SYNTHESIS OF NOVEL ANTIVIRAL AGENTS AND
FLUORESCENT MOLECULAR PROBES

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Chemistry
by
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RNA viruses cause a wide variety of diseases including SARS, influenza, hepatitis C and polio. Therapeutics for RNA virus infections are often limited because of the rapid development of antiviral drug resistance. RNA viruses are known to exhibit high error rates during replication and thus exist as quasispecies. To maintain the maximum adaptability, these viruses exist on the edge of “error catastrophe”, and small increases in the mutation frequency can cause a drastic decrease in viral infectivity. By taking advantage of the high mutation rate of RNA virus replication, a relatively new antiviral approach termed “lethal mutagenesis” can be used to increase the error rate of RNA viral replication to intolerable levels, resulting in the loss of viral viability.

Chapter one of this dissertation reviews current antiviral therapeutics and lethal mutagenesis as an antiviral strategy. The clinically used antiviral drug ribavirin represents an agent that functions as a lethal mutagen against poliovirus (PV) and hepatitis C virus (HCV). This drug is converted intracellularly to the 5'-triphosphate (RTP), which is a degenerate substrate of viral RNA-dependent RNA polymerases (RdRP). Once in the genome, ribavirin promotes mutagenesis by templating the incorporation of both C and U during multiple rounds of viral replication, leading to error catastrophe and decreased infectivity of the virus.

The work described herein includes efforts to design and synthesize novel antiviral nucleosides and probe their mechanism of action. In chapter two, we report the synthesis and antiviral effects of bioisosteric deaza analogues of 6-methyl-9-β-D-ribofuranosylpurine, a hydrophobic analogue of adenosine. Whereas the 1-deaza and 3-deaza analogues are essentially inactive in whole cell assays, a novel 7-deaza-6-methyl-9-β-D-ribofuranosylpurine analogue, structurally related to the natural product tubercidin, potently inhibited replication of poliovirus (PV) in HeLa cells (IC₅₀ = 11 nM) and dengue virus (DENV) in Vero cells (IC₅₀ = 62 nM) as
evidenced by plaque assays. Moreover, selectivity against PV over cytotoxic effects to HeLa cells was >100-fold after incubation for 7 h. We further found that the putative triphosphate metabolite of this 7-deaza analogue was effectively incorporated into RNA by PV RdRP.

Chapter three describes studies of the pyrazinecarboxamide compound T-1106 as an antiviral agent. Although this compound is active against several RNA viruses, its mechanism of action is poorly understood. Only a single patent has reported the synthesis of T-1106, and the coupling of its nucleobase with ribose leads to a mixture of α and β anomers. Improved Vorbrüggen coupling conditions were developed here to achieve a stereoselective synthesis of this compound. Treatment of PV infected HeLa cells with 1 mM T-1106 caused a dramatic decrease of viral titer, and the corresponding triphosphate was found to be incorporated by PV RdRP across all four natural nucleotides.

Chapter four focuses on another topic, development of new analogues of rhodamine as red fluorescent probes. As members of the xanthene class of dyes, rhodamines often exhibit high fluorescence quantum yields, pH-insensitivity, excellent photostability and photophysical properties. For these reasons, rhodamines are widely used as probes for labeling of biomolecules and construction of chemosensors. Three novel hydrophobic rhodamine analogues with good photophysical properties were designed and synthesized. The ability of biotinylated derivatives of these fluorophores to bind streptavidin fusion proteins expressed in living yeast cells was investigated.
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Chapter 1

Lethal Mutagenesis as an Antiviral Strategy

1.1 Overview

Many human diseases such as hepatitis A and C, AIDS, and the common cold are attributed to RNA viral infections. The high mutation rate of RNA viruses coupled with the emergence of new lethal RNA viruses have made current therapies inadequate for the treatment of many viral diseases. Additionally, lack of global vaccination has made it impossible to prevent some of these diseases. Therefore, new strategies are needed to combat these viruses. Lethal mutagenesis is a relatively new approach for the treatment of infections by RNA viruses. Due to the intrinsically high error rate of viral RNA-dependent RNA polymerases (RdRP) and natural selection, RNA viruses exist as quasispecies, a virus population in which each member differs from another by a finite number of changes in the genome sequence. However, there is an “error threshold” to the number of random viral mutations. Once this limit is exceeded, viral viability is lost and the virus population is sent into “error catastrophe.” To exploit this weakness, base-modified nucleoside analogues that can be phosphorylated may be incorporated into viral genomes by the promiscuous RdRP. These compounds will then increase viral mutation frequencies to intolerable levels and force the virus into error catastrophe and extinction. Ribavirin (29), a broad spectrum antiviral agent active against at least 40 RNA viruses, is a lethal mutagen to many RNA viruses including poliovirus (PV) and hepatitis C virus (HCV). Thus, introducing mutations into viral genomes represents a useful therapeutic approach to treat RNA virus infection. An overview of existing treatments for RNA virus infection, the mechanism of action of ribavirin, and the design of novel lethal mutagens is discussed in this chapter.
1.2 Diseases Related to RNA Viruses

A number of human diseases are caused by RNA viruses. Human immunodeficiency virus (HIV) has killed over 25 million people since its emergence in 1981. HIV infects around 0.6% of the population worldwide, and this virus has reduced average life expectancy by more than 20 years.\(^1\) Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two other important viral infections. These pathogens currently infect approximately 350 million\(^2\) and 200 million people\(^3\) worldwide, respectively. Chronic HBV and HCV infections are major causes of end-stage liver disease and hepatocellular carcinoma. Influenza virus, one of the major pathogens that causes respiratory viral infections, has a high rate of morbidity and mortality.\(^4\) As a seasonal epidemic, influenza virus can infect 10-20% of the global population, and the annual epidemic kills 0.25 to 0.5 million people worldwide.\(^5\) Meanwhile, new RNA viral diseases, such as SARS (severe acute respiratory distress),\(^6\) Ebola,\(^7\) West Nile,\(^8\) H5N1 influenza,\(^6\) and H1N1 influenza\(^9\) cause widespread panic. Emergence of new lethal RNA viral pathogens, coupled with resistance to current antiviral drugs, hastens the need for more effective therapeutics against RNA viruses.

1.3 Life Cycle of an RNA Virus: Poliovirus

Poliovirus (PV) is stable, easy to purify, and one of the most well characterized RNA viruses,\(^10\) making it ideal for laboratory studies. PV is a human enterovirus in the family of Picornaviridae. It is composed of a single-stranded positive-sense RNA genome and a protein capsid. The replication cycle (Figure 1-1) begins when PV binds to the poliovirus receptor (CD155) on a host cell. After intracellular internalization and viral uncoating, the genomic RNA
is available for protein synthesis by the host ribosome. A single polyprotein is synthesized, and it undergoes a series of cleavage reactions to produce all of the structural and non-structural viral proteins. The positive-sense genome is replicated via a negative stranded intermediate by PV RNA-dependent RNA polymerase (RdRP) or 3D\textsuperscript{pol}. This replication of genomic material happens in the cytoplasm on surfaces of cell-specific membrane vesicles. The newly formed RNAs are assembled with viral structural proteins to form new virions, which are ultimately released after cell lysis.\textsuperscript{11}

![Figure 1-1: Life cycle of poliovirus.\textsuperscript{11}](image)

### 1.4 Current Drugs and Targets for Blocking Viral Infection

The current pace of antiviral drug development is similar to the pace of antibiotic development 30 years ago. At present, 44 compounds are approved by the FDA for clinical use, and most of these drugs were approved in the past twenty years, since only five antiviral compounds were used clinically in the early 1990’s.\textsuperscript{12} For the treatment of HIV infection, 23
compounds are licensed targeting key viral enzymes including reverse transcriptase (Figure 1-2), protease, and integrase (Figure 1-3). Two compounds target viral entry into host cells (Figure 1-3).\textsuperscript{13}

**Nucleoside/Nucleotide reverse transcriptase inhibitors**

- Zidovudine (AZT, 1)
- Didanosine (ddl, 2)
- Zalcitabine (ddC, 3)
- Stavudine (d4T, 4)
- Lamivudine (3TC, 5)
- Abacavir (ABC, 6)
- Emtricitabine ([(-)-FTC, 7]
- Tenofovir disoproxil fumarate (8)

**Non-nucleoside reverse transcriptase inhibitors**

- Nevirapine (9)
- Delavirdine (10)
- Efavirenz (11)
- Etravirine (12)

Figure 1-2: Clinically approved HIV reverse transcriptase inhibitors.
Figure 1-3: Clinically approved HIV protease inhibitors, entry inhibitors, and an integrase inhibitor.
In addition to anti-HIV drugs, other antiviral therapeutics are used to treat HBV, HCV, influenza virus, HSV (herpes simplex virus), VZV (varicella-zoster virus), and CMV (cytomegalovirus) (Figure 1-4 and Figure 1-5). Lamivudine and tenofovir disoproxil fumarate are approved for treatment of both HIV infection and HBV infection. All five anti-HBV compounds target HBV RT (reverse transcriptase) and function as chain terminators. Ribavirin and interferon-α combination therapy is a standard treatment for chronic hepatitis C. Ribavirin is also used for the treatment of lassa fever and influenza virus. The mechanism of action of this compound is further discussed in Section 1.5.3. Amantadine and rimantadine inhibit influenza A virus by blocking the M2 ion channel, which is required for the uncoating of viral particles. Two structurally similar compounds, zanamivir and oseltamivir, are inhibitors of neuraminidase, an enzyme necessary for releasing viral particles from infected cells. Acyclovir, a guanosine analogue, is the first acyclic nucleoside approved for clinical use. Acyclovir, as well as other acyclic drugs, is metabolized intracellularly to the corresponding triphosphate, which works as a chain terminator and a potent inhibitor of viral DNA polymerase. The active metabolites of idoxuridine and trifluridine, the corresponding triphosphates, are incorporated into viral DNA by viral DNA polymerase, exerting antiviral effects by blocking correct base pairing. Foscarnet, a mimic of pyrophosphate, selectively inhibits the pyrophosphate binding site on viral DNA polymerase, without affecting human DNA polymerase at its effective concentration. Fomivirsen is a 21-mer phosphorothioate oligonucleotide, and is the first antisense antiviral approved by FDA. By binding to a coding segment of a viral gene, fomivirsen blocks translation of viral mRNA. A summary of these FDA approved antiviral drugs and the drug targets is shown in Table 1-1.
**Anti-HBV compounds**

Lamivudine (3TC, 5)

Tenofovir disoproxil fumarate (8)

Adefovir dipivoxil (26)

Entecavir (27)

Telbivudine (28)

**Anti-HCV compounds**

Ribavirin (29)

+ Interferon α

**Anti-influenza virus compounds**

Ribavirin (29)

Amantadine (30)

Rimantadine (31)

Zanamivir (32)

Oseltamivir (33)

**Figure 1-4:** Clinically used therapeutics against RNA virus infections.
**Anti-herpesvirus compounds**

**HSV and VZV inhibitors**

- Acyclovir (34)
- Valaciclovir (35)
- Penciclovir (36)

- Famciclovir (37)
- Idoxuridine (38)
- Trifluridine (39)

**CMV inhibitors**

- Ganciclovir (40)
- Valganciclovir (41)
- Cidofovir (42)

- Foscarnet (43)
- Fomiviren (44)

5'-GCG TTT GCT CTT CTT CTT GCG-3'

Figure 1-5: Clinically used therapeutics against DNA virus infections.
Despite of the rapid development of antiviral drugs, the need for new antiviral compounds still exists because of the emergence of resistance and new pathogenic viruses.

Table 1-1: FDA approved antiviral drugs.

<table>
<thead>
<tr>
<th>Virus</th>
<th>FDA approved antiviral drug</th>
<th>Drug target</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
<td><em>Nucleoside/Nucleotide reverse transcriptase inhibitors</em>&lt;br&gt; Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir, Emtricitabine, Tenofovir disoproxil fumarate</td>
<td>HIV RT</td>
</tr>
<tr>
<td></td>
<td><em>Non-nucleoside reverse transcriptase inhibitors</em>&lt;br&gt; Nevirapine, Delavirdine, Efavirenz, Etravirine</td>
<td>HIV RT</td>
</tr>
<tr>
<td></td>
<td><em>Protease inhibitors</em>&lt;br&gt; Saquinavir, Ritonavir, Indinavir, Nelfinavir, Amprenavir, Lopinavir, Fosamprenavir, Darunavir, Tipranavir, Atazanavir</td>
<td>HIV protease</td>
</tr>
<tr>
<td></td>
<td><em>Integrase inhibitor</em>&lt;br&gt; Raltegravir</td>
<td>HIV integrase</td>
</tr>
<tr>
<td></td>
<td><em>Entry inhibitors</em>&lt;br&gt; Maraviroc&lt;br&gt; Enfuvirtide</td>
<td>CCR5, Fusion</td>
</tr>
<tr>
<td>HBV</td>
<td>Lamivudine, Tenofovir disoproxil fumarate, Adefovir dipivoxil, Entecavir, Telbivudine</td>
<td>HBV RT</td>
</tr>
<tr>
<td>HCV</td>
<td>Ribavirin + Interferon α</td>
<td>IMPDH and lethal mutagen</td>
</tr>
<tr>
<td>Influenza</td>
<td>Amantadine, Rimantadine&lt;br&gt; Zanamivir, Oseltamivir</td>
<td>M2 ion channel, Neuraminidase</td>
</tr>
<tr>
<td>HSV and VZV</td>
<td>Acyclovir, Idoxuridine, Trifluridine, Famiclovir, Valaciclovir, Penciclovir</td>
<td>Viral polymerase</td>
</tr>
<tr>
<td>CMV</td>
<td>Ganciclovir, Valganciclovir, Cidofovir, Foscarnet, Fomivirsen</td>
<td>Viral polymerase, mRNA</td>
</tr>
</tbody>
</table>
Ideally, improved chemotherapeutics should be less susceptible to the development of viral resistance. Lethal mutagenesis has been explored as a therapeutic approach to accomplish this goal.

