofluorene 7 was shown to cleave DNA under oxidizing conditions, while 1-methoxydiazofluorene 17 cleaves under reducing thiol conditions and 4-aminodiazofluorene 25 requires no activation. These intrinsic properties allowed us to explore the antiproliferative activity from several avenues to achieve maximal activity. The studies were conducted at 100 nM, the solubility limit of the synthetic compounds in cell media. Although we could not calculate IC$_{50}$ values due to solubility issues, we did observe that 1-methoxydiazofluorene 17 had the highest activity of the three compounds tested. 1-Methoxydiazofluorene 17 demonstrated a time-dependent inhibition of HeLa cell proliferation and inhibited cell growth by 35-40% at 12 h. This activity is similar to the antiproliferative activity reported for the most active kinamycins.$^2$
1.5 Conclusion

Using a small library of synthesized diazo derivatives, we have delineated a MOA not precedent in the literature for these compounds. We found that the use of DTT effectively activated kinamycin D 2d and several diazofluorenes for DNA cleavage.\textsuperscript{27} In total, four major hypotheses have been proposed for the MOA of the kinamycins and lomaiviticins. Interestingly, they have varied tremendously in activating conditions: oxidation, increased electrophilicity, one electron reduction, and thiol-mediated reduction/activation. From these various activating conditions though, a clear trend has emerged that the diazo group is the obvious active site and that loss of N\textsubscript{2} forms a reactive site. How this reactive intermediate proceeds to damage DNA is not clearly understood, but continued work by our group and others will begin to shed light on this interesting and important topic.
1.6 Experimental Section

General Experimental and Procedures

All $^1$H NMR (400 MHz or 300 MHz) and $^{13}$C NMR (100 MHz or 75 MHz) spectra were recorded at 25.0 °C on a Varian Mercury spectrometer. Chemical shifts ($\delta$) are given in ppm relative to tetramethylsilane or the respective NMR solvent; coupling constants ($J$) are in hertz. Abbreviations used are s = singlet, bs = broad singlet, d = doublet, dd = doublet of doublets, t = triplet, dt = doublet of triplets, m = multiplet, and br = broad. Infrared spectra (KBr pellet) were taken using JASCO FT-IR-410 spectrophotometer. Wave numbers in cm$^{-1}$ are reported for characteristic peaks. FAB-MS spectra were measured via high-resolution fast atom bombardment using a matrix of nitrobenzyl alcohol. Silica gel (40 μm average particle size) was used for column chromatography. Toluene was purified by distillation over sodium/benzophenone, and anhydrous MeOH was purified by distillation over magnesium. All other reagents were used as purchased from commercial sources.

9-Fluorenone ($p$-tosyl)hydrazone (1-i). $p$-Toluenesulfonylhydrazide (0.98 g, 5.24 mmol) was added to a stirred suspension of 9-fluorenone (0.76 g, 4.2 mmol) in boiling CH$_3$CN (20 mL). The resulting mixture was refluxed for 0.5 h, the yellow crystals obtained upon cooling were filtered. Flash chromatography (2:1 / CH$_2$Cl$_2$:hexanes) afforded 9-fluorenone ($p$-
tosyl)hydrazone (1.20 g, 81%) as yellow crystals. IR (KBr) 3500, 3213, 3065, 1595, 1451, 1384, 1327, 1311, 1152 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.40 (s, 3H), 7.3 (m, 5H), 7.44 (t, 1H, \(J = 6.8\) Hz), 7.53 (d, 1H, \(J = 7.6\) Hz), 7.64 (d, 1H, \(J = 7.6\) Hz), 7.70 (d, 1H, \(J = 7.6\) Hz), 7.86 (d, 1H, \(J = 7.6\) Hz), 7.97 (d, 2H, \(J = 8.4\) Hz), 8.38 (bs, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.9, 119.9, 121.1, 122.4, 126.5, 128.4, 128.5, 128.7, 129.9, 130.7, 131.9, 134.9, 136.6, 140.0, 142.7, 144.8; HRMS (FAB) \(m/z\), ([M + H]\(^+\), C\(_{20}\)H\(_{16}\)N\(_2\)O\(_2\)S): calcd. 349.1011, found 349.1032.

9-Diazofluorene (7). A dioxane (4.5 mL) solution of 9-fluorenone (\(p\)-tosyl)hydrazone (73 mg g, 0.21 mmol) and aqueous 50% NaOH (2.3 mL) was vigorously stirred at 50 °C for 24 h under Ar. The mixture was then diluted with water (2.5 mL) and extracted quickly with EtOAc (3 x 2.5 mL). The combined organic layer was then washed with water (2.5 mL), dried (MgSO\(_4\)) and concentrated under reduced pressure. Flash chromatography (2:1 / CH\(_2\)Cl\(_2\):hexanes) gave 9-diazofluorene 7 (35 mg, 88%) as fine light red crystals. IR (KBr) 3040, 2066, 1439, 752, 722 cm\(^{-1}\); \(^1\)H NMR (300 MHz, acetone-\(d_6\)) \(\delta\) 7.34 (t, 2H, \(J = 7.8\) Hz), 7.42 (t, 2H, \(J = 7.8\) Hz), 7.67 (d, 2H, \(J = 7.5\) Hz), 8.06 (d, 2H, \(J = 7.8\) Hz); \(^13\)C NMR (100 MHz, acetone-\(d_6\)) \(\delta\) 119.7, 121.0, 121.1, 124.6, 124.7, 126.5, 126.6; HRMS (FAB) \(m/z\), ([M]\(^+\), C\(_{13}\)H\(_8\)N\(_2\)): calcd. 192.0687, found 192.0687.
**1-Amino-9-fluorenone (p-tosyl)hydrazone (1-xiv).** A 95% EtOH (7 mL) solution of 1-amino-9-fluorenone 16 (0.32 g, 1.64 mmol), p-toluenesulfonylhydrazide (0.45 g, 2.42 mmol) and conc. HCl (280 µL) was refluxed under Ar for 6 h, then cooled and concentrated under reduced pressure. The product was isolated by flash chromatography (CH₂Cl₂) to give 1-amino-9-fluorenone (p-tosyl)hydrazone (0.45 g, 76%) as yellow crystals. IR (KBr) 3469, 3358, 3195, 1618, 1456, 1311, 1164, 1089, 752 cm⁻¹; ¹H NMR (400 MHz, acetone-d₆) δ 2.41 (s, 3H), 6.06 (bs, 2H), 6.64 (d, 1H, J = 8.0 Hz), 6.99 (d, 1H, J = 8.0 Hz), 7.13 (t, 1H, J = 8.0 Hz), 7.35 (t, 1H, J = 8.0 Hz), 7.44 (m, 3H), 7.73 (d, 1H, J = 8.0 Hz), 7.94 (m, 2H), 8.16 (d, 1H, J = 8.0 Hz), 9.91 (bs, 1H); ¹³C NMR (100 MHz, acetone-d₆) δ 20.8, 108.7, 116.1, 120.6, 127.6, 127.9, 128.7, 129.8, 130.5, 131.5, 131.8, 135.7, 140.8, 142.6, 144.4, 146.1, 156.6; HRMS (FAB) m/z, ([M + H]⁺, C₂₀H₁₇N₃O₂S): calcd. 364.1120, found 364.1113.

**1-Amino-9-diazofluorene (24).** A MeOH (5 mL) solution of 1-amino-9-fluorenone (p-tosyl)hydrazone (0.10 g, 0.28 mmol) and NaOMe (37 mg, 0.69 mmol) was refluxed for 10 h under Ar. The mixture was then concentrated under reduced pressure. The product was
isolated by flash chromatography (1:1 / CH₂Cl₂:hexanes) to give 24 (15 mg, 26%) as a red solid. IR (KBr) 3396, 3318, 2923, 2050, 1589, 1435, 1339, 1285, 1152, 789, 714 cm⁻¹; ¹H NMR (400 MHz, acetone-d₆) δ 6.51 (bs, 2H), 6.80 (d, 1H, J = 8.4 Hz), 7.10 (d, 1H, J = 8.0 Hz), 7.24 (m, 2H), 7.45 (t, 1H, J = 7.2 Hz), 7.77 (d, 1H, J = 8.4 Hz), 8.41 (d, 1H, J = 7.6 Hz); ¹³C NMR (100 MHz, acetone-d₆) δ 109.1, 116.3, 120.6, 128.0, 128.2, 131.5, 132.5; LRMS (FAB) m/z, ([M]⁺, C₁₃H₉N₃): calcd. 207.0796, found 207.1410.

1-Hydroxy-9-fluorenone (1-ii). 1-Amino-9-fluorenone 16 (0.185 g, 0.95 mmol) was suspended in H₂O (6 mL) at 0 ºC and dissolved with the addition of conc. H₂SO₄ (11 mL). With vigorous stirring, a solution of sodium nitrite (0.091 g, 1.31 mmol) and H₂O (4 mL) was slowly added dropwise. The solution was stirred at 0 ºC for 2 h and then canulated into a boiling solution of conc. H₂SO₄/H₂O (32 mL/32 mL). Following addition, the solution was boiled for 15 min then cooled to room temperature and allowed to stir for an additional 4 h. The reaction mixture was then extracted with CH₂Cl₂ (3x50 mL) and the combined organic layer was washed with H₂O (50 mL), dried (Na₂SO₄), filtered and concentrated. The product was isolated by flash chromatography (0-15% EtOAc/hexanes) to give 1-hydroxy-9-fluorenone (0.129 g, 70%) as fine yellow needles. IR (KBr) 3368 (br, s), 1690, 1599 (vs) cm⁻¹; ¹H NMR (300 MHz, acetone-d₆) δ 6.78 (dd, 1H, J = 8.2 and 0.6 Hz), 7.22 (dd, 1H, J = 7.2 and 0.6 Hz), 7.37 (dt, 1H, J = 7.5 and 1.2 Hz), 7.44 (dd, 1H, J = 7.2 and 0.9 Hz), 7.57 (m,
2H), 7.70 (dt, 1H, $J = 7.5$ and 0.9 Hz), 8.76 (bs, 1H); $^{13}$C NMR (100 MHz, acetone-$d_6$) $\delta$ 113.8, 118.3, 119.3, 122.19, 124.47, 130.2, 135.0, 135.7, 138.4, 144.8, 145.2, 157.9, 195.4; HRMS (FAB) $m/z$, ([M + H]$^+$, C$_{13}$H$_8$O$_2$): calcd. 197.0603, found 197.0614.

1-Methoxy-9-fluorenone (1-iii). 1-Hydroxy-9-fluorenone (0.10 g, 0.51 mmol) was dissolved into 3 mL dry DMF under Ar and cooled to 0 ºC with stirring. Then, 60% NaH (0.03 g, 0.76 mmol) was added in portions and the solution was warmed to room temperature for 30 min. Methyl Iodide (0.07 mL, 1.1 mmol) was then added and stirred for 2 h after which the reaction was poured into H$_2$O (10 mL) and extracted with Et$_2$O (3 x 10 mL). The combined organic layers were then washed with H$_2$O and brine, dried (Na$_2$SO$_4$), filtered and concentrated. The product was isolated by flash chromatography (0-20% EtOAc/hexanes) to give 1-methoxy-9-fluorenone (0.103 g, 96%) as fine yellow-green needles. IR (KBr) 2837 (w), 1700 (vs), 1592 (vs), 1023 (m) cm$^{-1}$; $^1$H NMR (300 MHz, acetone-$d_6$) $\delta$ 3.94 (s, 3H), 7.01 (d, 1H, $J = 8.7$ Hz), 7.31 (dt, 1H, $J = 7.2$ and 1.2 Hz), 7.36 (dd, 1H, $J = 7.2$ and 1.2 Hz), 7.53 (m, 3H), 7.69 (dt, 1H, $J = 7.2$ and 1.2 Hz); $^{13}$C NMR (75 MHz, acetone-$d_6$) $\delta$ 55.6, 113.2, 113.9, 119.9, 120.7, 123.3, 129.4, 134.2, 134.5, 137.2, 143.3, 146.4, 158.6, 190.4; HRMS (FAB) $m/z$, ([M + H]$^+$, C$_{14}$H$_{10}$O$_2$): calcd. 211.0759, found 211.0759.
1-Methoxy-9-fluorenone (p-tosyl)hydrazone (1-iv). A THF (3.0 mL) solution of 1-methoxy-9-fluorenone (86.1 mg, 0.41 mmol), p-toluenesulfonylhydrazide (86.6 mg, 0.47 mmol) and conc. HCl (50 µL) was refluxed under Ar for 12 h, then cooled and dried under reduced pressure. The product was isolated by flash chromatography (0-50% EtOAc/hexanes) to give 1-methoxy-9-fluorenone (p-tosyl)hydrazone (0.131 g, 85%) as bright yellow crystals. IR (KBr) 3117 (br), 2841 (w), 1602, 1578 (s), 1351, 1168 (vs), 1051 (s) cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ 2.37 (s, 3H), 4.03 (s, 3H), 6.76 (d, 1H, \( J = 8.4 \) Hz), 7.27 (m, 6H), 7.48 (d, 1H, \( J = 7.5 \) Hz), 7.76 (d, 1H, \( J = 7.2 \) Hz), 7.94 (d, 2H, \( J = 8.1 \) Hz), 11.66 (s, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 21.8, 56.8, 111.3, 114.6, 118.1, 119.8, 122.3, 128.0, 128.5, 129.7, 129.9, 133.7, 136.4, 137.6, 138.5, 144.1, 144.7, 145.5, 153.0; HRMS (FAB) \( m/z \), ([M + H]+, C₂₁H₁₈N₂O₃S): calcd. 379.1116, found 379.1121.

1-Methoxy-9-diazofluorene (17). A MeOH (4 mL) solution of 1-methoxy-9-fluorenone (p-tosyl)hydrazone (69.2 mg, 0.18 mmol) and NaOMe (83.0 mg, 1.54 mmol) was refluxed for 2 h under Ar. After the reaction was complete, the mixture was dried under reduced pressure.
The product was isolated by flash chromatography (1:1 / CH₂Cl₂:hexanes) of the residue to give 17 (14.4 mg, 36%) as a red solid. IR (KBr) 2836 (w), 2071 (vs), 1572, 1447 (s), 1260 (vs), 1014 (m) cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 3.95 (s, 3H), 6.84 (d, 1H, J = 8.0 Hz), 7.26 (m, 1H), 7.31 (dt, 1H, J = 8.0 and 1.2 Hz), 7.38 (dt, 1H, J = 8.0 and 1.6 Hz), 7.48 (d, 1H, J = 8.0 Hz), 7.58 (d, 1H, J = 8.0 Hz), 7.93 (d, 1H, J = 8.0 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 55.7, 107.4, 114.1, 119.1, 121.5, 124.5, 125.7, 126.3; HRMS (FAB) m/z, ([M]+, C₁₄H₁₀N₂O): calcd. 222.0793, found 222.0777.

![Chemical structure of 1-Chloro-9-fluorenone hydrazone (1-xii).](image)

**1-Chloro-9-fluorenone hydrazone (1-xii).** To a solution of 1-chloro-9-fluorenone³³ (0.41 g, 1.9 mmol) in 1-butanol (2 mL), 85% aqueous hydrazine hydrate (0.25 g, 6.5 mmol) was added. After refluxing for 4 h, the mixture was concentrated under reduced pressure and flash chromatography (CH₂Cl₂) gave 1-chloro-9-fluorenone hydrazone (0.17 g, 38%) as light yellow crystals. IR (KBr) 3366, 3226, 2360, 2337, 1602, 1445, 1412, 1186, 742 cm⁻¹; ¹H NMR (300 MHz, acetone-d₆) δ 7.30 (m, 2H), 7.47 (m, 2H), 7.80 (m, 3H), 7.96 (m, 1H), 8.22 (m, 1H); ¹³C NMR (75 MHz, CDCl₃) δ 118.4, 120.6, 125.3, 128.2, 128.6, 128.9, 129.8, 130.1, 133.4, 138.6, 140.3, 140.5; HRMS (FAB) m/z, ([M + H]+, C₁₃H₉ClN₂): calcd. 229.0533, found 229.0522.
1-Chloro-9-diazofluorene (22). 1-chloro-9-fluorenone hydrazone (0.17 g, 0.73 mmol) was dissolved in toluene (10 mL) and yellow mercuric oxide (1.60 g, 7.4 mmol) was added. The resulting suspension was stirred for 96 h, filtered and dried (MgSO₄). The filtrate was concentrated under reduced pressure and flash chromatography (1:4 / CH₂Cl₂:petroleum ether) gave 22 (70.7 mg, 43%) as a red solid. IR (KBr) 3476, 2063, 1557, 1444, 1415, 742, 657 cm⁻¹; ¹H NMR (400 MHz, acetone-̃d₆) δ 7.27 (m, 3H), 7.39 (dt, 1H, J = 8.0 and 1.2 Hz), 7.46 (d, 1H, J = 8.0 Hz), 7.87 (dd, 1H, J = 8.0 and 1.2 Hz), 7.94 (d, 1H, J = 8.0 Hz); ¹³C NMR (100 MHz, acetone-̃d₆) δ 119.0, 119.7, 121.5, 125.0, 125.8, 126.8, 127.1, 127.2, 129.0, 130.5, 133.5; HRMS (FAB) m/z, ([M]⁺, C₁₃H₇ClN₂): calcd. 226.0298, found 226.0300.

1-Nitro-9-fluorenone (1-viii). Trifluoroacetic anhydride (1.50 mL, 10.5 mmol) in CH₂Cl₂ (3.0 mL) was slowly added to H₂O₂ (0.57 mL, 9.2 mmol) at 0 ºC while stirring under Ar. The solution was held at 0 ºC for 1.5 h, then a solution of 1-amino-9-fluorenone 16 (0.196 g, 1.00 mmol) and CH₂Cl₂ (3 mL) was added dropwise. The solution was held at 0 ºC for 2 h then warmed to room temperature and poured into H₂O (10 mL) and extracted with CH₂Cl₂ (2 x 10 mL). The combined organic layers were then washed with brine, dried (Na₂SO₄),
filtered and concentrated. The product was isolated by flash chromatography (0-5% EtOAc/hexanes) to give 1-nitro-9-fluorenone (0.131 g, 58%) as yellow-orange needles. IR (KBr) 1728, 1526, 1364 (vs) cm\(^{-1}\); \(^1\)H NMR (300 MHz, acetone-\(d_6\)) \(\delta\) 7.49 (t, 1H, \(J = 7.5\) Hz), 7.67 (m, 3H), 7.85 (m, 2H), 8.06 (d, 1H, \(J = 7.5\) Hz); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 120.8, 123.1, 123.6, 125.0, 125.3, 130.5, 133.4, 135.3, 135.6, 142.1, 146.3, 146.6, 187.5; LRMS (FAB) \(m/z\), ([M + H]\(^+\), C\(_{13}\)H\(_7\)NO\(_3\)): calcd. 226.0504, found 226.1187.

1-Nitro-9-fluorenone (\(p\)-tosyl)hydrazone (1-ix). A THF (2 mL) solution of 1-nitro-9-fluorenone (71 mg, 0.31 mmol), \(p\)-toluenesulfonylhydrazide (63 mg, 0.34 mmol) and conc. HCl (36.5 \(\mu\)L) was stirred at room temperature for 24 h, and then concentrated under reduced pressure. Flash chromatography (CH\(_2\)Cl\(_2\)) gave 1-nitro-9-fluorenone (\(p\)-tosyl)hydrazone (68 mg, 56%) as light yellow crystals. IR (KBr) 3433, 3248, 3216, 1536, 1453, 1371, 1342, 1167, 784, 678, 547 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.42 (s, 3H), 7.38 (d, 3H, \(J = 8.0\) Hz), 7.48 (m, 3H), 7.73 (d, 2H, \(J = 8.0\) Hz), 7.87 (d, 1H, \(J = 8.0\) Hz), 7.94 (d, 2H, \(J = 8.0\) Hz), 8.44 (bs, 1H); \(^13\)C NMR (100 MHz, acetone-\(d_6\)) \(\delta\) 20.9, 121.5, 121.8, 122.8, 127.8, 128.9, 129.2, 129.58, 129.64, 131.8, 132.2, 140.2, 144.5; LRMS (FAB) \(m/z\), ([M + H]\(^+\), C\(_{20}\)H\(_{15}\)N\(_3\)O\(_4\)S): calcd. 394.0862, found 394.1357.
1-Nitro-9-diazofluorene (20). A dioxane (2.5 mL) solution of 1-nitro-9-fluorenone (p-tosyl)hydrazone (43 mg, 0.11 mmol) and aqueous 50% NaOH (0.32 mL) was vigorously stirred at 50 °C for 24 h. The mixture was then diluted with H2O (2.0 mL) and extracted quickly with EtOAc (3x2.0 mL), the organic layer was washed with water (2.5 mL), dried (MgSO4), filtered and concentrated under reduced pressure. Flash chromatography (1:1 / CH2Cl2:hexanes) gave 20 (19.8 mg, 76%) as a light red solid. IR (KBr) 3442, 2082, 1559, 1514, 1448, 1420, 1301, 1207, 744 cm⁻¹; ¹H NMR (400 MHz, acetone-d6) δ 7.40 (dt, 1H, J = 8.0 and 1.6 Hz), 7.51 (m, 3H), 8.14 (dd, 1H, J = 8.0 and 0.8 Hz), 8.24 (dd, 1H, J = 8.0 and 0.8 Hz), 8.42 (dd, 1H, J = 8.0 and 0.8 Hz); ¹³C NMR (100 MHz, acetone-d6) δ 118.0, 121.5, 122.9, 124.2, 125.0, 127.0, 127.9; HRMS (FAB) m/z, ([M]+, C₁₃H₇N₃O₂): calcd. 237.0538, found 237.0541.

4-Amino-9-fluorenone (p-tosyl)hydrazone (1-xv). A 95% EtOH (3 mL) solution of 4-amino-9-fluorenone 18 (0.15 g, 0.80 mmol), p-toluenesulfonylhydrazide (0.22 g, 1.20 mmol) and conc. HCl (130 µL) was refluxed under Ar for 6 h, then cooled and concentrated under
reduced pressure. Flash chromatography (CH$_2$Cl$_2$) gave 4-amino-9-fluorenone (p-tosyl)hydrazone (0.20 g, 70%) as bright yellow crystals. As a mixture of E:Z isomers. IR (KBr) 3390, 3188, 1624, 1594, 1454, 1399, 1336, 1165, 1064, 724, 538 cm$^{-1}$; $^1$H NMR (400 MHz, acetone-d$_6$) See spectrum; $^{13}$C NMR (100 MHz, acetone-d$_6$) $\delta$ 20.9, 117.5, 120.8, 121.59, 121.62, 122.0, 122.5, 123.2, 123.6, 126.4, 127.5, 128.8, 129.1, 129.55, 129.60, 130.6, 130.8, 144.3; HRMS (FAB) m/z, ([M + H]$^+$, C$_{20}$H$_{17}$N$_3$O$_2$S): calcd. 364.1120, found 364.1114.

4-Amino-9-diazofluorene (25). A dioxane (2.5 mL) solution of 4-amino-9-fluorenone (p-tosyl)hydrazone (0.04 g, 0.11 mmol) and aqueous 50% NaOH (0.32 mL) was vigorously stirred at 50 °C for 24 h under Ar. EtOAc (10 mL) and H$_2$O (5 mL) were then added. The organic layer was washed with H$_2$O (5 mL), dried (Na$_2$SO$_4$), filtered and concentrated under reduced pressure. Flash chromatography (1:1 / CH$_2$Cl$_2$:hexanes) gave 25 (7.9 mg, 35%) as deep red crystals. IR (KBr) 3421, 2067, 1430, 719 cm$^{-1}$; $^1$H NMR (300 MHz, acetone-d$_6$) $\delta$ 5.16 (bs, 2H), 6.72 (d, 1H, $J = 7.8$ Hz), 6.94 (d, 1H, $J = 7.8$ Hz), 7.21 (t, 1H, $J = 8.1$ Hz), 7.33 (m, 2H), 7.61 (d, 1H, $J = 7.2$ Hz), 8.07 (d, 1H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, acetone-d$_6$) $\delta$ 110.5, 112.7, 119.4, 122.5, 124.8, 125.2, 127.4; HRMS (FAB) m/z, ([M]$^+$, C$_{12}$H$_9$N$_3$): calcd. 207.0796, found 207.0779.
4-Hydroxy-9-fluorenone (1-v). The product was isolated by flash chromatography (0-15% EtOAc/hexanes) to give 4-hydroxy-9-fluorenone (0.069 g, 73%) as fine red-orange needles. IR (KBr) 3183 (br, s), 1683, 1278 (s) cm\(^{-1}\); \(^1\)H NMR (300 MHz, acetone-\(d_6\)) \(\delta\) 7.16 (m, 3H), 7.29 (dt, 1H, \(J = 7.5\) and 1.2 Hz), 7.52 (dd, 1H, \(J = 7.5\) and 1.2 Hz), 7.57 (d, 1H, \(J = 6.3\) Hz), 7.88 (d, 1H, \(J = 7.5\) Hz), 9.35 (bs, 1H); \(^{13}\)C NMR (100 MHz, acetone-\(d_6\)) \(\delta\) 116.1, 123.8, 124.1, 124.7, 128.6, 129.6, 131.1, 134.1, 135.5, 136.5, 144.7, 154.0 193.8; HRMS (FAB) \(m/z\), ([M + H]\(^+\), \(C_{13}H_{8}O_2\)): calcd. 197.0603, found 197.0615.

4-Methoxy-9-fluorenone (1-vi). 4-Hydroxy-9-fluorenone (0.066 g, 0.34 mmol) was dissolved into 95% EtOH (0.75 mL) at 50 °C under Argon with stirring. To the solution was added \(H_2O\) (0.06 mL) then NaOH (0.02 g). Dimethyl sulfate (0.04 mL, 0.40 mmol) was added dropwise with an additional NaOH (0.02 g) added after addition. The solution was refluxed for 30 mins then cooled to room temperature. The solution was then poured into \(H_2O\) (5 mL) and extracted with CH\(_2\)Cl\(_2\) (3 x 5 mL). The combined organic layers were then
washed with H₂O and brine, dried (Na₂SO₄), filtered and concentrated. The product was isolated by flash chromatography (0-25% EtOAc/hexanes) to give 4-methoxy-9-fluorenone (0.067 g, 95%) as fine yellow needles. IR (KBr) 2851 (w), 1714, 1273 (vs), 1055 (m) cm⁻¹; ¹H NMR (300 MHz, acetone-δ₆) δ 4.04 (s, 1H), 7.21 (dd, 1H, J = 7.0 and 0.9 Hz), 7.31 (m, 3H), 7.51 (dd, 1H, J = 7.5 and 1.2 Hz), 7.57 (dt, 1H, J = 7.5 and 0.9 Hz), 7.84 (dt, 1H, J = 7.5 and 0.9 Hz); ¹³C NMR (75 MHz, acetone-δ₆) δ 56.1, 116.5, 119.1, 124.1, 124.9, 128.9, 131.4, 131.5, 134.0, 135.5, 136.1, 144.3, 156.4, 193.6; HRMS (FAB) m/z, ([M + H]⁺, C₁₄H₁₀O₂): calcd. 211.0759, found 211.0764.

4-Methoxy-9-fluorenone (p-tosyl)hydrazone (1-vii). A THF (2.50 mL) solution of 4-methoxy-9-fluorenone (79.40 mg, 0.38 mmol), p-toluenesulfonylhydrazide (77.54 mg, 0.42 mmol) and conc. HCl (45 µL) was refluxed under Ar for 6 h, then cooled and concentrated under reduced pressure. Flash chromatography (CH₂Cl₂) gave 4-methoxy-9-fluorenone (p-tosyl)hydrazone (0.10 g, 71%) as bright yellow crystals. IR (KBr) 3185, 2938, 1592, 1489, 1439, 1334, 1270, 1165, 1044, 797, 725, 674, 537 cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 2.41 (s, 3H), 4.02 (s, 3H), 7.07 (d, 1H, J = 8.4 Hz), 7.29 (m, 6H), 7.73 (dd, 1H, J = 15.6 and 7.6 Hz), 7.95 (m, 2H), 8.11 (d, 1H, J = 7.6 Hz), 10.16 (s, 1H); ¹³C NMR (100 MHz, acetone-δ₆) δ 20.8, 55.4, 113.4, 114.2, 115.1, 119.9, 121.5, 124.0, 124.3, 127.3, 127.4, 128.8, 129.6,
129.8, 130.7, 131.8, 135.9, 144.4; HRMS (FAB) m/z, (M + H)$^+$, C$_{21}$H$_{18}$N$_2$O$_3$S: calcd. 379.1116, found 379.1130.

4-Methoxy-9-diazofluorene (19). A dioxane (2.50 mL) solution of 4-methoxy-9-fluorenone ($p$-tosyl)hydrazone (41.58 mg, 0.11 mmol) and aqueous 50% NaOH (0.32 ml) was vigorously stirred at 50 °C for 24 h under Ar. After the reaction was over, EtOAc (10 mL) and H$_2$O (5 mL) were added. The organic layer was separated and washed with H$_2$O (5 mL), then dried (Na$_2$SO$_4$), filtered and concentrated under reduced pressure. Flash chromatography (1:1 / CH$_2$Cl$_2$:hexanes) gave 19 (10.00 mg, 41%) as a light red solid. IR (KBr) 3439, 2051, 1574, 1494, 1427, 1265, 1154, 1052, 782, 745, 716 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.05 (s, 3H), 6.81 (d, 1H, $J$ = 8.0 Hz), 7.13 (d, 1H, $J$ = 8.0 Hz), 7.34 (m, 3H), 7.49 (m, 1H), 8.27 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 55.6, 106.2, 112.1, 118.9, 124.87, 124.90, 125.6, 127.5; LRMS (FAB) m/z, ([M]$^+$, C$_{14}$H$_{10}$N$_2$O): calcd. 222.0793, found 222.1422.
4-Chloro-9-fluorenone hydrazone (1-xiii). To a solution of 4-chloro-9-fluorenone\textsuperscript{34} (0.12 g, 0.56 mmol) dissolved in 1-butanol (0.6 mL), 85% aqueous hydrazine hydrate (73 mg, 1.9 mmol) was added. After refluxing for 4 h, the mixture was concentrated under reduced pressure. Flash chromatography (CH\textsubscript{2}Cl\textsubscript{2}) gave 4-chloro-9-fluorenone hydrazone (94 mg, 74%) as light yellow crystals. As a mixture of E:Z isomers. IR (KBr) 3368, 3208, 3051, 2905, 1633, 1436, 1358, 792, 725 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (300 MHz, acetone-\textit{d}\textsubscript{6}) See spectrum; \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) \(\delta\) 120.8, 122.4, 123.9, 124.0, 127.8, 128.56, 128.60, 128.8, 128.9, 129.3, 130.8, 131.3, 132.1, 132.69; LRMS (FAB) \(m/z\), ([M + H]\textsuperscript{+}, C\textsubscript{13}H\textsubscript{9}ClN\textsubscript{2}): calcd. 229.0533, found 229.1150.

4-Chloro-9-diazofluorene (23). 4-Chloro-9-fluorenone hydrazone (0.12 g, 0.52 mmol) was dissolved in toluene (75 mL) and yellow mercuric oxide (0.31 g, 1.4 mmol) was added. The resulting suspension was stirred for 24 h, filtered and the filtrate dried (MgSO\textsubscript{4}). The filtrate was concentrated under reduced pressure and flash chromatography (1:4 / CH\textsubscript{2}Cl\textsubscript{2}:petroleum ether) gave 23 (80.0 mg, 66%) as a red solid. IR (KBr) 3448, 2065, 1440, 1420, 1185, 775, 736, 712 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (300 MHz, acetone-\textit{d}\textsubscript{6}) \(\delta\) 7.36 (m, 3H), 7.47 (t, 1H, \(J = 7.8\) Hz), 7.59 (d, 1H, \(J = 7.8\) Hz), 7.66 (d, 1H, \(J = 8.1\) Hz), 8.52 (d, 1H, \(J = 7.8\) Hz); \textsuperscript{13}C NMR (75 MHz,
acetone-$d_6$) $\delta$ 118.2, 119.5, 124.3, 124.8, 125.4, 127.1; HRMS (FAB) $m/z$, ([M]$^+$, C$_{13}$H$_7$ClN$_2$): calcd. 226.0298, found 226.0302.

4-Nitro-9-fluorenone (1-x). Trifluoroacetic anhydride (1.37 mL, 9.87 mmol) in CH$_2$Cl$_2$ (2.8 mL) was slowly added to H$_2$O$_2$ (0.53 mL, 8.65 mmol) at 0 °C while stirring under Argon. The solution was held at 0 °C for 1.5 h then a solution of 4-amino-9-fluorenone 18 (0.186 g, 0.94 mmol) and CH$_2$Cl$_2$ (5 mL) was added dropwise. The solution was held at 0 °C for 3 h and warmed to room temperature for 1 h then poured into H$_2$O (10 mL) and extracted with CH$_2$Cl$_2$ (2 x 10 mL). The combined organic layers were then washed with brine, dried (Na$_2$SO$_4$), filtered and concentrated. The product was isolated by flash chromatography (0-5% EtOAc/hexanes) to give 4-Nitro-9-fluorenone (0.143 g, 67%) as light-yellow needles. IR (KBr) 1722, 1525, 1356 (vs) cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.47 (q, 2H, $J = 7.2$ Hz), 7.60 (t, 1H, $J = 7.6$ Hz), 7.79 (d, 1H, $J = 7.2$ Hz), 7.95 (d, 1H, $J = 7.6$ Hz), 8.02 (t, 2H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 124.8, 126.0, 128.3, 129.85, 129.87, 131.0, 134.3, 135.6, 136.5, 137.0, 140.3, 144.9, 191.1; LRMS (FAB) $m/z$, ([M + H]$^+$, C$_{13}$H$_7$NO$_3$): calcd. 226.0504, found 226.1265.
4-Nitro-9-fluorenone hydrazone (1-xi). To a solution of 4-nitro-9-fluorenone (59 mg, 0.22 mmol) dissolved in 1-butanol (0.23 mL), 85% aqueous hydrazine hydrate (28 µL, 0.76 mmol) was added. After refluxing for 4 h under Ar, the mixture was concentrated under reduced pressure. Flash chromatography (CH$_2$Cl$_2$) gave 4-nitro-9-fluorenone hydrazone (36 mg, 68%) as light yellow crystals. IR (KBr) 3388, 3195, 1576, 1519, 1443, 1353, 718 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.67 (bs, 2H), 7.41 (t, 1H, $J = 8.0$ Hz), 7.50 (m, 2H), 7.84 (dd, 1H, $J = 8.0$ and 1.2 Hz), 7.97 (m, 1H), 8.02 (dd, 1H, $J = 8.0$ and 1.2 Hz), 8.15 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 124.3, 125.1, 125.2, 125.8, 128.0, 129.7, 130.2; LRMS (FAB) $m/z$, ([M + H]$^+$, C$_{13}$H$_9$N$_3$O$_2$): calcd. 240.0773, found 240.1715.

4-Nitro-9-diazofluorene (21). 4-nitro-9-fluorenone hydrazone (0.05 g, 0.21 mmol) was dissolved in toluene (3 mL) and yellow mercuric oxide (0.13 g, 0.62 mmol) was added. The resulting suspension was stirred for 24 h, filtered and the filtrate dried with MgSO$_4$ and concentrated under reduced pressure. Flash chromatography (1:2 / acetone:hexanes) gave 21.
(23 mg, 47%) as a light red solid. IR (KBr) 3442, 2067, 1517, 1445, 1430, 1354, 1337, 1306, 1218, 1156, 803, 742, 713 cm\(^{-1}\); \(^1\)H NMR (400 MHz, acetone-\(d_6\)) \(\delta\) 7.39 (dt, 1H, \(J = 8.0\) and 1.2 Hz), 7.54 (dt, 1H, \(J = 8.0\) and 1.2 Hz), 7.62 (t, 1H, \(J = 8.0\) Hz), 7.75 (dd, 1H, \(J = 8.0\) and 0.8 Hz), 7.89 (dd, 1H, \(J = 8.0\) and 1.2 Hz), 8.05 (dd, 1H, \(J = 8.0\) and 1.2 Hz), 8.15 (dd, 1H, \(J = 8.0\) and 0.8 Hz); \(^13\)C NMR (100 MHz, acetone-\(d_6\)) \(\delta\) 119.6, 120.4, 124.2, 125.0, 125.2, 126.3, 128.3; LRMS (FAB) \(m/z\), ([M]+, C\(_{13}\)H\(_7\)N\(_3\)O\(_2\)): calcd. 237.0538, found 237.1370.

1,4-Dimethoxy-9-fluorenone (\(p\)-tosyl)hydrazone (1-xvi). A THF (4 mL) solution of 1,4-dimethoxy-9-fluorenone\(^{31}\) (32.20 mg, 0.13 mmol), \(p\)-toluenesulfonylhydrazide (27.54 mg, 14.8 \(\mu\)mol) and concentrated HCl (15.85 \(\mu\)L) was refluxed for 6 h under Ar, then cooled and concentrated under reduced pressure. The product was isolated by flash chromatography (3:1 / CH\(_2\)Cl\(_2\):hexanes) to give 1,4-dimethoxy-9-fluorenone (\(p\)-tosyl)hydrazone (25.7 mg, 47%) as yellow crystals. IR (KBr) 3438, 3104, 2920, 2841, 1590, 1503, 1454, 1349, 1267, 1245, 1164, 1079, 1046, 662, 552 cm\(^{-1}\); \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 2.39 (s, 3H), 3.93 (s, 3H), 4.03 (s, 3H), 6.80 (d, 1H, \(J = 9.2\) Hz), 6.95 (d, 1H, \(J = 9.2\) Hz), 7.27 (m, 5H), 7.77 (d, 1H, \(J = 6.8\) Hz), 7.93 (m, 2H), 11.84 (s, 1H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 21.8, 56.2, 57.6, 112.3, 115.7, 121.8, 124.0, 127.6, 128.0, 129.7, 129.8, 144.0, 147.2; LRMS (FAB) \(m/z\), ([M + H]+, C\(_{22}\)H\(_{20}\)N\(_2\)O\(_4\)S): calcd. 409.1222, found 409.2147.
1,4-Dimethoxy-9-diazofluorene (29). An anhydrous MeOH (2 mL) solution of 1,4-dimethoxy-9-fluorenone (p-tosyl)hydrazone (25.7 mg, 62.9 µmol) and NaOMe (28.8 mg, 0.53 mmol) was refluxed for 2 h under Ar. After the reaction was complete, the solution was concentrated under reduced pressure and purified by flash chromatography (1:1 / CH₂Cl₂:hexanes) to give 29 (5 mg, 31%) as a red solid. IR (KBr) 3429, 2923, 2850, 2054, 1508, 1444, 1408, 1253, 1025, 785, cm⁻¹; ¹H NMR (400 MHz, CDCl₃) δ 3.90 (s, 3H), 3.99 (s, 3H), 6.70 (d, 1H, J = 8.8 Hz), 6.75 (d, 1H, J = 8.8 Hz), 7.34 (m, 2H), 7.44 (m, 1H); ¹³C NMR (100 MHz, CDCl₃) δ 56.0, 106.1, 107.6, 118.5, 124.6, 124.9, 125.5; HRMS (FAB) m/z, ([M]+, C₁₅H₁₂N₂O₂): calcd. 252.0899, found 252.0895.

1,4-Dihydroxy-9-fluorenone (1-xvii). 1,4-Dimethoxy-9-fluorenone⁴ (0.0671 g, 0.279 mmol) was dissolved in dry CH₂Cl₂ (6.5 mL) under Ar and cooled to –78 ºC. BBr₃ (1 M in CH₂Cl₂, 0.84 mL, 0.84 mmol) was added dropwise with stirring and the reaction mixture was warmed to room temperature overnight then quenched with H₂O after 20 h. The reaction mixture was washed with saturated NaHCO₃ (aq) and saturated Na₂S₂O₅ (aq), dried (Na₂SO₄), filtered and
concentrated yielding 1,4-Dihydroxy-9-fluorenone (0.0587 g, 100%) as a red solid that was taken on crude to the next step. Judged ≥95% pure by TLC, MS, and $^1$H NMR; IR (KBr) 3223 (br, s), 1675 (vs), 1611, 1597 (s), 1385 (m), 1285 (s) cm$^{-1}$; $^1$H NMR (300 MHz, DMSO- $d_6$) δ 6.65 (d, 1H, $J = 8.7$), 6.93 (d, 1H, $J = 8.7$), 7.26 (t, 1H, $J = 7.6$), 7.51 (m, 2H), 7.79 (d, 1H, $J = 7.6$), 7.79 (d, 1H, $J = 7.6$); HRMS (FAB) $m/z$, ([M]$^+$, C$_{13}$H$_8$O$_3$): calcd. 212.0473, found 212.0469.

9-Diazoquinofluorene (30). Crude 1,4-dihydroxy-9-fluorenone (29.45 mg, 0.14 mmol) was dissolved in 100% EtOH (10 mL) and anhydrous hydrazine (320.4 µL, 70 equiv) and the reaction mixture was refluxed for 0.5 h. The solution was then cooled to room temperature and the solvent was removed under reduced pressure. The solid was dissolved again in anhydrous EtOH, which was removed under reduced pressure to remove traces of hydrazine. The resulting crude hydrazone was then suspended in dry CH$_2$Cl$_2$ (4 mL) under Ar with Et$_3$N (0.38 mL) and Fetizon’s reagent (1.0 g, ~ 1.81 mmol, ~ 13 equiv) was added at once with stirring. The reaction mixture was stirred for 5-10 min at room temperature, then the solid was removed by gravity filtration and washed with CH$_2$Cl$_2$. The resulting solvent was removed under reduced pressure and the product was purified by flash chromatography (CH$_2$Cl$_2$) to give 30 (12.9 mg, 42%) as a red solid. IR (KBr) 2096, 1644 (vs), 1585 (m), 1509 (s), 1477 (w) cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$) δ 6.69 (m, 2H), 7.42 (m, 2H), 7.54 (m, 1H),
8.36 (m, 1H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 118.9, 125.3, 125.6, 127.2, 127.4, 130.2, 133.7, 136.4, 139.4, 182.6, 183.1; LRMS (FAB) $m/z$, ([M + H]$^+$, C$_{13}$H$_6$N$_2$O$_2$): calcd. 223.0508, found 223.1741.

**General Procedure for DNA Cleavage Assay**

Kinamycin D was isolated as previously described from S. murayamaensis (ATCC 21414) and dissolved in DMF.$^{35}$ Covalently closed, supercoiled DNA (pBR322) was used as supplied by New England Biolabs in a 10 mM Tris-HCl buffer (pH=8.0) containing 1.0 mM EDTA. DL-dithiothreitol (DTT), glutathione (GSH) and $\beta$-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH) were dissolved in dH$_2$O. Incubations with DNA were carried out in a microfuge tube with the total volume not exceeding 4 µL. The reactions were conducted in the dark at room temperature (25 ºC) or 37 ºC for 48 h. DNA samples were then mixed with 8 µL of 10X Ficoll® loading buffer (0.25% xylene cyanol FF, 0.25% bromophenol blue, 15% w/v Ficoll® in water) and loaded on a 0.7% agarose gel (containing 50 µL Ethidium bromide) and run at 50 V in a TBE buffer (pH=7.6), until bromophenol blue reached the bottom of the gel (2 h). The gel was photographed on a UV (302 nm) transilluminator with a Kodak EasyShare digital camera. DNA cleavage was quantitated with ImageQuant TL.
References


32. P. J. Proteau, Provided authentic sample of kinamycin D.


Appendix
Pulse Sequence: s2p1  
Solvent: CDCl3  
Ambient temperature:  
Mercury-400B, "nccmeric400"  
Pulsed 70° pulse  
RF power 1.28 W  
Width 2390.0 Hz  
32,000 scans  
Acquire 63.146146 MHz  
DECOUPLING: H1, 689.137141 MHz  
Power to all  
Continuously on  
Waistline modulated  
Line broadening 1.0 Hz  
Relative scale  
Total time 0 min, 0 sec
Pulse Sequence: eZpol
Solvent: Acetone
Ambient Temperature
Mercury-300B 4recht30B8

Pulse delay 1.00E-03
Pulse 55.4 degree
Acq. time 1.955 sec
VSpin 40.8 L Hz
16 repetitions
DRB rest: 4 Hz; 23.782506 MHz
D9 rest: 23.850 MHz
T1 ref 23708
Total time: 8 min, 69 sec
1D OBSERVE

Pulse sequence: szpul
Spin 1: 199.26 ppm
Temperature
Magnetic field: 15.00 T

Pulse 70.0 degrees
Echo time: 3.100 sec
Flip angle: 90 degrees

Saturate C, 50.84.61/5727 MHz
DECouple B1, 456.183/2146 MHz
Pulse Modulation

Data processing

Total time: 1 hr, 13 min, 55 sec
NH₂
\[ \text{N-NHTs} \]

1-xiv
Pulse Sequence: SI2PUL

Polypeptide analysis
Anion at temperature
Mercury-1986 "nucmerc460"

Pulse 70-8 degrees
Acq. Time: 1.698 sec
6400 repetitions

Resonance 30.564527 MHz
Decoupling 71.585448 MHz
Powers 90-90

Continuous on

Monitor 14 accumulated

Total time 1 hr, 27 min, 55 sec

1-xiv

\( \text{NH}_2 \quad \text{N-NHTe} \quad \text{NH}_2 \)

1-xiv
13C NMR

Pulse sequence: s2pul

Averaging: Automatic

Temperature: 298K

References: 0.25 ppm

Water suppression: Automatic

Residual water suppression: 10.2 ppm

Chemical shifts: 150.8, 128.4, 111.3, 108.7, 104.3, 14.3 ppm

Proton coupling: 8, 8, 8, 8, 8, 8, 8 Hz

Decoupling: Off

Power: 400 W

Mixing time: 3 sec

Data acquisition time: 10 sec

Number of scans: 32

Total run time: 2.5 min

Signal to noise: 200:1

Chemical structure:

24

NH₂

N₂
Pulse sequence: s2pm
Solvent: Acetone
Ambient temperature
Field: Liquid-Phase Fluoroenol
Mercury-300B: "chemical.c3b"
Relax. delay: 1,100 sec
Pulse flip angle: 90°
Data: 128 pts, 128 sec
FT: 256 pts, 128 sec
32 repetitions
RESOLVE: 102, 0.0508412 MHz
DATA PROCESSING
FT size: 512
Total time: 8 min, 6 sec
STANDARD IN OBSERVE

Pulse Sequence: szpu
Solvent: Acetone
Ambient temperature
Field: 300 MHz/9.4 T
Mercury-300B - cimoseride

Relax. delay: 1.200 sec
Pulse: 7.5 degrees
Wait time: 1.200 sec
32 repetitions

OBSERVE: 9.0, 9.0

DATA PROCESSED: 4K
Total time: 8 min. 36 sec.

1-iii

OMe

9 8 7 6 5 4 3 2 1 0 ppm
1.65 2.11
3.03 1.96
3.36
1H NMR

- Proton spectrum
- Chemical shift, 0.32 ppm
- Multiplicity, singlet
- Integration, 1H
- Resonance at 0.32 ppm

**Note:** The diagram shows a spectrum with a peak at 0.32 ppm, indicating the presence of a proton at that chemical shift.
STANDARD IN OBSERVE

Pulse Sequence: s/zp
Solute: CDCl3
Ambient Temperature
Mercury-30008 *nemeroc300*

Relax. delay 1.000 sec
Pulse 90.0 degrees

Width 0.106 MHz
16 repetitions
OBSERVE 81.259.718.101 MHz
DATA PROCESSING
FT 1/2 32768
Total time 2 min, 41 sec.

OMe
N-NH Ts

1-iv
NMR OBSERVE

Pulse Sequence: szpul

Pulse set 90°

Ampl. 00010

Ambient temperature
Mercury=40380 °C

Pulse 78.8 degrees

Relax. time 6.28 sec

Width 25000.0 Hz

128 repetitions

Sample cuj, 100.614055 MHz

Decoupled N1, 100.137141 MHz

Power 10 dB

Continuously on

With time modulated

Line broadening 1.0 Hz

Total time 1 hr, 37 min, 55 sec

OMe N-NHTs

1-iv
Pulse sequence: sdpul
B1 = 4.4, 50000 Gauss
Ambient temperature
Mercury = 60, 60.0°C

Pulse 70.0° degrees
Res. time 1.283 sec
Width 25.097 kHz
100 cycles

OBSERVE C, 100.6340505 MHz
DECOUP N1, 600.1371641 MHz
Power 470 mW
Continuous on

MAG Puls demodulated
Line broadening 1.0 Hz
At room temp
Total time 1 hr, 37 min, 55 sec
Pulse Sequence: s2pul
Cabinet: Anstec
Ambient temperature
Mercury-400DD "Micromercury"
Pulse 90.0 degree
Avg time 1.002 sec
Width 2.5000 Hz
Field strength
GND: 0.00 Hz
DECouple: 1 HZ 0.1592868 MHz
Sweep 1.00 Hz
Delay 0.01 sec
Spectrum on
WALTZ-16 modulated
NMR Processing:
Line broadening 1.0 Hz
FI size: 65536
Total time 1 hr 32 min 55 sec
Pulse Sequence: 2pul
Solvent: CDCl3
Ambient temperature: Mercury-60885 "nucmerc 009"
Relay delay: 2.000 sec
Polarization: 79.0 degrees
Act. time: 1.198 sec
Width: 25500.0 Hz
Jd=22.8 Hz
QS=0.0 Hz
QS=0.0 Hz
QS=0.0 Hz
Power: 46 dB
Continuously on
DATA PROCESSING
Line Broadening: 3.0 Hz
File size: 00004
Total time: 14 hr, 24 min, 52 sec

1-viii
1-ix

NO₂ N-NHTs

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**TSTANDS IN OOSERVE**

**Archive directory:** /project/home/standa/spears/ssys/data

**Sample directory:** /archive/12400020/12400020/

**File:** 12400020

**Date:** 2005/10/10

**Solvent:** CDCl₃

**Ambient temperature:** 298 K

**Mercury:** 400.686 MHz "n.c" numereted

**Relaxation delay:** 1.060 sec

**Acq. time:** 1.88 sec

**Width:** 5110.3 Hz

**640 repetitions**

**Instrument:** HA 300.125152 MHz

**Number of data points:** 32768

**Total time:** 8 min, 38 sec

---

1.98 1.13 3.29 4.21
1.82 1.32 1.50
3.00 3.00
Pulse Sequence: s2pul
Solvent: Acetone
Ambient temperature: Mercury=20.0C

Pulse 72.0 degrees
Rate: 1.233 sec
Width: 2500.0 Hz
228 repetitions

DECOUPLING: W1, 500.1364 Hz
Power: 40 dB
WIDEN: ON
MULTI-E modulation
LINE BROAD: 1.0 Hz

Total time 1 hr, 57 min, 55 sec
Pulse Sequence: t2pul

Solvent: dichloromethane

Ambient Temperature

Mercury-400B - "recrubex600"

Pulse 70.0 degrees

Fade time: 1.9. sec

Pitch: 60.0 MHz

2000 pulses

Observation 300, 106.616577 MHz

Decoupled: H1, 406.139248 MHz

Power: 40 dB

continuously on

WALTZ-16 modeled

T1r: 1.2 sec

T1e: 1.2 sec

Line broadening: 1.0 Hz

Power decouplers

Total time: 1 hr, 37 min, 55 sec
13C OBSERVE

Pulse Sequence: s2pul1
Cycles: 50,000
Ambient temperature: -50°C
Mercury = 408 hz, 40000 hz

Pulse 70.0 degrees
MLP 15000 hz
MLP 15000 hz
880 repetitions
OBSERVE C13, 100.6140505 MHz
DECOUPLE H1, 60.1230541 MHz
Power 40 dB
continuously on
UNIT was completed
Line broadening 1.8 Hz
Total time 1 hr, 37 min, 55 sec

25
Pulse Sequence: sipul

Sample: NMR sample
Ambient temperature: Room temperature
Mercury-3600 "Intumerc630"

Pulse 12.0 degrees
Delay 1186.71 sec
Spin 11577.66 Hz
Line experiment

Spectrum: C25, 75.49537 MHz
DECOUPLING: H2, 300.008450 MHz
Power 35 W
Continuously on
MULTI-11 modulated

Line broadening: 1.8 Hz
150 ppm interval
Total time: 4 hr, 31 min, 49 sec
STANDARD IN OBSERVE

Pulse Sequence: szpul
Solvent: Acetone
Chemical temperature
Mercury-300BB "excimercc30"

Relax. delay 1.000 sec
Pulse 37.1 degree
Width 14.65 Hz
X 2 repetitions
OBSERVE 1H, 39.1990349 Hz
Data processing
FT time 32768
Total time 3 min 52 sec

1-vi

OMe
Isocyanate

Instrument: spectrometer

Newcastle site

Sample temperature

Acetone-1,1,1-trifluoroethane-1,1,1-trifluoroethane

Pulse width 2.0 degrees

Acq. time 1.885 sec

Sweep 60 Hz

1600 repetitions

Sample rate 0.5125 GHz

Recycle time 8 sec

Continuously on

Wait-16 modulated

Data processing

Line-broadening 1.0 Hz

Total time 1 hr, 37 min, 55 sec

1-vii
Pulse Sequence: s2pul
Antenna: G6113
Ambient Temperature
Mercury-39066 "Nexismerc390"

Pulse 90.0 degrees
Mix time: 1.00 sec
Total time: 20.00 sec
Total experiments
Observe 315, 25.58 GHz MHz
Redundant 3.1, 25.5802026 MHz
Power 30 dB
Continuously on
WALTZ-16 modulated
Linewidth: 1.0 Hz
Residual Interferon
Total time 45 min, 4 sec
Pulse Sequence: 62pul
Solvent: Acetone
Temperature: Ambient
Mercury-199B "Hermes 300"
Relax delay 1.000 sec
Pulse 40.0 degrees
Data point 1.0 sec
Silent time 1.0 sec
6 replications
OBSERVE 51.296396 MHz
DATA PROCESSED
FT size 32768
Total time 6 min, 25 sec

23
**Spectrum Image**

- **Sample Name:** \textit{SampleName}\_20051280
- **Solvent:** DMSO-d6
- **Acquisition Parameters:**
  - Field Frequency: 600.108 MHz
  - Repetition Time: 1.000 sec
  - Pulse Delay: 1.000 sec
  - Pulse Angle: 65.0 degrees
  - Number of Scans: 6

**Chemical Structure:**

![Chemical Structure Image]

**Notes:**
- Peaks observed at specific ppm values.

1-xi

N-NH₂

\[
\text{NMR spectrum of 1-xi}
\]

1H NMR (400 MHz, CDCl₃) δ 7.77 (d, J = 8.0 Hz, 2H), 7.44 (d, J = 8.0 Hz, 2H), 7.31 (t, J = 7.2 Hz, 1H), 7.20 (t, J = 7.2 Hz, 1H), 7.16 (m, 2H), 7.07 (m, 2H), 7.00 (s, 1H), 6.88 (d, J = 8.0 Hz, 1H), 6.80 (d, J = 8.0 Hz, 1H), 3.56 (s, 3H), 2.80 (s, 3H).
Pulse Sequence: m2pul
Saturation: DMP10
Ambient temperature: -100°C
NMR: Varian 400 MHz

Pulse: 70.0 degrees

Data:

-98.190 Hz
-100.0 Hz

Spectra:

-98.190 Hz

Coupling HL: 160.00 Hz
Power: 48 dB
Continuously daq

Total time: 1 hr, 37 min, 55 sec

1-xi
88
Pulse Sequence: 52pul

Acquire: 90° 21.31 sec
52pul 1.413 sec
17.15 sec

HVCRCPS 52pul

Observe: 62.154 61.52727 MHz

Decouple: 46.1932408 MHz
Power 500 mW

Continuously on

Data Processing

Line broadening 1.0 Hz
Total time 1 hr, 37 min, 55 sec
Pulse Sequence: 52ųv1
Solvent: DMSO
Ambient temperature: Mercury-400SB "Acromer409"

Pulse 20.0 degrees
Acq. time 1.0 sec
No. of scans 40000
No. of repetitions

Observe 400.1371641 MHz
Decoupler H1, 400.1371641 MHz
Power: 10 W
Continuously on
WHIT: 16 modulated
Larmor frequency
Line broadening 1.0 Hz

Total time 1 hr, 37 min, 55 sec

1-xvi
93

Pulse Sequence: 1G72
Pulse: 70.6 degrees
Window: 1.45 sec
Wait: 2000.0 Hz
TR: 0.60 sec
DE: 10.5, 10.5 Hz
Pseudospin decoupling
WALTZ-16 modulated
Pulse repetition time: 1.0 Hz
FI dwell: 25550
Total time: 1 hr, 37 min, 55 sec

29

ppm
archive directory: report/chem/camosy/synergy/data
sample directory: strchns0_28Oct2013
file: PHM03
pulse sequence: e2pu1
solvent: CDCl3
ambient temperature: Mercury-400B
"hessmerc400"
relax. delay 1.000 sec
pulse 45.0 degrees
acq. time 1.993 s/c
width 5000.3 Hz
32 repetitions
spectral width 0.91415.213537 Hz
marker width 0.001150 Hz
fft size 32768
total time 1 min, 48 sec

![N2]

30
Pulse Sequence: 62pwI
Solvent: 2000.0
Ambient Temperature
Mercury-400Sb "octawera400"

Pulse 70.5 degrees
Acq. time 1.23 sec
Width 250.60 Hz

OBSERVE: 100.614015 MHz
DECOUPL: 90.000, 137.1661 MHz
Power 40 W

WALTz=18 modulated
Data Processing
Line Broadening 1.0 Hz
FF line 256k
Total time 1 hr, 37 min, 55 sec
CHAPTER 2
Kinamycin-mediated DNA Cleavage

2.1 Investigation into the Biomimetic Activation of Kinamycin D

As part of our research program directed toward understanding the mechanism by which the kinamycins/lomaiviticins cleave DNA, we have shown that kinamycin D cleaved DNA in the presence of dithiothreitol (DTT). Furthermore, we were able to recapitulate this activity with simple, electronically tuned diazofluorene analogues, and demonstrated that these simple analogues had anti-proliferative activity against HeLa cells.¹ The conditions used to affect DNA cleavage in the previous work were based on preliminary data and it was deemed prudent to fully delineate the effect that temperature, thiol concentration, and thiol identity have upon the extent of kinamycin-mediated DNA cleavage. From these studies, we demonstrate that kinamycin D displays potent DNA damaging capability under biomimetic conditions.²

Modest kinamycin-mediated DNA cleavage was observed after 48 hours when the reaction was initiated in the presence of a large excess of thiol ([DTT] = 1.0 M). Although these conditions successfully promoted kinamycin-dependent DNA cleavage, the large excess of DTT employed was not consummate with typical intracellular thiol concentrations. Glutathione (GSH) is the most abundant intracellular thiol with concentrations ranging from 0.1 to 10 mM.³⁵ Given this disparity, it was uncertain if our previous work was directly applicable to potential models of in vivo activity.
To address this discrepancy, a room temperature kinamycin D-mediated DNA cleavage study was performed in which the thiol identity (DTT or GSH), thiol concentration and kinamycin D concentration were varied. It was found that moderate DNA cleavage was observed at low mM concentrations of DTT (Figure 8a) and no cleavage with moderate µM concentrations of GSH (Figure 8c) at room temperature. The extent of DTT-mediated DNA cleavage under these conditions was similar to what was observed under 1.0 M DTT conditions.

Next, the effect temperature had upon the extent of DNA cleavage was examined. DNA cleavage experiments with kinamycin D at physiological temperature (37 °C) showed that potent DNA cleavage activity far surpassed cleavage results at room temperature (Figure 8b/d). The result with GSH is particularly significant because it represents a molecular ratio of 5:1/GSH:kinamycin D when [KinD] = 250 µM (Figure 8d, lane 9). Kinamycin D was also shown to display time-dependent DNA cleavage activity at 1 mM at 37 °C in both DTT and GSH (Figure 9). The plasmid was completely degraded within 24 hours in DTT while a minimal amount DNA remained at 24 in GSH (Figure 9). Alternative
biological reductants, such as NADPH, failed to induce kinamycin D-mediated DNA cleavage under these conditions as had been shown previously at room temperature.

Given that sulfides are a well-established source of $2e^-$ reductions, it is unlikely that under these biomimetic conditions a $1e^-$ reduction is occurring as Feldman has postulated. We have previously documented that simple nucleophiles will not promote kinamycin D-mediated DNA cleavage; therefore simple nucleophilic activation is also unlikely. To resolve this ambiguity, the LUMO of kinamycin D (without substituted D-ring) was modeled with Spartan (Figure 10). This model predicts that the LUMO of kinamycin D resides within the benzoquinone portion of the molecule. Therefore, it is reasonable to predict that under the mild reducing conditions employed in this study that a $2H^+/2e^-$ reduction occurs to reduce the benzoquinone to the hydroquinone 31 (Scheme 9).

Figure 9. Time-dependent Plasmid Cleavage Assay, top band is type II nicked DNA, middle band is type III linear DNA, bottom band is type I supercoiled DNA. Conditions: all lanes contain 714 ng pBR322 DNA, lanes 1-3 final [KinD] = 1.0 mM, assay run at 37° C. Lane 1, 48 h; lane 2, 24 h; lane 3, 12 h; lane 4, [KinD] = 0 mM for 48 h.

Figure 10. Kinamycin D (model) LUMO.
Once the hydroquinone is formed, there are two potential routes for DNA cleavage that parallel the previous proposed mechanisms (Scheme 10). The first is nucleophilic attack on the distal diazo nitrogen, which would subsequently undergo homolytic cleavage to generate a carbon-based radical 32 that would mediate DNA strand scission (Route A; Scheme 10). Construction of the LUMO map of the hydroquinone clearly shows the LUMO resides on the diazo group (Figure 11). This route proves viable based on the already preceded carbon-based radical hypotheses of both Dmitrienko, Eastman and the LUMO map of the hydroquinone (Figure 11).6, 7

Scheme 9. Reduction of kinamycin D.

The second route is protonation followed by spontaneous decomposition of the diazo group to generate an orthoquinone methide 33 (Route B; Scheme 10). To the best of our knowledge, there have been no published accounts of an orthoquinone methide mediating DNA cleavage under the conditions that were employed in this study. Orthoquinone methides have been proposed as the active alkylating species for the structurally similar anthracycline drugs but this has also been brought into question and alkylation does not, in itself, cause DNA cleavage.\textsuperscript{8,9} The hydroquinone LUMO map, coupled with the extensive DNA cleavage observed in this study suggests that once kinamycin D has been reduced, nucleophilic activation resulting in a carbon based radical is necessary to initiate DNA cleavage; however further mechanistic studies will be required to deconvolute these two pathways.