1.5 Lethal Mutagenesis as an Antiviral Strategy

1.5.1 Biological Basis of Lethal Mutagenesis

RNA viruses maintain an extraordinarily high mutation rate compared with microbes that have DNA-containing genomes. The mutation rate for RNA virus replication is generally accepted to be in the range of $10^{-4}$ to $10^{-5}$ misincorporations per nucleotide incorporated, compared with $10^{-7}$ for DNA virus replication and $10^{-8}$ to $10^{-10}$ for standard double-stranded DNA replication.22 That is roughly one mutation per genome per replication cycle for RNA viruses, compared with 0.003 for DNA based organisms.23 This high mutation rate of RNA viruses is caused by infidelity and the absence of a proofreading domain in most RdRPs (RNA-dependent RNA polymerases).24 Therefore, RNA viruses exist as quasispecies, consisting of genetically distinct yet related genotypes.25 This diversity within a population is necessary for RNA viruses to adapt to environmental changes and develop resistance to antiviral drugs and immunological defenses.26 However, there is an inherent upper limit, termed the “error threshold”, to the mutation frequency of RNA viruses. Once this limit is exceeded, the virus population is no longer genetically viable.27 Interestingly, most RNA viruses exist on the edge of this error threshold, allowing maximal genetic diversity, and leading to maximal adaptability.28 Lethal mutagenesis is an antiviral strategy based on increasing the viral mutation rate beyond the error threshold, yielding an inviable population.29 A typical single-stranded, positive-sense RNA virus is used as a model to illustrate the mechanism of lethal mutagenesis. After internalization into the cytoplasm,
the viral RNA is translated to produce the necessary viral proteins, including RdRP. Replication of viral RNA by RdRP is error-prone, generating approximately one mutation per progeny genome (Figure 1-6, left panel). Treating the host cell with a mutagen leads to additional mutations in the progeny genome, and therefore lowers the fitness of the viral population. (Figure 1-6, right panel)

1.5.2 Nucleoside Analogues as Antiviral Lethal Mutagens

Based on studies demonstrating the presence of quasispecies and the error threshold, lethal mutagenesis has been explored to develop anti-HIV compounds. Loeb and coworkers used
5-hydroxy-2'-deoxycytidine (45) to mutate HIV-1 into loss of infectivity.\textsuperscript{30} They also showed that the 5'-triphosphate of 45 was a substrate for HIV RT (reverse transcriptase), and replication of HIV in the presence of 45 resulted in a concomitant increase of G to A substitutions. These results demonstrate the feasibility of lethal mutagenesis as an antiviral strategy against HIV. Another deoxynucleoside analogue, 5-aza-5,6-dihydro-2'-deoxycytidine (KP1212, 46) was developed by Daifuku and coworkers as a potent anti-HIV agent in 2005.\textsuperscript{31} Compound 46 inhibited HIV-1 with an IC\textsubscript{50} of 10 nM and therapeutic index of 100,000. This compound increased the mutation rate of HIV-1 by 50-100\%. In addition to the deoxyribonucleoside approach, a ribonucleoside strategy was proposed, because mutagenic ribonucleosides can be incorporated into the HIV genome by RNA polymerase II of the host cell, conferring an antiviral effect in the latter portion of the viral life cycle.\textsuperscript{32} The antiviral activity of 5-azacytidine (47) against HIV-1 was demonstrated,\textsuperscript{33} but the antiviral activity was not due to the incorporation of the ribonucleoside during transcription in the latter stage of viral life cycle. Instead, the compound was reduced to 5-aza-2'-deoxycytidine and incorporated into the viral genome by RT, leading to increased mutation frequency and loss of viral infectivity.\textsuperscript{34}

Ribonucleoside analogues have been used to promote lethal mutagenesis in other RNA viruses. 5-Fluorouridine (48) effectively mutagenizes foot-and-mouth disease virus (FMDV)\textsuperscript{35} and lymphocytic choriomeningitis virus (LCMV)\textsuperscript{36} into extinction. In a cell-free poliovirus (PV) synthesis assay, the triphosphate of the pyrimidine analogue rP (49) can be incorporated and
exhibit ambiguous base-pairing capacity, because 49 can mimic cytidine or uridine through tautomerization of the nucleobase to the amino or imino form. However, 49 does not show antiviral activity in cell culture, due to the inability of the nucleoside to be converted to the nucleoside monophosphate. This problem was bypassed using the nucleobase of 49, which is antiviral and mutagenic against PV, presumably because of rP nucleotide formation by a nucleotide salvage pathway. The strategy of achieving ambiguous base-pairing property through tautomerization was successfully employed in a series of N-6-modified purine nucleoside analogues, with JA28 (50) and JA30 (51) providing the most potent antiviral compounds against PV and coxsackievirus (CVB3). These two compounds increase the mutation rate of PV by more than 30-fold over a single passage, resulting in a two to three order of magnitude reduction in viral titer. However, ribavirin is the most broad spectrum lethal mutagen, and the lethal mutagenesis mechanism of ribavirin has been demonstrated against PV, VSV, HCV, GV virus B, HIV-1, FMDV, LCMV and hantaan virus.
1.5.3 Ribavirin as a Lethal Mutagen

Ribavirin is the only broad spectrum antiviral nucleoside employed clinically. Ribavirin was first evaluated as an antiviral agent by Sidwell and coworkers at ICN Pharmaceuticals in 1972, and it showed broad spectrum activity against a series of RNA and DNA viruses. Early mechanistic studies suggested that ribavirin monophosphate (RMP) inhibited inosine monophosphate dehydrogenase (IMPDH), an enzyme required for de novo synthesis of GTP in cells. Since GTP is essential for translation of mRNAs and replication of RNA virus genomes, a decrease in the concentration of GTP could inhibit virus multiplication and lead to broad-spectrum activity. However, the decrease of GTP concentration could not account for the antiviral activity with various viruses, and not all IMPDH inhibitors are antiviral agents.
Alternate mechanisms, including inhibition of viral capping, inhibition of polymerase and immunomodulation have been proposed.

Studies by Crotty and coworkers suggest lethal mutagenesis as an alternative mechanism of action of ribavirin against PV. They showed that ribavirin is converted intracellularly to ribavirin triphosphate, is incorporated into the viral genome by PV RdRP, and causes transition mutations. Accumulation of mutations pushes PV over the error threshold, leading to loss of infectivity. Direct incorporation of ribavirin triphosphate is proved by using a primer-extension assay, in which end labeled symmetrical primers are extended in vitro by PV RdRP. Ribavirin is incorporated by the polymerase and is templated by both CMP and UMP equally, with rates of incorporation nearing mispairing by natural nucleosides. Upon incorporation into RNA, ribavirin templates both CTP and UTP equally due to free rotation about the carboxamide bond. This ambiguous base-pairing process introduces G to A and A to G transition mutations into the viral genome (Figure 1-9). This mechanism of action is further supported by a guanidine resistance assay. The assay is developed based on the fact that wild type poliovirus does not replicate in the presence of 2 mM guanidine because guanidine inhibits the viral 2C ATPase, which is essential for replication. However, a single C to U transition mutation in this 2C gene is sufficient to confer resistance of the virus to guanidine. In this assay, guanidine resistant variants increased in a dose dependent manner in the presence of ribavirin, indicating that ribavirin was a viral mutagen.
1.6 Design Elements of Lethal Mutagens

Treatment of HCV infection with ribavirin and interferon-α requires high doses, leading to undesirable side effects. Moreover, this combination therapy is not always effective. Demonstration of lethal mutagenesis as an effective antiviral strategy offers a new approach for development of novel ribonucleoside analogues with increased activity and greater selectivity to the virus than to host cells. There are four criteria necessary for ribonucleoside analogues to function effectively as lethal mutagens (Figure 1-10): (1) the nucleoside must be transported across the plasma membrane; (2) cellular kinases need to phosphorylate the nucleoside to the corresponding nucleoside triphosphate; (3) the generated nucleoside triphosphate needs to be recognized by the polymerase and incorporated into the viral genome; and (4) the incorporated
nucleoside should exhibit ambiguous base pairing properties and induce mutagenesis. Additionally, host-cell cytotoxicity effects should be minimized. Discussed herein is a brief overview of the current understanding of these processes.

1.6.1 Intracellular Transport of Nucleosides

Although some artificial nucleosides, such as AZT, are transported through the plasma membrane by passive diffusion,52 most nucleosides cannot diffuse into cells due to their hydrophilicity. Alternatively, hydrophilic nucleosides are transported into cells by integral membrane proteins called nucleoside transporters. These transporters include two main categories: equilibrative nucleoside transporters (ENTs) and concentrative nucleoside transporters (CNTs).53 ENTs transport nucleosides down their concentration gradient, while CNTs take up nucleosides together with sodium against a nucleoside concentration gradient. Studies on the structure-function relationships controlling nucleoside recognition by nucleoside transporters have demonstrated some specificity of transporters.54 However, most nucleoside analogues without dramatic modification to the sugar moiety or the nucleobase, will be transported into cells, given the large number of known nucleoside transporters.55

Figure 1-10: Design elements of lethal mutagens.

1.6.1 Intracellular Transport of Nucleosides

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1.6.2 Phosphorylation of Nucleoside Analogues by Cellular Kinases

Intracellular phosphorylation of nucleosides to their 5'-triphosphates is essential to activate lethal mutagens. This conversion occurs sequentially through monophosphate and diphosphate intermediates. The first phosphorylation step to generate the monophosphate is generally rate-limiting, and enzymes catalyzing this step have the greatest specificity compared to kinases involved in subsequent steps. Steric effects and the hydrogen bonding pattern of substrates are two major recognition elements controlling the specificity of kinases. Nucleoside monophosphate kinase and nucleoside diphosphate kinase convert mono and diphosphorylated nucleosides to phosphorylated products. Both of these enzymes have very broad substrate specificity.

1.6.3 Incorporation of Nucleotides by the Polymerase and Ambiguous Base Templating

Polymerase recognition of unnatural nucleosides and ambiguous base-pairing have been studied extensively in order to develop more effective antiviral therapies. Two primary classes of base-modified nucleosides can increase mutagenesis. The first contains universal nucleobases with minimal hydrogen bonding capacity; these compounds utilize hydrophobic interactions, and can be incorporated opposite any of the four natural nucleotides. The other category includes nucleosides with ambiguous hydrogen bonding properties, either by tautomerization or rotation of a functional group of the base. Some non-hydrogen bonding nucleosides have been studied, and most are deoxyribonucleosides that are stable in duplex DNA. These studies suggest strategies for the development of universal ribonucleosides as lethal mutagens. Nucleosides that rely on
ambiguous hydrogen bonding properties to induce mutagenesis tend to be more structurally similar to natural nucleosides than the more hydrophobic universal nucleosides. Thus, they are more likely to fulfill the required transport, phosphorylation to the triphosphate and viral polymerase recognition steps. However, they are also more likely to hit endogenous cellular targets, leading to undesired effects, such as cytotoxicity.

1.7 Advantages of Lethal Mutagenesis

The emergence of resistance to antiviral drugs remains a problem in the development of effective therapeutics. In the presence of antiviral drugs, viral replication facilitates the selection of drug-resistant variants, rendering the antiviral therapeutic ineffective. Therefore, developing new antiviral strategies that are less susceptible to resistance is highly favored. Because error-prone RNA virus replication is necessary to maintain viral fitness, treating the virus with lethal mutagens is unlikely to induce drug resistance. A possible mechanism of resistance is the selection of a polymerase with higher fidelity, resulting in less incorporation of the mutagen. Recently, two separate research groups identified ribavirin-resistant PV variants with exactly the same mutation,\textsuperscript{59} and the polymerase fidelity of this mutated variant was increased by approximately threefold. Subsequent research into this resistant variant showed that it had restricted tropism and lessened pathogenesis in a mammalian host,\textsuperscript{60} suggesting that resistance by increasing polymerase fidelity disrupted the optimal level of quasispecies diversity. Therefore, even if resistance arises to a lethal mutagen, the resulting virus is more susceptible to the changes in environment, immunology, or other antiviral drugs. Not surprisingly, a combination of a mutagen and traditional viral inhibitors can drive a virus population into extinction, whereas either treatment alone might not be sufficient.\textsuperscript{35a} Interestingly, mutation in the ribavirin resistant PV variant occurs at a location on the enzyme remote from the catalytic site, indicating that the
active site is not the sole mechanism of fidelity control. This mutation may change the overall enzyme conformational dynamics, perturbing the active site to affect fidelity. Thus, targeting the enzyme with small molecules that change the conformational dynamics and fidelity of viral polymerases would possibly lead to antiviral effect. However, further studies are needed to elucidate factors that contribute to these effects. Encouraged by existing lethal mutagens and the relatively low risk of developing drug resistant variants, the development of novel lethal mutagens is an important area of research.