\textbf{2.2 Conclusion}

We have demonstrated that kinamycin D has potent DNA cleavage activity under biomimetic conditions.\textsuperscript{2} Additionally, while this research was in progress, Hasinoff and Dmitrienko also showed that kinamycin F cleaves DNA at 37 °C using 5 mM GSH.\textsuperscript{10} Based upon the mild conditions that were employed in this study in conjunction with our LUMO calculations, it is probable that the first step of kinamycin D-mediated DNA cleavage is a
$2H^+/2e^-$ reduction to the corresponding hydroquinone. LUMO calculations and previous results from our research group suggest that nucleophilic activation is subsequently required to mediate DNA cleavage; however further experiments are necessary to substantiate this mechanism.
2.3 Experimental Section

**General Procedure for DNA Cleavage Assay**

Kinamycin D was isolated as previously described from *S. murayamaensis* (ATCC 21414) and dissolved in DMF. Covalently closed, supercoiled DNA (pBR322) was used as supplied by New England Biolabs in a 10 mM Tris-HCl buffer (pH=8.0) containing 1.0 mM EDTA. DL-dithiothreitol (DTT), glutathione (GSH) and β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) were dissolved in dH₂O. Incubations with DNA were carried out in a microfuge tube with the total volume not exceeding 4 µL. The reactions were conducted in the dark at room temperature (25 °C) or 37 °C for 48 h. DNA samples were then mixed with 8 µL of 10X Ficoll® loading buffer (0.25% xylene cyanol FF, 0.25% bromophenol blue, 15% w/v Ficoll® in water) and loaded on a 0.7% agarose gel (containing 50 µL Ethidium bromide) and run at 50 V in a TBE buffer (pH=7.6), until bromophenol blue reached the bottom of the gel (2 h). The gel was photographed on a UV (302 nm) transilluminator with a Kodak EasyShare digital camera. DNA cleavage was quantitated with ImageQuant TL.

**Dose Response DNA Cleavage Assay**

Followed general procedure. Cleavage assay with 0.57 mM GSH at 37 °C required the total volume to be increased to 8 µL while all concentrations of components were adjusted accordingly.
Time-Dependent DNA Cleavage Assay

Followed general procedure. 48h time point setup first, followed by 24h and 12h time points respectively. Cleavage assay with 0.57 mM GSH required the total volume to be increased to 8 µL while all concentrations of components were adjusted accordingly.
References


CHAPTER 3

2-Aminoimidazoles as Modulators of Bacterial Biofilms

3.1 Bacterial Biofilms

Recent developments in the fields of bacteriology and infectious disease have revealed that bacteria often exist as intertwined communities rather than as independent planktonic microorganisms. These surface associated microcolonies have come to be known as biofilms and are ubiquitous in nature. It has been estimated that approximately 80% of the world’s microbial biomass resides in the biofilm state. Bacterial biofilms are often described as surface-associated complex communities of bacteria encased in a protective extracellular matrix. Pathogenic infections commonly persist due to the respective bacteria’s ability to form robust biofilms which are much less susceptible to traditional means of antiseptic and antibiotic therapy. Persistent infections of indwelling medical devices is also of grave concern as eradication of these biofilms is virtually impossible. Additionally, given the combination of high morbidity and mortality rates of infectious diseases due to biofilm virulence, a clear and decisive avenue is needed to pave the way towards new anti-biofilm modulators.

*Pseudomonas aeruginosa* is an opportunistic Gram-negative γ-proteobacteria that is relatively innocuous to healthy individuals and is ubiquitously present throughout the environment. However, *P. aeruginosa* is the second most common pathogenic bacteria in hospital-acquired pneumonia and is a serious threat to cystic fibrosis (CF) patients. A combination of the genetic disposition and abnormal composition of respiratory airways in CF patients in conjunction with the virulence of *P. aeruginosa* commonly leads to chronic
infection which then portends increased morbidity and mortality rates. The inability to treat CF patients with chronic \textit{P. aeruginosa} infections has been directly correlated to the virulence of \textit{P. aeruginosa} biofilms.\textsuperscript{8,10}

Another opportunistic \(\gamma\)-proteobacterium that has become a serious threat over the last decade due to its multi-drug resistance is \textit{Acinetobacter baumannii}.\textsuperscript{14} This Gram-negative bacterium is also quite benign to the healthy populous, but onset of an \textit{A. baumannii} infection can life-threatening. \textit{A. baumannii} biofilms are particularly hardy, being able to survive for weeks on inanimate objects thus making eradication especially problematic.\textsuperscript{15} Approximately 25\% of all hospital swabs are positive for \textit{A. baumannii}.\textsuperscript{16}

\textbf{3.1.1 Structure and Properties}

Specifically, biofilms are a microbially derived sessile community characterized by cells attached to a surface, or each other and are embedded in a matrix of self-produced extracellular polymeric substances and exhibit altered phenotypes in respect to growth rate and gene transcription (\textbf{Figure 12}).\textsuperscript{5,17} The alteration of the bacterial phenotype has a profound impact on the medical community because this is often displayed as increased resistance to antibiotics and the host immune response leading to chronic infections. Developed biofilms are not structurally homogeneous layers of cells, but rather, heterogenous microcolonies of cells within the biofilm matrix.\textsuperscript{5} The tendency for these microcolonies to break away from mature biofilms is of great concern to the medical community as these microcolonies retain all of the biofilm genetic traits (i.e. antibiotic resistance).
Biofilms ultimately begin to form when a signal causes a switch from the planktonic state to a sessile growth phase. Quorum sensing (QS) is known as a way for cells to communicate with each other through signaling molecules and one of the predominant uses for QS is the determination of cell density necessary to form biofilms. Communication based on QS is commonly triggered by N-acyl homoserine lactones (AHL) in gram-negative bacteria.

### 3.1.2 Influence and Effect on Society

Biofilms underpin a significant list of problems encountered in the agricultural, engineering, and medical sectors of the global economy. The NIH estimates that approximately 3 out of 4 microbial infections that occur in the body are biofilm mediated. Biofilms also underlie high morbidity rates in patients who suffer from cystic fibrosis as described above. Bacteria that reside within the biofilm state display different phenotypes.
than their planktonic brethren and become more resistant to many antibiotics and biocides that would often lead to their eradication\textsuperscript{6,17}.

As most pathogenic bacteria exist as biofilm communities, which hinder antibiotic effectiveness and adorn resistant traits, the discovery and development of anti-biofilm modulating compounds should be of utmost importance to the medical community. Anti-biofilm modulators have the ability to inhibit biofilm formation in a non-toxic manner and/or disperse pre-existing biofilms into the planktonic state thus presenting the possibility of dosing a potent antibiotic to disrupt pathogenesis and eradicate the infection more effectively.

### 3.2 Chemical Controls of Biofilms

Despite the prevalence of biofilms in our society, examples of molecular scaffolds that inhibit biofilm formation are scarce (Figure 13). These limited examples include the homoserine lactones (31) which are naturally occurring signaling molecules that elicit their activity through disruption of bacterial communication\textsuperscript{20}, brominated furanones (32) which were originally isolated from the macroalga \textit{Delisea pulchra}\textsuperscript{21,22} and ursene triterpenes (33) from the plant \textit{Diospyros dendo} (Figure 1)\textsuperscript{23}.

![Figure 13. Molecules Known to Inhibit Biofilm Formation.](image-url)
Interest in the chemical control of biofilms has been growing steadily over the last few decades. Many synthetic biofilm modulating compounds have been based on AHLs in hopes of having agonistic/antagonist properties. Unfortunately, many of the synthetic AHL analogues are much less potent than the endogenous AHLs which may lead to toxicity issues upon further analogue development. Blackwell has provided a great contribution by synthesizing a diverse array of AHLs and assaying them against several biofilm bacteria strains, including *P. aeruginosa*. Through her work, it was discovered that only minor changes in the aliphatic side chain of the AHLs could modify their activity considerably. It is also worth noting that screening of a 66,095 compound library by Clardy for anti-biofilm activity resulted in only 61 active compounds with only 30 of those possessing IC$_{50}$ values less than 20 µM.

### 3.3 Oroidin as a Scaffold for Biofilm-Active Analogue Generation

Our research has been focused on the generation of small molecule libraries that have been based on the oroidin scaffold. The activity of oroidin has been documented in a limited number of studies involving bacterial attachment and colonization. Oroidin has also been shown to inhibit biofouling driven by the marine α-proteobacteria *R. salexigens*. Due to this structural simplicity and documented anti-biofilm activity, oroidin was chosen as a lead compound for structure-activity-relationship (SAR) analysis in order to identify new structural motifs capable of disrupting biofilms.
Several diverse and functionalized libraries have been developed in our lab from oroidin with some being based directly on the scaffold while others only borrow minimal features (Figure 14). From these synthetic libraries, many potent and effective anti-biofilm modulators have been identified and assayed against a number of medically relevant biofilm forming bacteria.29-35 One of the most unique approaches involved reversal of the amide bond moiety which connects the pyrrolecarboxamide tail to the 2-aminoimidazole (2-Al) head of oroidin 34 (Figure 15).

Figure 14. Oroidin as a Scaffold for Anti-biofilm Analogues.

Figure 15. Retrosynthetic Analysis of Oroidin and the RA Scaffold.
Of direct comparison, previous analogue generation based on oroidin involved derivatization achieved through acylation of the alkyl amine of 35 by various trichloroacetyl pyrroles. Several of the major limitations associated with this pathway include: the lack of compatibility of the system with many trichloroacetyl esters, solubility of the parent 2-AI, and the cumbersome purification of acylated intermediates. Notably, the acylated analogues required large amounts of methanol saturated with ammonia to purify due to the very polar and unprotected 2-aminoimidazole. Efforts to recapitulate and improve upon the anti-biofilm activity of oroidin employing a reverse amide motif is described below.
References


CHAPTER 4

A Reverse Amide (RA) Approach to Oroidin Analogues

Implementation of a Reverse Amide (RA) approach would allow for a more facile generation of analogues that would be otherwise inaccessible through the oroidin pathway. Additionally, many of the handicaps would be eliminated using a reverse amide approach. Installation of the RA bond could be obtained through direct aminolysis of an appropriate alkyl ester or through couplings of a carboxylic acid intermediate (Figure 16). These intermediates could be accessed through α-bromoketones which are quickly derived from the requisite acid chloride through diazomethane homologation. The use of an α-bromoketone allows for condensation with Boc-guanidine to produce a Boc-protected 2-AI instead of having to work with the problematic unprotected 2-AI headgroup implemented in previous studies. In addition, significant diversity is attainable by incorporating any commercially available amine with a common RA intermediate scaffold (38 and 39) far surpassing the diversity available in trichloroacetyl esters available to couple with 35.

Figure 16. Synthetic Accessibility of RA Analogues.
4.1 Synthesis of a Pilot RA Library

Scaffold synthesis began with treatment of the commercially available acid chloride 40 with diazomethane (Scheme 11).\(^1\) Quenching with concentrated HCl or HBr delivered the corresponding α-haloketones in excellent yields which were isolable by column chromatography. Installation of the protected 2-aminoimidazole moiety was achieved through a Boc-guanidine condensation in DMF at ambient temperature to yield 38. Significantly higher yields for this step were obtained when two equivalents of sodium iodide were added to the reaction mixture and this represents a significant improvement over previous reports.\(^2\), \(^3\) It was also observed during this sequence that the α-bromoketone afforded higher yields than its α-chloro counterpart in the cyclization reaction.

**Scheme 11.** 1st Generation Synthesis of RA Scaffold. *Reaction conditions:* (a) \(\text{CH}_2\text{N}_2\), Et\(_2\)O/CH\(_2\)Cl\(_2\), 0 °C ii. conc. HCl (90%) or conc. HBr (93%) (b) Boc-guanidine, NaI, DMF, 65% (c) LiOH, MeOH-THF-H\(_2\)O (3:1:1) then 1N HCl to pH = 5, 94% (d) AlMe\(_3\), NHR\(_1\)R\(_2\), DCE, 0 °C to 60 °C (e) TFA, CH\(_2\)Cl\(_2\) (f) 2M HCl in Et\(_2\)O.

The first approach to the RA scaffold relied heavily on the aminolysis of intermediate 38 since this would afford the Boc-protected RA precursors in a single synthetic step. After deprotection with TFA and HCl salt exchange, isolation of the targets would require only filtration with no need for further purification. Based upon the seminal paper published by Weinreb on the transformation, trimethylaluminum was used as the Lewis acid to affect the
direct aminolysis reaction. Numerous reaction factors were taken into account such as choice of solvent, equivalents of aluminum-amine complex, reagent order of addition, time, and temperature. Despite all of the conditions tested, the highest yielding reaction occurred in only 55% yield when aniline was used as the amine partner. Triazabicyclo[4.4.0]deca-5-ene (TBD) was also examined as a potential catalyst to promote the direct aminolysis of ester 38. Heating both starting materials in the presence of 30 mol % of TBD in toluene at elevated temperatures for extended periods of time failed to produce any desired product as evident by TLC analysis.

Due to the problems encountered utilizing aminolysis, we opted for a more conventional route to access the RA scaffold through the intermediacy of an activated carboxylic acid. Unfortunately, saponification of the methyl ester 38 proved problematic on this system as cleavage of the Boc group was observed under the basic conditions of both LiOH/MeOH/THF/H2O or LiI/pyridine. Decomposition of the methyl ester was also observed when TMSOK in methylene chloride or (Bu3Sn)2O in toluene at either ambient temperature or reflux were employed as the saponification agents.

Persuaded by these results that the current route required revision, a second generation approach to our core scaffold was envisioned (Scheme 12). This approach relied on a different protecting group strategy, substituting the methyl ester for a benzyl ester which, in the case of another failed attempt at aminolysis, would undergo hydrogenolysis under mild conditions to deliver the corresponding Boc-protected acid 43. Synthesis began with the known mono benzyl ester acid 42 which was transformed into the benzyl protected α-bromoketone by conversion to its acid chloride followed by diazomethane homologation.
and concomitant quench with concentrated HBr. Cyclization of this intermediate afforded the Boc-protected 2-AI 39 in 66% yield. All attempts at direct aminolysis of benzyl ester 39 resulted in sluggish reactions that were plagued by the formation of multiple side products.

![Scheme 12. 2nd Generation Synthesis of RA Scaffold; Reaction conditions: (a) i. (COCl)₂, DMF (cat.), CH₂Cl₂ ii. CH₂N₂, Et₂O/CH₂Cl₂, 0 °C iii. conc. HBr, 88% (b) Boc-guanidine, DMF, 66% (c) H₂ (1 atm), 10% Pd/C, THF, 98% (d) EDC, HOBt, NHR₁R₂, DMF (e) TFA, CH₂Cl₂ (f) 2M HCl in Et₂O](image)

Given the failure of the direct aminolysis conversion, the two-step approach to the RA scaffold was investigated. Deprotection proceeded as planned and was accomplished by subjecting 39 to a hydrogen atmosphere at balloon pressure which cleanly afforded pure Boc-protected acid 43 in near quantitative yield (98%). With the acid prepared and available on a multi-gram scale, attempts to install the key amide bond were assessed. A number of activating agents were scanned including DCC, EDC, HCTU, CDI, and cyanuric chloride to affect the transformation. Of those listed only EDC and HCTU were able to give consistent and tangible results. EDC was chosen over HCTU due to ease of purification in separating side products during column chromatography. It was during this optimization that the limitation of the synthetic route was identified to be the reactivity of the Boc group. A significant quantity of a Boc-protected starting amine was isolated and characterized, signifying the lability of the Boc-group due to Boc-transfer under the reaction conditions regardless of which activating agent was used.

With two routes in hand to generate the RA scaffold, we assembled the focused library outlined in **Table 4.** EDC/HOBt couplings of acid 43 were used to generate most of
the linear alkyl chain analogues (28-34%) while aminolysis of the methyl ester intermediate 28 furnished the remaining compounds (11-55%) in the library (Table 4). The final step of the synthetic approach required removal of the Boc-group, which proceeded at room temperature in TFA/DCM. The resulting trifluoroacetate salts of each target were then exchanged for their HCl counterparts before characterization and assessment of their biological activity.

Table 4. Completion of the RA Library.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Conditions</th>
<th>Coupled Product</th>
<th>Target</th>
</tr>
</thead>
<tbody>
<tr>
<td>isobutylamine</td>
<td>a</td>
<td>44</td>
<td>53</td>
</tr>
<tr>
<td>hexylamine</td>
<td>b</td>
<td>45</td>
<td>54</td>
</tr>
<tr>
<td>octylamine</td>
<td>b</td>
<td>46</td>
<td>55</td>
</tr>
<tr>
<td>decylamine</td>
<td>a</td>
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</tr>
<tr>
<td>dododecylamine</td>
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<td>57</td>
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<tr>
<td>cyclopentylamine</td>
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<td>49</td>
<td>58</td>
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<tr>
<td>morpholine</td>
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<tr>
<td>2-aminopyrimidine</td>
<td>a</td>
<td>52</td>
<td>61</td>
</tr>
</tbody>
</table>

Reaction conditions: (a) AlMe₃, 38, DCE, 0 °C to 60 °C (b) 43, EDC, HOBt, DMF (c) TFA, CH₂Cl₂ (d) 2M HCl in Et₂O

4.2 Inhibition of *Pseudomonas aeruginosa* Biofilms

*P. aeruginosa* is an opportunistic γ-proteobacterium that is a serious threat to immunocompromised patients and is frequently isolated from patients found in intensive care units suffering from severe burns or other traumas. For cystic fibrosis patients, the onset of colonization by this bacterium is of great concern. Morbidity rates of patients who suffer from the disease are directly correlated to the virulence of *P. aeruginosa* biofilms.⁷, ⁸ The
speed and prevalence with which multidrug resistant (MDR) strains are appearing puts pressure on the medical community to find ways to combat the aggressive nature of this bacterium.9

Members of the reverse amide library along with oroidin10 were initially screened at 500 µM in a 96-well format using a crystal violet reporter assay to assess each compound’s ability to inhibit the formation of PAO1 or PA14 biofilms (Figure 17).11 Compounds 35, 62, and 41 were used as controls in the assays and all showed only marginal inhibition. There was a remarkable range of activities among the RA compounds analyzed in the inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Inhibition vs. PAO1</th>
<th>% Inhibition vs. PA14</th>
</tr>
</thead>
<tbody>
<tr>
<td>oroidin (34)</td>
<td>&gt; 95</td>
<td>&gt; 95</td>
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<tr>
<td>35</td>
<td>14 ± 3</td>
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<tr>
<td>61</td>
<td>31 ± 4</td>
<td>55 ± 5</td>
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Figure 17. Preliminary Inhibition Data at 500 µM. All values are averages of at least experiments.

Members of the reverse amide library along with oroidin10 were initially screened at 500 µM in a 96-well format using a crystal violet reporter assay to assess each compound’s ability to inhibit the formation of PAO1 or PA14 biofilms (Figure 17).11 Compounds 35, 62, and 41 were used as controls in the assays and all showed only marginal inhibition. There was a remarkable range of activities among the RA compounds analyzed in the inhibition
assay. Similar activities were observed between PAO1 and PA14, although most compounds were slightly more potent against PA14. Interestingly, this trend is opposite to our previously reported bromoageliferin analogues.¹² These screens also suggested that the aliphatic chain derivatives (54-57) and oroidin 34 were very potent inhibitors of *P. aeruginosa* biofilms.

Subsequently the aliphatic derivatives (54-57) and oroidin 34 were selected for IC₅₀ value determination against PAO1 and PA14 (Table 5). The generation of dose-response curves for compounds 54-57 revealed a correlation between the length of the carbon chain and the potency of the compound. This trend is apparent when the IC₅₀ values are plotted as a function of chain length in both PAO1 and PA14 (Figure 18). Increasing the chain length from six to twelve carbons effectively increased the inhibition activity over a full order of magnitude in both strains. All linear carbon chain analogues were significantly more potent than oroidin (PAO1 IC₅₀ = 190 µM, PA14 IC₅₀ = 166 µM). The most active RA analogue identified was 57 (PAO1 IC₅₀ = 2.84 µM, PA14 IC₅₀ = 2.26 µM), effectively demonstrating that changes to certain portions of the natural product have the ability to dramatically increase biological activity.

**Table 5.** PAO1 and PA14 IC₅₀ Values.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PAO1 IC₅₀ (µM)</th>
<th>PA14 IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oroidin (34)</td>
<td>190 ± 9</td>
<td>166 ± 23</td>
</tr>
<tr>
<td>54 (n = 5)</td>
<td>32.7 ± 6.5</td>
<td>39.9 ± 13.0</td>
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<td>55 (n = 7)</td>
<td>18.4 ± 2.3</td>
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<tr>
<td>56 (n = 9)</td>
<td>7.79 ± 1.52</td>
<td>7.48 ± 1.60</td>
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<tr>
<td>57 (n = 11)</td>
<td>2.84 ± 0.93</td>
<td>2.26 ± 0.83</td>
</tr>
</tbody>
</table>
To validate that our compounds were true inhibitors of biofilm formation and not acting as bactericidal agents, growth curves were performed at the determined IC50 values for PAO1 and PA14 with the dodecyl-based analogue 57 and oroidin 34. Bacterial cell densities for both strains remained unchanged when grown in the presence or absence of either the natural product 34 or 57 throughout a 24-hour time period (appendix).

4.3 Dispersion of Established P. aeruginosa Biofilms

While the focus has predominantly been on designing small molecules that inhibit the formation of bacterial biofilms, the more significant challenge is the development of a small molecule that will disperse established biofilms. Treatment of chronic infections is commonly hindered by the presence of established biofilms that impart increased resistance to conventional antibiotics.13 Small molecules able to disperse established biofilms are, therefore, of great interest to the medical community. To test for the ability to disperse
established biofilms, PAO1 and PA14 were allowed to form biofilms for 24 hours in the absence of compound. After this time the media was discarded. The wells were washed and fresh media was added containing varying concentrations of 57 and then incubated at 37 °C for 24 hours. RA analogue 57 displayed significant anti-biofilm activity, dispersing established PAO1 and PA14 biofilms with EC$_{50}$ values of 32.8 ± 4.7 µM and 21.3 ± 3.9 µM respectively (Figure 19).

![Figure 19. Dispersion of Established P. aeruginosa Biofilms with 57.](image)

4.4 Conclusion

We have identified several reverse amide (RA) analogues that possess potent anti-biofilm properties. These compounds are based on a reverse amide scaffold which switches the directionality of the amide bond frequently found in many members of the oroidin class of marine alkaloids. The synthetic path taken to access these derivatives allows for rapid access and simplified purification of all library members. Clearly, the most potent derivatives were those that contained linear carbon chains of various lengths from the amide nitrogen. The most active of these compounds, 57, has also been shown to disperse established P. aeruginosa biofilms at low micromolar concentrations, making it a highly noteworthy
addition to the limited number of small molecules known to possess such characteristics.¹⁴-¹⁶

It also remains a goal to identify even more potent compounds with structures similar to the most active analogues identified in this study while gaining mechanistic insight into how these compounds elicit their anti-biofilm effects and will be the subject of the following chapter.
4.5 Experimental Section

Stock solutions (100, 10, 1 mM) of all compounds assayed for biological activity were prepared in DMSO and stored at room temperature. The amount of DMSO used in both inhibition and dispersion screens did not exceed 1% (by volume). *P. aeruginosa* strains PAO1 and PA14 were graciously supplied by the Wozniak group at Wake Forest University School of Medicine.

**General Static Inhibition Assay Protocol for *Pseudomonas aeruginosa***.

An overnight culture of the wild type strain was subcultured at an OD$_{600}$ of 0.01 into LBNS along with a predetermined concentration of the small molecule to be tested for biofilm inhibition. Samples were then aliquoted (100 µL) into the wells of a 96-well PVC microtiter plate. The microtiter dishes were covered and sealed before incubation under stationary conditions at 37 °C for 24 hours. After that time, the medium was discarded and the plates thoroughly washed with water. The wells were then inoculated with a 0.1% aqueous solution of crystal violet (100 µL) and allowed to stand at ambient temperature for 30 minutes. Following another thorough washing with water the remaining stain was solubilized with 200 µL of 95% ethanol. Biofilm inhibition was quantitated by measuring the OD$_{540}$ for each well by transferring 125 µL of the ethanol solution into a fresh polystyrene microtiter dish for analysis.

**General Static Dispersion Assay Protocols for *Pseudomonas aeruginosa***.
An overnight culture of the wild type strain was subcultured at an OD\textsubscript{600} of 0.05 into LBNS and then aliquoted (100 µL) into the wells of a 96-well PVC microtiter plate. The microtiter dishes were covered and sealed before incubation under stationary conditions at room temperature to allow formation of the biofilms. After 24 hours the medium was discarded and the plates thoroughly washed with water. Fresh medium containing the appropriate concentration of compound was then added to the wells. The plates were again sealed and this time incubated under stationary conditions at 37 °C. After 24 hours, the media was discarded from the wells and the plates washed thoroughly with water. The wells were inoculated with a 0.1% aqueous solution of crystal violet (100 µL) and allowed to stand at ambient temperature for 30 minutes. Following another thorough washing with water the remaining stain was solubilized with 200 µL of 95% ethanol. Biofilm dispersion was quantitated by measuring the OD\textsubscript{540} for each well by transferring 125 µL of the ethanol solution into a fresh polystyrene microtiter dish for analysis. Percent dispersion was calculated by comparison of the OD\textsubscript{540} for established biofilm (untreated) versus treated established biofilm under identical conditions.

**Chemistry**

All reagents including anhydrous solvents used for the chemical synthesis of the library were purchased from commercially available sources and used without further purification unless otherwise noted. All reactions were run under either a nitrogen or argon atmosphere. Flash silica gel chromatography was performed with 60Å mesh standard grade silica gel from Sorbtech. $^1$H and $^{13}$C NMR spectra were obtained using
Varian 300 MHz or 400 MHz spectrometers. NMR solvents were purchased from Cambridge Isotope Labs and used as is. Chemical shifts are given in parts per million relative to DMSO-\textsubscript{d6} (δ 2.50) and CDCl\textsubscript{3} (δ 7.27) for proton spectra and relative to DMSO-\textsubscript{d6} (δ 39.51) and CDCl\textsubscript{3} (δ 77.21) for carbon spectra with an internal TMS standard. High-resolution mass spectra were obtained at the North Carolina State Mass Spectrometry Laboratory for Biotechnology. ESI experiments were carried out on Agilent LC-TOF mass spectrometer.

![Chemical Structure](image)

**6-Bromo-5-oxohexanoic acid methyl ester (4-i).** Methyl glutaryl chloride (2.5 mL, 18.23 mmol) was dissolved into anhydrous dichloromethane (10 mL) and added dropwise to a 0 °C solution of CH\textsubscript{2}N\textsubscript{2} (55.0 mmol generated from Diazald\textsuperscript{®}/KOH) in diethyl ether (150 mL). This solution was stirred at 0 °C for 1.5 h at which time the reaction was quenched via the dropwise addition of 48% HBr (7.5 mL). The reaction mixture was diluted with dichloromethane (25 mL) and immediately washed with sat. NaHCO\textsubscript{3} (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO\textsubscript{4}), filtered and concentrated. The crude oil was purified via flash column chromatography (10-30% EtOAc/hexanes) to obtain 4-i (3.76 g, 93%) as a colorless oil. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 3.91 (s, 2H), 3.68 (s, 3H), 2.76 (t, 2H, J = 7.2 Hz), 2.38 (t, 2H, J = 7.2 Hz), 1.95 (quint, 2H, J = 7.2 Hz); \textsuperscript{13}C NMR (75 MHz, CDCl\textsubscript{3}) δ 201.4, 173.4, 51.7, 38.7, 34.2, 32.9, 19.1; HRMS (ESI) calcd for C\textsubscript{7}H\textsubscript{12}O\textsubscript{3}Br (MH)+ 222.9964, found 222.9964.

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6-Chloro-5-oxohexanoic acid methyl ester (4-ii). Using the same general procedure as used above but instead quenching with conc. HCl afforded the chloro derivative 4-ii (2.93 g, 90%) as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.13 (s, 2H), 3.67 (s, 3H), 2.69 (t, 2H, $J = 7.2$ Hz), 2.38 (t, 2H, $J = 7.2$ Hz), 1.94 (quint., 2H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 201.9, 173.3, 51.6, 48.2, 38.5, 32.7, 18.6; HRMS (ESI) calcd for C$_7$H$_{12}$O$_3$Cl (MH)$^+$ 179.0469, found 179.0476.

6-Bromo-5-oxohexanoic acid benzyl ester (4-iii). Monobezylesterbutanoic acid 42 (3.0 g, 13.6 mmol) was dissolved in anhydrous dichloromethane (70 mL) at 0 °C and a catalytic amount of DMF was added. To this solution was added oxalyl chloride (3.6 mL, 41.3 mmol) dropwise and the solution was then warmed to room temperature. After 1 h, the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting solid was dissolved into anhydrous dichloromethane (10 mL) and added dropwise to a 0 °C solution of CH$_2$N$_2$ (42.0 mmol generated from Diazald®/KOH) in diethyl ether (120 mL). This solution was stirred at 0 °C for 1.5 h at which time the reaction was quenched via the dropwise addition of 48% HBr (4.7 mL). The reaction mixture was diluted with dichloromethane (25 mL) and immediately washed with sat.
NaHCO$_3$ (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO$_4$), filtered and concentrated. The crude oil was purified by flash column chromatography (0-30% EtOAc/hexanes) to obtain 4-iii (3.57 g, 88%) as a colorless oil. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 7.35 (m, 5H), 5.12 (s, 2H), 3.85 (s, 2H), 2.73 (t, 2H, $J = 6.8$ Hz), 2.42 (t, 2H, $J = 6.8$ Hz), 1.96 (quint, 2H, $J = 6.8$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 201.4, 172.9, 136.2, 128.8, 128.48, 128.45, 66.5, 38.7, 34.1, 33.2, 19.2; HRMS (ESI) calcd for C$_{13}$H$_{16}$O$_3$Br (MH)$^+$ 299.0277, found 299.0279.

2-Amino-4-(3-methoxycarbonylpropyl)imidazole-1-carboxylic acid tert-butyl ester (38). 6-Bromo-5-oxohexanoic acid methyl ester (2.3 g, 10.3 mmol), Boc-guanidine (4.92 g, 30.9 mmol),$^{17}$ and NaI (3.07 g, 20.6 mmol) were dissolved in DMF (30 mL) and allowed to stir at room temperature. After 24 h the DMF was removed under reduced pressure and the residue was taken up in ethyl acetate (100 mL) and washed with water (3 x 50 mL) and brine (50 mL) before being dried (Na$_2$SO$_4$), filtered and evaporated to dryness. The resulting oil was purified by flash column chromatography (50-100% EtOAc/hexanes) to obtain a yellow oil. Trituration of the viscous oil with cold hexanes (20 mL) produced a precipitate, which upon filtration yielded 38 (1.89 g, 65%) as a pale yellow solid. $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.53 (s, 1H), 5.6 (br s, 2H), 2.41 (t, 2H, $J = 7.2$ Hz), 2.37 (t, 2H, $J = 7.2$ Hz), 1.93 (quint., 2H, $J = 7.2$ Hz), 1.58 (s, 9H); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 174.1, 150.1, 149.6, 138.4, 107.2, 84.8, 51.6, 33.7, 28.2, 27.7, 23.8;
HRMS (ESI) calcd for C$_{13}$H$_{22}$N$_{3}$O$_{4}$ (MH)$^{+}$ 284.1604, found 284.1606.