1.8 References

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Chapter 2

Synthesis of a Hydrophobic Mimic of Adenosine that Potently Inhibits Replication of Polio and Dengue Viruses

2.1 Overview

Bioisosteric deaza analogues of 6-methyl-9-β-D-ribofuranosylpurine, a hydrophobic analogue of adenosine, were synthesized and evaluated for antiviral activity as potential lethal mutagens. Hydrophobicity of the nucleobase was modulated in an effort to achieve ambiguous base-pairing properties. The overall shape and size of adenosine was maintained to maximize the probability of the nucleosides being recognized by cellular kinases and viral RdRP (RNA-dependent RNA polymerase). Therefore, three deazapurine ribonucleoside analogues, 1DA6MePN (58) 3DA6MePN (59) and 7DA6MePN (60) were synthesized and evaluated as antiviral agents compared with 6MePN (54) and the natural product tubercidin (55). Whereas the 1-deaza (58) and 3-deaza (59) analogues were essentially inactive in whole cell assays, the novel 7-deaza analogue 60, structurally related to tubercidin (55), potently inhibited replication of poliovirus (PV) in HeLa cells (IC₅₀ = 11 nM) and dengue virus (DENV) in Vero cells (IC₅₀ = 62 nM) by plaque assay. Selectivity against PV over cytotoxic effects to HeLa cells was >100-fold after incubation for 7 h. The corresponding triphosphates of 6MePN (54) and 7DA6MePN (60) were both incorporated rapidly into the primer-template by PV RdRP. This efficient incorporation is likely to be responsible for the observed biological activity.
2.2 Pathogens

In this chapter, we study the efficacy of synthetic compounds against poliovirus (PV) and dengue virus (DENV). Poliovirus, a human enterovirus, is the causative agent of poliomyelitis. Poliovirus contains a positive-strand RNA genome, and is a member of the family *Picornaviridae*. There are three slightly different serotypes of poliovirus (PV 1-3). Since effective vaccines are used, no therapeutic treatment of poliovirus exists. However, the recent emergence of polio cases in the US due to reversion of the vaccine to wild type virus, and the appearance of post-poliomyelitis syndrome, warrant the development of effective antivirals against PV. Dengue virus, a mosquito-borne flavivirus, induces an infectious disease called dengue fever. Dengue fever occurs in tropical and subtropical regions, particularly in Asia, Africa and Latin America, and approximately 40 million people are infected each year. DENV is a positive-strand RNA virus that belongs to the family of *Flaviviridae*, and it includes four closely related but antigenically different viral serotypes (DENV 1-4). There is no vaccine or therapeutic treatment for dengue virus infections.

2.3 Existing Adenosine Mimics as Antiviral Agents

Synthetic mimics of adenosine (53) and adenosine triphosphate (ATP) constitute an important class of therapeutics. Imatinib (Gleevec) is an example of a drug that by engaging the ATP recognition site blocks phosphorylation of substrates by the BCR-ABL protein tyrosine kinase, inhibiting the proliferation of cells that cause chronic mylogenous leukemia. Similarly, antiviral agents such as Adefovir, a sugar-modified analogue of adenosine, are phosphorylated intracellularly and subsequently inhibit actions of viral polymerases. Many adenosine mimics are
substrates of adenosine kinase and other kinases, and are converted to phosphorylated metabolites that exhibit potent biological activities.6

Although sugar-modified mimics of adenosine have been extensively studied,6a, 7 relatively little is known about how structural modifications to the nucleobase of adenosine collectively impact both cellular proliferation and antiviral activity. However, subtle changes to the adenosine nucleobase can confer substantial antiproliferative and antiviral effects. For example, bioisosteric replacement of the exocyclic amino group of 53 with a methyl group yields 6-methyl-9-/β-D-ribofuranosylpurine (54), a potent but non-selective and highly cytotoxic inhibitor of replication of a number of viruses, including herpes simplex virus-1 (HSV-1) and vaccinia virus, but the mechanism of action of 54 is poorly understood.8

Numerous other mimics of adenosine bearing deazapurine modifications are known.9 The natural product tubercidin (55), representing a 7-deazapurine analogue, is a potent antineoplastic10 and antiviral agent.11 Tubercidin is phosphorylated intracellularly to the corresponding nucleoside triphosphate,12 is incorporated into DNA and RNA, and exhibits some polymerase inhibition activity.13 However, this compound is non-selective and too toxic for clinical use as an antiviral agent.11 Interest in the broad-spectrum antiviral activity and cytotoxicity of tubercidin has led to a number of studies of nucleobase and sugar-modified tubercidin analogues.14 Olsen and coworkers identified the tubercidin analogue 7-deaza-2'-C-methyladenosine (56) as an anti-HCV agent.13b This compound inhibits replication of hepatitis C virus (HCV, IC50 = 0.108 µM), without appreciable cytotoxicity. The structurally similar 7-deaza-2'-C-ethynyladenosine (57, NID008) was recently identified as a potent inhibitor of dengue virus type 2 (DENV2, IC50 = 0.64 µM).15 A series of 7-deazapurine ribonucleosides with alkyl, aryl, or heteroaryl groups in the 6-position and H, F, or Cl substitution at the 7-position have also been evaluated in a HCV replicon system.16 However, the decrease in the replicon reporter signal caused by some of these compounds was attributed to cytostatic effects. Other deaza adenosine analogues such as 1-deazapurine and 3-
deazapurine exhibit some antiproliferative activity against certain mammalian cell lines, and 3-deazapurine is known to inhibit HIV replication in peripheral blood mononuclear cells. 

2.4 Design of Deazapurine Ribonucleosides as Lethal Mutagens

Previous studies of tubercidin and other deazapurine adenosine analogues have revealed the importance of heteroatoms in the nucleobase in biologically active compounds. In order to generate lethal mutagens, ambiguous base-pairing properties are desired. To decrease the specificity of recognition by polymerase, we replaced the amino group in the 6-position of adenosine with a hydrophobic methyl group. Additionally, individual nitrogen atoms of the heterocycle were replaced with a similarly sized carbon atom to mimic the shape of adenosine and alter bioactivity. This bioisosterism approach was designed to maximize the probability of the nucleosides being recognized by cellular kinases and viral RdRP (RNA-dependent RNA polymerase). We hypothesize that this modification might also separate the undesirable toxicity.
of 54 from its desirable antiviral activity, because removing a nitrogen atom from the nucleobase might reduce recognition by cellular targets of adenosine. Thus, three deazapurine ribonucleoside analogues, 1DA6MePN (58) 3DA6MePN (59) and 7DA6MePN (60) were synthesized and evaluated as antiviral nucleosides.

![Structures of deazapurine nucleoside analogues](image)

Figure 2-2: Structures of deazapurine nucleoside analogues.

### 2.5 Chemical Synthesis of Deazapurine Nucleoside Analogues

#### 2.5.1 Chemical Synthesis of 1DA6MePN

We employed the fusion glycosylation method to synthesize compound 58 (Figure 2-3). The nucleobase 7-methyl-3H-imidazo[4,5-b]pyridine (66) was prepared in 5 steps from 2-aminopicoline (61). Substitution with bromine was used to block the 5-position of 61, permitting selective nitration at the 3-position. Catalytic hydrogenation afforded diamine 64, which was cyclized with formic acid, and dehalogenated with a palladium / platinum catalyst to yield 66.

Another approach for the preparation of 58 was recently published, but the previously reported method generates a mixture of isomers at the 7- and 9-positions. To avoid this limitation, we used
the fusion coupling method to exclusively generate the 9- substituted isomer of 67. Deprotection of 67 under basic conditions afforded 58.

2.5.2 Chemical Synthesis of 3DA6MePN

To synthesize 59, the readily prepared 4-chloro-1H-imidazo[4,5-c]pyridine (68) was coupled with β-D-ribofuranose 1,2,3,5-tetraacetate in the presence of p-toluenesulfonic acid. Cross coupling of 69 with trimethyl aluminum followed by deacetylation of 70 in methanolic ammonia afforded 3-deaza-6-methyl-9-β-D-ribofuranosylpurine (59, Figure 2-4). Another route to 59 was recently reported in the literature.
2.5.3 Chemical Synthesis of 7DA6MePN

The synthesis of the novel nucleoside 7-deaza-6-methyl-9-β-D-ribofuranosylpurine (60) employed a solid-liquid phase transfer glycosylation method (Figure 2-5). Treatment of commercially available 4-methyl-7-pyrrolo[2,3-d]pyrimidine in toluene with KOH and the phase transfer catalyst TDA-1\textsuperscript{23} allowed alkylation upon dropwise addition of halogenose 72, prepared in situ. Exchange of the acetonide of 73 with acetyl protecting groups allowed efficient cross coupling with trimethyl aluminum and deprotection to yield 60.
To probe whether a free 5'-OH was required for the bioactivity of 7DA6MePN, the 5'-methoxy-analogue was synthesized (Figure 2-6). The 2' and 3' positions in 7DA6MePN (60) were protected using acetone, 2,2-dimethoxypropane and p-toluenesulfonic acid, and 77 was generated in 78% yield. The 5' hydroxyl group was deprotonated with sodium hydride, and methylated with methyl iodide to produce the protected intermediate 78 in high yield. The final deprotection in aqueous trifluoroacetic acid yielded 5'OMe7DA6MePN (79) in 78% yield.
Compounds 54 and 55 are known to be highly cytotoxic or cytostatic to mammalian cells. To compare these compounds with analogues 58-60 and 79, HeLa S3 cells were treated with these compounds for 7 h, 24 h, and 48 h. Cellular viability was evaluated with a luciferase assay that quantifies the abundance of ATP present in living cells (Figure 2-7). These experiments confirmed previous reports that although both 54 and 55 are highly cytotoxic, 54 is ~5-fold less toxic than 55 after treatment of cells for 48 h. In contrast, compounds 58, 59 and 79 showed essentially no cytotoxicity below 10 µM under these conditions. Interestingly, compound 60 showed a unique pattern of cytotoxicity. Unlike compounds 54 and 55, essentially no cytotoxic effects of 60 to HeLa cells were observed after treatment for 7 h. Only after treatment for longer periods of 24 h or 48 h was cytotoxicity of 60 evident, with cytotoxicity of 60 becoming similar to 54 only after 48 h in culture.
Figure 2-7: Cytotoxicity to HeLa S3 cells after treatment with 54, 55, 58-60 and 79 for 7 h (A), 24 h (B) or 48 h (C).
2.7 Antiviral Activity of Adenosine Mimics against Poliovirus

As shown in Figure 2-8, compounds 54, 55, 58-60 and 79 were evaluated against infectious PV in a whole-cell assay. HeLa S3 cells were pretreated with these compounds for 1 h before administration of a high multiplicity of infection (MOI) of PV. After treatment of cells with PV for 15 min, fresh media containing these compounds was added, the infection was allowed to progress for another 6 h, and the cell-associated viral titer was determined as previously reported. Under these conditions, as listed in Section 2.9, compounds 54 and 55 inhibited replication of PV (IC50 (54) = 1.01 µM; IC50 (55) = 0.030 µM). The known compounds 58 and 59 were inactive against PV at concentrations below 10 µM. However, the novel nucleoside 60 proved to be the most potent inhibitor of PV replication in this series of compounds (IC50 = 0.011 µM). Methylation of the 5'-hydroxyl group of 60 to afford 79 eliminated the antiviral effect, suggesting that like other structurally related compounds, intracellular phosphorylation of 60 is required to generate a biologically active metabolite.