4-(2-Amino-1H-imidazol-4-yl) butyric acid hydrochloride (19). To 2-amino-4-(3-methoxycarbonylpropyl)imidazole-1-carboxylic acid tert-butyl ester 38 (50 mg, 0.176 mmol) was added methanol (0.60 mL), tetrahydrofuran (0.20 mL), and water (0.20 mL). Lithium hydroxide (9 mg, 0.352 mmol) was then added and the reaction was stirred at room temperature for 30 min. The pH of the solution was carefully adjusted to pH = 5 with a 1N aqueous solution of HCl before being evaporated to dryness. The crude product was purified via a silica gel plug (100% MeOH sat. NH$_3$) to deliver the product as its corresponding free base. The hydrochloride salt was obtained through addition of a single drop of concentrated HCl to a methanolic solution (2 mL) of the free base. Rotary evaporation of this solution afforded 41 (34 mg, 94%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.25 (s, 1H), 12.13 (br s, 1H), 11.77 (s, 1H), 7.33 (s, 2H), 6.54 (s, 1H), 2.43 (t, 2H, $J$ = 7.2 Hz), 2.21 (t, 2H, $J$ = 7.2 Hz), 1.73 (m, 2H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 174.0, 146.9, 126.0, 108.6, 32.8, 23.5, 23.1; HRMS (ESI) calcd for C$_7$H$_{12}$N$_3$O$_2$ (MH)$^{+}$ 170.0924, found 170.0927.
2-Amino-4-(3-benzyloxy carbonylpropyl)imidazole-1-carboxylic acid tert-butyl ester (39). 6-bromo-5-oxohexanoic acid benzyl ester (3.42 g, 11.99 mmol) and Boc-guanidine (5.73 g, 35.97 mmol) were dissolved in DMF (35 mL) and allowed to stir at room temperature. After 48 h the DMF was removed under reduced pressure and the residue was taken up in ethyl acetate (100 mL) and washed with water (3 x 50 mL) and brine (50 mL) before being dried (Na₂SO₄), filtered and evaporated to dryness. The resulting oil was purified by flash column chromatography (30-100% EtOAc/hexanes) to obtain the title compound 39 (2.79 g, 66%) as a colorless oil which solidified upon prolonged standing. ¹H NMR (400 MHz, CDCl₃) δ 7.35 (m, 5H), 6.51 (s, 1H), 5.91 (s, 2H), 5.12 (s, 2H), 2.41 (m, 4H), 1.94 (quint., 2H, J = 7.2 Hz), 1.57 (s, 9H); ¹³C NMR (75 MHz, CDCl₃) δ 173.5, 150.3, 149.6, 138.3, 136.4, 128.7, 128.29, 128.27, 107.1, 84.7, 86.2, 33.8, 28.2, 27.6, 23.8; HRMS (ESI) calcd for C₁₉H₂₆N₃O₄ (MH)⁺ 360.1917, found 360.1919.

2-Amino-4-(3-carboxypropyl)imidazole-1-carboxylic acid tert-butyl ester (43). To a solution of anhydrous THF (2 mL) and 10% Pd/C (12 mg) was added 2-amino-4-(3-benzyloxy carbonylpropyl)imidazole-1-carboxylic acid tert-butyl ester 39 (101 mg, 0.281 mmol). Air was removed from the system and the reaction was back flushed with hydrogen. This process was repeated three times before setting the reaction under a hydrogen balloon at atmospheric pressure and temperature for 1 h. After that time the
reaction was filtered through a Celite® pad and the filter cake was washed with THF (8 mL). The filtrate was concentrated under reduced pressure to afford the title compound 43 (75 mg, 98%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.52 (s, 1H), 6.42 (br s, 2H), 2.52 (t, 2H, $J = 5.4$ Hz), 2.18 (t, 2H, $J = 5.4$ Hz), 1.71 (m, 2H), 1.53 (s, 9H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 175.0, 150.0, 149.0, 138.3, 105.9, 84.1, 39.2, 38.9, 33.7, 27.5, 27.1, 23.5; HRMS (ESI) calcd for C$_{12}$H$_{20}$N$_3$O$_4$ (MH)$^+$ 270.1448, found 270.1452.

**General aminolysis procedure:** To a stirring 0 °C solution of amine (0.704 mmol) in anhydrous 1,2-dichloroethane (1 mL) was added dropwise a 2M solution of AlMe$_3$ in PhCH$_3$ (0.351 mL, 0.704 mmol). The solution was stirred for 10 min before the addition of 2-Amino-4-(3-methoxycarbonylpropyl)imidazole-1-carboxylic acid tert-butyl ester 38 (100 mg, 0.352 mmol) in several portions. Once dissolution was complete, the reaction was warmed to 60 °C and stirred until completion as evident by TLC analysis. The reaction was then cooled back down to 0 °C before being diluted with dichloromethane (5 mL) and quenched with water (1 mL). The resulting viscous solution was warmed to ambient temperature and Celite® was added. After stirring for 5 min, the mixture was filtered and the filtrate washed with brine (2 x 3 mL), dried (Na$_2$SO$_4$), and evaporated to dryness. The crude product was purified via flash column chromatography (2-10% MeOH/CH$_2$Cl$_2$) to afford pure product.
2-Amino-4-(3-isobutylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (44) White solid (46 mg, 40%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.75 (m, 1H), 6.51 (s, 1H), 6.37 (br s, 2H), 2.85 (t, $J = 6.4$ Hz), 2.22 (t, $J = 6.8$ Hz), 2.07 (t, $J = 7.2$ Hz), 1.61 – 1.73 (m, 3H), 1.53 (s, 9H), 0.81 (d, $J = 6.4$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 171.8, 149.9, 149.0, 138.4, 105.4, 84.0, 45.9, 34.9, 28.1, 27.5, 27.2, 24.1, 20.1; HRMS (ESI) calcd for C$_{16}$H$_{29}$N$_4$O$_3$ (MH)$^+$ 325.2234, found 325.2238.

2-Amino-4-(3-decylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (47). Tan solid (24 mg, 16%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.74 (m, 1H), 6.52 (s, 1H), 6.49 (br s, 2H), 3.00 (q, $J = 6.8$ Hz), 2.22 (t, $J = 6.8$ Hz), 2.04 (t, $J = 6.8$ Hz), 1.71 (quint., $J = 6.8$ Hz), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (s, 14H), 0.85 (t, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 171.6, 149.8, 148.9, 137.9, 105.9, 84.2, 38.3, 34.9, 31.3, 29.2, 29.03, 28.97, 28.8, 28.7, 28.0, 27.5, 27.1, 26.4, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C$_{22}$H$_{41}$N$_4$O$_3$ (MH)$^+$ 409.3173, found 409.3175.
2-Amino-4-(3-cyclopentylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (49). White solid (54 mg, 45%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 7.72 (d, 1H, \(J = 6.4\) Hz), 6.5 (s, 1H), 6.38 (s, 2H), 3.97 (m, 1H), 2.21 (t, 2H, \(J = 7.2\) Hz), 2.02 (t, 2H, \(J = 7.2\) Hz), 1.73 (m, 4H), 1.61 (m, 2H), 1.53 (s, 9H), 1.47 (m, 2H), 1.32 (m, 2H); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 171.3, 149.9, 149.0, 138.4, 105.9, 84.0, 50.0, 34.8, 32.3, 28.1, 27.5, 27.2, 24.0, 23.4; HRMS (ESI) calcd for C\(_{17}\)H\(_{29}\)N\(_4\)O\(_3\) (MH\(^+\)) 337.2234, found 337.2235.

2-Amino-4-(4-morpholin-4-yl-4-oxobutyl)imidazole-1-carboxylic acid tert-butyl ester (50). Tan solid (33 mg, 27%). \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 6.52 (s, 1H), 6.39 (s, 2H), 3.52 (m, 4H), 3.41 (m, 4H), 2.28 (m, 4H), 1.42 (quint., 2H, \(J = 7.2\) Hz) 1.53 (s, 9H); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 171.4, 150.6, 149.6, 139.2, 106.6, 84.8, 66.9, 46.1, 32.2, 28.2, 27.9, 24.2; HRMS (ESI) calcd for C\(_{16}\)H\(_{27}\)N\(_4\)O\(_4\) (MH\(^+\)) 339.2026, found 339.2027.
2-Amino-4-(3-phenylcarbamoylpropyl)imidazole-1-carboxylic acid tert-buty l ester (51). White solid (66 mg, 55%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 9.88 (s, 1H), 7.59 (d, 2H, $J = 8.1$ Hz), 7.27 (t, 2H, $J = 7.5$ Hz), 7.00 (t, 1H, $J = 7.2$ Hz), 6.55 (s, 1H), 6.44 (br s, 2H), 2.97 (m, 4H), 1.82 (m, 2H), 1.53 (s, 9H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.0, 149.7, 148.9, 139.3, 138.2, 128.5, 122.8, 119.0, 105.9, 84.0, 38.4, 35.7, 27.4, 27.0, 23.72; HRMS (ESI) calcd for C$_{18}$H$_{25}$N$_4$O$_3$ (MH)$^+$ 345.1921, found 345.1920.

2-Amino-4-[3-(pyrimidin-2-ylcarbamoyl)propyl]imidazole-1-carboxylic acid tert-butyl ester (52). Tan solid (19 mg, 11%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.51 (s, 1H), 8.63 (d, 2H, $J = 4.8$ Hz), 7.15 (t, 1H, $J = 4.8$ Hz), 6.54 (s, 1H), 6.40 (s, 2H), 2.49 (t, 2H, $J = 7.2$ Hz), 2.29 (t, 2H, $J = 7.2$ Hz), 1.80 (quint., 2H, $J = 7.2$ Hz), 1.53 (s, 9H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.3, 158.1, 157.6, 149.8, 148.9, 138.3, 116.4, 105.8, 84.0, 35.8, 27.4, 27.1, 23.4; HRMS (ESI) calcd for C$_{16}$H$_{23}$N$_6$O$_3$ (MH)$^+$ 347.1826, found 347.1827.

General EDC/HOBt procedure: 2-Amino-4-(3-carboxypropyl)imidazole-1-carboxylic
acid tert-butyl ester 43 (100 mg, 0.371 mmol), 1-hydroxybenzotriazole (100 mg, 0.742 mmol) and N-(3-dimethylaminopropyl)-N' -ethylcarbodiimide hydrochloride (142 mg, 0.742 mmol) were dissolved in anhydrous DMF (3 mL). The appropriate amine coupling partner (1.48 mmol) was then added and the solution was stirred at ambient temperature until completion was evident by TLC analysis. The reaction was concentrated under reduced pressure and the residue partitioned between ethyl acetate (20 mL) and water (10 mL). The organic layer was successively washed with water (3 x 10 mL), a 10% aqueous solution of citric acid (2 x 10 mL), sat. NaHCO₃ (2 x 10 mL), and brine (10 mL) before being dried (Na₂SO₄) and evaporated to dryness. The crude product was purified via flash column chromatography (2-10% MeOH/CH₂Cl₂) to afford the target compound.

2-Amino-4-(3-hexylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (45). Pale yellow solid (41 mg, 32%). ¹H NMR (300 MHz, DMSO-d₆) δ 7.73 (m, 1H), 6.50 (s, 1H), 6.39 (s, 2H), 2.99 (q, 2H, J = 6.3 Hz), 2.21 (t, 2H, J = 7.5 Hz), 2.04 (t, 2H, J = 7.2 Hz), 1.70 (m, 2H), 1.53 (s, 9H), 1.31 (m, 3H), 1.23 (br s, 7H), 0.85 (t, 3H, J = 5.1 Hz); ¹³C NMR (75 MHz, DMSO-d₆) δ 171.7, 149.9, 149.0, 138.4, 105.8, 84.0, 38.6, 34.9, 31.0, 29.1, 27.5, 27.2, 26.1, 24.1, 22.1, 13.9; HRMS (ESI) calcd for C₁₈H₃₃N₄O₃ (MH)⁺ 353.2547, found 353.2549.
2-Amino-4-(3-octylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (46). White solid (48 mg, 34%). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.73 (m, 1H), 6.50 (s, 1H), 6.38 (s, 2H), 2.99 (q, 2H, $J = 5.4$ Hz), 2.21 (t, 2H, $J = 7.5$ Hz), 2.04 (t, 2H, $J = 7.2$ Hz), 1.73 (m, 2H), 1.53 (s, 9H), 1.36 (m, 4H), 1.23 (br s, 10H), 0.85 (t, 3H, $J = 5.1$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.7, 150.0, 149.0, 138.4, 105.8, 84.1, 38.4, 34.9, 31.3, 29.2, 28.7, 27.5, 27.2, 26.4, 24.1, 22.1, 14.0; HRMS (ESI) calcd for C$_{20}$H$_{37}$N$_4$O$_3$ (MH)$^+$ 381.2860, found 381.2861.

2-Amino-4-(3-dodecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (48). White solid (44 mg, 28%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.73 (t, 1H, $J = 5.6$ Hz), 6.50 (s, 1H), 6.38 (s, 2H), 3.00 (q, 2H, $J = 5.6$ Hz), 2.21 (t, 2H, $J = 7.6$ Hz), 2.04 (t, 2H, $J = 7.6$ Hz), 1.71 (quint., 2H, $J = 7.6$ Hz), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (s, 18H), 0.85 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.6, 149.8, 148.9, 138.4, 105.8, 84.0, 38.3, 34.9, 31.2, 29.1, 28.9, 28.6, 27.5, 27.2, 26.3, 24.0, 22.0, 13.8, 13.3; HRMS (ESI) calcd for C$_{24}$H$_{45}$N$_4$O$_3$ (MH)$^+$ 437.3486, found 437.3487.
4-(2-Amino-1H-imidazol-4-yl)-N-isobutylbutyramide hydrochloride (53). A solution of 2-amino-4-(3-isobutylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 44 (76 mg, 0.234 mmol) in anhydrous dichloromethane (1 mL) was cooled to 0 °C. TFA (1 mL) was charged into the flask and the reaction stirred for 5 h. After that time the reaction was evaporated to dryness and toluene (2 mL) was added. Again the mixture was concentrated and the process repeated. The resulting TFA salt was dissolved in dichloromethane (1 mL) and 2M HCl in diethyl ether (0.50 mL) was added followed by cold diethyl ether (8 mL). The precipitate was collected by filtration and washed with diethyl ether (3 mL) to yield the target compound 53 (59 mg, 97%) as a tan solid. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 12.14 (s, 1H), 11.70 (s, 1H), 7.89 (m, 1H), 7.34 (br s, 2H), 6.55 (s, 1H), 2.84 (t, 2H, $J = 6.6$ Hz), 2.38 (t, 2H, $J = 7.5$ Hz), 2.10 (t, 2H, $J = 7.5$ Hz), 1.60 – 1.79 (m, 3H), 0.82 (d, 6H, $J = 6.3$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.5, 146.8, 126.3, 108.7, 46.0, 34.4, 28.1, 23.9, 23.6, 20.2; HRMS (ESI) calcd for C$_{11}$H$_{21}$N$_4$O (MH)$^+$ 225.1709, found 225.1711.

4-(2-Amino-1H-imidazol-4-yl)-N-hexylbutyramide hydrochloride (54). Using the
same general procedure as used for the synthesis of 53, 2-amino-4-(3-hexylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 45 (90 mg, 0.255 mmol) gave 54 (70 mg, 96%) as a pale yellow foam. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 11.96 (s, 1H), 11.54 (s, 1H), 7.81 (m, 1H), 7.29 (br s, 2H), 6.56 (s, 1H), 3.01 (m, 2H), 2.40 (t, 2H, $J = 7.8$ Hz), 2.07 (t, 2H, $J = 7.2$ Hz), 1.73 (m, 2H), 1.23 – 1.36 (m, 8H), 0.85 (m, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 171.4, 146.7, 126.4, 108.7, 38.4, 34.4, 31.0, 29.1, 26.1, 23.9, 23.6, 22.1, 14.0; HRMS (ESI) calcd for C$_{13}$H$_{25}$N$_4$O (MH)$^+$ 253.2022, found 253.2025.

4-(2-Amino-1H-imidazol-4-yl)-N-octylbutyramide hydrochloride (55). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(3-octylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 46 (50 mg, 0.131 mmol) gave 55 (39 mg, 93%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 12.13 (s, 1H), 11.69 (s, 1H), 7.87 (m, 1H), 7.33 (br s, 2H), 6.55 (s, 1H), 2.99 (q, 2H, $J = 6.3$ Hz), 2.38 (t, 2H, $J = 7.5$ Hz), 2.07 (t, 2H, $J = 7.5$ Hz), 1.73 (m, 2H), 1.35 (m, 2H), 1.23 (m, 10H), 0.85 (t, 3H, $J = 6.3$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.3, 146.8, 126.3, 108.6, 38.4, 34.4, 31.2, 29.1, 28.62, 28.56, 26.4, 23.9, 23.6, 22.0, 13.9; HRMS (ESI) calcd for C$_{15}$H$_{29}$N$_4$O (MH)$^+$ 281.2335, found 281.2339.
4-(2-Amino-1H-imidazol-4-yl)-N-decybutyramide hydrochloride (56). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(3-decylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 47 (32 mg, 0.078 mmol) gave 56 (27 mg, 99%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.07 (s, 1H), 11.64 (s, 1H), 7.85 (s, 1H), 7.32 (br s, 2H), 6.56 (s, 1H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.38 (t, 2H, $J = 7.2$ Hz), 2.07 (t, 2H, $J = 7.2$ Hz), 1.73 (quint., 2H, $J = 7.2$ Hz), 1.36 (m, 2H), 1.23 (s, 14H), 0.85 (t, 3H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 171.3, 146.7, 126.4, 108.7, 38.4, 34.4, 31.3, 29.2, 29.04, 28.99, 28.8, 28.7, 26.5, 23.9, 23.6, 22.1, 14.0; HRMS (ESI) calcd for $C_{17}H_{33}N_4O$ (MH)$^+$ 309.2648, found 309.2647.

4-(2-Amino-1H-imidazol-4-yl)-N-dodecybutyramide hydrochloride (57). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(3-dodecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 48 (20 mg, 0.046 mmol) gave 57 (16 mg, 94%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.03 (s, 1H), 11.60 (s, 1H), 7.83 (t, 1H, $J = 6.4$ Hz), 7.31 (s, 2H), 6.56 (s, 1H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.38 (t, 2H, $J = 7.2$ Hz), 2.07 (t, 2H, $J = 7.2$ Hz), 1.73 (quint., 2H, $J = 7.2$ Hz),
1.36 (m, 2H), 1.23 (s, 18H), 0.85 (t, 2H, \( J = 6.4 \) Hz); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \( \delta \) 171.2, 146.7, 126.3, 108.6, 38.3, 34.32, 31.2, 29.0, 28.9, 28.61, 28.55, 26.3, 23.8, 23.5, 21.9, 13.8; HRMS (ESI) calcd for \( \text{C}_{19}\text{H}_{37}\text{N}_4\text{O} \) (MH\(^+\)) 337.2961, found 337.2964.

4-(2-Amino-1H-imidazol-4-yl)-N-cyclopentylbutyramide hydrochloride (58). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(3-cyclopentylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 49 (100 mg, 0.297 mmol) gave 58 (78 mg, 96%) as a pale yellow foam. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 11.99 (s, 1H), 11.57 (s, 1H), 7.79 (d, 1H, \( J = 7.2 \) Hz), 7.30 (s, 2H), 6.56 (s, 1H), 3.97 (m, 1H), 2.37 (t, 2H, \( J = 7.2 \) Hz), 2.05 (t, 2H, \( J = 7.2 \) Hz), 1.69 – 1.79 (m, 4H), 1.59 (m, 2H), 1.47 (m, 2H), 1.31 (m, 2H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) \( \delta \) 171.0, 146.7, 126.4, 108.7, 50.1, 34.4, 32.3, 23.8, 23.6, 23.4; HRMS (ESI) calcd for \( \text{C}_{12}\text{H}_{21}\text{N}_4\text{O} \) (MH\(^+\)) 237.1709, found 237.1711.

4-(2-Amino-1H-imidazol-4-yl)-1-morpholin-4-yl-butan-1-one hydrochloride (59). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(4-
morpholin-4-yl-4-oxobutyl)imidazole-1-carboxylic acid tert-butyl ester 50 (44 mg, 0.133 mmol) gave 59 (25 mg, 70%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.1 (s, 1H), 11.64 (s, 1H), 7.33 (s, 2H), 6.58 (s, 1H), 3.54 (m, 4H), 3.42 (m, 4H), 2.43 (t, 2H, $J = 7.2$ Hz), 2.33 (t, 2H, $J = 7.2$ Hz), 1.75 (quint., 2H, $J = 7.2$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 170.3, 146.7, 126.4, 108.6, 66.0, 45.3, 31.1, 23.6, 23.2; HRMS (ESI) calcd for C$_{11}$H$_{19}$N$_4$O$_2$ (MH)$^+$ 239.1502, found 239.1503.

![Chemical structure of 51 and 60]

4-(2-Amino-1H-imidazol-4-yl)-N-phenylbutyramide hydrochloride (60). Using the same general procedure as used for the synthesis of 53, 2-amino-4-(3-phenylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester 51 (80 mg, 0.232 mmol) gave 60 (64 mg, 99%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.03 (s, 1H), 11.60 (s, 1H), 9.98 (s, 1H), 7.59 (d, 2H, $J = 8.0$ Hz), 7.33 (br s, 2H), 7.28 (t, 2H, $J = 8.0$ Hz), 7.02 (t, 1H, $J = 7.6$ Hz), 6.61 (s, 1H), 2.44 (m, 2H), 2.32 (t, 2H, $J = 6.8$ Hz), 1.85 (m, 2H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 170.7, 146.8, 139.3, 137.2, 128.6, 126.4, 123.0, 119.1, 108.8, 35.3, 23.6, 23.5; HRMS (ESI) calcd for C$_{13}$H$_{17}$N$_4$O (MH)$^+$ 245.1396, found 245.1401.
4-(2-Amino-1H-imidazol-4-yl)-N-pyrimidin-2-yl-butyramide hydrochloride (61).

Using the same general procedure as used for the synthesis of 53, 2-amino-4-[3-(pyrimidin-2-ylcarbamoyl)propyl]imidazole-1-carboxylic acid tert-butyl ester 52 (50 mg, 0.144 mmol) gave 61 (41 mg, 99%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.06 (s, 1H), 11.64 (s, 1H), 10.59 (s, 1H), 8.64 (d, 2H, $J = 4.8$ Hz), 7.34 (s, 2H), 7.17 (t, 1H, $J = 4.8$ Hz), 6.60 (s, 1H), 2.51 (m, 2H), 2.46 (t, 2H, $J = 7.2$ Hz), 1.83 (quint., 2H, $J = 7.2$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 158.2, 157.5, 146.7, 129.0, 126.3, 116.5, 108.7, 35.4, 23.5, 23.1; HRMS (ESI) calcd for C$_{11}$H$_{15}$N$_6$O (MH)$^+$ 247.1301, found 247.1304.
References


Representative Growth Curves

**Growth Curve at IC₅₀ = 190 µM**

- PAOI wt
- PAOI w/ cmpd

**Growth Curve at IC₅₀ = 166 µM**

- PAI4 wt
- PAI4 w/ cmpd

**Growth Curve at IC₅₀ = 2.84 µM**

- PAOI wt
- PAOI w/ cmpd

**Growth Curve at IC₅₀ = 2.26 µM**

- PAI4 wt
- PAI4 w/ cmpd
4-iii
49
CHAPTER 5

Second Generation RA Library – A Detailed SAR Study

The first generation RA library identified the dodecyl-based RA analogue 57 as being both a very potent inhibitor and disruptor of *P. aeruginosa* biofilms (PA14 biofilm inhibition IC$_{50}$ = 2.26 ± 0.83 µM, PA14 biofilm dispersion EC$_{50}$ = 21.3 ± 3.9 µM).$^1$ However, additional compounds were needed to fully complete the aliphatic chain length study to identify the most active chain for anti-biofilm potency (Table 6). Following the completion of the chain length study, the design for a second generation RA library would be based on derivatization of the hexyl RA analogue 54. This analogue was chosen as a template for additional SAR analysis due to its relatively moderate activity profile (PA14 IC$_{50}$ = 39.9 ± 13.0 µM), availability, and overall ease of synthesis across the desired scaffolds for SAR consideration. The SAR for 54 was subsequently divided into six separate but overlapping themes: deletion of the amide bond, linker chain modification, sliding of the amide bond, increased substitution of the amide bond, reversal of the amide bond directionality to mimic oroidin, and examination of a triazole isostere (Figure 20).

**Table 6. Examining the Aliphatic Chain Length.**

<table>
<thead>
<tr>
<th>1st Generation</th>
<th>Proposed 2nd Generation</th>
</tr>
</thead>
<tbody>
<tr>
<td>54 ($n = 5$)</td>
<td>62 ($n = 10$)</td>
</tr>
<tr>
<td>55 ($n = 7$)</td>
<td>63 ($n = 12$)</td>
</tr>
<tr>
<td>56 ($n = 9$)</td>
<td>64 ($n = 13$)</td>
</tr>
<tr>
<td>57 ($n = 11$)</td>
<td>65 ($n = 15$)</td>
</tr>
<tr>
<td></td>
<td>66 ($n = 17$)</td>
</tr>
</tbody>
</table>
Initially, it was planned to evaluate all of the target analogues against *Pseudomonas aeruginosa* PA14 biofilms. When active compounds were identified, IC₅₀ values would then be determined. Additionally, the most active analogues in each sub-class would then be tested for anti-biofilm activity against *Acinetobacter baumannii* biofilms. Pending the identification of active compounds in these screens, *A. baumannii* IC₅₀ values would also be determined. Several of the most active analogues would then be assayed for their ability to disperse established PA14 and/or *A. baumannii* biofilms respective of their biofilm inhibition activity profile.

### 5.1 Reaching the Limit of the Aliphatic Chain Length Effect

The first goal of the study was to further investigate the full extent of anti-biofilm activity attainable by lengthening the aliphatic side chain. To this end, we synthesized the targets following our previously established chemistry to furnish 64-66 as their HCl salts (Table 7).
Table 7. Synthesis and Anti-biofilm Activity of 2nd Generation Aliphatic RA Analogues.

<table>
<thead>
<tr>
<th>Amine</th>
<th>Coupled Product (yield)</th>
<th>Target (yield)</th>
<th>PA14 IC\textsubscript{50} [\mu M]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetradecylamine</td>
<td>67 (24%)</td>
<td>64 (92%)</td>
<td>2.88 ± 1.19</td>
</tr>
<tr>
<td>Hexadecylamine</td>
<td>68 (19%)</td>
<td>65 (92%)</td>
<td>50.3</td>
</tr>
<tr>
<td>Octadecylamine</td>
<td>69 (13%)</td>
<td>66 (94%)</td>
<td>109.9</td>
</tr>
<tr>
<td>Undecylamine</td>
<td>70 (31%)</td>
<td>62 (98%)</td>
<td>4.25 ± 0.67</td>
</tr>
<tr>
<td>Tridecylamine</td>
<td>71 (25%)</td>
<td>63 (99%)</td>
<td>0.73 ± 0.08</td>
</tr>
</tbody>
</table>

*Reaction conditions: (a) EDC, HO\textsub{B}t, DMF (b) TFA, CH\textsub{2}Cl\textsub{2} (c) 2M HCl/Et\textsub{2}O.*

The anti-biofilm activity of these analogues left much to be desired, as the hexa- and octa-decyl based analogues 65 and 66 were not nearly as active as the dodecyl-based RA analogue (PA14 IC\textsub{50} values of 50.3 \mu M and 109.9 \mu M, respectively). Tetradecyl RA analogue 64 was slightly less active than 57 with a PA14 IC\textsub{50} = 2.88 ± 1.19 \mu M, concluding that the break point for activity for the RA scaffold should be a tridecyl RA analogue.

With that hypothesis, both 62 and 63 were synthesized and assayed for their ability to inhibit PA14 biofilm formation (Table 7). The undecyl RA analogue 62 corresponded well with the previously reported aliphatic data (PA14 IC\textsub{50} = 4.25 ± 0.67 \mu M) as it was slightly less active than the dodecyl RA analogue 57 but slightly more active than the decyl RA analogue 56. As expected, the tridecyl RA analogue 63 was an extremely potent anti-biofilm modulator with an IC\textsub{50} value of 729 ± 85 nM against PA14. This makes 63 over 3-fold more active than 57 in reference to inhibition of PA14 biofilms and brings the activity profile of this class of compounds into the high nanomolar region for the first time. Growth curves were performed in the presence and absence of 63 with PA14 to ensure that the anti-biofilm
activity was not a result of a bactericidal effect and, gratifyingly, bacterial cell densities remained the same throughout a 24-hour time period (appendix).

These results show that by incrementally increasing the aliphatic chain length for the RA scaffold up to a certain point, one of the most potent anti-biofilm modulators ever presented in the literature with inhibitory activity in the high nanomolar range can be obtained. It is also worth noting the high correlation between the aliphatic chain length and the activity profile of the aliphatic RA analogues (Figure 21). As newly identified 2-aminoimidazole based compounds increase in potency, the stringencies for an active compound have become higher, and yet, all of the aliphatic RA derivatives are still more active than the parent natural product oroidin.\(^1\), \(^2\) Having now identified the most active aliphatic RA side-chain and the break-point in activity, interest turned to how modifications of the core scaffold would tune activity.

![Figure 21. Correlation of Aliphatic RA Analogues.](image-url)
5.2 Deletion of the Amide Bond

From a synthetic standpoint, removal of the amide bond has a great advantage in simplifying the synthetic sequence for this class of compounds, most notably circumventing the low yielding amide bond formation step. A quick search of commercially available acid chlorides revealed a large number of building blocks that, when derivatized, would be directly comparable to the RA scaffold. It also allowed for additional functionality such as points of unsaturation along the chains to be incorporated into the molecules. Ultimately, if these compounds were to show promise, further analogue development could easily be executed throughout the well-established chemistry of olefin metathesis.3, 4

Starting with a sampling of commercially available acid chlorides, we elected to synthesize several analogues that would mimic the aliphatic RA derivatives in overall length. Several unsaturated intermediates were also prepared. The 2-AI targets were quickly

Scheme 13. Deletion of the Amide Bond. Reaction conditions: (a) i: CH$_2$N$_2$, Et$_2$O/CH$_2$Cl$_2$, 0 °C; ii: Conc. HBr (b) Boc-guanidine, NaI, DMF (c) TFA, CH$_2$Cl$_2$ (d) 2M HCl in Et$_2$O.
accessed with no unexpected issues (Scheme 13). Briefly, the acid chlorides were homologated with diazomethane followed by quenching with concentrated HBr to afford the requisite α-bromoketones. The α-bromoketones were then condensed with Boc-guanidine in the presence of NaI followed by deprotection in the presence of acid and salt exchange to afford the target compounds (Scheme 13).

Table 8. Anti-biofilm Activity of Amide Deletion Sub-class against PA14.

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>72</td>
<td>14.3 ± 2.2</td>
</tr>
<tr>
<td>73</td>
<td>18.6 ± 0.7</td>
</tr>
<tr>
<td>74</td>
<td>18.2 ± 1.3</td>
</tr>
</tbody>
</table>

Anti-biofilm assessment of these analogues lacking the amide bond exhibited a range of potencies. While the pentadecyl, heptadecyl and unsaturated analogues 75-78 were completely inactive, the shorter chain aliphatic derivatives showed a steep increase in inhibitory activity against PA14 biofilm formation. The isopropyl-based fatty acid 79 also exhibited modest activity, inhibiting biofilm formation 48% at 100 µM. The most active analogue obtained was the nonyl 2-AI fatty acid 72 with a PA14 IC₅₀ value of 14.3 ± 2.2 µM (Table 8). This was followed closely by the undecyl and tridecyl analogues (IC₅₀ values of 18.6 ± 0.7 µM and 18.2 ± 1.3 µM, respectively). Analogue 72 was chosen for PA14 growth curve analysis to represent this class and in the presence or absence of 72, PA14 cellular densities remained the same throughout a 24-hour time period (appendix).