Figure 2-8: Antiviral effects of compounds against poliovirus. HeLa S3 cells were incubated with compounds for 1 h at the concentrations shown, and subsequently infected with 10^6 PFU of PV. After infection for 15 min, fresh media containing compounds was added and the infection continued for 6 h. Cell-associated virus was titered by plaque assay. Data obtained by Dr. Hyung Suk Oh.
2.8 Antiviral Activity of Adenosine Mimics against Dengue Virus

The antiviral activities of compounds 54, 58-60 and 79 were further evaluated in a dengue replicon assay (Figure 2-9). In this assay, BHK-21 cells stably expressing a luciferase-driven DENV2 replicon were treated with these compounds for 24 h. The cells were subsequently lysed, and the activity of luciferase was quantified. As listed in Section 2.9, compound 60 was the most potent inhibitor (IC$_{50}$ = 0.88 µM), and compound 55 showed some inhibition of replicon activity (IC$_{50}$ = 5.46 µM), but compounds 58, 59, and 79 showed essentially no inhibition below 10 µM. To probe the effect of the novel nucleoside 60 further, this compound was evaluated in an infectivity assay with live dengue virus. Vero cells infected with DENV2 were treated with 60 for 24 h, and the viral titer was determined by counting viral plaques present after cell lysis$^{25}$ and through quantification of DENV2 RNA by real time PCR.$^{26}$ In these assays, compound 60 was a highly potent inhibitor of DENV2 replication (plaque assay IC$_{50}$ (60) = 62 nM; real time PCR IC$_{50}$ (60) = 39 nM).
Figure 2-9: Panel A: Dengue replicon assay. BHK-21 cells expressing DENV2 replicon were treated with nucleosides for 24 h. After cell lysis, the luciferase signal was measured. Panel B: Infectivity assay of compound 60 with infectious dengue virus. Data obtained by Dr. Ratree Takhampunya.
2.9 Summary of Biological Activities

Table 2-1: Summary of biological evaluation of compounds 54, 55, 58-60 and 79.

<table>
<thead>
<tr>
<th>Compd</th>
<th>HeLa IC_{50} (7 h, µM)</th>
<th>HeLa IC_{50} (24 h, µM)</th>
<th>HeLa IC_{50} (48 h, µM)</th>
<th>PV IC_{50} (µM)</th>
<th>DENV2 IC_{50} (µM)</th>
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<tr>
<td>54</td>
<td>17.66</td>
<td>6.68</td>
<td>2.20</td>
<td>1.01</td>
<td>5.46 (replicon)</td>
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<tr>
<td>55</td>
<td>1.44</td>
<td>0.819</td>
<td>0.400</td>
<td>0.030</td>
<td>ND</td>
</tr>
<tr>
<td>58</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>&gt;10</td>
<td>&gt;10 (replicon)</td>
</tr>
<tr>
<td>59</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>&gt;10 (replicon)</td>
</tr>
<tr>
<td>60</td>
<td>&gt;100</td>
<td>0.986^a</td>
<td>0.224</td>
<td>0.011</td>
<td>0.877 (replicon)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.062 (plaque)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.039 (PCR)</td>
</tr>
<tr>
<td>79</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;10</td>
<td>&gt;10 (replicon)</td>
</tr>
</tbody>
</table>

^aViability of cells was 33% after treatment with 60 at 100 µM. ND: not determined

2.10 Evaluation of Mutagenicity of 6MePN (54) and 7DA6MePN (60) by a Guanidine Resistance Assay

To examine whether the antiviral mechanism of action of 54 or 60 might involve lethal viral mutagenesis, these compounds were compared with ribavirin in a guanidine resistance assay. The assay is based on the fact that replication of PV is blocked by 2 mM guanidine because of inhibition of a key enzyme, 2C protease, but a single mutation will confer resistance of PV to guanidine, generating Gua^r mutants. Ribavirin, a clinically employed antiviral drug, becomes phosphorylated intracellularly, mimics purine ribonucleotides, and becomes misincorporated into the genome of RNA viruses, resulting in loss of function due to error catastrophe. However, compared to ribavirin, neither 54 nor 60 increased the frequency of viral mutagenesis (Table 2-2), indicating that the antiviral activity of these compounds against PV is...
mechanistically distinct from ribavirin. The three compounds were tested at different concentrations that correlate with their antiviral potency.

Table 2-2: Formation of guanidine-resistant plaques (Gua’ plagues) by 54, 60 and ribavirin (a mutagenic control) in a previously reported guanidine resistance assay of viral mutagenesis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ribavirin</th>
<th>Ribavirin</th>
<th>54</th>
<th>54</th>
<th>60</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conc. (µM)</td>
<td>150</td>
<td>300</td>
<td>1.62</td>
<td>3.24</td>
<td>0.046</td>
<td>0.092</td>
</tr>
<tr>
<td>Gua’ plagues</td>
<td>150</td>
<td>&gt;250</td>
<td>12</td>
<td>22</td>
<td>19</td>
<td>17</td>
</tr>
</tbody>
</table>

The number of gua’ plagues is 13 when cell are only treated with guanidine. Data obtained by Dr. Hyung Suk Oh.

2.11 Synthesis of Triphosphates of 6MePN and 7DA6MePN

To probe the mechanism of action, the triphosphates 6MePRTP (83) and 7DA6MePRTP (84) were elaborated from hydrogen phosphonates using a one-pot triphosphate synthesis route previously developed by our research group. Nucleosides 54 and 60 were protected at the 2’ and 3’ positions, and the 5’ hydroxyl group was reacted with excess phosphorous trichloride. Subsequent hydrolysis and deprotection in aqueous trifluoroacetic acid afforded the corresponding hydrogen phosphonates, which were subjected to one-pot triphosphate synthesis conditions to generate triphosphates 83 and 84.
2.12 Incorporation of 6MePRTP (83) and 7DA6MePRTP (84) by PV RdRP

A previously reported primer-extension assay\textsuperscript{29} was used to study the \textit{in vitro} incorporation of 6MePRTP (83) and 7DA6MePRTP (84) into RNA by poliovirus RNA-dependent RNA polymerase (PV RdRP, 3D\textsuperscript{pol}). In this assay, both 83 and 84 became incorporated opposite uracil and cytosine, but were not efficiently incorporated opposite adenine and guanine (Figure 2-11 and Figure 2-12). As expected for compounds structurally related to ATP, the incorporation efficiency of both 83 and 84 across from uracil was comparable to natural ATP on this time scale. Compared with GTP, incorporation opposite cytosine after reaction for 10 min was ~40\% for 83 and ~60\% for 84, illustrating the importance of the exocyclic amino group of ATP for discrimination of the complementary nucleobase in RNA by PV RdRP.
Figure 2-11: Incorporation of 6MePRTP (83) and 7DA6MePRTP (84) into double-stranded RNA by PV RdRP. Complexes of RNA template and PV RdRP were incubated for 3 min, 83, 84 or the natural NTP (0.5 mM) was added respectively, and the reaction was allowed to proceed at 30 °C for 1, 5 or 10 min. Data obtained by Dr. Eric D. Smidansky.

Figure 2-12: Incorporation of 6MePRTP (83) and 7DA6MePRTP (84) into double-stranded RNA by PV 3Dpol. Data obtained by Dr. Eric D. Smidansky.
Kinetic parameters were calculated to quantify the incorporation efficiency of 83 and 84. As shown in Table 2-3, compared with incorporation of ATP into the symmetrical RNA template S/S-U \( (k_{pol} = 86.7 \pm 3.7 \text{ s}^{-1}) \),\(^{24}\) incorporation of 83 \( (k_{pol} = 10.6 \pm 0.3 \text{ s}^{-1}) \) and 84 \( (k_{pol} = 6.3 \pm 0.3 \text{ S}^{-1}) \) was efficient, and these values are more than 40-fold higher than the \( k_{pol} \) value for ribavirin. The apparent dissociation constant \( (K_{d, app}) \) of 83 \( (K_{d, app} = 172 \pm 17 \mu \text{M}) \) and 84 \( (K_{d, app} = 223 \pm 34 \mu \text{M}) \) from the PV RdRP RNA primer/template complex was comparable to ATP \( (K_{d, app} = 133 \pm 18 \mu \text{M}) \).

Table 2-3: Incorporation efficiency against U by PV RdRP.

<table>
<thead>
<tr>
<th>Nucleoside TP</th>
<th>ATP(^{[a]})</th>
<th>Ribavirin TP(^{[a]})</th>
<th>6MePRTP (83)(^{[b]})</th>
<th>7DA6MePRTP (84)(^{[b]})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_{d, app} (\mu \text{M}) )</td>
<td>133±18</td>
<td>310±30</td>
<td>172±17</td>
<td>223±34</td>
</tr>
<tr>
<td>( k_{pol} (\text{S}^{-1}) )</td>
<td>86.7±3.7</td>
<td>0.013±0.001</td>
<td>10.6±0.3</td>
<td>6.3±0.3</td>
</tr>
<tr>
<td>( k_{pol}/K_{d, app} (\text{S}^{-1} \mu \text{M}^{-1}) )</td>
<td>0.67</td>
<td>0.0002</td>
<td>0.06</td>
<td>0.05</td>
</tr>
</tbody>
</table>

\(^{[a]}\) Previously reported values.\(^{24}\) \(^{[b]}\) Data obtained by Dr. Eric D. Smidansky.

2.13 6MePRTP (83) and 7DA6MeRTP (84) do not Terminate the Growing RNA Chain

Although other antiviral nucleosides are known to function by causing termination of the growing RNA chain,\(^{30}\) both 83 and 84 were capable of extending RNA oligomers to the +4 position (Figure 2-13), indicating that neither of these compounds functions as a RNA chain terminators.
2.14 Steady State Incorporation of 6MePRTP (83) and 7DA6MeRTP (84)

To investigate the effects of triphosphates 83 and 84 on PV RdRP-RNA primer/template complexes, these compounds were assessed in steady state incorporation assays (Figure 2-14). The concentration of products of incorporation of 83, 84, ATP, and GTP templated by uracil or cytosine was plotted against time, and the calculated $k_{cat}$ (quotient of slope/y-intercept from linear fits to data points), which is related to the dissociation rate constant of the RdRP from the RNA primer/template, was compared (Figure 2-14). These experiments revealed that both 83 and 84 destabilize PV RdRP-RNA primer/template complexes, as evidenced by increases in $k_{cat}$ compared with natural correct nucleotides (ATP opposite U; GTP opposite C). The slopes of incorporation of both 83 and 84 were constant over time, indicating that neither 83 nor 84 inhibit PV RdRP.
2.15 Crystal Structure of PV RdRP

The complete crystal structure of PV RdRP (3Dpol) was solved by the Peersen group\textsuperscript{31} at 2.0 Å resolution, and it follows the usual right hand structure composing of a thumb, palm, and
fingers domain (Figure 2-15, Panel A). The protein adopts a “closed” conformation where interactions between the fingers and thumb domains encircle the active site and create an NTP binding pocket. The co-crystal structure of 3Dpol-GTP complex was determined (Figure 2-15, Panel B), and hydrogen bonding interaction between the 1-position nitrogen atom on the nucleobase and the protein was verified. However, according to the reported crystal structure, the 3-position and 7-position nitrogen atoms on the nucleobase do not interact with the protein directly. The lack of hydrogen bonding between the 1-position nitrogen atom of 1DA6MePN (58) and PV RdRP will cause ineffective interaction, which may eliminate the biological activity of this nucleoside. Both 3DA6MePN (59) and 7DA6MePN (60) have retained the 1-position nitrogen atom, but only 60 shows effective inhibitory activity against PV.

![Figure 2-15: (A): Crystal structure of poliovirus 3Dpol. (B): Electron density map and model of the GTP molecule bound to 3Dpol.](image)

### 2.16 Discussion

Examined collectively, the kinetics of incorporation of triphosphates 83 and 84 into RNA, the known structure-activity relationships governing phosphorylation of ATP, tubercidin, and related compounds, and the lack of biological activity of control compound 79 suggest that triphosphate 84 represents the active antiviral metabolite of compound 60. This compound (84)
functions as an efficient substrate of PV RdRP, and it likely engenders antiviral effects not by inhibiting the viral polymerase or by chain termination of viral RNA but through a downstream mechanism that may involve RNA-RNA or protein-RNA interactions, or possibly effects on host enzymes essential for viral replication. Once incorporated into RNA, the product of 84 may alternatively function as a defective templating base in duplex synthesis because of the missing 7-deaza hydrogen bond involved in Watson-Crick base pairing. This RNA modification may lead to slow or defective synthesis of complementary RNA, or stalling or dissociation of PV RdRP when an analogue-templated site is encountered, or possibly result in other effects that block successful replication of the viral genome.

Interestingly, the cytotoxicity profiles of compounds 54, 55, 58-60 and 79 differ substantially. The cytotoxicity of tubercidin (55) is thought to derive from suppression of a number of cellular processes including mitochondrial respiration, de novo purine synthesis, processing of rRNA, tRNA methylation and protein and RNA synthesis.¹¹ Whereas 54 and 55 show pronounced effects on proliferation of HeLa cells after treatment for only 7 h, compounds 58-60 and 79 show little cytotoxicity during that time period. Significant cytotoxic effects of 60 are evident only after 24 to 48 h. These results suggest that subtle modifications to the adenine heterocycle show promise for separation of antiviral from cytotoxic effects on host cells.

2.17 Conclusions and Future Directions

A novel 7-deaza derivative of 6-methyl-9-β-D-ribofuranosylpurine (60) was synthesized. This compound (60) exhibits highly potent activity against PV and DENV2 in cell culture assays and possesses an improved cytotoxicity profile compared to 6-methyl-9-β-D-ribofuranosylpurine (54) and tubercidin (55). Studies of the putative triphosphate metabolites of 54 and 60 revealed that these compounds (83, 84) do not inhibit the viral polymerase, do not serve as RNA chain
terminators, and are not lethal mutagens, but rather are efficient substrates for PV RdRP, suggesting antiviral mechanisms of action that involve downstream effects on RNA-RNA interactions, RNA-protein interactions, or perturbation of endogenous targets in host cells.