In summary, removal of the amide bond from the aliphatic chain of the RA scaffold is not entirely detrimental to anti-biofilm activity. The undecyl 2-AI fatty acid 73 is directly comparable to the hexyl RA derivative’s (54) overall chain length, thus showing that removal
of the amide bond led to activity which was well over 2-fold more active than 54. Two additional derivatives were also shown to be potent inhibitors of PA14 biofilm formation. However, there appeared to be a markedly steep cutoff point for activity within this subset of molecules. This could possibly be due to the adoption of pseudo-surfactant properties causing changes in conformation and thus affecting overall solubility at the concentrations examined.

An intriguing comparison of these molecules can be made to N-acyl homoserine lactones (AHLs) (Figure 22). AHLs are well known modulators of bacterial quorum sensing in *Pseudomonas aeruginosa*, allowing the bacteria to effectively communicate with one another to make coordinated efforts in regulating virulence and other biological functions including biofilm development. The structural similarities of exhibiting a polar head group with a non-polar tail of these two types of molecules could lend credence to the possibility that similar molecular pathways are being conserved. Despite the steep activity cut-off for these analogues and possible bactericidal properties, the overall attractiveness of simplifying the synthetic sequence as demonstrated to obtain multi-gram quantities of such potent PA14 biofilm inhibitors can not be overlooked.
5.3 Linker Chain Modification and Sliding of the Amide Bond

Examination of the majority of compounds comprising the oroidin class of natural products often reveals a three-carbon methylene linker between the 2-aminoimidazole head group and the pyrrole carboxamide group. This three-carbon linker was previously confirmed to be of utmost importance for maximum biological activity in the context of \textit{P. aeruginosa} anti-biofilm activity.\textsuperscript{2} Although this aspect was not detailed in the previous RA library, it was deemed necessary to revisit this structural modification and fully investigate the spacer effect in the RA class of molecules. Therefore, the two-carbon and four-carbon Boc-2AI carboxylic acid homologues necessary for amide bond coupling were synthesized (Scheme 14). Briefly, succinic anhydride was opened with benzyl alcohol followed by diazomethane/\(\alpha\)-bromo homologation of the acid chloride to afford the \(\alpha\)-bromo succinic benzyl ester. The \(\alpha\)-bromoketone was then condensed with Boc-guanidine followed by hydrogenolysis of the benzyl ester to afford the two-carbon Boc-2AI carboxylic acid \textbf{80}. Similarly, the four-carbon homologue was synthesized from the known mono-benzyl ester of dipentanoic acid following the same procedure to deliver the intermediate acid \textbf{81} (Scheme 14).\textsuperscript{8}

![Scheme 14. Synthesis of Boc-2AI Linker Acids. Reaction conditions: (a) BnOH, TEA, DMAP, CH\(_2\)Cl\(_2\), 87% (b) \(i\): (COCl)\(_2\), DMF (cat.), CH\(_2\)Cl\(_2\); \(ii\): CH\(_3\)N\(_2\), Et\(_2\)O/CH\(_2\)Cl\(_2\), 0 °C; \(iii\): Conc. HBr (c) Boc-guanidine, DMF (d) H\(_2\) (1 atm), 10% Pd/C, THF.](image)

With these scaffolds in hand, coupling to hexylamine under carbodiimide conditions afforded derivatives comparable to the hexyl RA analogue in that they were either a carbon
atom shorter or longer in overall length from the 2-AI motif (Table 9). These compounds were deprotected cleanly and were then assayed for their ability to inhibit PA14 biofilm formation. The two methylene unit analogue 82 had much lower activity than the parent three carbon linker displaying only 32% inhibition at 100 µM. Similarly, the four methylene unit linker 83 only inhibited PA14 biofilm formation 28% at 100 µM (Table 9).

Table 9. Synthesis and Anti-biofilm Activity of Modified Linker Analogues.

<table>
<thead>
<tr>
<th>Target</th>
<th>Biofilm Inhibition at 100 µM vs PA14 [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>82</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>83</td>
<td>28 ± 5</td>
</tr>
</tbody>
</table>

Reaction conditions: (a) Hexylamine, EDC, HOBt, DMF (b) TFA, CH₂Cl₂ (c) 2M HCl/Et₂O.

Following the use of hexylamine as the coupling partner, we elected to investigate a few additional amines employing the two-carbon scaffold due to availability (Table 10). The two compounds prepared were chosen to delineate whether a phenyl ring could mimic the activity of the aliphatic chains and recapitulate potency. It was anticipated that the p-bromo phenethyl RA analogue may follow the same trend observed in other studies with increasing anti-biofilm activity correlating to an increased degree of bromination. Gratifyingly, the p-bromo phenethyl RA analogue exhibited very potent activity with an IC₅₀ value of 14.7 ± 3.9 µM against PA14 while the phenbutyl RA analogue was markedly less active with an IC₅₀ value of 58.9 ± 14.8 µM. Again, to demonstrate the non-toxic nature of these analogues on planktonic bacteria, growth curve experiments with the p-bromo analogue 84 were performed.
Both the treated and untreated cell densities remained relatively unchanged during a 24-hour
time period (appendix).

Table 10. Synthesis and Anti-biofilm Activity of Phenyl Amine Analogues.

<table>
<thead>
<tr>
<th>Target</th>
<th>PA14 IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>84</td>
<td>14.7 ± 3.9</td>
</tr>
<tr>
<td>85</td>
<td>58.9 ± 14.8</td>
</tr>
</tbody>
</table>

Reaction conditions: (a) EDC, HOBt, DMF (b) TFA, CH₂Cl₂ (c) 2M HCl/Et₂O.

Ultimately either the addition or a removal of a single methylene unit to the linker of
the RA scaffold with the hexylamide in place drastically reduced anti-biofilm potency.
However, it was shown that activity could be regained with addition of a phenyl ring.
Compounds possessing increasing degrees of bromination similar to 84 are of further interest
and are currently being formulated and evaluated for anti-biofilm properties within the lab.
We envisioned using the hexyl RA analogue as our starting point and shifting the amide bond one carbon in each direction along the chain (Figure 23). This would be complementary to the linker chain modification study (vide supra) and would allow some insight into what intramolecular interactions may be occurring that affect anti-biofilm activity within the class on compounds.

**Table 11.** Synthesis and Anti-biofilm Activity of Slider Analogues.

<table>
<thead>
<tr>
<th>Target</th>
<th>Biofilm Inhibition at 100 µM vs PA14 [%]</th>
<th>PA14 IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>86</td>
<td>74 ± 2</td>
<td>42.9 ± 7.9</td>
</tr>
<tr>
<td>87</td>
<td>24 ± 5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

*Reaction conditions: (a) EDC, HOBt, DMF (b) TFA, CH₂Cl₂ (c) 2M HCl/Et₂O. n.d. = not determined*

Using the aforementioned two- and four-carbon carboxylic acids, 80 was coupled to heptylamine while 81 was coupled to pentylamine to afford the two analogues both having identical total chain lengths in reference to the parent hexyl RA analogue (Table 11). Evaluation of these two analogues for anti-biofilm activity against PA14 showed that the two-carbon analogue 86 (PA14 IC₅₀ = 43.8 ± 10.2 µM) was only slightly less active than the parent three-carbon hexyl RA 54, but this difference was negligible due to associated error (Table 11). The four-carbon analogue 87 was incredibly less active, only being able to inhibit PA14 biofilm formation 24% at 100 µM.
5.4 Increased Substitution of the Amide Bond

As previously shown, using long linear aliphatic amides on a RA 2-AI scaffold afforded compounds that were extremely active at inhibiting PA14 biofilm formation. Another intriguing option involved installation of an additional aliphatic chain to the molecule in hopes that it would be able to further enhance activity. Therefore, both a secondary and tertiary dihexyl RA derivative were synthesized to simultaneously test for the affect of additional aliphatic chains as well as the necessity of the NH amide proton for anti-biofilm activity. The synthesis of the secondary dihexyl derivative followed our previously established chemistry (Scheme 15). Coupling the three-carbon and two-carbon carboxylic acids with the known branched secondary hexaheptylamine\textsuperscript{10} under EDC conditions followed by deprotection and salt exchange afforded the target secondary dihexyl RA analogues 89 and 88, respectively. Synthesis of the tertiary dihexyl RA analogue was simply executed through opening of glutaric anhydride with dihexylamine and moving the acid intermediate through identical chemistry to deliver the final target.
Upon biological evaluation of these analogues, the tertiary derivative 90 displayed moderate activity against PA14 biofilms with an IC$_{50}$ value of 59.7 ± 12.3 µM (Table 12). It is notable that within experimental errors reported, that this activity is almost identical to the mono-hexyl RA analogue 54. Evaluation of the three-carbon secondary dihexyl RA analogue 89 against inhibiting PA14 biofilms, however, gave an IC$_{50}$ value of 15.6 ± 1.8 µM which was over 2-fold more active than the mono-hexyl RA analogue 54. The two-carbon secondary dihexyl RA analogue 88 was also active at inhibiting PA14 biofilms with an IC$_{50}$ = 18.2 ± 1.3 µM, essentially identical to the three carbon homologue. Growth curve experiments with compound 89 validated that cellular densities remained relatively identical over a 24-hour time period (appendix). Upon examination of this subset of compounds, it becomes clear that the activity increases with the increased substitution alpha to the amide but, conversely, the activity is still retained when the NH amide proton is replaced with an aliphatic chain.

**Scheme 15.** Synthesis of Additionally Substituted Analogues. Reaction conditions: (a) EDC, HOBr, DMF (b) TFA, CH$_2$Cl$_2$ (c) 2M HCl/Et$_2$O (d) dihexylamine, TEA, DMAP, CH$_2$Cl$_2$ (87%) (e) i: (COCl)$_2$, DMF (cat.), CH$_2$Cl$_2$; ii: CH$_3$N$_2$, Et$_2$O/CH$_2$Cl$_2$, 0 °C; iii: Conc. HBr (f) Boc-guanidine, DMF (27% over 2 steps) (g) TFA, CH$_2$Cl$_2$ (h) 2M HCl/Et$_2$O (94%).
Table 12. Anti-biofilm Activity of Additionally Substituted RA Analogues against PA14.

<table>
<thead>
<tr>
<th></th>
<th>PA14 IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>18.2 ± 1.3</td>
</tr>
<tr>
<td>89</td>
<td>15.6 ± 1.8</td>
</tr>
<tr>
<td>90</td>
<td>59.7 ± 12.3</td>
</tr>
</tbody>
</table>

5.5 Reversal of the Amide Bond to Mimic Oroidin

The deletion of, sliding, and additional substitution to the amide functionality were all efforts to elucidate if the aforementioned region of the molecule was critical to eliciting anti-biofilm activity. At this juncture, there have been quite a range of activities for the compounds presented. Some modifications in the structure are well tolerated within the context of anti-biofilm activity while others are not. Throughout the synthetic efforts on this project, one aspect had eluded our best efforts; construction of a novel “natural” dihydrooroidin scaffold capable of being acylated by more traditional means other than the established chemistry of using trichloroesters. As previously discussed, the use of 4-(3-aminopropyl)-2-aminoimidazole 62 in acylation reactions involving the oroidin class of molecules leaves much to be desired. Upon close examination of the literature, a new approach was developed to circumvent these problems while also again taking advantage of the ability to synthesize a large number of chemically unique derivatives. The idea was to develop acylation chemistry around a masked amine relative of our established Boc-2AI carboxylic acid intermediates.

Advancement of the known azido carboxylic acid proceeded smoothly to afford the Boc-2AI azide 91 (Scheme 16). This intermediate was reduced with Pd/C under a hydrogen
atmosphere followed by *in-situ* acylation employing heptanoic anhydride to afford the “natural” amide analogue 92 in a very high yield over two-steps in comparison to the yields traditionally obtained with 62. This pathway also allowed access to a Boc-protected derivative of 62 not accessible through the established routes to this intermediate. Deprotection afforded the “natural” amide hexyl cousin of 54, thus obtaining a compound that contained the amide directionality seen in the natural products.

Assays performed investigating this analogue against PA14 proved very interesting as it was completely inactive within the concentrations tested, yielding < 5% biofilm inhibition at 100 µM. This result is in stark contrast to the activity reported for 54 and is very instructive to possibly understanding that the amide directionality coupled with an appropriate aliphatic chain has a profound impact on anti-biofilm properties. Further development and tuning of this methodology is currently under further investigation to increase chemical diversity around this portion of the molecule.

5.6 Examination of a Triazole Isostere

*En route* to synthesizing the natural amide hexyl analogue, it was also observed that construction of a triazole isostere was possible through utilization of the pendant azide functionality. The overall dipolar moment of a triazole system is larger than that of an
amide bond, thus making its hydrogen bonding donor and acceptor properties more pronounced.\textsuperscript{13, 14} Due to the recent success of a small library of triazole substrates in inhibiting and dispersing various medically relevant biofilms,\textsuperscript{15} an analogue of this nature would be deemed a valuable addition to the SAR study. To this end, the Boc-2AI azide 91 was reacted with 1-octyne in a [3+2] copper-catalyzed click reaction followed by deprotection to smoothly afford the triazole hexyl isostere 93. (Table 13). Gratifyingly, the triazole analogue 93 was observed to possess an IC\textsubscript{50} value of 26.9 ± 4.0 µM, allowing for retainment of activity in comparison with 54. A PA14 growth curve was also performed in the presence and absence of 93. Over a 24-hour time period, both the treated and untreated cell densities remained relatively unchanged (appendix). Additional libraries taking advantage of both the speed and diversity of the click reaction with the newly developed scaffold 91 are currently being generated and are under investigation for anti-biofilm properties.

| Table 13. Synthesis and Anti-biofilm Activity of a Triazole Isostere. |
| | Target | Reaction conditions: (a) 1-octyne, CuSO\textsubscript{4} \cdot 5H\textsubscript{2}O (20 mol\%), Na Ascorbate (10 mol\%), H\textsubscript{2}O/EtOH (1/1) (55\%) (b) TFA, CH\textsubscript{2}Cl\textsubscript{2} (c) 2M HCl/Et\textsubscript{2}O (99\%). |
| | 93 | PA14 IC\textsubscript{50} [µM] |
| | 26.9 ± 4.0 |

5.7 PA14 Biofilm Inhibition: Activity Profile Comparisons

Overall, the activity of the hexyl RA analogue 54 was able to be tuned in various ways along a defined SAR route to increase anti-biofilm activity (Figure 24). Although this study was designed around specific modifications, one may also look at the compounds
across each sub-class to help give a better understanding of what is necessary for an exceptional anti-biofilm modulator. One of the most revealing observations about the current Reverse Amide SAR study was the activity of the “natural” amide hexyl congener 92 in comparison to the RA derivative 54. The natural amide 92 was completely inactive, unable to inhibit PA14 biofilm formation even at the highest concentration tested (100 µM). This result confirmed that the reversal of the amide bond directionality was partly responsible for the activity of this particular class of compounds, not withstanding the fatty acid derivatives. Interestingly, analogue 86 (which bears the amide carbonyl towards the side of the 2-AI motif) was an active biofilm modulator in stark contrast to 92 (Figure 25).

Figure 24. PA14 inhibition IC₅₀ values from selected SAR analogues.

Additionally, exchange of the amide bond with a triazole surrogate to produce 93 showed that activity could be slightly enhanced. These observations again, may be hinting towards a crucial placement of the amide carbonyl/triazole moiety to elicit a maximum biological effect through possible intra- or intermolecular interactions. Both the two-carbon
(86) and four-carbon (87) analogues were less active than the parent three-carbon hexyl RA analogue.

While sliding the amide bond and changing the chain length tended to reduce activity, increasing the substitution of the amide bond or removing it altogether tended to cause an increase in activity. Synthesis of a secondary dihexyl derivative 89 increased the activity 2-fold. While this may be attributable to an increase in the longest chain (7C’s for 35, 6C’s for 4) the activity corresponds closely to that of the octyl RA analogue indicating that the increased substitution is modulating the activity. Additionally, the tertiary amide derivative 90 is only slightly less active then the mono-hexyl RA analogue 54.

Deletion of the amide bond provided a few active fatty acid 2-AI analogues which displayed increased anti-biofilm activity in reference to their RA relatives. There was, however, a clear cut-off point for the potency of this class of molecules as only the shorter chain saturated derivatives were active. This is partly explainable by making a comparison to naturally occurring signaling molecules found in P. aeruginosa which share similar structural characteristics (AHLs). It is probable that these shorter chain fatty acid derivatives may elicit their biological effect in a different manner than the RA linear alkyl chain compounds.
5.8 *Acinetobacter baumannii* Biofilm Inhibition

To this point, we have exclusively employed PA14 as the bacterial strain of choice to evaluate the current library as novel small molecule anti-biofilm agents. However, it was deemed necessary to deviate from *Pseudomonas* and assay our most active and structurally diverse analogues against the Gram-negative γ-proteobacterium *Acinetobacter baumannii*.

Nine of the most active analogues obtained from the *Pseudomonas* biofilm inhibition assays underwent a preliminary screen for biofilm inhibition activity against *A. baumannii* (*Table 14*). Three of these derivatives (84, 90, and 93) showed only marginal activity at 100 µM which was in stark contrast to the potency observed for these analogues against PA14. Tridecyl RA 63, the most active analogue with activity in the high nanomolar range against PA14, was noticeably less potent (albeit still extremely active) against *A. baumannii* (IC$_{50}$ = 25.5 ± 3.2 µM). The analogues derived from the sub-class of compounds in which the amide bond had been removed (72-74) displayed the greatest activity out of any assayed against *Acinetobacter*. It was observed that as the chain length increased, potency increased culminating in the most potent derivative 74 (IC$_{50}$ = 13.2 ± 0.7 µM).
Table 14. Anti-biofilm Activity against *A. baumannii*.

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Biofilm Inhibition at 100 µM vs *A. baumannii [%]</th>
<th>*A. baumannii IC₅₀ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>≥ 95</td>
<td>25.5 ± 3.2</td>
</tr>
<tr>
<td>72</td>
<td>≥ 95</td>
<td>42.5 ± 0.8</td>
</tr>
<tr>
<td>73</td>
<td>≥ 95</td>
<td>13.2 ± 0.7</td>
</tr>
<tr>
<td>74</td>
<td>≥ 95</td>
<td>8.04 ± 0.32</td>
</tr>
<tr>
<td>84</td>
<td>18 ± 3</td>
<td>n.d.</td>
</tr>
<tr>
<td>88</td>
<td>≥ 95</td>
<td>50.7 ± 0.7</td>
</tr>
<tr>
<td>89</td>
<td>≥ 95</td>
<td>37.7 ± 0.1</td>
</tr>
<tr>
<td>90</td>
<td>34 ± 4</td>
<td>n.d.</td>
</tr>
<tr>
<td>93</td>
<td>22 ± 4</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not determined.

Growth curves were performed with several of the active compounds (63, 73, 74, 89) at their respective IC₅₀ values and, surprisingly, the compounds were eliciting some of their anti-biofilm properties through a bacteriostatic effect as evidenced by the lack of bacterial growth in the presence of the compounds. *A. baumannii* growth was suppressed by the analogues for at least 9 hours, but by the 24 hour time-point, the treated cell densities are nearly the same as the untreated. This lead us to run growth curves at the analogues respective IC₃₀ values (63: 14.8 µM, 73: 10.2 µM, 74, 6.27 µM, 89: 36.7 µM) as a means to determine the toxicity of these compounds at lower concentrations. At the IC₃₀ value, growth was evident after only 3 hours but was still slower to grow compared to the untreated. After
24 hours at the IC$_{30}$, the cell densities are, again, identical to the untreated. This appears to indicate that the RA class of compounds has a very small window between inhibiting biofilm formation and inhibiting planktonic \textit{A. baumannii} growth. Overall, these compounds are still eliciting a biofilm inhibitory effect which is unique to this class of molecules. Their \textit{A. baumannii} biofilm inhibitory activity may be modulated by slight bacteriostatic effects, but during the course of the assay (24h), the treated bacteria have enough time to become homeostatic in respect to the untreated controls as evidenced by the growth curve.

Despite the overall decrease in ability to inhibit \textit{Acinetobacter} biofilm formation in comparison to PA14, the inhibition data presented is still noteworthy. Although the \textit{A. baumannii} anti-biofilm modulating properties of the RA class of compounds may be due, in part, to bacteriostatic effects, their activity lends hope to the reverse amide library’s ability to possibly inhibit other medically relevant biofilm forming bacteria thus solidifying their importance as novel small molecules in the anti-biofilm arena.

\textbf{5.9 Dispersion of \textit{P. aeruginosa} and \textit{A. baumannii} Biofilms}

Perhaps a more important characteristic of small molecules known to inhibit biofilm formation is their ability to disperse pre-existing biofilms. This is significant from a biomedical standpoint as treatment for infections usually begins after biofilm formation has been initiated and current therapies may be of no use. It was shown previously that our most active reverse amide derivative \textbf{57} (obtained from the first generation library) acted as both a superior inhibitor and dispersal agent against \textit{Pseudomonas} PAO1 and PA14 biofilms. With the discovery of additional biofilm inhibitors, it was hoped that this success could be
duplicated with the current library. PA14 and *A. baumannii* biofilms were first allowed to form in the absence of compound before being dosed with the most active derivatives encompassing various structural characteristics and potencies against both strains of bacteria.

The stringency applied to the dispersion screens was identical to that used in the inhibition assays. Dispersal activity was first assessed at 100 µM to determine which compounds would then be moved on further for EC$_{50}$ value determination. One general trend observed for dispersion activity was that all biofilm dispersion EC$_{50}$ values were higher than the respective compounds biofilm inhibition IC$_{50}$ values regardless of the bacterial strain employed.

**Table 15. Selected Compounds with Anti-biofilm Dispersing Properties.**

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Dispersion at 100 µM [%]</th>
<th>EC$_{50}$ [µM]</th>
<th>Dispersion at 100 µM [%]</th>
<th>EC$_{50}$ [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>73 ± 2</td>
<td>54.0 ± 6.3</td>
<td>38 ± 1</td>
<td>75.4 ± 13.1</td>
</tr>
<tr>
<td>72</td>
<td>≥ 95</td>
<td>64.3 ± 3.4</td>
<td>39 ± 3</td>
<td>131 ± 10</td>
</tr>
<tr>
<td>73</td>
<td>≥ 95</td>
<td>66.5 ± 4.9</td>
<td>72 ± 1</td>
<td>68.0 ± 2.2</td>
</tr>
<tr>
<td>74</td>
<td>24 ± 4</td>
<td>n.d.</td>
<td>37 ± 3</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>84</td>
<td>18 ± 3</td>
<td>n.d.</td>
<td>&lt; 5</td>
<td>n.d.</td>
</tr>
<tr>
<td>88</td>
<td>n.d.</td>
<td>n.d.</td>
<td>77 ± 1</td>
<td>59.8 ± 1.8</td>
</tr>
<tr>
<td>89</td>
<td>≥ 95</td>
<td>26.3 ± 2.3</td>
<td>65 ± 3</td>
<td>61.3 ± 2.6</td>
</tr>
<tr>
<td>93</td>
<td>33 ± 6</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.d. = not determined.
The hope of duplicating the dispersal activity of 57 with the newly identified most active PA14 biofilm inhibitor 63 was not realized as surprisingly this compound only showed marginal dispersal activity against PA14 with an EC$_{50}$ value of 54.0 ± 6.3 µM. The only other compound of exceptional note was the secondary dihexyl derivative 89 whose EC$_{50}$ dispersal activity is, within error, just as potent as 57. Two of the shorter chain fatty acid analogues 72 and 73 also showed moderate biofilm dispersing properties while the triazole analogue 93, which was a potent PA14 biofilm inhibitor, did not possess the same dispersal activity.

The most active small molecules identified in the dispersal of existing Acinetobacter baumannii biofilms were those bearing the dihexyl side chains on the two (88) or three (89) carbon amide bond linker (EC$_{50}$ values of 59.8 ± 1.8 and 61.3 ± 2.6 µM, respectively). Closely following in potency was the fatty acid derivative 73 and the tridecyl reverse amide analogue 63.

5.10 Conclusion

Overall, the most concrete information obtained from the SAR study involved the activities of the analogues bearing the oroidin family amide directionality and its replacement with a triazole isostere. Intriguingly, no anti-biofilm activity was observed for the natural amide congener 92 which is in contrast compared to the parent hexyl RA 54. Also, a direct amide-bond directionality comparison can be made with analogues 86 and 92. Analogue 86 was shown to be nearly as active as the hexyl RA 54, while 92, with the natural amide orientation, displayed no activity. Additionally, the triazole isostere 93 recapitulated the activity of RA 54 which will allow for further SAR analysis.
With the exception of the fatty acids, the data indicates that reversal of the amide bond is partly responsible for the anti-biofilm activity of this class of compounds. It remains to be seen whether other derivatives bearing the amide orientation present in the natural products have the ability to be as potent as those within the RA scaffold. Exploitation of the click reaction and reductive acylation methodology outlined above will ultimately allow this point to be addressed head-on and is currently under intensive investigation.

Further SAR information indicates that increased substitution of the amide bond is an effective means of increasing activity. Applying this to the newly identified tridecyl RA analogue 63, it may be possible to further enhance its activity and efforts to address this issue are currently being formulated.

Additionally, removal of the amide bond entirely allowed for the facile multi-gram generation of several fatty acid 2-AI derivatives (72-74) that were shown to possess potent anti-biofilm activity. Albeit, the range of active analogues does not mirror that of the RA aliphatic chain sub-class, but the three active fatty acid analogues identified were active anti-biofilm modulators. Additionally, the fatty acid 2-AI analogues were shown to possess some bacteriostatic properties during the early stages of A. baumannii biofilm development.

Despite the inability of our most active PA14 biofilm inhibitor (tridecyl RA analogue 63) to become the most potent PA14 biofilm dispersal agent, the dispersal activity of selected members from the library is noteworthy due to the potency of analogue 35 in being able to disperse both PA14 and A. baumannii biofilms. It is also interesting that a few analogues which were active in the inhibition assays lacked significant dispersal activity (i.e.
compounds 84 and 93). This trend is not without precedence as other anti-biofilm molecules have demonstrated similar activity profiles against various bacterial biofilms.\textsuperscript{9,15,16}

In conclusion, we have demonstrated that the Reverse Amide class of 2-aminoimidazoles has provided a fertile avenue for the exploration and development of numerous biofilm modulators. Information obtained with the SAR profile from this class of compounds should allow for further analogue tuning and ultimately facilitate the construction of even more potent 2-AI anti-biofilm derivatives. With the lack of new classes of antibiotics and increased multi-drug resistance, the need for alternative approaches to mitigate infectious diseases is sorely needed.
5.11 Experimental Section

Stock solutions (100, 50, 10, 1 mM) of all compounds assayed for biological activity were prepared in DMSO and stored at room temperature. The amount of DMSO used in both inhibition and dispersion screens did not exceed 1% (by volume). *P. aeruginosa* PA14 was graciously supplied by the Wozniak group at Wake Forest University School of Medicine and by the O’Toole group at Dartmouth Medical School. *A. baumannii* (ATCC 19606) was purchased from ATCC.

**General Static Inhibition Assay Protocol for *P. aeruginosa* and *A. baumannii*.**

An overnight culture of the wild type strain was subcultured at an OD$_{600}$ of 0.01 into LBNS (PA14) or LB (*A. baumannii*) along with a predetermined concentration of the small molecule to be tested for biofilm inhibition. Samples were then aliquotted (100 µL) into the wells of a 96-well PVC microtiter plate. The microtiter dishes were covered and sealed before incubation under stationary conditions at 37 °C for 24 hours. After that time, the medium was discarded and the plates thoroughly washed with water. The wells were then inoculated with a 0.1% aqueous solution of crystal violet (100 µL) and allowed to stand at ambient temperature for 30 minutes. Following another thorough washing with water the remaining stain was solubilized with 200 µL of 95% ethanol. Biofilm inhibition was quantitated by measuring the OD$_{540}$ for each well by transferring 125 µL of the ethanol solution into a fresh polystyrene microtiter dish for analysis.
General Static Dispersion Assay Protocols for *P. aeruginosa* and *A. baumannii*.

An overnight culture of the wild type strain was subcultured at an OD$_{600}$ of 0.05 into LBNS (PA14) or LB (*A. baumannii*) and then aliquotted (100 µL) into the wells of a 96-well PVC microtiter plate. The microtiter dishes were covered and sealed before incubation under stationary conditions at room temperature to allow formation of the biofilms. After 24 hours the medium was discarded and the plates thoroughly washed with water. Fresh medium containing the appropriate concentration of compound was then added to the wells. The plates were again sealed and this time incubated under stationary conditions at 37 °C. After 24 hours, the media was discarded from the wells and the plates washed thoroughly with water. The wells were inoculated with a 0.1% aqueous solution of crystal violet (100 µL) and allowed to stand at ambient temperature for 30 minutes. Following another thorough washing with water the remaining stain was solubilized with 200 µL of 95% ethanol. Biofilm dispersion was quantitated by measuring the OD$_{540}$ for each well by transferring 125 µL of the ethanol solution into a fresh polystyrene microtiter dish for analysis. Percent dispersion was calculated by comparison of the OD$_{540}$ for established biofilm (untreated) versus treated established biofilm under identical conditions.

Chemistry

All reagents including anhydrous solvents used for the chemical synthesis of the library were purchased from commercially available sources and used without further purification unless otherwise noted. All reactions were run under either a nitrogen or argon atmosphere. Flash silica gel chromatography was performed with 60Å mesh standard grade silica gel from
Sorbtech. $^1$H and $^{13}$C NMR spectra were obtained using Varian 300 MHz or 400 MHz spectrometers. NMR solvents were purchased from Cambridge Isotope Labs and used as is. Chemical shifts are given in parts per million relative to DMSO-$d_6$ ($\delta$ 2.50), CD$_3$OD ($\delta$ 3.34) and CDCl$_3$ ($\delta$ 7.27) for proton spectra and relative to DMSO-$d_6$ ($\delta$ 39.51), CD$_3$OD ($\delta$ 49.86) and CDCl$_3$ ($\delta$ 77.21) for carbon spectra with an internal TMS standard. High-resolution mass spectra were obtained at the North Carolina State Mass Spectrometry Laboratory for Biotechnology. ESI experiments were carried out on Agilent LC-TOF mass spectrometer.

**Finalizing the RA Chain Length**

**General EDC/HOBt procedure:** 2-Amino-4-(3-carboxypropyl)imidazole-1-carboxylic acid tert-butyl ester 43 (0.10 g, 0.371 mmol), 1-hydroxybenzotriazole (0.10 g, 0.742 mmol) and $N$-(3-dimethylaminopropyl)-$N'$-ethylcarbodiimide hydrochloride (0.142 g, 0.742 mmol) and DIPEA (0.26 mL, 1.48 mmol) were dissolved in anhydrous DMF (3 mL) and allowed to stir for 15 mins. The appropriate amine coupling partner (0.78 mmol) was then added and the solution was stirred at ambient temperature until completion was evident by TLC analysis. The reaction was concentrated under reduced pressure and the residue partitioned between ethyl acetate (20 mL) and water (10 mL). The organic layer was successively washed with water (3 x 10 mL), 1M HCl (3 x 10 mL), sat. NaHCO$_3$ (2 x 10 mL), and brine (10 mL) before being dried (Na$_2$SO$_4$) and evaporated to dryness. The crude product was purified via flash column chromatography (2-10% MeOH/CH$_2$Cl$_2$) to afford the target compounds.
2-Amino-4-(3-tetradecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (67). Using the general EDC/HOBt procedure, carboxylic acid 43 yielded a tan solid (0.042 g, 24%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.72 (m, 1H), 6.50 (s, 1H), 6.37 (s, 2H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.21 (t, 2H, $J = 6.8$ Hz), 2.04 (t, 2H, $J = 7.6$ Hz), 1.71 (m, 2H), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (bs, 22H), 0.85 (t, 3H, $J = 5.2$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 171.6, 149.9, 148.9, 138.3, 105.8, 84.0, 38.3, 34.9, 31.3, 29.2, 29.1, 29.0, 28.7, 28.0, 27.9, 27.5, 27.2, 26.4, 24.1, 22.1, 14.0; HRMS (ESI) calcd for C$_{26}$H$_{48}$N$_4$O$_3$ (M)$^+$ 464.3726, found 464.3742.