Some key observations from this study can be applied to the future design of antiviral nucleosides. First, analogues of the natural nucleosides have the potential advantages that they can be recognized by the cellular machinery efficiently, so these compounds have more chances to show biological activities. Second, subtle substitutions on the natural nucleobase can reveal drastic changes in biological activity, and some of these modifications may separate the undesired cytotoxicity from the desired antiviral activity. Third, further separation of the cytotoxicity from the antiviral effect is still needed. Therefore, future study may focus on modification of the nucleobase of pyrimidine nucleosides, and this modification may lead to less cytotoxic compounds than adenosine analogues, because pyrimidine nucleotide will hit less host targets than ATP analogues. Additionally, since modification of adenosine analogues on the 2’ position can generate biologically active compounds (Section 2.3), this modification can be applied in the 7-deaza analogue we studied to potentially reduce the cytotoxicity further.

2.18 Experimental Section

2.18.1 Chemical Synthesis

*General Synthesis Information.* Chemical reagents and solvents were obtained from Acros, Aldrich, TCI or Fisher without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). Reactions were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated
with 0.25 mm silica gel 60 F254 (EMD Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with 20% sulfuric acid in ethanol. Nuclear magnetic resonance (NMR) spectroscopy employed a Bruker DRX 400 spectrometer. NMR peaks are reported as parts per million (ppm) referenced to internal CHCl₃, MeOH or H₂O peaks. Mass spectral data was obtained from The University of Kansas Mass Spectrometry Facility. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Column chromatography employed silica gel (ICN SiliTech, 32-63 µm) or Sephadex LH-20 (GE Healthcare, column dimensions: 2.5 cm × 40 cm). Purification by preparative HPLC employed an Agilent 1100 series instrument equipped with an Atlantis C18 preparative column (19 × 150 mm, 5 µm; Waters Corporation). The HPLC flow rate was maintained at 25 mL/min for the duration of the run. Determination of purity of compounds 54 and 58-60 by analytical HPLC used a PRP-1 (polystyrene-divinylbenzene) reverse-phase column (4.1 × 250 mm, 7 µm; Hamilton) and a gradient of 1% to 99% CH₃CN (containing 0.1% TFA) in nanopure water (containing 0.1% TFA, 0 to 30 min) followed by 99% CH₃CN (containing 0.1% TFA) from 30-40 min at a flow rate of 0.8 mL/min. Analysis of purity of compounds 83 and 84 by HPLC used an Aquasil column (4.6 × 250 mm, 5 µm; Thermoelectron Corporation) at a flow rate of 1.0 mL/min with a gradient of 1% to 50% CH₃CN in aqueous KH₂PO₄ (100 mM) over 30 min followed by 50% CH₃CN in aqueous KH₂PO₄ from 30-40 min.

6-Methyl-9-β-D-ribofuranosylpurine (54)
This compound was prepared as previously reported,\textsuperscript{8b} and the purity was verified by analytical reverse-phase HPLC.

Analytical reverse-phase HPLC profile of 6MePN (54) after recrystallization. Retention time = 9.5 min. Purity = 98% Absorbance wavelength = 254 nm.

5-Bromo-2-amino-4-methylpyridine (62)\textsuperscript{32}

2-Amino-4-methylpyridine (Aldrich, 61, 2.49 g, 23.0 mmol) in CH\textsubscript{2}Cl\textsubscript{2} (30 mL) was cooled to -45 °C with dry ice in a bath of CH\textsubscript{3}CN. 1,3-Dibromo-5,5-dimethylhydantoin (6.58 g, 23.0 mmol) was added and the mixture was stirred at -45 °C for 30 min. Saturated aqueous NaHCO\textsubscript{3} (40 mL) was added, the mixture was allowed to warm to room temperature (22 °C), and CH\textsubscript{2}Cl\textsubscript{2} (100 mL) was added. The organic layer was separated, dried over anhydrous sodium sulfate and evaporated. The resulting residue was recrystallized from EtOAc to yield 62 (3.53 g, 82%) as a pale brown solid. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) \( \delta \) 8.10 (s, 1H), 6.42 (s, 1H), 4.37 (s, 2H), 2.29 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) \( \delta \) 149.2 (\( \times \)3), 110.3 (\( \times \)2), 22.3.
5-Bromo-4-methyl-3-nitropyridin-2-amine (63)\textsuperscript{32}

5-Bromo-2-amino-4-methylpyridine (62, 2.43 g, 13.0 mmol) was dissolved in concentrated H\textsubscript{2}SO\textsubscript{4} (20 mL), and the solution was cooled to -10 °C with a bath of ice and NaCl. HNO\textsubscript{3} (14 mL) was added dropwise, the reaction mixture was stirred for 30 min and allowed to warm to room temperature (22 °C). The mixture was poured into an ice / water mixture (50 mL), and the pH was adjusted to 3 by addition of concentrated aqueous ammonium hydroxide (22%). A light yellow precipitate was collected by filtration, washed with water and dried \textit{in vacuo}. The resulting solid was dissolved in H\textsubscript{2}SO\textsubscript{4} (20 mL), and the mixture was stirred at room temperature (22 °C) for 2 h. The reaction solution was poured onto ice and neutralized with aqueous ammonium hydroxide (22%). After extraction with CH\textsubscript{2}Cl\textsubscript{2} (3×70 mL), the combined organic layers were dried over anhydrous sodium sulfate and evaporated. The crude product was recrystallized from CH\textsubscript{2}Cl\textsubscript{2} to afford 63 (1.93 g, 64%) as yellowish brown solid. \textsuperscript{1}H NMR (400 MHz, CDCl\textsubscript{3}) δ 8.30 (s, 1H), 5.88 (s, 2H), 2.55 (s, 3H); \textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) δ 153.4 (×2), 151.5, 144.1, 111.8, 20.4.

\textsuperscript{5-Bromo-4-methylpyridine-2,3-diamine (64)\textsuperscript{14}}

SnCl\textsubscript{2} (6.23 g, 27.6 mmol) was dissolved in concentrated aqueous hydrochloric acid, and the mixture was cooled to 0 °C. 5-Bromo-4-methyl-3-nitropyridin-2-amine (63, 1.60 g, 6.90
mmol) was added to this solution in four portions, the reaction mixture was warmed to room temperature (22 °C) and placed in an oil bath maintained at 100 °C for 1 h. After cooling, the solution was adjusted to pH = 11 with aqueous NaOH solution (40%). The precipitate was collected by filtration, washed with water, and the crude product was further purified by flash chromatography (eluent: hexane, EtOAc, 1:1) to yield 64 (0.989 g, 66%) as a light brown solid.

$^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.49 (s, 1H), 2.34 (s, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 147.3, 135.4, 127.6, 120.1, 111.8, 15.2.

6-Bromo-7-methyl-3H-imidazo[4,5-b]pyridine (65)$^{34}$

5-Bromo-4-methylpyridine-2,3-diamine (64, 0.695 g, 3.20 mmol) was dissolved in formic acid (5 mL) and heated to reflux for 1 h. The formic acid was removed in vacuo, and the residue was recrystallized from methanol to give 65 (250 mg) as an orange solid. Chromatographic purification of the mother liquor yielded another 220 mg of the product (yield: 69%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.43 (s, 1H), 8.35 (s, 1H), 2.68 (s, 3H); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 145.0 (×6), 17.1.

7-Methyl-3H-imidazo[4,5-b]pyridine (66)$^{35}$

6-Bromo-7-methyl-3H-imidazo[4,5-b]pyridine (65, 403 mg, 1.90 mmol) in aqueous NaOH (1%, 20 mL) containing PtO$_2$ (29.5 mg, 0.130 mmol) and palladium on carbon (10%, 298
mg, 0.280 mmol) was hydrogenated in a Parr apparatus for 30 min at 50 psi. The catalyst was removed by filtration through celite, and the filtrate was neutralized by dropwise addition of aqueous hydrochloric acid (2 M). After extraction with CH₂Cl₂ (3 × 50 mL), the organic layers were combined, dried over anhydrous sodium sulfate and evaporated in vacuo. The crude product was recrystallized from CH₂Cl₂ to afford 66 (148 mg, 59%) as a white solid. ¹H NMR (400 MHz, CD₃OD) δ 8.34 (s, 1H), 8.22 (d, 1H, J = 5.0 Hz), 7.12 (d, 1H, J = 5.0 Hz), 2.61 (s, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 143.7 (×2), 142.8, 119.2 (×3), 15.1.

7-Methyl-3-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine
(1-deaza-6-methyl-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purine, 67)

A mixture of 7-methyl-3H-imidazo[4,5-b]pyridine (66, 70.2 mg, 0.520 mmol), β-D-ribofuranose 1,2,3,5-tetraacetate (Aldrich, 318 mg, 1.00 mmol), and p-toluenesulfonic acid (19.0 mg, 0.10 mmol) was heated to 170 °C under reduced pressure (12-15 mm Hg) with stirring for 20 min. The resulting solid was neutralized with saturated aqueous NaHCO₃ and extracted with CH₂Cl₂ (4×40 mL). The combined organic layers were dried over anhydrous sodium sulfate, and removed in vacuo to afford a residue that was purified by flash column chromatography (eluent: hexane, EtOAc, 4:1) to yield 67 (101 mg, 49%) as a white foam. mp 53-55 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.27 (d, 1H, J = 4.9 Hz), 8.17 (s, 1H), 7.09 (d, 1H, J = 4.9 Hz), 6.30 (d, 1H, J₁',₂' = 5.3 Hz), 6.04 (dd, 1H, J₂',₁' = 5.3 Hz, J₂',₂' = 5.4 Hz), 5.74 (dd, 1H, J₃',₂' = 5.4 Hz, J₃',₄' = 2.8 Hz), 4.49-4.37 (m, 3H), 2.68 (s, 3H), 2.15 (s, 3H), 2.13 (s, 3H), 2.08 (s, 3H); ¹³C NMR (100
MHz, CDCl₃) δ 170.4, 169.6, 169.4, 145.9, 144.6, 141.2, 140.1, 135.8, 120.1, 86.3, 80.0, 73.0, 70.8, 63.2, 20.8, 20.6, 20.4, 16.2; IR (film) ν max 3412, 2912, 2846, 1748, 1608, 1498, 1432, 1372, 1228, 1092, 1045 cm⁻¹; HRMS (ESI+) m/z 392.1444 (M⁺, C₁₈H₂₂N₃O₇ requires 392.1458).

4-Chloro-1-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine
(3-deaza-6-chloro-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purine, 69)

A mixture of 4-chloro-1H-imidazo[4,5-c]pyridine (68, prepared by a previously published route,²¹ 100 mg, 0.650 mmol), β-D-ribofuranose 1,2,3,5-tetraacetate (Aldrich, 413 mg, 1.30 mmol), and p-toluenesulfonic acid (24.7 mg, 0.130 mmol) was fused at 170 °C under reduced pressure (12-15 mm Hg) with stirring for 20 min. The resulting melt was neutralized with saturated aqueous NaHCO₃ solution and extracted three times with CH₂Cl₂ (3 × 60 mL). The organic layers were combined, dried over anhydrous sodium sulfate, and solvent was removed in vacuo to provide a residue that was purified by flash chromatography (eluent: hexane, EtOAc, 1:2) to yield 69 (104 mg, 39%) as a white foam.¹ H NMR (400 MHz, CDCl₃) δ 8.29 (s, 1H), 8.27 (d, 1H, J = 5.6 Hz), 7.56 (d, 1H, J = 5.6 Hz), 6.10 (d, 1H, J₁',₂' = 5.3 Hz), 5.53 (t, 1H, J₂',₁' = J₂',₃' = 5.3 Hz), 5.41 (dd, 1H, J₃',₂' = 5.3 Hz, J₃',₄' = 5.0 Hz), 4.54 (ddd, 1H, J₄',₃' = 5.0 Hz, J₄',₅' = 2.9, 2.6 Hz), 4.49 (dd, 1H, J₆',₅' = 12.6 Hz, J₆',₄' = 2.9 Hz), 4.42 (dd, 1H, J₇',₆' = 12.6 Hz, J₇',₅' = 2.6 Hz), 2.20 (s, 3H), 2.18 (s, 3H), 2.13 (s, 3H);¹³ C NMR (100 MHz, CDCl₃) δ 170.1, 169.5, 169.3, 143.5, 142.2, 141.8, 138.7, 138.4, 106.0, 87.5, 80.6, 73.6, 69.9, 62.5, 20.8, 20.5, 20.4.
4-Methyl-1-(2’,3’,5’-tri-O-acetyl-β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine
(3-deaza-6-methyl-9-(2’,3’,5’-tri-O-acetyl-β-D-ribofuranosyl)purine, 70).