2-Amino-4-(3-hexadecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (68). Using the general EDC/HOBt procedure, carboxylic acid 43 yielded a tan solid (0.034 g, 19%). $^1$H NMR (400 MHz, DMSO-$d_6$) δ 7.72 (m, 1H), 6.50 (s, 1H), 6.39 (s, 2H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.21 (t, 2H, $J = 7.2$ Hz), 2.04 (t, 2H, $J = 7.2$ Hz), 1.71 (m, 2H), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (bs, 26H), 0.85 (t, 3H, $J = 5.2$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 171.6, 149.9, 148.9, 138.3, 105.8, 84.0, 38.3, 34.9, 31.3, 29.1, 29.0, 28.7, 27.5, 27.2, 26.4, 24.1, 22.1, 14.0; HRMS (ESI) calcd for C$_{28}$H$_{52}$N$_4$O$_3$ (M)$^+$
2-Amino-4-(3-octadecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (69). Using the general EDC/HOBt procedure, carboxylic acid 43 afforded a tan solid (0.025 g, 13%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.73 (m, 1H), 6.50 (s, 1H), 6.38 (s, 2H), 3.00 (q, 2H, J = 6.4 Hz), 2.21 (t, 2H, J = 7.2 Hz), 2.03 (t, 2H, J = 7.2 Hz), 1.70 (m, 2H), 1.53 (s, 9H), 1.35 (m, 2H), 1.22 (bs, 30H), 0.85 (t, 3H, J = 6.0 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.6, 149.8, 148.9, 138.4, 105.8, 84.00, 38.3, 24.9, 31.2, 29.1, 29.0, 28.6, 27.9, 27.5, 27.2, 26.3, 24.0, 22.0, 13.9; HRMS (ESI) calcd for C$_{30}$H$_{56}$N$_4$O$_3$ (M)$^+$ 520.4352, found 520.4354.

2-Amino-4-(3-undecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (70). Using the general EDC/HOBt procedure, carboxylic acid 43 (0.150 g, 0.58 mmol) yielded 70 (0.073 g, 31%) as a tan solid. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.72 (t, 1H, J = 7.2 Hz), 6.50 (s, 1H), 6.37 (s, 2H), 3.00 (q, 2H, J = 6.9 Hz), 2.21 (t, 2H, J = 6.9 Hz), 2.04 (t, 2H, J = 7.2 Hz), 1.70 (quin, 2H, J = 7.5 Hz), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (bs, 16H), 0.85 (t, 3H, J = 6.9 Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.7, 149.9, 148.9,
2-Amino-4-(3-tridecylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (71). Using the general EDC/HOBt procedure, carboxylic acid 43 (0.150 g, 0.580 mmol) afforded 71 (0.062 g, 25%) as a tan solid. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.72 (t, 1H, $J = 7.2$ Hz), 6.50 (s, 1H), 6.37 (s, 2H), 3.00 (q, 2H, $J = 6.9$ Hz), 2.21 (t, 2H, $J = 6.9$ Hz), 2.04 (t, 2H, $J = 7.2$ Hz), 1.70 (quint, 2H, $J = 7.5$ Hz), 1.53 (s, 9H), 1.36 (m, 2H), 1.23 (bs, 20H), 0.85 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.7, 149.9, 149.0, 138.4, 105.8, 84.1, 38.4, 34.9, 31.3, 29.2, 29.0, 28.7, 27.5, 27.2, 26.4, 24.1, 22.1, 14.0; HRMS (ESI) calcd for $C_{25}H_{46}N_4O_3$ ($M^+$) 450.3570, found 450.3576.

4-(2-Amino-1H-imidazol-4-yl)-N-undecylbutyramide hydrochloride (62). A solution of 70 (0.027 g, 0.064 mmol) in anhydrous dichloromethane (1 mL) was cooled to 0 °C. TFA (1.0 mL) was added to the reaction and was stirred for 6 h. After that time the solution was evaporated to dryness and toluene (2 mL) was added. The toluene was then
evaporated to dryness and this process was repeated two times. The resulting TFA salt was dissolved in dichloromethane (1 mL) and 2M HCl in diethyl ether (0.5 mL) was added followed by cold diethyl ether (8 mL). The precipitate was collected by filtration and washed with diethyl ether (3 mL) to yield 62 (0.023 g, 99%) as a white amorphous solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.09 (s, 1H), 11.65 (s, 1H), 7.85 (m, 1H), 7.30 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 5.7$ Hz), 2.38 (t, 2H, $J = 7.5$ Hz), 2.07 (t, 2H, $J = 7.5$ Hz), 1.73 (quint, 2H, $J = 7.5$ Hz), 1.36 (m, 2H), 1.23 (bs, 16H), 0.85 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 171.3, 146.7, 126.3, 108.6, 38.4, 34.4, 31.3, 29.1, 29.0, 28.7, 28.7, 26.4, 23.9, 23.6, 22.1, 13.9; HRMS (ESI) calcd for C$_{18}$H$_{34}$N$_4$O (M)$^+$ 322.2732, found 322.2743.

4-(2-Amino-1H-imidazol-4-yl)-N-tridecylbutyramide hydrochloride (63). Using the same general procedure as used for the synthesis of 62, 71 (0.028 g, 0.062 mmol) afforded 63 (0.024 g, 99%) as a white amorphous solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.08 (s, 1H), 11.66 (s, 1H), 7.85 (m, 1H), 7.30 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 5.7$ Hz), 2.38 (t, 2H, $J = 7.5$ Hz), 2.07 (t, 2H, $J = 7.5$ Hz), 1.73 (quint, 2H, $J = 7.5$ Hz), 1.36 (m, 2H), 1.23 (bs, 20H), 0.85 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 171.3, 146.7, 126.3, 108.6, 38.4, 34.4, 31.3, 29.1, 29.0, 28.98, 28.7, 28.7, 26.4, 23.9, 23.6, 22.1, 13.9; HRMS (ESI) calcd for C$_{20}$H$_{38}$N$_4$O (M)$^+$ 350.3045, found 350.3052.
4-(2-Amino-1H-imidazol-4-yl)-N-tetradecylbutyramide hydrochloride (64). Using the same general procedure as used for the synthesis of 62, 67 (0.030 g, 0.064 mmol) gave 64 (0.024 g, 92%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.05 (s, 1H), 11.62 (s, 1H), 7.83 (m, 1H), 7.31 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.37 (t, 2H, $J = 7.2$ Hz), 2.07 (t, 2H, $J = 7.2$ Hz), 1.73 (m, 2H), 1.35 (m, 2H), 1.23 (bs, 22H), 0.85 (t, 3H, $J = 6.4$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.3, 146.7, 126.4, 108.7, 38.4, 34.4, 31.3, 29.1, 29.03, 28.99, 28.7, 28.7, 26.4, 23.9, 23.6, 22.1, 13.9; HRMS (ESI) calcd for C$_{21}$H$_{40}$N$_4$O (M)$^+$ 364.3203, found 364.3196.

4-(2-Amino-1H-imidazol-4-yl)-N-hexadecylbutyramide hydrochloride (65). Using the same general procedure as used for the synthesis of 62, 68 (0.029 g, 0.059 mmol) yielded 65 (0.023 g, 92%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.02 (s, 1H), 11.59 (s, 1H), 7.82 (m, 1H), 7.31 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 6.4$ Hz), 2.37 (t, 2H, $J = 7.2$ Hz), 2.07 (t, 2H, $J = 7.2$ Hz), 1.73 (m, 2H), 1.35 (m, 2H), 1.23 (bs, 26H), 0.85 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.3, 146.7, 126.4, 108.7, 38.4, 34.4,
31.3, 29.1, 28.99, 28.96, 28.7, 28.7, 26.4, 23.8, 23.6, 22.1, 13.9; HRMS (ESI) calcd for C$_{23}$H$_{44}$N$_4$O (M)$^+$ 392.3515, found 392.3513.

4-(2-Amino-1H-imidazol-4-yl)-N-octadecylbutyramide hydrochloride (66). Using the same general procedure as used for the synthesis of 62, 69 (0.028 g, 0.054 mmol) afforded 66 (0.023 g, 94%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.02 (s, 1H), 11.60 (s, 1H), 7.82 (s, 1H), 7.31 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 6.6$ Hz), 2.37 (t, 2H, $J = 6.6$ Hz), 2.06 (t, 2H, $J = 7.2$ Hz), 1.73 (m, 2H), 1.35 (m, 2H), 1.23 (bs, 30H), 0.85 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 171.3, 146.7, 126.4, 108.7, 38.4, 34.4, 31.3, 29.1, 29.0, 28.74, 28.70, 26.4, 23.9, 23.6, 22.1, 14.0; HRMS (ESI) calcd for C$_{25}$H$_{48}$N$_4$O (M)$^+$ 420.3828, found 420.3838.

1-Bromoundecan-2-one (5-vi). Decanoyl chloride (2.25 mL, 11.0 mmol) was dissolved in anhydrous dichloromethane (10 mL) and added dropwise to a 0 ºC solution of CH$_2$N$_2$ (33.0 mmol generated from Diazald®/KOH) in diethyl ether (100 mL). This solution was stirred at 0 ºC for 1.5 h at which time the reaction was quenched via the dropwise addition of 48% HBr (4.0 mL). The reaction mixture was diluted with dichloromethane.
(25 mL) and immediately washed with sat. NaHCO₃ (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO₄), filtered and concentrated. The crude oil was purified via flash column chromatography (0-8% Et₂O/hexanes) to obtain 5-vi (2.31 g, 84%) as a white solid. ¹H NMR (400 MHz, DMSO-𝑑₆) δ 4.32 (s, 2H), 2.56 (t, 2H, J = 7.2 Hz), 1.47 (m, 2H), 1.23 (bs, 12H), 0.85 (t, 3H, J = 6.0 Hz); ¹³C NMR (100 MHz, DMSO-𝑑₆) δ 201.7, 39.1, 36.9, 31.3, 28.9, 28.8, 28.7, 28.4, 23.2, 22.1, 14.0; HRMS (ESI) calcd for C₁₁H₂₁BrO (M)⁺ 248.0776, found 248.0787.

1-Bromotridecan-2-one (5-vii). Using the same general procedure as used for 5-vi, dodecanoyl chloride (4.72 mL, 19.9 mmol) gave a crude oil which was purified by flash column chromatography (0-10% Et₂O/hexanes) to yield 5-vii (4.59 g, 83%) as a white solid. ¹H NMR (300 MHz, DMSO-𝑑₆) δ 4.32 (s, 2H), 2.56 (t, 2H, J = 7.2 Hz), 1.47 (m, 2H), 1.24 (bs, 16H), 0.85 (t, 3H, J = 6.6 Hz); ¹³C NMR (75 MHz, DMSO-𝑑₆) δ 201.7, 39.0, 36.9, 31.3, 29.0, 28.9, 28.8, 28.7, 28.4, 23.2, 22.1, 14.0; HRMS (ESI) calcd for C₁₃H₂₅BrO (M)⁺ 276.1089, found 276.1098.

1-Bromopentadecan-2-one (5-viii). Using the same general procedure as used for 5-vi,
tetradecanoyl chloride (2.97 mL, 11.0 mmol) gave a crude oil which was purified by flash column chromatography (0-8% Et₂O/hexanes) to obtain 5-viii (3.08 g, 92%) as a white solid. ¹H NMR (400 MHz, DMSO-d₆, 60 °C) δ 4.27 (s, 2H), 2.57 (t, 2H, J = 7.2 Hz), 1.50 (m, 2H), 1.25 (bs, 20H), 0.86 (t, 3H, J = 6.0 Hz); ¹³C NMR (100 MHz, DMSO-d₆, 60 °C) δ 201.4, 38.9, 36.1, 31.0, 28.8, 28.7, 28.7, 28.6, 28.5, 28.4, 28.2, 23.0, 21.8, 13.6; HRMS (ESI) calcd for C₁₅H₂₉BrO (M)⁺ 304.1402, found 304.1415.

1-Bromoheptadecan-2-one (5-ix). Using the same general procedure as used for 5-vi, hexadecanoyl chloride (3.33 mL, 11.0 mmol) gave a crude oil which was purified by flash column chromatography (0-8% Et₂O/hexanes) to afford 5-ix (3.18 g, 87%) as a white solid. ¹H NMR (400 MHz, DMSO-d₆, 75 °C) δ 4.25 (s, 2H), 2.58 (t, 2H, J = 7.2 Hz), 1.51 (m, 2H), 1.26 (bs, 24H), 0.87 (t, 3H, J = 6.0 Hz); ¹³C NMR (100 MHz, DMSO-d₆, 75 °C) δ 201.34, 38.87, 35.83, 30.92, 28.64, 28.48, 28.36, 28.29, 28.11, 22.97, 21.68, 13.46; HRMS (ESI) calcd for C₁₇H₃₃BrO (M)⁺ 332.17148, found 332.17254.

1-Bromononadecan-2-one (5-x). Using the same general procedure as used for 5-vi, octadecanoyl chloride (3.3 g, 11.0 mmol) gave a crude solid which was triturated with a 1:1
EtOAc/CH$_2$Cl$_2$ mixture and filtered to obtain 5-x (3.61 g, 91%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$, 80 °C) $\delta$ 4.22 (s, 2H), 2.58 (t, 2H, $J = 7.2$ Hz), 1.52 (m, 2H), 1.26 (bs, 28H), 0.87 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$, 61 °C) $\delta$ 201.4, 38.9, 36.1, 31.0, 28.8, 28.6, 28.5, 28.4, 28.2, 23.0, 21.8, 13.6; HRMS (ESI) calcd for C$_{19}$H$_{37}$BrO (M)$^+$ 360.2028, found 360.2036.

(Z,Z)-1-Bromononadeca-10,13-dien-2-one (5-xvii). Using the same general procedure as used for 5-vi, octadecanoyl chloride (1.0 g, 3.34 mmol) gave a crude oil which was purified by flash column chromatography (0-8% Et$_2$O/hexanes) to yield 5-xvii (0.94 g, 78%) as a colorless oil. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 5.32 (m, 4H), 4.31 (s, 2H), 2.74 (t, 2H, $J = 5.4$ Hz), 2.57 (t, 2H, $J = 4.2$ Hz), 2.02 (q, 4H, $J = 6.6$ Hz), 1.48 (quint, 2H, $J = 6.6$ Hz), 1.27 (bs, 14H), 0.859 (t, 3H, $J = 6.6$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 201.6, 129.7, 129.7, 127.7, 127.7, 39.1, 36.7, 30.9, 29.0, 28.7, 28.6, 28.5, 28.4, 26.6, 25.2, 23.2, 22.0, 13.9; HRMS (ESI) calcd for C$_{19}$H$_{33}$BrO (M)$^+$ 356.1715, found 356.1716.

(Z)-1-Bromoocadec-10-en-2-one (5-xx). Using the same general procedure as used for 5-vi, octadecanoyl chloride (3.30 g, 11.0 mmol) gave a crude oil which was purified by
flash column chromatography (0-20% Et<sub>2</sub>O/hexanes) to afford 5-xx (3.89 g, 98%) as a colorless oil which solidified upon standing. <sup>1</sup>H NMR (300 MHz, DMSO-<em>d<sub>6</sub></em>) δ 5.31 (t, 2H, <em>J</em> = 4.2 Hz), 4.29 (s, 2H), 2.56 (t, 2H, <em>J</em> = 6.9 Hz), 1.97 (q, 4H, <em>J</em> = 6.0 Hz), 1.47 (t, 2H, <em>J</em> = 6.6 Hz), 1.24 (bs, 20H), 0.85 (t, 3H, <em>J</em> = 6.6 Hz); <sup>13</sup>C NMR (75 MHz, DMSO-<em>d<sub>6</sub></em>) δ 201.5, 129.6, 129.5, 39.0, 38.7, 36.7, 31.3, 29.1, 29.1, 28.9, 28.74, 28.70, 28.65, 28.5, 28.4, 26.6, 23.2, 22.1, 13.9; HRMS (ESI) calcd for C<sub>19</sub>H<sub>35</sub>BrO (M)<sup>+</sup> 358.1871, found 358.1866.

(E)-1-Bromo-9-methyldec-7-en-2-one (5-xviii). Using the same general procedure as used for 5-vi octadecanoyl chloride (1.50 g, 7.95 mmol) gave a crude oil which was purified by flash column chromatography (0-10% Et<sub>2</sub>O/hexanes) to obtain 5-xviii (1.92 g, 98%) as an amber oil. <sup>1</sup>H NMR (300 MHz, DMSO-<em>d<sub>6</sub></em>) δ 5.34 (m, 2H), 4.31 (s, 2H), 2.57 (t, 2H, <em>J</em> = 7.2 Hz), 2.20 (m, 1H), 1.93 (q, 2H, <em>J</em> = 6.0 Hz), 1.48 (m, 2H), 1.28 (m, 2H), 0.92 (d, 6H, <em>J</em> = 6.6 Hz); <sup>13</sup>C NMR (75 MHz, DMSO-<em>d<sub>6</sub></em>) δ 201.6, 137.4, 126.4, 38.9, 36.8, 31.6, 30.4, 28.4, 22.7, 22.5; HRMS (ESI) calcd for C<sub>11</sub>H<sub>19</sub>BrO (M)<sup>+</sup> 246.0619, found 246.0614.

2-Amino-4-nonylimidazole-1-carboxylic acid tert-butyl ester (5-xi). 1-bromo-undecan-
2-one (0.748 g, 3.0 mmol), Boc-guanidine (1.40 g, 9.0 mmol), and NaI (0.90 g, 6.0 mmol) were dissolved in DMF (15 mL) and allowed to stir at room temperature. After 72 h, the DMF was removed under reduced pressure. The residue was taken up in ethyl acetate (50 mL), washed with water (3 x 25 mL) and brine (25 mL) then dried (Na₂SO₄). Concentration gave an oil which was purified by flash column chromatography (0-50% EtOAc/hexanes) to afford 5-xi (0.408 g, 44%) as a tan solid. ¹H NMR (400 MHz, DMSO-
_d₆) δ 6.47 (s, 1H), 6.41 (s, 2H), 2.21 (t, 2H, J = 7.2 Hz), 1.51 (s, 9H), 1.47 (m, 2H), 1.22 (bs, 12H), 0.84 (t, 3H, J = 6.0 Hz); ¹³C NMR (100 MHz, DMSO-
_d₆) δ 149.9, 149.0, 138.9, 105.4, 83.9, 31.3, 29.0, 28.9, 28.7, 27.8, 27.7, 27.5, 22.1, 13.9; HRMS (ESI) calcd for C₁₇H₂₃N₃O₂ (M)+ 309.2416, found 309.2426.

2-Amino-4-undecylimidazole-1-carboxylic acid tert-butyl ester (5-xii). Using the same general procedure as used for 5-xi, 1-bromotridecan-2-one (4.0 g, 14.4 mmol) gave a crude oil which was purified by flash column chromatography (0-30% EtOAc/hexanes) to yield 5-xii (2.23 g, 46%) as a tan solid. ¹H NMR (400 MHz, DMSO-
_d₆) δ 6.49 (s, 1H), 6.37 (s, 2H), 2.22 (bs, 2H), 1.52 (s, 9H), 1.48 (m, 2H), 1.23 (bs, 16H), 0.86 (t, 3H, J = 6.0 Hz); ¹³C NMR (75 MHz, DMSO-
_d₆) δ 149.9, 149.0, 138.9, 105.5, 84.0, 31.3, 29.0, 28.9, 28.7, 27.8, 27.7, 27.5, 22.1, 14.0; HRMS (ESI) calcd for C₁₉H₃₅N₃O₂ (M)+ 337.2729, found 337.2730.
2-Amino-4-tridecylimidazole-1-carboxylic acid tert-butyl ester (5-xiii). Using the same general procedure as used for 5-xi, 1-bromopentadecan-2-one (0.916 g, 3.0 mmol) gave a crude oil which was purified by flash column chromatography (0-60% EtOAc/hexanes) to provide 5-xiii (0.473 g, 43%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$, 75 °C) $\delta$ 6.48 (s, 1H), 6.22 (s, 2H), 2.24 (t, 2H, $J = 7.2$ Hz), 1.54 (s, 9H), 1.5 (m, 2H), 1.25 (bs, 20H), 0.86 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$, 75 °C) $\delta$ 149.5, 148.7, 138.8, 105.3, 83.7, 31.0, 28.6, 28.6, 28.5, 28.4, 28.3, 27.5, 27.4, 27.3, 21.7, 13.4; HRMS (ESI) calcd for C$_{21}$H$_{39}$N$_3$O$_2$ (M)$^+$ 365.3042, found 365.3048.

2-Amino-4-pentadecylimidazole-1-carboxylic acid tert-butyl ester (5-xiv). Using the same general procedure as used for 5-xi, 1-bromoheptadecan-2-one (1.0 g, 3.0 mmol) gave a crude oil which was purified by flash column chromatography (0-60% EtOAc/hexanes) to obtain 5-xiv (0.628 g, 53%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$, 75 °C) $\delta$ 6.49 (s, 1H), 6.20 (s, 2H), 2.24 (t, 2H, $J = 7.2$ Hz), 1.54 (s, 9H), 1.50 (m, 2H), 1.25 (bs, 24H), 0.86 (t, 3H, $J = 6.4$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$, 75 °C)
δ 149.4, 148.7, 138.8, 105.3, 83.7, 30.9, 28.6, 28.6, 28.5, 28.34, 28.27, 27.5, 27.4, 27.3, 21.7, 13.4; HRMS (ESI) calcd for C_{23}H_{43}N_{3}O_{2} (M)^+ 393.3355, found 393.3362.

2-Amino-4-heptadecylimidazole-1-carboxylic acid tert-butyl ester (5-xv). Using the same general procedure as used for 5-xi, 1-bromononadecan-2-one (1.14 g, 3.15 mmol), with additional DMF (25 mL) for solubility, gave a crude oil which was purified by trituration (MeOH:H_{2}O / 1:20) to obtain 5-xv (1.10 g, 83%) as a white solid. ^1H NMR (400 MHz, DMSO-d_6, 80 °C) δ 6.49 (s, 1H), 6.19 (s, 2H), 2.24 (t, 2H, J = 7.2 Hz), 1.54 (s, 9H), 1.50 (m, 2H), 1.25 (bs, 28H), 0.87 (t, 3H, J = 6.8 Hz); ^13C NMR (100 MHz, DMSO-d_6, 80 °C) δ 149.5, 148.7, 138.9, 105.3, 83.76, 30.9, 28.6, 28.5, 28.4, 28.3, 27.6, 27.52, 27.45, 27.3, 21.7, 13.4; HRMS (ESI) calcd for C_{25}H_{47}N_{3}O_{2} (M)^+ 421.3668, found 421.3678.

(Z,Z)-2-Amino-4-heptadeca-8,11-dienylimidazole-1-carboxylic acid tert-butyl ester (5-xviii). Using the same general procedure as used for 5-xi, 1-bromononadeca-10,13-dien-2-one (0.660 g, 1.85 mmol) gave a crude oil which was purified by flash column chromatography (0-30% EtOAc/hexanes) to obtain 5-xviii (0.382 g, 50%) as a dark brown
oil. \( ^{1} \)H NMR (400 MHz, DMSO-\( d_{6} \)) \( \delta \) 6.47 (s, 1H), 6.37 (s, 2H), 5.31 (m, 4H), 2.72 (t, 2H, \( J = 6.0 \) Hz), 2.21 (t, 2H, \( J = 7.2 \) Hz), 2.00 (q, 4H, \( J = 6.8 \) Hz), 1.52 (s, 9H), 1.48 (m, 2H), 1.25 (bs, 14H), 0.84 (t, 3H, \( J = 6.4 \) Hz); \( ^{13} \)C NMR (100 MHz, DMSO-\( d_{6} \)) \( \delta \) 149.9, 149.0, 138.9, 129.7, 127.7, 105.5, 83.9, 30.9, 29.0, 28.74, 28.71, 28.6, 27.8, 27.7, 27.5, 26.6, 25.2, 22.0, 13.9; HRMS (ESI) calcd for C\(_{25}\)H\(_{43}\)N\(_{3}\)O\(_{2}\) (M\(^{+}\)) 417.3355, found 417.3360.

(Z)-2-Amino-4-heptadec-8-enylimidazole-1-carboxylic acid tert-butyl ester (5-xxi). Using the same general procedure as used for 5-xi, 1-bromooctadec-10-en-2-one (1.0 g, 2.78 mmol) gave a crude oil which was purified by flash column chromatography (20-100% EtOAc/hexanes) to afford 5-xxi (0.578 g, 50%) as pale yellow oil. \( ^{1} \)H NMR (400 MHz, DMSO-\( d_{6} \)) \( \delta \) 6.48 (s, 1H), 6.36 (s, 2H), 5.31 (t, 2H, \( J = 4.4 \) Hz), 2.21 (t, 2H, \( J = 7.2 \) Hz), 1.97 (m, 4H), 1.52 (s, 9H), 1.48 (m, 2H), 1.25 (bs, 10H), 1.23 (bs, 10H), 0.84 (t, 3H, \( J = 6.0 \) Hz); \( ^{13} \)C NMR (100 MHz, DMSO-\( d_{6} \)) \( \delta \) 149.9, 149.0, 138.9, 129.7, 105.5, 84.0, 31.3, 29.1, 28.9, 28.7, 28.60, 28.55, 27.8, 27.7, 27.5, 26.6, 22.1, 14.0; HRMS (ESI) calcd for C\(_{25}\)H\(_{45}\)N\(_{3}\)O\(_{2}\) (M\(^{+}\)) 419.3512, found 419.3515.
(E)-2-Amino-4-(7-methyloct-5-enyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxiv).

Using the same general procedure as used for 5-xi, 1-bromo-9-methyldec-7-en-2-one (1.0 g, 4.05 mmol) gave a crude oil which was purified by flash column chromatography (0-80% EtOAc/hexanes) to yield 5-xxiv (0.594 g, 48%) as yellow oil. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 6.49 (s, 1H), 6.38 (s, 2H), 5.34 (m, 2H), 2.22 (m, 3H), 1.94 (q, 2H, $J = 6.4$ Hz), 1.52 (s, 9H), 1.48 (m, 2H), 1.31 (m, 2H), 0.92 (d, 6H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 149.9, 149.0, 138.9, 137.3, 126.8, 105.6, 84.0, 31.8, 30.4, 28.8, 27.5, 27.4, 22.6; HRMS (ESI) calcd for C$_{17}$H$_{29}$N$_3$O$_2$ (M)$^+$ 307.2260, found 307.2262.

4-Nonyl-1H-imidazol-2-yl amine hydrochloride (72). A solution of 5-xi (0.20 g, 0.65 mmol) in anhydrous dichloromethane (6 mL) was cooled to 0 °C. TFA (6 mL) was added and the mixture was stirred for 6 h. The mixture was evaporated to dryness and toluene (2 mL) was added. Again the mixture was concentrated and the process repeated. The resulting TFA salt was dissolved in dichloromethane (1 mL) and 2M HCl in diethyl ether (1.0 mL) was added followed by cold diethyl ether (8 mL). The precipitate was collected by filtration and washed with diethyl ether (3 mL) to yield the target compound 72 (0.159 g, 99%) as a brown amorphous solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.19 (s, 1H), 11.76 (s, 1H), 7.35 (s, 2H), 6.54 (s, 1H), 2.37 (t, 2H, $J = 7.2$ Hz), 1.49 (m, 2H), 1.22 (bs, 12H), 0.83 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 146.8, 126.9, 108.4,
31.3, 28.9, 28.7, 28.4, 27.6, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C_{21}H_{23}N_{3} (M)^+ 209.1892, found 209.1901.

4-Undecyl-1H-imidazol-2-ylamine hydrochloride (73). Using the same general procedure as used for the synthesis of 72, 2-amino-4-undecylimidazole-1-carboxylic acid tert-butyl ester (2.23 g, 6.60 mmol) afforded 73 (1.80 g, 99%) as a brown amorphous solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.19 (s, 1H), 11.72 (s, 1H), 7.34 (s, 2H), 6.54 (s, 1H), 2.37 (d, 2H, $J$ = 5.6 Hz), 1.50 (m, 2H), 1.24 (bs, 16H), 0.85 (t, 3H, $J$ = 6.0 Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 146.8, 126.8, 108.4, 31.3, 29.1, 29.02, 28.96, 28.8, 28.7, 28.4, 27.6, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C_{14}H_{28}N_{3} (M)^+ 237.2205, found 237.2209.

4-Tridecyl-1H-imidazol-2-ylamine hydrochloride (74). Using the same general procedure as used for the synthesis of 72, 2-amino-4-tridecylimidazole-1-carboxylic acid tert-butyl ester (0.20 g, 0.55 mmol) gave 74 (0.158 g, 96%) as a brown amorphous solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.19 (s, 1H), 11.68 (s, 1H), 7.34 (s, 2H), 6.54 (s, 1H), 2.37 (d, 2H, $J$ = 5.6 Hz), 1.50 (m, 2H), 1.24 (bs, 16H), 0.85 (t, 3H, $J$ = 6.0 Hz), 31.3, 28.9, 28.7, 28.4, 27.6, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C_{21}H_{23}N_{3} (M)^+ 209.1892, found 209.1901.

\[
\begin{array}{c}
\text{H}_2\text{N} - \text{N} - \text{CH}_3 \\
\text{Boc} \\
5-xii \\
\end{array} & \xrightarrow{\text{Boc}} & 
\begin{array}{c}
\text{H}_2\text{N} - \text{N} - \text{CH}_3 \\
\text{H}\cdot\text{HCl} \\
\text{73} \\
\end{array}
\]

\[
\begin{array}{c}
\text{H}_2\text{N} - \text{N} - \text{CH}_3 \\
\text{Boc} \\
5-xiii \\
\end{array} & \xrightarrow{\text{Boc}} & 
\begin{array}{c}
\text{H}_2\text{N} - \text{N} - \text{CH}_3 \\
\text{H}\cdot\text{HCl} \\
\text{74} \\
\end{array}
\]
2.38 (t, 2H, J = 7.2 Hz), 1.50 (m, 2H), 1.23 (bs, 20H), 0.84 (t, 3H, J = 7.2 Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 146.8, 126.8, 108.4, 31.3, 29.08, 29.05, 28.96, 28.7, 28.69, 28.4, 27.6, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C$_{16}$H$_{31}$N$_3$ (M)$^+$ 265.2518, found 265.2529.

4-Pentadecyl-1H-imidazol-2-ylamine hydrochloride (75). Using the same general procedure as used for the synthesis of 72, 2-amino-4-pentadecylimidazole-1-carboxylic acid tert-butyl ester (0.20 g, 0.51 mmol) yielded 75 (0.167 g, 99%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.18 (s, 1H), 11.65 (s, 1H), 7.32 (s, 2H), 6.53 (s, 1H), 2.38 (t, 2H, J = 7.2 Hz), 1.50 (m, 2H), 1.23 (bs, 24H), 0.84 (t, 3H, J = 7.6 Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 146.7, 126.8, 108.3, 31.3, 29.1, 29.03, 28.98, 28.7, 28.71, 28.4, 27.6, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C$_{18}$H$_{35}$N$_3$ (M)$^+$ 293.2831, found 293.2844.