A mixture of 4-chloro-1-(2’,3’,5’-tri-O-acetyl-β-D-ribofuranosyl)-1H-imidazo[4,5-c]pyridine (69, 78.2 mg, 0.190 mmol), Pd(PPh₃)₄ (43.9 mg, 0.0380 mmol) and trimethyl aluminum (2 M solution in hexanes, 0.190 mL, 0.380 mmol) in THF (5 mL) was heated to reflux for 7 h. Saturated aqueous NH₄Cl was added slowly to quench the reaction, and the volatile fraction was removed in vacuo. After extraction with EtOAc (3 × 40 mL), the organic layers were combined, washed with saturated aqueous sodium chloride, dried over anhydrous sodium sulfate, and evaporated to a colorless oil, which was purified by flash chromatography (eluent: CH₂Cl₂, MeOH, 30:1) to give 70 (45.4 mg, 61%) as a white foam. mp 51-53 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.37 (d, 1H, J = 5.7 Hz), 8.17 (s, 1H), 7.40 (d, 1H, J = 5.7 Hz), 6.09 (d, 1H, J₁’,₂’ = 5.6 Hz), 5.57 (t, 1H, J₂’,₁’ = J₂’,₃’ = 5.6 Hz), 5.44 (dd, 1H, J₃’,₂’ = 5.6 Hz, J₃’,₄’ = 4.4 Hz), 4.51 (ddd, 1H, J₄’,₃’ = 4.4 Hz, J₄’,₅’ = 2.9, 2.8 Hz), 4.47 (dd, 1H, J₅’ₐ,₄’ = 2.9 Hz), 4.42 (dd, 1H, J₅’ₜ,₄’ = 2.8 Hz), 2.91 (s, 3H), 2.20 (s, 3H), 2.18 (s, 3H), 2.11 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 170.2, 169.6, 169.3, 152.8, 142.3, 140.4, 139.9, 137.0, 104.1, 87.1, 80.5, 73.3, 70.2, 62.8, 20.8, 20.6, 20.4, 19.9; IR (film) ν max 3390, 2939, 1748, 1603, 1438, 1374, 1223, 1048 cm⁻¹; HRMS (ESI⁺) m/z 392.1461 (M⁺, C₁₈H₂₂N₃O₇ requires 392.1458).
4-Chloro-7-(5'-O-tert-butyldimethylsilyl-2',3'-O-isopropylidene-β-D-ribofuranosyl) Pyrrolo[2,3-d]pyrimidine(7-deaza-6-chloro-9-(5'-O-tert-butyldimethylsilyl-2',3'-O-isopropylidene-β-D-ribofuranosyl)purine, 73)

A solution of 5'-O-tert-butyldimethylsilyl-2',3'-O-isopropylidene-D-ribofuranose\(^{36}\) (71, 2.74 g, 9.00 mmol) and CCl\(_4\) (1.15 mL, 11.8 mmol) in toluene was cooled to -10 °C and treated dropwise with hexamethylphosphorous triamide (1.80 mL, 9.90 mmol). After stirring at -10 °C for 30 min, the reaction mixture was transferred to a separatory funnel and washed quickly with ice cold saturated aqueous sodium chloride solution. The organic layer was dried over anhydrous magnesium sulfate and the crude product 72 (5'-O-tert-butyldimethylsilyl-2',3'-O-isopropylidene-α-D-ribofuranosyl chloride) was used directly in the glycosylation step. To a mixture of 4-chloropyrrolo[2,3-d]pyrimidine (1.07 g, 7.00 mmol), KOH (0.511 g, 9.10 mmol), and TDA-1 (2.40 mL, 7.50 mmol) in toluene was added 72. The reaction mixture was stirred for 20 h at room temperature (22 °C), washed with saturated aqueous sodium chloride solution. The organic layer was dried over anhydrous sodium sulfate and evaporated to a colorless oil, which was purified by flash chromatography (eluent: hexane, EtOAc, 20:1) to give 73 (1.12 g, 35%) as a white foam. \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.68 (s, 1H), 7.59 (d, 1H, \(J = 3.7\) Hz), 6.64 (d, 1H, \(J = 3.7\) Hz), 6.42 (d, 1H, \(J_{1',2'} = 3.0\) Hz), 5.08 (dd, 1H, \(J_{2',1'} = 3.0\) Hz, \(J_{2',3'} = 6.2\) Hz), 4.97 (dd, 1H, \(J_{3',2'} = 6.2\) Hz,
$J_{3',4'} = 3.0$ Hz), 4.37 (ddd, 1H, $J_{4',3'} = 3.0$ Hz, $J_{4',5'} = 3.4$, 3.6 Hz), 3.91 (dd, 1H, $J_{gem} = 11.2$ Hz, $J_{5,a,4'} = 3.4$ Hz), 3.81 (dd, 1H, $J_{gem} = 11.2$ Hz, $J_{5,b,4'} = 3.6$ Hz), 1.66 (s, 3H), 1.40 (s, 3H), 0.92 (s, 9H), 0.08 (s, 3H), 0.07 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 152.2, 150.9, 127.1, 118.3, 114.3, 100.4, 90.6, 86.2, 85.1, 80.9, 63.4, 27.4 ($\times 2$), 25.9 ($\times 3$), 25.5, 18.4, -5.4, -5.5.

![Image of 4-Chloro-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidine](image)

**4-Chloro-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidine**

(7-deaza-6-chloro-9-β-D-ribofuranosylpurine, 74).

Compound 73 (1.12 g, 0.240 mol) was dissolved in aqueous TFA (30 mL, 70%), and the solution was stirred at room temperature (22 °C) for 20 min. The solvent was removed **in vacuo** and the residue was purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 9:1) to yield a white solid (74, 650 mg, 88%). $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.58 (s, 1H), 7.86 (d, 1H, $J = 3.8$ Hz), 6.71 (d, 1H, $J = 3.8$ Hz), 6.26 (d, 1H, $J_{1',2'} = 6.0$ Hz), 4.56 (dd, 1H, $J_{2',1'} = 6.0$ Hz, $J_{2',1'} = 5.6$ Hz), 4.30 (dd, 1H, $J_{3',2'} = 5.6$ Hz, $J_{3',4'} = 3.6$ Hz), 4.10 (ddd, 1H, $J_{4',3'} = 3.6$ Hz, $J_{4',5'} = 3.1$, 3.5 Hz), 3.84 (ddd, 1H, $J_{gem} = 12.2$ Hz, $J_{5,a,4'} = 3.1$ Hz), 3.75 (dd, 2H, $J_{gem} = 12.2$ Hz, $J_{5,b,4'} = 3.5$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 151.5, 151.2, 150.0, 128.6, 118.3, 99.7, 88.9, 85.6, 74.6, 70.9, 61.8.

![Image of structure](image)
6-Chloro-7-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine
(7-deaza-6-chloro-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purine, 75).

A solution of 74 (300 mg, 1.00 mmol) in dry pyridine (5 mL) was cooled to 4 °C, and Ac₂O (0.501 mL, 5.30 mmol) was added dropwise. The mixture was allowed to warm to room temperature (22 °C) and continuously stirred for 4 h. The reaction solution was cooled to 4 °C, and H₂O (10 mL) was added to quench the reaction. The reaction was extracted with CH₂Cl₂ (4 × 40 mL), and the combined organic layers were washed with H₂O (2 × 30 mL), dried over anhydrous sodium sulfate, evaporated to dryness, and the product was purified by flash chromatography (eluent: hexane, EtOAc, 3:1) to yield a white foam (75, 344 mg, 84%). ¹H NMR (400 MHz, CDCl₃) δ 8.67 (s, 1H), 7.43 (d, 1H, J = 3.8 Hz), 6.70 (d, 1H, J = 3.8 Hz), 6.46 (d, 1H, J₁,₂ = 6.0 Hz), 5.76 (dd, 1H, J₂,₁ = 6.0 Hz, J₂,₃ = 5.8 Hz), 5.57 (dd, 1H, J₃,₂ = 5.8 Hz, J₃,₄ = 4.4 Hz), 4.44-4.34 (m, 3H), 2.16 (s, 3H), 2.15 (s, 3H), 2.05 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 170.3, 169.7, 169.4, 152.6, 151.5, 151.2, 126.2, 118.5, 101.5, 85.9, 79.9, 73.1, 70.7, 63.3, 20.8, 20.6, 20.4.

4-Methyl-7-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine
(7-deaza-6-methyl-9-(2',3',5'-tri-O-acetyl-β-D-ribofuranosyl)purine, 76).

A mixture of 75 (285 mg, 0.690 mmol), Pd(PPh₃)₄ (160 mg, 0.140 mmol) and trimethyl aluminum (2 M solution in hexanes, 0.690 mL, 1.380 mmol) in THF (10 mL) was heated to reflux for 7 h. Saturated aqueous NH₄Cl was added slowly to quench the reaction, and the volatile
fraction was removed in vacuo. After extraction with EtOAc (3 × 50 mL), the organic layers were combined, washed with saturated aqueous sodium chloride solution, dried over anhydrous sodium sulfate and evaporated to a colorless oil, which was purified by flash chromatography (eluent: hexane, EtOAc, 1:3) to give 76 (165 mg, 61%) as a white foam. mp 48-49 °C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) δ 8.80 (s, 1H), 8.34 (d, 1H, \(J = 3.8\) Hz), 6.66 (d, 1H, \(J = 3.8\) Hz), 6.52 (d, 1H, \(J_{1',2'} = 6.0\) Hz), 5.79 (dd, 1H, \(J_{2',1'} = 6.0\) Hz, \(J_{2',3'} = 5.8\) Hz), 5.60 (dd, 1H, \(J_{3',2'} = 5.8\) Hz, \(J_{3',4'} = 3.9\) Hz), 4.44-4.36 (m, 3H), 2.75 (s, 3H), 2.18 (s, 3H), 2.17 (s, 3H), 2.06 (s, 3H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) δ 170.4, 169.8, 169.5, 159.9, 151.7, 150.9, 124.5, 118.7, 101.5, 85.3, 79.6, 73.0, 70.8, 63.4, 21.6, 20.8, 20.6, 20.4; IR (film) ν max 3434, 3132, 2928, 2846, 1748, 1684, 1586, 1514, 1429, 1374, 1229, 1095, 1042 cm\(^{-1}\); HRMS (ESI+) \(m/z\) 392.1443 (M\(^+\), \(C_{18}H_{22}N_{3}O_{7}\) requires 392.1458).

General procedure for deprotection of compounds 67, 70 and 76 (0.220 mmol) to yield 58, 59, and 60. These compounds were treated with ammonia in methanol (7 N, 5 mL), and stirred at room temperature (22 °C) for 4 h. The solvent was removed in vacuo and the residue was purified by flash column chromatography (eluent: CH\(_2\)Cl\(_2\), MeOH, 9:1). The resulting solid was further purified by recrystallization from methanol.

7-Methyl-3-\(\beta\)-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine
(1-deaza-6-methyl-9-\(\beta\)-D-ribofuranosylpurine, 58).
After deprotection of 67, compound 58 was obtained as a white solid (52.5 mg, 90%). mp 198-200 °C; 1H NMR (400 MHz, CD3OD) δ 8.55 (s, 1H), 8.22 (d, 1H, J = 5.0 Hz), 7.21 (d, 1H, J = 5.0 Hz), 6.06 (d, 1H, J1', 2' = 6.4 Hz), 4.82 (dd, 1H, J2', 1' = 6.4 Hz, J2', 3' = 5.2 Hz), 4.34 (dd, 1H, J3', 2' = 5.2 Hz, J3', 4' = 2.6 Hz), 4.19 (ddd, 1H, J4', 3' = 2.6 Hz, J4', 5' = 2.4, 2.6 Hz), 3.89 (dd, 1H, Jgem = 12.5 Hz, J5a, 4 = 2.4 Hz), 3.76 (dd, 1H, Jgem = 12.5 Hz, J5b, 4 = 2.6 Hz), 2.66 (s, 3H); 13C NMR (100 MHz, CD3OD) δ 145.1, 143.5, 143.4, 140.4, 135.6, 119.7, 90.0, 73.7, 71.3, 62.1, 20.6, 14.9; IR (film) ν max 3271, 2912, 2852, 1610, 1501, 1206, 1081 cm⁻¹; HRMS (ESI⁺) m/z 266.1138 (M⁺, C12H16N3O4 requires 266.1141).

Analytical reverse-phase HPLC profile of 1DA6MePN (58) after recrystallization. Retention time = 10.6 min. Purity = 98%. Absorbance wavelength = 254 nm.

4-Methyl-1-β-D-ribofuranosyl-1H-imidazo[4,5-c]pyridine
(3-deaza-6-methyl-9-β-D-ribofuranosylpurine, 59).
After deprotection of 70, compound 59 was obtained as a white solid (47.8 mg, 82%). mp 232-234 °C; \( ^1 \text{H NMR (400 MHz, CD}_3\text{OD)} \delta \) 8.62 (s, 1H), 8.21 (d, 1H, \( J = 5.8 \text{ Hz) }, 7.69 (d, 1H, \( J = 5.8 \text{ Hz) }, 5.97 (d, 1H, J_{1', 2'} = 6.0 \text{ Hz) }, 4.44 (dd, 1H, J_{2', 1'} = 6.0 \text{ Hz, J}_{2', 3'} = 5.2 \text{ Hz) }, 4.28 (dd, 1H, \( J_{3', 2'} = 5.2 \text{ Hz, J}_{3', 4'} = 3.4 \text{ Hz) }, 4.15 (ddd, 1H, J_{4', 3'} = 3.4 \text{ Hz, J}_{4', 5'} = 2.9, 3.2 \text{ Hz) }, 3.87 (dd, 1H, \( J_{5', 4'} = 2.9 \text{ Hz) }, 3.79 (dd, 1H, J_{\text{gem}} = 12.2 \text{ Hz, J}_{5', a, 4'} = 3.2 \text{ Hz) }, 2.80 (s, 3H); \( ^{13} \text{C NMR (100 MHz, CD}_3\text{OD) \delta } 150.8, 142.9, 140.6, 139.0, 137.8, 105.4, 89.7, 86.1, 74.9, 70.4, 61.2, 17.8; \text{ IR (film)} \nu \text{ max } 3308, 2918, 2846, 1605, 1495, 1470, 1451, 1311, 1218, 1081, 1056 \text{ cm}^{-1}; \text{ HRMS (ESI+) } m/z 266.1134 (M^+, C_{12}H_{16}N_3O_4 \text{ requires } 266.1141).


4-Methyl-7-\( \beta \)-D-ribofuranosylpyrrolo[2,3-d]pyrimidine

(7-deaza-6-methyl-9-\( \beta \)-D-ribofuranosylpurine, 60).

After deprotection of 76, compound 60 was obtained as a white solid (52.7 mg, 90%). mp 175-176 °C; \( ^1 \text{H NMR (400 MHz, CD}_3\text{OD) \delta } 8.61 (s, 1H), 7.70 (d, 1H, J = 3.8 \text{ Hz) }, 6.74 (d, 1H, \( J = 3.8 \text{ Hz) }, 5.72 (d, 1H, J_{5', 4'} = 2.9 \text{ Hz) }, 4.39 (dd, 1H, J_{4', 3'} = 3.4 \text{ Hz, J}_{4', 5'} = 2.7, 3.0 \text{ Hz) }, 3.87 (dd, 1H, J_{5', a, 4'} = 3.2 \text{ Hz) }, 2.76 (s, 3H); \( ^{13} \text{C NMR (100 MHz, CD}_3\text{OD) \delta } 150.8, 142.9, 140.6, 139.0, 137.8, 105.4, 89.7, 86.1, 74.9, 70.4, 61.2, 17.8; \text{ IR (film)} \nu \text{ max } 3308, 2918, 2846, 1605, 1495, 1470, 1451, 1311, 1218, 1081, 1056 \text{ cm}^{-1}; \text{ HRMS (ESI+) } m/z 249.0989 (M^+, C_{11}H_{15}N_3O_4 \text{ requires } 249.0992).
= 3.8 Hz), 6.20 (d, 1H, \( J_{1',2'} = 6.2 \) Hz), 4.61 (dd, 1H, \( J_{2',1'} = 6.2 \) Hz, \( J_{2',3'} = 5.5 \) Hz), 4.29 (dd, 1H, \( J_{3',2'} = 5.5 \) Hz, \( J_{3',4'} = 3.3 \) Hz), 4.10 (ddd, 1H, \( J_{2',3'} = 3.3 \) Hz, \( J_{2',5'} = 3.0 \) Hz, \( J_{3',4'} = 3.3 \) Hz), 3.84 (dd, 1H, \( J_{gem} = 12.2 \) Hz, \( J_{5'a,4'} = 3.0 \) Hz), 3.74 (dd, 1H, \( J_{gem} = 12.2 \) Hz, \( J_{5'b,4'} = 3.3 \) Hz), 2.72 (s, 3H); \( _{13} \text{C NMR} \) (100 MHz, DMSO-\( d_6 \)) \( \delta \) 175.0, 159.4, 149.8, 127.3, 119.0, 100.0, 89.0, 85.6, 74.3, 71.0, 62.0, 20.7; IR (film) \( \nu \) max 3293, 2923, 1594, 1569, 1514, 1432, 1361, 1232, 1125, 1084, 1042 cm\(^{-1}\); HRMS (ESI+) \( m/z \) 266.1133 (M\(^+\), C\(_{12}\)H\(_{16}\)N\(_3\)O\(_4\) requires 266.1141).

Analytical reverse-phase HPLC profile of 7DA6MePN (60) after recrystallization. Retention time = 9.6 min. Purity = 98%. Absorbance wavelength = 254 nm.

4-Methyl-7-(2',3'-O-isopropylidene-\( \beta \)-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine

(7-deaza-6-methyl-9-(2',3'-O-isopropylidene-\( \beta \)-D-ribofuranosyl)purine, 77).

Compound 60 (450 mg, 1.70 mmol) was dissolved in acetone (5 mL). 1,2-Dimethoxy propanol (5 mL) and \( p \)-toluenesulfonic acid (442 mg, 4.20 mmol) were added. The mixture was stirred at room temperature (22°C) for 20 min. MeOH (20 mL) was added to dilute the solution,
and Amberlite IRA-400 (OH) resin (from Supelco) was used to modify the pH value to 7. The resin was removed by filtration and washed with MeOH (30 mL), and the filtrate was evaporated in vacuo. The product was purified by flash chromatography (eluent: \(\text{CH}_2\text{Cl}_2\), MeOH, 25:1) to afford 404 mg 77 as a white foam (78%). mp 67-68 °C; \(^1\)H NMR (400 MHz, CD\(_3\)OD) \(\delta\) 8.64 (s, 1H), 7.69 (d, 1H, \(J = 3.8\) Hz), 6.74 (d, 1H, \(J = 3.8\) Hz), 6.34 (d, 1H, \(J_{1', 2'} = 3.6\) Hz), 5.21 (dd, 1H, \(J_{2', 1'} = 3.6\) Hz, \(J_{2', 3'} = 6.3\) Hz), 5.03 (dd, 1H, \(J_{3', 2'} = 6.3\) Hz, \(J_{3', 4'} = 2.9\) Hz), 4.30 (dd, 1H, \(J_{4', 3'} = 2.9\) Hz, \(J_{4', 5'} = 3.9\) Hz), 2.72 (s, 3H), 1.63 (s, 3H), 1.39 (s, 3H); \(^13\)C NMR (100 MHz, CD\(_3\)OD) \(\delta\) 159.4, 150.1, 149.9, 127.3, 118.7, 114.0, 100.2, 90.6, 85.8, 84.0, 81.3, 62.0, 26.2, 24.2, 19.5; IR (film) \(\nu\max\) 3275, 2984, 2928, 1676, 1588, 1566, 1427, 1374, 1210, 1073 cm\(^{-1}\); HRMS (ESI+) \(m/z\) 306.1443 (\(M^+\), \(\text{C}_{15}\text{H}_{20}\text{N}_3\text{O}_4\)) requires 306.1454.

4-Methyl-7-(2',3'-O-isopropylidene-5'-O-methyl-\(\beta\)-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (7-deaza-6-methyl-9-(2',3'-O-isopropylidene-5'-O-methyl-\(\beta\)-D-ribofuranosyl)purine, 78)

4-Methyl-7-(2',3'-O-isopropylidene-\(\beta\)-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (77, 44.0 mg, 0.144 mmol) was dissolved in CH\(_3\)CN (1 mL), and cooled to 4 °C. NaH (11.6 mg, 0.288 mmol) was added, and the resulting suspension was stirred at 4 °C for 0.5 h before adding MeI (0.180 mL, 0.288 mmol). The mixture was stirred at room temperature (22 °C) for 2 h. MeOH (3 mL) was added to quench excess NaH. After removal of solvent in vacuo, the crude product was purified by flash chromatography (eluent: hexane, EtOAc, 1:1) to afford 40.0 mg of a colorless
oil (86%). $^1$H NMR (400 MHz, CDCl$_3$) δ 8.78 (s, 1H), 7.38 (d, 1H, $J = 3.7$ Hz), 6.58 (d, 1H, $J = 3.7$ Hz), 6.38 (d, 1H, $J_{1',2'} = 2.9$ Hz), 5.21 (dd, 1H, $J_{2',1'} = 2.9$ Hz, $J_{2',3'} = 6.4$ Hz), 5.00 (dd, 1H, $J_{3',2'} = 6.4$ Hz, $J_{3',4'} = 3.4$ Hz), 4.39 (ddd, 1H, $J_{4',3'} = 3.4$ Hz, $J_{4',5'} = 3.9, 4.8$ Hz), 3.63 (dd, 1H, $J_{gem} = 10.3$ Hz, $J_{5',4'} = 3.9$ Hz), 3.59 (dd, 1H, $J_{gem} = 10.3$ Hz, $J_{5',3'} = 4.8$ Hz), 3.39 (s, 3H), 2.73 (s, 3H), 1.66 (s, 3H), 1.39 (s, 3H); $^{13}$C NMR (100 MHz, CDCl$_3$) δ 159.5, 151.5, 150.3, 125.9, 118.6, 114.4, 100.4, 94.0, 84.7, 84.5, 81.5, 72.9, 59.3, 27.3, 25.4, 21.5; IR (film) ν max 2984, 2928, 1586, 1512, 1460, 1374, 1347, 1215, 1092 cm$^{-1}$; HRMS (ESI+) m/z 320.1595 (M$^+$, C$_{16}$H$_{22}$N$_3$O$_4$ requires 320.1610).

![Chemical structure](image)

4-Methyl-7-(5′-O-methyl-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine

(7-deaza-6-methyl-9-(5′-O-methyl-β-D-ribofuranosyl)purine, 79).

Compound 78 (40.0 mg, 0.125 mmol) was treated with aqueous TFA (70%, 1 mL), and stirred at room temperature (22 °C) for 15 min. The solvent was removed in vacuo, and the residue was purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 20:1) to yield 30.4 mg of a white foam (87%). mp 63-65 °C; $^1$H NMR (400 MHz, CD$_2$OD) δ 8.89 (s, 1H), 8.01 (d, 1H, $J = 3.9$ Hz), 7.06 (d, 1H, $J = 3.9$ Hz), 6.42 (d, 1H, $J_{1',2'} = 5.6$ Hz), 4.48 (dd, 1H, $J_{2',1'} = 5.6$ Hz, $J_{2',3'} = 5.3$ Hz), 4.29 (dd, 1H, $J_{3',2'} = 5.3$ Hz, $J_{3',4'} = 3.6$ Hz), 4.19 (ddd, 1H, $J_{4',3'} = 3.6$ Hz, $J_{4',5'} = 3.0, 3.4$ Hz), 3.71 (dd, 1H, $J_{gem} = 10.8$ Hz, $J_{5',4'} = 3.0$ Hz), 3.63 (dd, 1H, $J_{gem} = 10.8$ Hz, $J_{5',3'} = 3.4$ Hz), 3.45 (s, 3H), 2.91 (s, 3H); $^{13}$C NMR (100 MHz, CD$_2$OD) δ 155.3, 150.5, 145.3, 129.5, 118.1, 102.3, 88.0, 84.3, 75.3, 72.2, 71.2, 58.2, 16.8; IR (film) ν max 3354, 3110, 2923, 2890, 2066,
1682, 1643, 1602, 1515, 1463, 1201, 1131 cm$^{-1}$; HRMS (ESI$^+$) $m/z$ 280.1285 (M$^+$, $\text{C}_{13}\text{H}_{18}\text{N}_3\text{O}_4$ requires 280.1297).


General procedure for synthesis of the hydrogen phosphonates 81 and 82 is listed below. To a solution of phosphorus trichloride (0.500 mL, 5.73 mmol) in anhydrous CH$_2$Cl$_2$ at -20 °C, ketal protected nucleosides 77 or 80 (0.570 mmol) was added and the solution was stirred for 1 h at -20 °C. The reaction was warmed to room temperature (22 °C) and stirred for 4 h. The solvent was evaporated in vacuo and the residue was treated with aqueous TFA (2 mL, 30%) for 20 min. The reaction mixture was concentrated in vacuo, the crude product was dissolved in aqueous triethylammonium bicarbonate (TEAB) buffer (1 mL, 10 mM, pH = 8), and the product was purified by Sephadex LH-20 column (2.5 cm × 40 cm). Elution with 10 mM aqueous TEAB buffer and lyophilization yielded the hydrogen phosphonates as the triethylammonium salt.
6-Methyl-9-β-D-ribofuranosylpurine 5'-H-phosphonate (81).