4-Heptadecyl-1H-imidazol-2-ylamine hydrochloride (76). Using the same general procedure as used for the synthesis of 72, 2-amino-4-heptadecylimidazole-1-carboxylic acid tert-butyl ester (0.40 g, 0.95 mmol) afforded 76 (0.320 g, 94%) as a white solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.10 (bs, 1H), 11.70 (bs, 1H), 7.33 (s, 2H), 6.54 (s, 1H), 2.37 (t,
2H, $J = 7.6\text{ Hz})$, 1.50 (m, 2H), 1.23 (bs, 28H), 0.84 (t, 3H, $J = 6.0\text{ Hz}$); $^{13}\text{C NMR (75 MHz, DMSO-}d_6\text{)} \delta 146.8, 126.8, 108.4, 31.3, 29.1, 28.8, 28.4, 7.2, 24.0, 22.1, 14.0; \text{ HRMS (ESI) calcd for C}_{20}H_{39}N_3 (M^+)^{32.3144, \text{ found 321.3146.}}$

(Z,Z)-4-Heptadeca-8,11-dienyl-1H-imidazol-2-ylamine hydrochloride (77). Using the same general procedure as used for the synthesis of 72, (Z,Z)-2-amino-4-heptadeca-8,11-dienylimidazole-1-carboxylic acid tert-butyl ester (0.323 g, 0.77 mmol) gave 77 (0.270 g, 99%) as a brown oil. $^1\text{H NMR (400 MHz, DMSO-}d_6\text{)} \delta 12.10 (s, 1H), 11.64 (s, 1H), 7.32 (s, 2H), 6.54 (s, 1H), 5.33 (m, 4H), 2.73 (t, 2H, $J = 6.0\text{ Hz})$, 2.38 (t, 2H, $J = 7.2\text{ Hz})$, 2.02 (q, 4H, $J = 5.6\text{ Hz})$, 1.50 (m, 2H), 1.27 (bs, 14H), 0.85 (t, 3H, $J = 6.0\text{ Hz}$); $^{13}\text{C NMR (100 MHz, DMSO-}d_6\text{)} \delta 146.7, 129.7, 127.7, 126.8, 108.4, 30.9, 29.0, 28.7, 28.6, 28.4, 27.6, 26.6, 25.2, 24.0, 22.0, 14.0; \text{ HRMS (ESI) calcd for C}_{20}H_{35}N_3 (M^+)^{317.2831, \text{ found 317.2836.}}$

(Z)-4-Heptadec-8-enyl-1H-imidazol-2-ylamine hydrochloride (78). Using the same general procedure as used for the synthesis of 72, (Z)-2-amino-4-heptadec-8-enylimidazole-1-carboxylic acid tert-butyl ester (0.528 g, 1.26 mmol) afforded 78 (0.447 g, 100%) as a
brown oil. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.10 (s, 1H), 11.75 (s, 1H), 7.35 (s, 2H), 6.52 (s, 1H), 5.31 (m, 2H), 2.37 (t, 2H, $J = 7.2$ Hz), 1.97 (m, 4H), 1.50 (m, 2H), 1.25 (bs, 10H), 1.22 (bs, 10H), 0.84 (t, 3H, $J = 6.0$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 146.8, 129.64, 129.61, 126.8, 108.4, 31.3, 29.14, 29.12, 28.9, 28.7, 28.6, 28.4, 27.6, 26.62, 26.59, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C$_{20}$H$_{37}$N$_3$ (M)$^+$ 319.2988, found 319.3009.

(E)-4-(7-Methyloct-5-enyl)-1H-imidazol-2-ylamine hydrochloride (79). Using the same general procedure as used for the synthesis of 72, (E)-2-amino-4-(7-methyloct-5-enyl)imidazole-1-carboxylic acid tert-butyl ester (0.479 g, 1.56 mmol) yielded 79 (0.35 g, 93%) as a brown oil. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 12.18 (s, 1H), 11.71 (s, 1H), 7.34 (s, 2H), 6.54 (s, 1H), 5.35 (m, 2H), 2.39 (t, 2H, $J = 7.6$ Hz), 2.20 (m, 1H), 1.94 (q, 2H, $J = 6.4$ Hz), 1.50 (quint, 2H, $J = 7.6$ Hz), 0.92 (d, 6H, $J = 6.8$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 146.8, 137.5, 126.7, 126.5, 108.4, 31.5, 30.4, 28.3, 27.1, 23.8, 22.5; HRMS (ESI) calcd for C$_{12}$H$_{21}$N$_3$ (M)$^+$ 207.1736, found 207.1738.

Succinic acid monobenzyl ester (5-xxv). Succinic anhydride (5.0 g, 50.0 mmol) was dissolved in anhydrous dichloromethane (40 mL). To this solution was added benzyl
alcohol (5.69 mL, 55.0 mmol), triethylamine (7.50 mL, 55.0 mmol), and a catalytic amount of DMAP. The resulting clear solution was stirred at ambient temperature for 15 h upon which all volatiles were removed by rotary evaporation. The crude residue was taken up in diethyl ether (200 mL) and was washed with 2N NaOH (2 x 75 mL). The aqueous extracts were carefully acidified to pH = 2 with concentrated HCl and then extracted with diethyl ether (2 x 100 mL). The combined extracts were (Mg(SO₄)), filtered, and concentrated to afford **5-xxv** (9.06 g, 87%) as a white solid with no further purification needed. ¹H NMR (400 MHz, DMSO-ᵈ) δ 12.26 (s, 1H), 7.32 (m, 5H), 5.08 (s, 2H), 2.56 (m, 2H), 2.49 (m, 2H); ¹³C NMR (100 MHz, DMSO-ᵈ) δ 173.5, 172.1, 136.3, 128.5, 128.0, 127.9, 65.6, 28.8, 28.7; HRMS (ESI) calcd for C₁₁H₁₂O₄ (M)⁺ 208.0736, found 208.0736.

5-Bromo-4-oxopentanoic acid benzyl ester (5-xxvi). Succinic acid monobenzyl ester (2.0 g, 9.6 mmol) was dissolved in anhydrous dichloromethane (30 mL) at 0 ºC and a catalytic amount of DMF was added. To this solution was added oxalyl chloride (2.5 mL, 28.8 mmol) dropwise and the solution was then warmed to room temperature. After 1 h, the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting solid was dissolved into anhydrous dichloromethane (10 mL) and added dropwise to a 0 ºC solution of CH₂N₂ (28.8 mmol generated from Diazald®/KOH) in diethyl ether (75 mL). This solution was stirred at 0 ºC for 1.5 h at which time the
reaction was quenched via the dropwise addition of 48% HBr (3.5 mL). The reaction mixture was diluted with dichloromethane (25 mL) and immediately washed with sat. NaHCO₃ (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO₄), filtered and concentrated. The resulting yellow oil 5-xxvi (2.59 g, 95%) was carried onto the next step without further purification. ¹H NMR (400 MHz, DMSO-d₆) δ 7.36 (m, 5H), 5.08 (s, 2H), 4.39 (s, 2H), 2.88 (t, 2H, J = 6.0 Hz), 2.60 (t, 2H, J = 6.0 Hz); ¹³C NMR (75 MHz, DMSO-d₆) δ 200.4, 171.8, 136.1, 128.4, 127.9, 127.8, 65.5, 36.3, 34.2, 27.8; HRMS (ESI) calcd for C₁₂H₁₃BrO₃ (M)+ 284.0048, found 284.0056.

2-Amino-4-(2-benzyloxycarbonyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxvii). 5-Bromo-4-oxopentanoic acid benzyl ester (1.0 g, 3.51 mmol) and Boc-guanidine (1.66 g, 10.5 mmol) were dissolved in DMF (10 mL) and allowed to stir at room temperature. After 48 h the DMF was removed under reduced pressure and the residue was taken up in ethyl acetate (50 mL) and washed with water (3 x 25 mL) and brine (25 mL) before being dried (Na₂SO₄), filtered and evaporated to dryness. The resulting oil was purified by flash column chromatography (20-100% EtOAc/hexanes) to obtain 5-xxvii (0.725 g, 60%) as a yellow solid. ¹H NMR (400 MHz, DMSO-d₆) δ 7.32 (m, 5H), 6.54 (s, 1H), 6.44 (s, 2H), 5.09 (s, 2H), 2.58 (m, 4H), 1.52 (s, 9H); ¹³C NMR (100 MHz, DMSO-d₆) δ 172.2, 150.0, 148.9, 137.2, 136.3, 128.4, 127.9, 127.7, 105.9,
84.1, 65.3, 32.3, 27.5, 23.3; HRMS (ESI) calcd for C\textsubscript{18}H\textsubscript{23}N\textsubscript{3}O\textsubscript{4} (M)\textsuperscript{+} 345.1689, found 345.1689.

2-Amino-4-(2-carboxyethyl)imidazole-1-carboxylic acid tert-butyl ester (80). To a solution of anhydrous THF (50 mL) and 10% Pd/C (0.20 g) was added 5-xxvi (2.0 g, 5.79 mmol). Air was removed from the system and the reaction was back flushed with hydrogen. This process was repeated three times. The mixture was kept under a hydrogen balloon at atmospheric pressure and temperature for 1 h. After that time the reaction was filtered through a Celite\textsuperscript{®} pad and the filter cake was washed with THF (10 mL). The filtrate was concentrated under reduced pressure to afford 80 (1.35 g, 92%) as a white solid. \(^1\)H NMR (400 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 6.54 (s, 1H), 6.44 (s, 2H), 2.46 (m, 4H), 1.53 (s, 9H); \(^1\)C NMR (100 MHz, DMSO-\textit{d}\textsubscript{6}) \(\delta\) 174.0, 150.0, 148.9, 137.6, 105.8, 84.2, 32.5, 27.5, 23.3; HRMS (ESI) calcd for C\textsubscript{11}H\textsubscript{17}N\textsubscript{3}O\textsubscript{4} (M)\textsuperscript{+} 255.1219, found 255.1219.

7-Bromo-6-oxoheptanoic acid benzyl ester (5-xxix). Using the same general procedure as used for 5-xxvi, hexanedioic acid monobenzyl ester\textsuperscript{8} (2.6 g, 11.0 mmol) gave a crude amber oil 5-xxix (2.65 g, 77%) which was used in the next step without further
purification. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.27 (m, 5H), 5.03 (s, 2H), 3.77 (s, 2H), 2.58 (m, 2H), 2.30 (m, 2H), 1.58 (q, 4H, $J$ = 3.6 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 201.8, 173.2, 136.1, 128.8, 128.4, 77.7, 77.2, 76.8, 66.4, 39.5, 34.3, 34.1, 24.4, 23.4; HRMS (ESI) calcd for C$_{14}$H$_{17}$BrO$_3$ (M)$^+$ 312.0361, found 312.0367.

2-Amino-4-(4-benzyloxycarbonylbutyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxx). Using the same general procedure as used for 5-xxvii, 5-xxix (1.50 g, 4.79 mmol) gave a crude oil which was purified by flash column chromatography (40-100% EtOAc/hexanes) to afford 5-xxx (0.896 g, 50%) as an amber oil. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.35 (m, 5H), 6.51 (s, 1H), 6.38 (s, 2H), 5.07 (s, 2H), 2.36 (t, 2H, $J$ = 6.9 Hz), 2.24 (t, 2H, $J$ = 6.9 Hz), 1.52 (m, 13H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 172.7, 149.8, 148.9, 138.5, 136.3, 128.4, 127.91, 127.85, 105.6, 84.0, 65.3, 33.3, 27.5, 27.3, 27.2, 24.1; HRMS (ESI) calcd for C$_{20}$H$_{27}$N$_3$O$_4$ (M)$^+$ 373.2002, found 373.2006.

2-Amino-4-(4-carboxybutyl)imidazole-1-carboxylic acid tert-butyl ester (81). Using the same general procedure as used for 80, 5-xxx (0.84 g, 2.25 mmol) gave the title
compound 81 (0.538 g, 84%) as a white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 6.52 (s, 1H), 6.40 (s, 2H), 2.20 (m, 4H), 1.50 (m, 13H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 174.6, 149.9, 148.9, 138.6, 105.7, 84.0, 33.6, 27.5, 27.3, 24.2; HRMS (ESI) calcd for C$_{13}$H$_{21}$N$_3$O$_4$ (M)$^+$ 283.1532, found 283.1532.

2-Amino-4-(2-hexylcarbamoylethyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxi). Using the general EDC/HOBt procedure, carboxylic acid 80 yielded 5-xxxi (0.021 g, 8%) as a yellow foam. $^1$H NMR (300 MHz, CDCl$_3$) δ 6.57 (s, 1H), 6.06 (s, 1H), 5.67 (bs, 2H), 3.20 (q, 2H, $J$ = 6.9 Hz), 2.69 (t, 2H, $J$ = 7.2 Hz), 2.47 (t, 2H, $J$ = 7.2 Hz), 1.57 (s, 9H), 1.43 (m, 2H), 1.25 (bs, 6H), 0.87 (t, 3H, $J$ = 6.6 Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) δ 172.6, 150.2, 149.6, 137.4, 107.5, 85.1, 39.6, 35.9, 31.7, 29.7, 28.2, 26.7, 24.4, 22.8, 14.2; HRMS (ESI) calcd for C$_{17}$H$_{31}$N$_4$O$_3$ (M)$^+$ 338.2318, found 338.2323.

2-Amino-4-(4-hexylcarbamoylbutyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxii). Using the general EDC/HOBt procedure, carboxylic acid 81 afforded 5-xxxii (0.044 g, 17%) as a colorless oil. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 7.73 (t, 1H, $J$ = 5.4 Hz), 6.50 (s,
1H), 6.38 (s, 2H), 3.00 (q, 2H, \( J = 6.6 \) Hz), 2.23 (m, 2H), 2.03 (m, 2H), 1.53 (s, 9H), 1.47 (m, 4H), 1.35 (m, 2H), 1.23 (m, 4H), 0.84 (t, 3H, \( J = 6.6 \) Hz); \(^{13}\)C NMR (75 MHz, DMSO-\( d_6 \)) \( \delta \) 171.8, 149.8, 148.9, 138.7, 105.6, 84.0, 38.3, 35.3, 31.0, 29.1, 27.5, 27.4, 26.0, 25.0, 22.0, 13.9; HRMS (ESI) calcd for C\(_{19}\)H\(_{34}\)N\(_4\)O\(_3\) (M\(^+\)) 366.2631, found 366.2632.

2-Amino-4-{2-[2-(4-bromophenyl)ethyl]carbamoyl}ethyl]imidazole-1-carboxylic acid tert-butyl ester (5-xxxiii). Using the general EDC/HOBt procedure, carboxylic acid 80 yielded 5-xxxiii (0.019 g, 6%) as an off-white solid. \(^1\)H NMR (300 MHz, CDCl\(_3\)) \( \delta \) 7.39 (d, 2H, \( J = 8.1 \) Hz), 7.03 (d, 2H, \( J = 8.4 \) Hz), 6.58 (s, 1H), 6.41 (m, 1H), 3.45 (q, 2H, \( J = 6.3 \) Hz), 2.72 (q, 2H, \( J = 6.9 \) Hz), 2.47 (t, 4H, \( J = 6.9 \) Hz), 1.59 (s, 9H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 172.2, 150.0, 148.9, 138.2, 134.0, 131.8, 130.7, 120.4, 107.5, 86.5, 40.5, 35.3, 35.0, 28.1, 23.1; HRMS (ESI) calcd for C\(_{19}\)H\(_{25}\)BrN\(_4\)O\(_3\) (M\(^+\)) 436.1110, found 436.1112.

2-Amino-4-[2-(4-phenylbutyl)carbamoyl]ethyl]imidazole-1-carboxylic acid tert-butyl ester (5-xxxiv). Using the general EDC/HOBt procedure, carboxylic acid 80 gave 5-xxxiv (0.081 g, 27%) as a yellow foam. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \( \delta \) 7.26 (t, 2H, \( J = 7.6 \) Hz),
7.15 (m, 2H), 6.55 (s, 1H), 6.28 (bs, 1H), 5.75 (bs, 2H), 3.22 (q, 2H, $J = 6.4$ Hz), 2.66 (t, 2H, $J = 7.2$ Hz), 2.59 (t, 2H, $J = 7.6$ Hz), 2.45 (t, 2H, $J = 7.2$ Hz), 1.55 (m, 11H), 1.47 (m, 3H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.2, 149.8, 148.9, 142.1, 138.1, 128.21, 128.17, 125.6, 105.6, 84.0, 38.1, 34.7, 34.0, 28.8, 28.3, 27.5, 24.0; HRMS (ESI) calcd for C$_{21}$H$_{31}$N$_4$O$_3$ (M)$^+$ 386.2318, found 386.2319.

2-Amino-4-(2-heptylcarbamoylthethyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxv). Using the general EDC/HOBt procedure, carboxylic acid 80 (0.115 g, 0.450 mmol) gave 5-xxxv (0.044 g, 27%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.78 (m, 1H), 6.50 (s, 1H), 6.38 (s, 2H), 3.00 (q, 2H, $J = 5.6$ Hz), 2.46 (t, 2H, $J = 8.0$ Hz), 2.28 (t, 2H, $J = 7.2$ Hz), 1.52 (s, 9H), 1.35 (m, 2H), 1.22 (bs, 8H), 0.85 (m, 3H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 171.2, 149.8, 148.9, 138.1, 105.6, 84.1, 38.4, 34.0, 31.2, 29.2, 28.4, 27.5, 26.3, 24.0, 22.1, 14.0; HRMS (ESI) calcd for C$_{19}$H$_{32}$N$_4$O$_3$ (M)$^+$ 352.2474, found 352.2489.

2-Amino-4-(4-pentylcarbamoylbutyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxvi). Using the general EDC/HOBt procedure, carboxylic acid 81 afforded 5-xxxvi (0.012
g, 5%) as an amber oil. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 6.52 (s, 1H), 5.66 (s, 2H), 5.55 (m, 1H), 3.23 (q, 2H, $J = 6.0$ Hz), 2.38 (t, 2H, $J = 6.6$ Hz), 2.18 (t, 2H, $J = 7.2$ Hz), 1.66 (m, 4H), 1.59 (s, 9H), 1.50 (m, 2H), 1.29 (m, 4H), 0.89 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 173.1, 150.1, 149.6, 138.7, 107.0, 84.9, 39.7, 36.9, 29.9, 29.6, 29.3, 28.2, 28.1, 27.9, 25.6, 22.6, 14.2; HRMS (ESI) calcd for C$_{18}$H$_{32}$N$_4$O$_3$ (M)$^+$ 352.2474, found 352.2481.

3-(2-Amino-1H-imidazol-4-yl)-N-hexylpropionamide hydrochloride (82). Using the same general procedure as used for the synthesis of 62, 5-xxx (0.020 g, 0.062 mmol) gave 82 (0.017 g, 100%) as a tan foam. $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 6.38 (s, 1H), 3.05 (t, 2H, $J = 6.6$ Hz), 2.66 (t, 2H, $J = 6.6$ Hz), 2.37 (t, 2H, $J = 6.6$ Hz), 1.36 (m, 2H), 1.19 (bs, 6H), 0.79 (m, 3H); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 174.1, 148.6, 128.0, 110.2, 40.6, 35.3, 32.8, 30.5, 27.8, 23.7, 21.7, 14.5; HRMS (ESI) calcd for C$_{12}$H$_{22}$N$_4$O (M)$^+$ 238.1794, found 238.1795.

5-(2-Amino-1H-imidazol-4-yl)pentanoic acid hexylamide hydrochloride (83). Using the same general procedure as used for the synthesis of 62, 5-xxxii (0.036 g, 0.098 mmol) gave 83 (0.029 g, 100%) as a colorless oil. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$
11.99 (s, 1H), 11.55 (s, 1H), 7.78 (m, 1H), 7.29 (s, 2H), 6.55 (s, 1H), 3.00 (q, 2H, $J = 6.9$ Hz), 2.39 (bs, 2H), 2.06 (bs, 2H), 1.48 (bs, 4H), 1.36 (m, 2H), 1.28 (bs, 4H), 0.85 (m, 3H); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 171.6, 146.6, 126.7, 108.5, 38.3, 34.9, 30.9, 29.1, 27.2, 26.0, 24.6, 23.7, 22.0, 13.9; HRMS (ESI) calcd for C$_{14}$H$_{26}$N$_4$O (M)$^+$ 266.2107, found 266.2109.

3-(2-Amino-1H-imidazol-4-yl)-N-[2-(4-bromophenyl)ethyl]propionamide hydrochloride (84). Using the same general procedure as used for the synthesis of 62, 5-xxxv (0.019 g, 0.043 mmol) gave 84 (0.016 g, 100%) as a brown foam. $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 7.29 (d, 2H, $J = 7.8$ Hz), 6.99 (d, 2H, $J = 8.1$ Hz), 6.33 (s, 1H), 3.27 (t, 2H, $J = 7.2$ Hz), 2.61 (m, 4H), 2.33 (t, 2H, $J = 6.6$ Hz); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 174.2, 139.9, 132.6, 131.9, 128.0, 121.2, 110.1, 41.7, 36.0, 35.2, 21.6; HRMS (ESI) calcd for C$_{14}$H$_{17}$BrN$_4$O (M)$^+$ 336.0586, found 336.0591.

3-(2-Amino-1H-imidazol-4-yl)-N-(4-phenylbutyl)propionamide hydrochloride (85).

Using the same general procedure as used for the synthesis of 62, 5-xxxiv (0.080 g, 0.206
mmol) gave 85 (0.066 g, 100%) as a tan foam. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 11.91 (s, 1H), 11.59 (s, 1H), 7.97 (m, 1H), 7.34 (s, 2H), 7.25 (m, 2H), 7.17 (d, 3H, $J = 7.2$ Hz), 6.50 (s, 1H), 3.05 (q, 2H, $J = 6.0$ Hz), 2.58 (m, 4H), 2.35 (t, 2H, $J = 7.2$ Hz), 1.53 (quint, 2H, $J = 7.2$ Hz), 1.38 (quint, 2H, $J = 7.2$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 170.6, 146.6, 142.1, 128.2, 128.2, 126.2, 125.6, 108.5, 38.2, 34.7, 33.5, 28.7, 28.3, 20.2; HRMS (ESI) calcd for C$_{16}$H$_{22}$N$_4$O (M)$^+$ 286.1794, found 286.1799.

3-(2-Amino-1H-imidazol-4-yl)-N-heptylpropionamide hydrochloride (86). Using the same general procedure as used for the synthesis of 62, 5-xxxv (0.031 g, 0.088 mmol) gave the title compound 86 (0.025 g, 99%) as a tan amorphous solid. $^1$H NMR (400 MHz, DMSO-$d_6$) δ 11.88 (s, 1H), 11.56 (s, 1H), 7.93 (m, 1H), 7.33 (s, 2H), 6.51 (s, 1H), 3.02 (q, 2H, $J = 6.4$ Hz), 2.62 (t, 2H, $J = 7.2$ Hz), 2.35 (t, 2H, $J = 7.2$ Hz), 1.36 (m, 2H), 1.23 (bs, 8H), 0.86 (t, 3H, $J = 6.4$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) δ 170.6, 146.6, 126.2, 108.6, 38.5, 33.5, 31.2, 29.1, 28.4, 26.3, 22.0, 20.2, 14.0; HRMS (ESI) calcd for C$_{13}$H$_{24}$N$_4$O (M)$^+$ 252.1950, found 252.1959.
5-(2-Amino-1H-imidazol-4-yl)pentanoic acid pentyamide hydrochloride (87). Using the same general procedure as used for the synthesis of 62, 5-xxxvi (0.012 g, 0.034 mmol) gave 87 (0.009 g, 100%) as an amber oil. $^1$H NMR (300 MHz, CD$_3$OD) $\delta$ 6.50 (s, 1H), 3.15 (t, 2H, $J = 6.9$ Hz), 2.51 (t, 2H, $J = 6.6$ Hz), 2.22 (t, 2H, $J = 6.9$ Hz), 1.63 (m, 4H), 1.49 (m, 2H), 1.32 (m, 4H), 0.91 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, CD$_3$OD) $\delta$ 176.3, 149.0, 129.3, 110.3, 40.9, 37.0, 30.7, 30.6, 29.3, 26.7, 25.7, 23.9, 14.8; HRMS (ESI) calcd for C$_{13}$H$_{24}$N$_4$O (M)$^+$ 252.1950, found 252.1951.

![Chemical Structure](image1)

2-Amino-4-[2-(1-hexylheptylcarbamoyl)ethyl]imidazole-1-carboxylic acid tert-butyl ester (5-xxxvii). Using the general EDC/HOBt procedure, carboxylic acid 80 yielded 5-xxxvii (0.176 g, 51%) as an off-white solid. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.46 (d, 1H, $J = 8.7$ Hz), 6.50 (s, 1H), 6.36 (s, 2H), 3.63 (m, 1H), 2.47 (t, 2H, $J = 7.2$ Hz), 2.28 (t, 2H, $J = 7.2$ Hz), 1.52 (s, 9H), 1.20 (m, 20H), 0.84 (t, 6H, $J = 7.2$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 170.8, 149.8, 148.9, 138.1, 105.7, 84.0, 47.7, 34.7, 34.0, 31.3, 28.6, 27.5, 25.4, 24.2, 22.1, 14.0; HRMS (ESI) calcd for C$_{24}$H$_{44}$N$_4$O$_3$ (M)$^+$ 436.3413, found 436.3414.

![Chemical Structure](image2)
2-Amino-4-[3-(1-hexylheptylcarbamoyl)propyl]imidazole-1-carboxylic acid tert-butyl ester (5-xxxviii). Using the general EDC/HOBt procedure, carboxylic acid 43 afforded 5-xxxviii (0.035 g, 21%) as a tan solid. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.43 (d, 1H, $J$ = 8.8 Hz), 6.48 (s, 1H), 6.38 (s, 2H), 3.66 (m, 1H), 2.21 (t, 2H, $J$ = 7.2 Hz), 2.04 (t, 2H, $J$ = 7.2 Hz), 1.71 (m, 2H), 1.52 (m, 9H), 1.23 (m, 20H), 0.84 (m, 6H); $^{13}$C NMR (100 MHz, DMSO-$d_6$) $\delta$ 171.3, 149.9, 148.9, 138.4, 105.7, 84.0, 47.7, 35.1, 34.7, 31.3, 31.1, 28.6, 27.5, 27.2, 25.5, 24.2, 22.1, 22.0, 14.0; HRMS (ESI) calcd for C$_{25}$H$_{46}$N$_4$O$_3$ (M)$^+$ 450.3567, found 450.3562.

![Diagram](image)

4-(Dihexylcarbamoyl)butyric acid (5-xxxix). Glutaric anhydride (1.00 g, 8.76 mmol) was dissolved in anhydrous dichloromethane (12 mL). To this solution was added dihexylamine (2.27 mL, 9.64 mmol), triethylamine (1.30 mL, 9.64 mmol), and a catalytic amount of DMAP. The resulting clear solution was stirred at ambient temperature for 15 h upon which all volatiles were removed by rotary evaporation. The crude residue was partitioned between ethyl acetate (150 mL) and a 1N HCl aqueous solution (100 mL). The organic layer was subsequently washed with 1N HCl (3 x 50 mL), saturated NaHCO$_3$ (2 x 75 mL), and brine (1 x 50 mL), and then dried over anhydrous Na$_2$SO$_4$. Concentration to dryness to afforded 5-xxxix (2.44 g, 93%) as a viscous oil which required no further purification. $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 3.28 (t, 2H, $J$ = 7.5 Hz),
3.19 (t, 2H, \( J = 7.5 \) Hz), 2.41 (m, 4H), 1.95 (t, 2H, \( J = 6.9 \) Hz), 1.51 (m, 4H), 1.27 (bs, 12H), 0.88 (m, 6H); \(^{13}\)C NMR (75 MHz, CDCl\(_3\)) \( \delta \) 177.6, 172.3, 48.3, 46.3, 33.7, 32.1, 31.8, 31.7, 29.3, 27.9, 26.9, 26.7, 22.7, 20.7, 14.2, 14.1; HRMS (ESI) calcd for C\(_{17}\)H\(_{33}\)NO\(_3\) (M\(^+\)) 299.2460, found 299.2462.

6-Bromo-5-oxohexanoic acid dihexylamide (5-xxxx). 4-(Dihexylcarbamoyl)butyric acid (1.61 g, 5.40 mmol) was dissolved in anhydrous dichloromethane (15 mL) at 0 °C and a catalytic amount of DMF was added. To this solution was added oxalyl chloride (1.41 mL, 16.2 mmol) dropwise and the solution was then warmed to room temperature. After 1 h, the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting oil was dissolved in anhydrous dichloromethane (10 mL) and added dropwise to a 0 °C solution of CH\(_2\)N\(_2\) (16.2 mmol generated from Diazald\(^\circledR\)/KOH) in diethyl ether (50 mL). This solution was stirred at 0 °C for 1.5 h at which time the reaction was quenched via the dropwise addition of 48% HBr (2.5 mL). The reaction mixture was diluted with dichloromethane (25 mL) and immediately washed with sat. NaHCO\(_3\) (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO\(_4\)), filtered and concentrated. The crude yellow oil 5-xxxx (0.78 g, 38%) was subsequently used in the next step without any further purification. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \( \delta \) 4.33 (s, 2H), 2.45 (t, 2H, \( J = 8.8 \) Hz), 2.75 (t, 2H, \( J = 7.6 \) Hz), 2.41 (t, 2H, \( J = 7.6 \) Hz), 1.59 (m, 4H),
2-Amino-4-(3-dihexylcarbamoylpropyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxxi). 6-Bromo-5-oxohexanoic acid dihexylamide (0.78 g, 2.07 mmol) and Boc-guanidine (0.988 g, 6.21 mmol) were dissolved in DMF (8 mL) and allowed to stir at room temperature. After 72 h, the DMF was removed under reduced pressure and the residue was taken up in ethyl acetate (100 mL). The solution was washed with water (3 x 50 mL) and brine (50 mL) and then dried (Na₂SO₄), filtered and evaporated to dryness. The resulting oil was purified by flash column chromatography (30-100% EtOAc/CH₂Cl₂) to obtain 5-xxxxi (0.643 g, 71%) as a white foam. ¹H NMR (400 MHz, DMSO-d₆) δ 6.47 (s, 1H), 6.41 (s, 2H), 3.42 (t, 4H, J = 7.6 Hz), 2.36 (m, 4H), 1.51 (bs, 13H), 1.25 (bs, 14H), 0.84 (t, 6H, J = 6.4 Hz); ¹³C NMR (100 MHz, DMSO-d₆) δ 184.0, 169.0, 150.0, 148.8, 137.0, 106.2, 103.1, 84.9, 84.1, 48.9, 30.8, 28.1, 27.7, 27.5, 25.4, 22.0, 20.9, 13.8; HRMS (ESI) calcd for C₂₄H₄₄N₄O₃ (M)⁺ 436.3413, found 436.3412.

3-(2-Amino-1H-imidazol-4-yl)-N-(1-hexylheptyl)propionamide hydrochloride (88).
Using the same general procedure as used for the synthesis of 62, 5-xxxvii (0.130 g, 0.297 mmol) gave 88 (0.107 g, 97%) as an amber oil. \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 11.92 (s, 1H), 11.62 (s, 1H), 7.65 (d, 1H, \(J = 8.7\) Hz), 7.36 (s, 2H), 6.51 (s, 1H), 3.65 (m, 1H), 2.63 (t, 2H, \(J = 7.2\) Hz), 2.36 (t, 2H, \(J = 7.2\) Hz), 1.25 (m, 20H), 0.84 (t, 6H, \(J = 7.2\) Hz); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 170.2, 146.6, 126.2, 108.5, 48.0, 34.5, 33.5, 31.2, 28.5, 25.4, 22.0, 20.4, 13.9; HRMS (ESI) calcd for \(\text{C}_{19}\text{H}_{36}\text{N}_4\text{O}\) (M)\(^+\) 336.2889, found 336.2894.

![Chemical structure](image)

4-(2-Amino-1H-imidazol-4-yl)-N-(1-hexylheptyl)butyramide hydrochloride (89).

Using the same general procedure as used for the synthesis of 62, 5-xxxviii (0.032 g, 0.071 mmol) afforded 89 (0.027 g, 99%) as a tan oil. \(^1\)H NMR (300 MHz, DMSO-\(d_6\)) \(\delta\) 11.99 (s, 1H), 11.57 (s, 1H), 7.52 (d, 1H, \(J = 8.7\) Hz), 7.31 (s, 2H), 6.55 (s, 1H), 3.70 (m, 1H), 2.38 (t, 2H, \(J = 7.2\) Hz), 2.08 (t, 2H, \(J = 7.2\) Hz), 1.73 (m, 2H), 1.24 (m, 20H), 0.84 (m, 6H); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 171.0, 146.7, 126.5, 108.7, 47.8, 38.4, 34.6, 31.3, 31.1, 28.6, 25.5, 24.2, 23.6, 22.1, 14.0; HRMS (ESI) calcd for \(\text{C}_{20}\text{H}_{38}\text{N}_4\text{O}\) (M)\(^+\) 350.3046, found 350.3033.
4-(2-Amino-1H-imidazol-4-yl)-N,N-dihexyl-butyramide hydrochloride (90). Using the same general procedure as used for the synthesis of 62, 5-xxxxi (0.562 g, 1.29 mmol) yielded 90 (0.451 g, 94%) as a brown foam. $^1$H NMR (300 MHz, DMSO-$d_6$) δ 12.21 (s, 1H), 11.67 (s, 1H), 7.39 (s, 2H), 6.50 (s, 1H), 3.43 (t, 4H, $J = 7.5$ Hz), 2.45 (m, 4H), 1.57 (m, 4H), 1.25 (bs, 14H), 0.85 (t, 6H, $J = 6.6$ Hz); $^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 146.8, 125.4, 109.0, 84.0, 49.1, 30.8, 28.1, 25.4, 24.3, 22.0, 20.6, 13.9; HRMS (ESI) calcd for C$_{19}$H$_{36}$N$_4$O (M$^+$) 336.2889, found 336.2897.

5-Azido-1-bromopentan-2-one (5-xxxxiii). 4-Azido-butyric acid$^{11}$ (2.15 g, 16.7 mmol) was dissolved in anhydrous dichloromethane (80 mL) at 0 ºC and a catalytic amount of DMF was added. To this solution was added oxalyl chloride (4.4 mL, 50 mmol) dropwise and the solution was then warmed to room temperature. After 1 h, the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting oil was dissolved into anhydrous dichloromethane (10 mL) and added dropwise to a 0 ºC solution of CH$_2$N$_2$ (50 mmol generated from Diazald$^{\text{®}}$/KOH) in diethyl ether (125 mL). This solution was stirred at 0 ºC for 1.5 h at which time the reaction was quenched via the dropwise addition of 48% HBr (6.0 mL). The reaction mixture was diluted with
dichloromethane (25 mL) and immediately washed with sat. NaHCO₃ (3 x 25 mL) and brine (2 x 25 mL) before being dried (MgSO₄), filtered and concentrated. The crude amber oil obtained (3.10 g, 90%) upon concentration was pure and used in the following steps without further purification. ¹H NMR (300 MHz, DMSO-dma) δ 4.35 (s, 2H), 3.33 (t, 2H, J = 6.9 Hz), 2.66 (t, 2H, J = 7.2 Hz), 1.75 (quint, 2H, J = 6.9 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 201.3, 50.6, 36.6, 34.3, 23.3; HR MS (ESI) calcd for C₅H₈BrN₃O (M⁺) 204.9851, found 204.9850.

2-Amino-4-(3-azidopropyl)imidazole-1-carboxylic acid tert-butyl ester (91). 5-Azido-1-bromopentan-2-one (0.87 g, 4.22 mmol) and Boc-guanidine (2.0 g, 12.7 mmol) were dissolved in DMF (15 mL) and allowed to stir at room temperature. After 24 h, the DMF was removed under reduced pressure and the residue was taken up in ethyl acetate (50 mL). The mixture was then washed with water (3 x 25 mL) and brine (25 mL) before being dried (Na₂SO₄), filtered and evaporated to dryness. The resulting oil was purified by flash column chromatography (10-100% EtOAc/hexanes) to obtain 91 (0.70 g, 63%) as a white solid. ¹H NMR (300 MHz, CDCl₃) δ 6.53 (s, 1H), 5.98 (s, 2H), 3.30 (t, 2H, J = 6.9 Hz), 2.43 (t, 2H, J = 7.2 Hz), 1.87 (quint, 2H, J = 6.9 Hz); ¹³C NMR (75 MHz, CDCl₃) δ 150.6, 149.6, 137.6, 107.1, 84.8, 50.9, 28.1, 27.8, 25.3; HRMS (ESI) calcd for C₁₁H₁₈N₆O₂ (M⁺) 266.1491, found 266.1499.
2-Amino-4-(3-heptanoylaminopropyl)imidazole-1-carboxylic acid tert-butyl ester (5-xxxxiv). To a mixture of anhydrous THF (4 mL) and 10% Pd/C (0.010 g) was added 2-amino-4-(3-azidopropyl)imidazole-1-carboxylic acid tert-butyl ester 91 (0.10 g, 0.38 mmol). Air was removed from the system and the reaction was back flushed with hydrogen. This process was repeated three times before setting the reaction under a hydrogen balloon at atmospheric pressure and temperature for 8 h. After that time heptanoic anhydride (0.104 mL, 0.39 mmol) was added dropwise to the reaction which was allowed to stir overnight. The reaction was then filtered through a Celite® pad and the filter cake was washed with THF (20 mL). The filtrate was concentrated under reduced pressure to afford the crude product which was purified by flash column chromatography (0-10% MeOH/CH₂Cl₂) to obtain 5-xxxxiv (0.093 g, 70%) as a white solid. ¹H NMR (300 MHz, DMSO-d₆) δ 7.76 (m, 1H), 6.52 (s, 1H), 6.38 (s, 2H), 3.02 (q, 2H, J = 6.9 Hz), 2.35 (t, 2H, J = 7.5 Hz), 2.03 (t, 2H, J = 7.5 Hz), 1.60 (quint, 2H, J = 7.2 Hz), 1.53 (s, 9H), 1.46 (m, 2H), 1.24 (m, 6H), 0.85 (t, 3H, J = 6.6 Hz); ¹³C NMR (75 MHz, DMSO-d₆) δ 171.9, 149.9, 148.9, 138.4, 105.7, 84.0, 38.1, 35.4, 31.0, 28.3, 27.9, 27.5, 25.3, 25.2, 22.0, 13.9; HRMS (ESI) calcd for C₁₈H₃₂N₄O₃ (M)⁺ 352.2474, found 352.2488.
Heptanoic acid [3-(2-Amino-1H-imidazol-4-yl)propyl]amide hydrochloride (92). A solution of 5-xxxxiv (0.041 g, 0.12 mmol) in anhydrous dichloromethane (1 mL) was cooled to 0 °C. TFA (1 mL) was added and the mixture was stirred for 7 h. After that time the reaction was evaporated to dryness and toluene (2 mL) was added. Again the mixture was concentrated and the process repeated. The resulting TFA salt was dissolved in dichloromethane (1 mL) and 2M HCl in diethyl ether (0.25 mL) was added followed by cold diethyl ether (8 mL). The precipitate was collected by filtration and washed with diethyl ether (3 mL) to yield 92 (0.033 g, 99%) as a tan amorphous solid. \(^{1}H\) NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 12.08 (s, 1H), 11.61 (s, 1H), 7.91 (m, 1H), 7.33 (s, 2H), 6.57 (s, 1H), 3.03 (q, 2H, \(J = 6.8\) Hz), 2.39 (t, 2H, \(J = 6.8\) Hz), 2.05 (t, 2H, \(J = 6.4\) Hz), 1.62 (t, 2H, \(J = 6.4\) Hz), 1.47 (m, 2H), 1.23 (bs, 6H), 0.85 (t, 3H, \(J = 6.4\) Hz); \(^{13}C\) NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 172.2, 146.7, 126.3, 108.7, 37.5, 35.4, 31.0, 28.3, 27.8, 25.3, 22.0, 21.4, 13.9; HRMS (ESI) calcd for C\(_{13}\)H\(_{24}\)N\(_4\)O (M\(^+\)) 252.1950, found 252.1957.

2-Amino-4-[3-(4-hexyl-[1,2,3]triazol-1-yl)propyl]imidazole-1-carboxylic acid tert-butyl ester (5-xxxxv). Azide 91 (0.108 g, 0.406 mmol) was dissolved in a 1:1 mixture of
ethanol (2 mL) and water (2 mL). To this solution was added sodium-L-ascorbate (0.017 g, 0.081 mmol), CuSO$_4$·5H$_2$O (0.010 g, 0.041 mmol), and 1-octyne (0.054 g, 0.487 mmol). The reaction was stirred at room temperature and monitored by TLC analysis. When completion of the reaction was evident, the ethanol was removed by rotary evaporation. The aqueous residue was diluted with water (50 mL) and EtOAc (100 mL). The organic layer was washed with sat. NaHCO$_3$ (3 x 50 mL) and brine (50 mL) before being dried (Na$_2$SO$_4$), filtered, and evaporated to dryness. Purification of the crude product via flash column chromatography (0-10% MeOH/CH$_2$Cl$_2$) afforded 5-xxxxv (0.084 g, 55%) as an amber oil. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 7.84 (s, 1H), 6.55 (s, 1H), 6.41 (s, 2H), 4.30 (t, 2H, $J = 6.9$ Hz), 2.58 (t, 2H, $J = 7.5$ Hz), 2.22 (t, 2H, $J = 6.9$ Hz), 2.02 (quint, 2H, $J = 6.9$ Hz), 1.55 (m, 11H), 1.27 (m, 6H), 0.85 (t, 3H, $J = 6.9$ Hz); $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ 149.9, 148.9, 146.8, 137.5, 121.6, 106.0, 84.1, 48.7, 31.0, 28.9, 28.5, 28.2, 27.5, 25.0, 24.6, 22.0, 13.9; HRMS (ESI) calcd for C$_{19}$H$_{32}$N$_6$O$_2$ (M)$^+$ 376.2587, found 376.2595.

4-[3-(4-Hexyl-[1,2,3]triazol-1-yl)propyl]1H-imidazol-2-ylamine hydrochloride (93).

Using the same general procedure as used for the synthesis of 62, 5-xxxxv (0.054 g, 1.43 mmol) gave 93 (0.045 g, 100%) as an amber oil. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ 12.12 (s, 1H), 11.68 (s, 1H), 7.88 (s, 1H), 7.37 (s, 2H), 6.59 (s, 1H), 4.32 (t, 2H, $J = 6.9$ Hz), 2.59 (t, 2H, $J = 7.5$ Hz), 2.38 (t, 2H, $J = 7.2$ Hz), 2.06 (quint, 2H, $J = 7.5$ Hz), 1.57 (m,
2H), 1.27 (m, 6H), 0.85 (t, 3H, J = 6.9 Hz); $^{13}$C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\) 146.8, 146.8, 125.5, 121.8, 108.9, 48.3, 31.0, 28.9, 28.2, 28.0, 25.0, 22.0, 21.2, 13.9; HRMS (ESI) calcd for C$_{14}$H$_{24}$N$_6$ (M)$^+$ 276.2062, found 276.2066.
References


Appendix
Representative Growth Curves

Growth Curve
at IC_{50} = 729 nM

Growth Curve
at IC_{50} = 14.3 μM

Growth Curve
at IC_{50} = 15.6 μM
Growth Curve
at IC$_{30}$ = 36.7 µM

![Growth Curve Diagram]

- A. baumannii wt
- A. baumannii w/ cmpd
277
62
5-vii
5-vii
5-xi
5-xv
5-xxi
\[ \begin{align*} 
\text{H}_2\text{N-} & \text{N} \quad \text{CH}_3 \\
\text{N} \quad \text{N} \\
\text{HCl} \\
\end{align*} \]

75
5-xxx
5-xxxiii
5-xxxiii
5-xxxv
5-xxxvi
5-xxxix
5-xxxxi
5-xxxxiii
$\text{5-xxxxiv}$
CHAPTER 6
Synthesis and Anti-HIV Properties of a TAK-779 Analogue

6.1 Multivalent Displays in Biology

It has been suggested that biological systems exploit multivalency in the synthesis of high-affinity ligands because they allow an organism to take advantage of an existing set of monovalent ligands without the need for evolving completely new molecules for every required function. Multivalent displays are ubiquitous in biology, and can confer dramatically enhanced affinity. For example, binding between multiple hemagglutinin (HA) ligands of an influenza virus and sialic acid (SA) surface receptors of an erythrocyte during viral infection is estimated to occur with an affinity of $10^{13} \text{ M}^{-1}$, while the association constant for a single SA-HA interaction is $10^3 \text{ M}^{-1}$.

In the development of therapeutics, a multivalent binding strategy could offer many advantages. Synthetic systems that present multiple low-affinity biomolecule-binding ligands may prove to be more synthetically accessible and could allow one to tune binding affinity over several orders-of-magnitude. Moreover, nanoparticles that are comparable in size to proteins and present multiple protein-binding ligands may be effective at disrupting protein/protein interactions that drive disease pathogenesis. This is significant because these interactions are typically considered to be “undruggable” by traditional small-molecule inhibitors. Multivalent linear polymers, dendrimers, proteins, and liposomes have successfully targeted pathogenic biomolecule targets. Of particular note are SA-coated liposomes and dendrimers that bind to HA on the surface of an influenza virus with affinity enhancements of $10^2 – 10^6$.1
Particle-based displays of multiple ligands have the additional advantage of creating a high local concentration of binding molecules. Consequently, binding equilibrium between a surface-bound ligand and receptor favors formation of more ligand-receptor pairs. For instance, DNA hybridization is thermodynamically favored by one order-of-magnitude if one of the single-stranded DNA sequences is conjugated to a 10 nm diameter gold nanoparticle surface.\textsuperscript{3, 4}

### 6.2 Synthesis of TAK-779 Analogue: SDC-1721

We employed 2.0 nm diameter, mercaptobenzoic acid-modified gold particles as a nanoscale platform to construct our multivalent therapeutic. These particles have a proposed empirical formula of [Au\textsubscript{144}(HSC\textsubscript{6}H\textsubscript{4}COOH)\textsubscript{52}], and are related to the [Au\textsubscript{102}(HSC\textsubscript{6}H\textsubscript{4}COOH)\textsubscript{44}] monolayer-protected gold nanoparticles that were recently characterized by X-ray crystallography.\textsuperscript{5} Importantly, this class of gold particles is similar to proteins and dendrimers in that they are atomically precise and monodisperse nanoscale molecules.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{scheme17.png}
\caption{Synthesis of SDC-1721. Reaction conditions: (a) i. (COCl)\textsubscript{2}, DMF (cat.), CH\textsubscript{2}Cl\textsubscript{2} ii. 4-[(N-Boc)-aminomethyl]aniline, THF (86%) (b) TFA, CH\textsubscript{2}Cl\textsubscript{2} (91%) (c) Thiol-dPEG\textsuperscript{4}-acid, DCC, HO\textsubscript{2}t, DMF (67%).}
\end{figure}
SDC-1721 was synthesized using a modification of the synthetic approach to TAK-779 (Scheme 17) and coupled to the 2.0 nm diameter gold nanoparticles via a simple ligand exchange reaction (Figure 26). The average number of SDC-1721 molecules per particle was determined to be 12, and the particle size was verified by transmission electron microscopy. Examination of the micrographs revealed no substantial differences in core size or dispersion when compared to the 2.0 nm diameter gold nanoparticles prior to ligand exchange.

![Figure 26. TAK-779 and SDC-1721.](image)

### 6.3 Inhibition of HIV Fusion

The 2.0 nm diameter mercaptobenzoic acid-coated gold nanoparticles were conjugated to SDC-1721, a derivative of TAK-779, a known CCR5 antagonist. CCR5 serves as the principal entry co-receptor for most commonly transmitted strains of HIV-1, and those that predominate in the early years of infection. A significant amount of structure-activity relationship (SAR) data has been generated with regard to TAK-779, most notably that the quaternary ammonium salt is essential for high-affinity binding and effective inhibition of HIV fusion. TAK-779 homologues that lack this quaternary ammonium salt are devoid of activity. Unfortunately, the quaternary ammonium salt imbues TAK-779 with poor
pharmacological properties, such as significant irritation at the injection site, which led to an exhaustive search for alternate small molecule CCR5 antagonists. As an alternative approach and proof-of-concept, we synthesized SDC-1721, a TAK-779 homologue that lacks the quaternary ammonium salt moiety, to show that it is possible to conjugate a low-affinity, biologically inactive small molecule to a gold nanoparticle to create biologically active multivalent gold nanoparticle therapeutics. The ability to transform small molecules that are not therapeutically viable into potential therapeutics by simply conjugating them to a gold nanoparticle scaffold could greatly accelerate the discovery of effective new drug formulations.

To evaluate the antiviral activity of the nanoparticle conjugate, phytohaemagglutinin (PHA) stimulated peripheral blood mononuclear cells (PBMCs) were infected with the CCR5-tropic HIV-1 clone JR-CSF in the presence or absence of the test compounds. On day 7 post-infection, supernatants were collected and HIV-1 capsid p24 antigen was measured by ELISA. We first verified inhibition of HIV-1 infection by TAK-779 using this experimental protocol. As shown in Figure 27a, TAK-779 inhibited HIV-1 replication with an IC₅₀ of 10 nM. The IC₅₀ for TAK-779 against four different CCR5-tropic viral isolates ranged from 1.6 – 3.7 nM. However, JR-CSF was not one of the viruses tested. With a similar virus, JR-FL, TAK-779 revealed an IC₅₀ of 20 nM. Sensitivity to CCR5 entry inhibitors is impacted by receptor expression levels and HIV-1 envelope/receptor affinity, mediated by both cellular and viral determinants. Thus, JR-CSF appears to be inherently less sensitive to TAK-779.
We next tested whether, as had been reported, the quaternary ammonium group was essential to TAK-779 activity. SDC-1721 did not inhibit viral replication (Figure 27b). Conjugation of SDC-1721 ((SDC-1721)-NP) to gold nanoparticles at an average ratio of 12:1 restored activity with an IC₅₀ of 10 nM (Figure 27c). To ensure that the inhibitory effect was not due to the gold particle, a glutathione-modified gold nanoparticle (GSH-NP) control was tested. The GSH-NP is unable to inhibit viral replication (Figure 27d). In addition, we verified that multivalent display of SDC-1721 on the nanoparticle surface was required for activity by assaying (SDC-1721)-NP conjugates with an average ratio of 0.93/1. This nanoparticle preparation was unable to inhibit viral replication up to 300 µM. There was no difference in cell viability between samples at these concentrations.

We further verified that inhibition of viral replication was specific for viral entry and was not secondary to a unique property acquired by the SDC-1721-modified gold nanoparticles. This was assayed by performing single-cycle infection experiments using the TZM-bl luciferase reporter gene assay system. This cell line is engineered to express high levels of CCR5 and CD4 and contains a luciferase reporter gene under control of the HIV-1
promoter, which is inducible in trans by the viral protein Tat.\textsuperscript{13} TZM-bl cells were infected with JR-CSF in the presence or absence the test compounds. 48 hours post-infection, luciferase activity was measured as relative luminescence units (RLU). SDC-1721-modified gold nanoparticles inhibited viral entry as well as TAK-779 (Figure 27e). At 48 hours, production of viral progeny is insignificant, thus establishing inhibition of viral replication at the stage of viral entry.

6.4 Conclusion

In conclusion, we have documented the first application of small-molecule coated gold nanoparticles as effective inhibitors of HIV fusion. These nanoscale drug conjugates recapitulate the activity of TAK-779, namely inhibition of viral fusion, which, in turn, leads to inhibition of viral replication. The results demonstrate that therapeutically inactive monovalent small organic molecules may be converted into highly active drugs by simply conjugating them to gold nanoparticles.
6.5 Experimental Section

Chemistry

All \textsuperscript{1}H NMR and \textsuperscript{13}C NMR spectra were recorded at 25.0 °C on a Varian Mercury spectrometer. Chemical shifts (\(\delta\)) are given in ppm relative to tetramethylsilane or the respective NMR solvent; coupling constants (\(J\)) are in hertz (Hz). Abbreviations used are s = singlet, bs = broad singlet, d = doublet, t = triplet, and m = multiplet. FAB-MS spectra were measured via high-resolution fast atom bombardment using a matrix of nitrobenzyl alcohol. Silica gel (40 \(\mu\)m average particle size) was used for column chromatography. All other reagents were used as purchased from commercial sources unless otherwise noted.

\[\text{N-(4-N-BOCmethylphenyl)-2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-carboxamide (6-i).}\]

2-(4-Methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-carboxylic acid\textsuperscript{6} (322 mg, 1.16 mmol) was suspended in CH\textsubscript{2}Cl\textsubscript{2} (5.5 mL) at 0 °C and a catalytic amount of DMF was added (~0.01 mL). To this slurry was added oxalyl chloride (0.3 mL, 3.4 mmol) dropwise and the slurry dissipated and became homogeneous. This solution was warmed to room temperature and allowed to stir for 1.5 h at which time the solvent and excess oxalyl chloride were removed under reduced pressure. The resulting white solid was dissolved in THF (7.5 mL) and added dropwise via syringe to a solution of 4-[(N-Boc)-aminomethyl]aniline (285 mg, 1.28 mmol) in THF (4.0 mL) and TEA (0.47 mL) at 0 °C.
The solution was allowed to warm to room temperature overnight then the solvent was removed under reduced pressure and the resulting solid was taken up in EtOAc (100 mL) and H₂O (100 mL). The H₂O was extracted with additional EtOAc (3x75 mL) and the organic layer was washed with H₂O, sat. NaHCO₃, brine, dried (Na₂SO₄), filtered and concentrated. Concentration was facilitated by adding EtOAc (3x50 mL) to obtain a fine white powder. This powder was subsequently filtered and washed with cold EtOAc to obtain 6-ii (479 mg, 86%) as a fine white powder. Rᵣ 0.6 (50% EtOAc/hexanes); ¹H NMR (300 MHz, CDCl₃) δ 7.63 (s, 1H), 7.55 (d, 2H, J = 8.4 Hz), 7.44 (m, 5H), 7.23 (m, 4H), 4.82 (bs, 1H), 4.28 (d, 2H, J = 5.7 Hz), 2.87 (m, 2H), 2.71 (t, 2H, J = 6.3 Hz), 2.39 (s, 3H), 2.16 (m, 2H), 1.46 (s, 9H) ppm; ¹³C NMR (75 MHz, CDCl₃) δ 168.1, 155.9, 141.3, 139.3, 137.9, 137.6, 137.4, 137.3, 135.0, 134.7, 134.6, 130.8, 130.0, 129.7, 128.4, 127.0, 126.9, 120.4, 44.5, 34.9, 30.7, 28.7, 28.3, 21.4, 0.4 ppm; HRMS (FAB) m/z, ([M + H]⁺, C₃₁H₃₄N₂O₃): calcd. 483.2648, found 483.2644.

N-(4-Aminomethylphenyl)-2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-carboxamide (6-iii). N-(4-N-BOCmethylphenyl)-2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-carboxamide (110 mg, 0.23 mmol) was suspended in CH₂Cl₂ (9.0 mL) at room temperature and TFA (2.3 mL) was added slowly to the slurry. The reaction mixture was stirred overnight and then quenched with sat. NaHCO₃ followed by dilution with
EtOAc:CH$_2$Cl$_2$ (1:2, 300 mL). The organic layer was then washed with 1 N NaOH, brine, dried (Na$_2$SO$_4$), filtered and concentrated. Concentration was facilitated by adding EtOAc (3x20 mL) to obtain a fine white powder. This powder was subsequently filtered and washed with cold EtOAc to obtain the title compound (80 mg, 91%) as a fine white powder. $R_f$ 0.05 (10% MeOH/CH$_2$Cl$_2$); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.62 (s, 1H), 7.55 (d, 2H, $J$ = 8.7 Hz), 7.45 (m, 4H), 7.40 (s, 1H), 7.28 (d, 2H, $J$ = 8.4 Hz), 7.20 (d, 2H, $J$ = 7.2 Hz), 3.81 (s, 2H), 2.87 (m, 2H), 2.71 (t, 2H, $J$ = 6.6 Hz), 2.39 (s, 3H), 2.15 (m, 2H), 1.74 (bs, 2H) ppm; $^{13}$C NMR (75 MHz, CDCl$_3$) $\delta$ 163.0, 136.3, 134.3, 133.3, 133.0, 132.6, 132.2, 131.9, 129.8, 129.5, 125.8, 125.0, 124.7, 122.9, 122.0, 121.9, 115.5, 29.9, 25.7, 25.0, 23.3, 16.4 ppm; HRMS (FAB) m/z, ([M + H]$^+$, C$_{26}$H$_{26}$N$_2$O): calcd. 383.2123, found 383.2133.

TAK-779 Analogue (SDC-1721). $N$-(4-Aminomethylphenyl)-2-(4-methylphenyl)-6,7-dihydro-5H-benzocyclohepten-8-carboxamide (180 mg, 0.47 mmol) was dissolved in DMF (5.0 mL) at room temperature and DCC (209 mg, 1.0 mmol), HOBT (128 mg, 0.95 mmol), and Thiol-dPEG4™-acid (132 µL, 0.47 mmol) were added consecutively. The reaction was stirred for 24 h at room temperature then filtered and concentrated under reduced pressure. Flash column chromatography (0-5% MeOH/CH$_2$Cl$_2$) of the crude material gave SDC-1721 (204 mg, 67%) as a white amorphous solid. $R_f$ 0.37 (10% MeOH/CH$_2$Cl$_2$); $^1$H NMR (300 MHz, CDCl$_3$) $\delta$ 7.95 (s, 1H), 7.55 (d, 2H, $J$ = 8.7 Hz), 7.44 (m, 5H), 7.21 (m, 4H).
$1H, J = 5.7 Hz), 4.40 (d, 2H, $J = 5.4 Hz), 3.7 (t, 2H, $J = 5.7 Hz), 3.56 (m, 16H), 2.85 (m, 2H), 2.66 (m, 4H), 2.5 (t, 2H, $J = 5.7 Hz), 2.38 (s, 3H), 2.13 (m, 2H); ^{13}C NMR (75 MHz, CDCl$_3$) $\delta$ 171.6, 168.2, 141.3, 139.2, 137.9, 137.6, 137.4, 137.2, 134.56, 134.65, 134.5, 130.7, 130.0, 129.6, 128.3, 126.9, 126.8, 120.5, 73.0, 70.8, 70.7, 70.5, 70.44, 70.36, 67.5, 43.1, 37.2, 34.9, 30.7, 28.2, 24.5, 21.4 ppm; HRMS (FAB) m/z, ([M + H]$^+$, C$_{37}$H$_{46}$N$_2$O$_6$S): calcd. 647.3155, found 647.3202.

**Preparation of [Au$_{144}$(SC$_6$H$_4$COOH)$_{52}$]**

A concentrated gold-thiol polymer stock solution composed of 1 mM HAuCl$_4$, 3.4 mM 4-mercaptobenzoic acid, and 50% aqueous methanol is adjusted to pH ~13 using sodium hydroxide. This solution is then allowed to sit overnight at room temperature in a sealed vessel. Once fully reacted, the solution is diluted to a final Au(III) concentration of 10 µM and a final methanol concentration of 27% (v/v) with the addition of methanol and water. Sodium borohydride (10 mM, aqueous) was then added in a 3:1 excess and after 16 hours of reaction time methanol was added to the reaction to precipitate the product. The product is collected by filtration, dissolved in water and then precipitated with methanol to obtain pure [Au$_{144}$(SC$_6$H$_4$COOH)$_{52}$].

**Preparation of [Au$_{144}$(SDC-1721)$_4$(SC$_6$H$_4$COOH)$_{48}$] ((SDC-1721)-NP)**

[Au$_{144}$(SC$_6$H$_4$COOH)$_{52}$] (0.84 mg, 0.0235 µmol) and SDC-1721 (3.8 mg, 5.87 µmol) were dissolved in 1.25 mL of 80% methanol, 10% water, and 10% glycerol. After 3 hours, the supernatant was removed and the product was washed with methanol (3x1.0 mL) to
remove excess SDC-1721 and displaced p-mercaptobenzoic acid to obtain pure (SDC-1721)-NP.

**Preparation of (GSH-NP)**

\[ [\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}] \] (0.84 mg, 0.0235 µmol) and glutathione (GSH) (1.8 mg, 5.87 µmol) were dissolved in 1.25 mL of water, and allowed to react at room temperature for 3 hours. The product was precipitated and washed with methanol (3x1 mL) and collected by centrifugation at 6000 x g to obtain pure GSH-NP.

**Characterization of [Au_{144}(SDC1721)_4(SC_6H_4COOH)_{48}] ((SDC-1721)-NP)**

Beer’s law extinction coefficients for SDC-1721-coated gold nanoparticles, \([\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}]\), and p-mercaptobenzoic acid at 262 nm are \(2.6 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}\), \(7.6 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}\), and \(3.26 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}\), respectively. The Beer’s law extinction coefficient for \([\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}]\) at 510 nm is \(4.36 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}\); SDC-1721 and p-mercaptobenzoic acid have no appreciable absorbance at 510 nm.

Because only the gold core has appreciable absorbance at 510 nm, the absorbance at 510 nm was used to determine the concentration of \([\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}]\). Since the extinction coefficients of SDC-1721-coated gold nanoparticles and \([\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}]\) are much larger than that of p-mercaptobenzoic acid at 261 nm, the problem was treated as a system of two absorbers rather than three.

Thus:

\[
(1) \ A_{\text{total, 261 nm}} = A_{[\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}], 261 \text{ nm}} + A_{\text{SDC-1721, 261 nm}}
\]
The assumption stated above is that term one on the RHS of equation (2) does not change significantly when \(p\)-mercaptobenzoic acid ligands are replaced by SDC-1721 ligands on the surface of the gold nanoparticle. Thus, every term is known except \([SDC-1721]\). Solving using measured absorbance values results in \([SDC-1721] = 276 \ \mu \text{M}, [\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}] = 22.7 \ \mu \text{M},\) therefore \([SDC-1721]/[\text{Au}_{144}(\text{SC}_6\text{H}_4\text{COOH})_{52}] = 12.2.\)

**Transmission electron microscopy**

5 \(\mu\)L of a solution of (SDC-1721)-NP was dispensed onto a 400 mesh carbon coated copper TEM grid (Electron Microscopy Sciences). The mixture was allowed to incubate on the grid for 1 minute and then side-blotted with Whatman #1 filter paper. The grid was examined in a FEI CM12 microscope, operating at 100 kV accelerating voltage, in the University of North Carolina - Chapel Hill school of dentistry.
PBMC infection assay

PMBC’s were collected from donors at the UNC Chapel Hill Medical School. Because of the considerable variation that is inherent in the infection of primary PBMCs, we performed assays in triplicate using PMBCs isolated from a single donor who maintains substantial CCR5 surface expression after PHA stimulation (data not shown).

Cell proliferation assay

Cell proliferation was determined by the MTT cell proliferation assay (Roche Diagnostics, Indianapolis, IN)

1 Drug/Particle Experiment
Nanoparticle preparations that contain 0.93 SDC-1721 molecules/particle were unable to inhibit HIV.

Figure 29. Quantitation of HIV p24 Protein as a Function of Treatment. Tak = TAK-779. NP1 = 0.93 SDC-1721 molecules/particle. Each number is the respective compound indicates concentration. Mock = no virus control, JR-CSF = no drug, positive virus control.
References


6-ii
6-ii
6-iii