The triethylammonium salt form of this compound (159 mg, 71%) was synthesized from 6-methyl-9-(2',3'-O-isopropylidene-β-D-ribofuranosyl)purine (80, 174 mg, 0.570 mmol). $^1$H NMR (300 MHz, D$_2$O) $\delta$ 8.96 (s, 1H), 8.83 (s, 1H), 6.59 (d, 1H, $J_{P,H} = 640$ Hz), 6.17 (d, 1H, $J_{1',2'} = 4.8$ Hz), 4.56 (dd, 1H, $J_{2',1'} = 4.8$ Hz, $J_{2',3'} = 4.4$ Hz), 4.40 (dd, 1H, $J_{3',2'} = 4.4$ Hz, $J_{3',4'} = 4.5$ Hz), 4.22 (m, 1H), 4.02 (m, 2H), 3.05 (q, 6H, $J = 7.4$ Hz, N(CH$_2$CH$_3$)$_3$), 2.87 (s, 3H), 1.13 (t, 9H, $J = 7.4$ Hz, N(CH$_2$CH$_3$)$_3$); $^{13}$C NMR (75 MHz, D$_2$O) $\delta$ 156.0, 152.2, 148.0, 147.6, 132.6, 88.9, 84.4 (d, $J_{c,p} = 31$ Hz), 74.7, 70.5, 63.0 (d, $J_{c,p} = 4$ Hz), 47.0, 16.1, 8.6; $^{31}$P NMR (145.8 MHz, D$_2$O), 7.5 (s); HRMS (ESI-) m/z 329.0639 (M$, \text{C}_{11}\text{H}_{14}\text{N}_4\text{O}_6\text{P}$ requires 329.0651).

4-Methyl-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidine 5'-H-phosphonate (7-deaza-6-methyl-9-β-D-ribofuranosylpurine 5'-H-phosphonate, 82)

4-Methyl-7-(2',3'-O-isopropylidene-β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (77, 174 mg, 0.570 mmol) was used as the starting material to yield 82 as a colorless glassy solid (139 mg, 62%). $^1$H NMR (400 MHz, D$_2$O) $\delta$ 8.37 (s, 1H), 7.55 (d, 1H, $J = 3.8$ Hz), 6.62 (d, 1H, $J = 3.8$ Hz), 6.61 (d, 1H, $J_{P,H} = 638$ Hz), 6.19 (d, 1H, $J_{1',2'} = 6.4$ Hz), 4.57 (dd, 1H, $J_{2',1'} = 6.4$ Hz, $J_{2',3'} = 6.4$ Hz).
= 5.6 Hz), 4.36 (dd, 1H, \( J_{3', 2'} = 5.6 \text{ Hz}, J_{3', 4'} = 3.2 \text{ Hz} \)), 4.22 (ddd, 1H, \( J_{4', 3'} = 3.2 \text{ Hz}, J_{4', 5'} = 1.5, 1.2 \text{ Hz} \)), 3.98 (dd, 1H, \( J_{\text{gem}} = 2.8 \text{ Hz}, J_{5', 4'} = 1.5 \text{ Hz} \)), 3.97 (dd, 1H, \( J_{\text{gem}} = 2.8 \text{ Hz}, J_{5'h, 4'} = 1.2 \text{ Hz} \)), 3.05 (q, 6H, \( J = 7.4 \text{ Hz}, N(CH_2CH_3)_3 \)), 2.46 (s, 3H), 1.13 (t, 9H, \( J = 7.4 \text{ Hz}, N(CH_2CH_3)_3 \))\); \(^{13}\)C NMR (100 MHz, D\(_2\)O) \( \delta \) 154.7, 150.3, 143.7, 130.3, 118.4, 104.1, 87.1, 84.0 (d, \( J_{c, p} = 8 \text{ Hz} \)), 74.3, 70.6, 63.1 (d, \( J_{c, p} = 4 \text{ Hz} \)), 46.6, 16.7, 8.2; \(^{31}\)P NMR (162 MHz, D\(_2\)O), 6.72 (s); HRMS (ESI-) \( m/z \) 328.0706 (M, \( C_{12}H_{15}N_3O_6P \) requires 328.0689).

General procedure for synthesis of the triphosphonates 83 and 84 followed a route developed by our research group.\(^{28}\) Prior to the reaction, tris(tetra-n-butylammonium) hydrogen pyrophosphate (prepared as previously described,\(^{37}\) dried over P\(_2\)O\(_5\), and stored at -20 °C, 116 mg, 0.13 mmol) and hydrogen phosphonate 81 or 82 (55.0 mg, 0.130 mmol) were dried overnight under vacuum at 22 °C (room temperature) in two separate round bottom flasks. Anhydrous DMF (1.5 mL) and pyridine (250 µL, 3.00 mmol) were added to the round bottom flask containing the hydrogen phosphonate. After the solid was dissolved, TMSCl (132 µL, 1.04 mmol) was added by gas tight microsyringe. After 5 min, a solution of I\(_2\) in DMF (0.2 M, 910 µL) was added dropwise. The mixture was stirred for 5 min at 22 °C before solution of tris(tetra-n-butylammonium) hydrogen pyrophosphate in DMF (0.5 mL) was added. The reaction mixture was stirred at room temperature for 30 min and concentrated \emph{in vacuo}. The residue was dissolved in cold deionized water (1 mL) by sonication for 1 min. The precipitated iodine was removed by filtration through a small plug of cotton inserted in the bottom of a 12 cm glass Pasteur pipet. The product was purified by Sephadex LH-20 column with ice-cold aqueous TEAB buffer (10 mM, pH = 8) as the eluent. The fractions containing the triphosphate were identified by mass spectrometry, combined and lyophilized to yield the crude product, which was further purified by preparative reverse-phase HPLC with a linear gradient of 0% to 10% CH\(_3\)CN in TEAB buffer (10 mM, pH = 7.5, adjusted with acetic acid) over 30 min. Repeated lyophilization and resuspension
in deionized water (3 mL × 3) yielded the corresponding triphosphate as the triethylammonium salt.

6-Methyl-9-β-D-ribofuranosylpurine 5′-triphosphate (83).

This compound was synthesized from 81, and obtained as a colorless glassy solid (32.5 mg, 28%). 1H NMR (360 MHz, D$_2$O) δ 8.70 (s, 1H), 8.66 (s, 1H), 6.12 (d, 1H, $J = 6.0$ Hz), 4.73 (dd, 1H), 4.51 (dd, 1H), 4.28 (m, 1H), 4.13 (m, 2H), 3.05 (q, 18H, $J = 7.3$ Hz), 2.68 (s, 3H), 1.13 (t, 27H, $J = 7.3$ Hz); 31P NMR (145.8 MHz, D$_2$O) δ -9.7 (d, $J_{p,p} = 19.8$ Hz), -10.6 (d, $J_{p,p} = 19.8$ Hz), -22.3 (t, $J_{p,p} = 19.8$ Hz); HRMS (ESI-) m/z 504.9900 (M -, C$_{11}$H$_{16}$N$_4$O$_{13}$P$_3$ requires 504.9927).

Analytical reverse-phase HPLC profile of compound 84. Retention time = 4.9 min. Purity = 98%. Absorbance wavelength = 254 nm.
4-Methyl-7-β-D-ribofuranosylpyrrolo[2,3-d]pyrimidine 5'-triphosphate
(7-deaza-6-methyl-9-β-D-ribofuranosylpurine 5'-triphosphate, 84)

This compound was synthesized from 82, and obtained as a colorless glassy solid (17.4 mg, 15%). $^1$H NMR (400 MHz, D$_2$O) δ 8.56 (s, 1H), 7.76 (d, 1H, $J = 3.8$ Hz), 6.82 (d, 1H, $J = 3.8$ Hz), 6.30 (d, 1H, $J_{1',2'} = 7.0$ Hz), 4.65 (dd, 1H, $J_{2',1'} = 7.0$ Hz, $J_{2',3'} = 4.7$ Hz), 4.48 (dd, 1H, $J_{3',2'} = 4.7$ Hz, $J_{3',4'} = 2.8$ Hz), 4.27-4.26 (m, 1H), 4.18-4.04 (m, 2H), 3.08 (q, 18H, $J = 7.3$ Hz), 2.65 (s, 3H), 1.15 (t, 27H, $J = 7.3$ Hz); $^{31}$P NMR (162 MHz, D$_2$O) δ -10.2 (d, $J_{p,p} = 19.9$ Hz), -11.3 (d, $J_{p,p} = 19.9$ Hz), -23.1 (t, $J_{p,p} = 19.9$ Hz); HRMS (ESI-) m/z 503.9962 (M-, C$_{18}$H$_{27}$N$_3$O$_{13}$P$_3$ requires 503.9974).

Analytical reverse-phase HPLC profile of compound 84. Retention time = 4.9 min. Purity = 95%. Absorbance wavelength = 254 nm.
2.18.2 Cytotoxicity Assays

HeLa S3 cells (ATCC# CCL-2.2) were maintained in DMEM/F-12 media supplemented with 2% dialyzed fetal bovine serum and 1% penicillin/streptomycin (1×, Invitrogen). HeLa S3 cells (4 × 10³) in 100 µL media were loaded on a 96-well plate 24 h before use. Cells were incubated with ribonucleosides at various concentrations for 7 h, 24 h and 48 h respectively at 37 °C. All wells were adjusted to a final concentration of 0.1% DMSO. Media was removed and cells were washed with PBS (100 µL). 100 µL media, 75 µL PBS and 25 µL CellTiter-Glo reagent (Promega) were added to each well, and the 96-well plate was shaken on a titer plate shaker for 2 min to detach the cells. The cell suspension (200 µL) was transferred to an opaque 96-well plate according to the Promega protocol provided with the reagent. After incubation at room temperature (22 °C) for 10 min, the luminescence of the samples was measured with a Packard Fusion microtiterplate reader. The IC₅₀ values were calculated by with a nonlinear four-parameter curve fit (GraphPad Prism 5.0) of luciferase activity vs. concentration of nucleoside.

2.18.3 Antiviral Assay against Poliovirus

HeLa S3 cells (1 × 10⁵) were plated 1 day prior to treatment in a 24-well plate. Cells were pretreated with different concentrations of nucleosides in fresh media containing 0.1% DMSO. After 1 h incubation at 37 °C, media was removed and cells were infected with PV (1 × 10⁶ PFU) in PBS (100 µL). Plates were incubated for 15 min at room temperature (22 °C), PBS was removed by aspiration, and fresh media containing the specified amount of nucleoside was added. The infection was allowed to proceed at 37 °C for 6 h. Cells were washed with PBS and collected by treatment with trypsin. Cells were pelleted by centrifugation, resuspended in PBS (500 µL), and subjected to 3 freeze-thaw cycles. Cell debris was removed by centrifugation and the
supernatant containing the cell-associated virus was saved. Viral titer was determined by applying serial dilutions of supernatant to HeLa S3 monolayers (plated in 6-well plates 1 day before at 5 × 10⁵ cells/well) and overlaying with growth media containing low melting point agarose (1%). Plates were incubated for 2 days at 37 °C. The agarose was removed and plaques were visualized by staining with crystal violet (1%) in aqueous ethanol (20%). The IC₅₀ values were calculated with a nonlinear four-parameter curve fit (GraphPad Prism 5.0) of viral titer vs. concentration of nucleoside.

2.18.4 Antiviral Assay against Dengue Virus (DENV2)

**DENV2 replicon assay.** BHK-21 cells stably expressing the DENV2 replicon were thawed and cultured in DMEM/F-12 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (1×, Invitrogen). Cells (6 × 10⁵) were seeded in a 48-well plate 24 h before use. Nucleosides at different concentrations in media containing 1% DMSO were used to treat cells for 24 h. Cells were washed with PBS once and lysed by adding lysis buffer (Promega, 60 µL/well) and passing the lysate through 27-gauge needle fit with 1 mL syringe. 20 µL of lysate was added into opaque 96-well reader plate, and the Luciferase activity was measured by Luminometer (Centro LB 960, Berthold Technologies).

**DENV2 infectivity plaque assay.**²⁵,³⁸ BHK-21 cells (5 × 10⁵) were plated one day prior to treatment in a 12-well plate. Cells were infected with DENV2, and treated with nucleosides at different concentrations in media containing 1% DMSO for 24 h at 37 °C. Cell culture supernatant was covered with a first overlayer of media containing 0.9% low-melting point agarose, 1 × EMEM and 0.5% FBS, and incubated at 37 °C for 6 days. A second layer containing 0.9% low melting point agarose, 1% NaCl, 0.015% neutral red dye was added, and the plate was incubated at 37 °C overnight. The number of plaques were counted and calculated as PFU/ml.
The IC\textsubscript{50} values were calculated with a nonlinear four-parameter curve fit (GraphPad Prism 5.0) of viral titer vs. concentration of nucleoside.

**DENV2 infectivity real time PCR assay.** The DENV2 RNA copy number was measured using primers and probe specific to DENV2 NS1 region. The primers and probe were designed using the Beacon designer 4.0 program. PCR amplification and real-time data collection and analysis were performed according to iQ5 multicolor real-time PCR detection system (Bio-Rad). Briefly, a reverse-transcriptase (RT) reaction was carried out using a RT kit from Bio-Rad (iScript cDNA synthesis). 5 µL of RNA samples was mixed with iScript cDNA synthesis mixture and incubated at 42 °C for 30 min. 2 µL of cDNA product was subjected to PCR using conditions as previously reported.\textsuperscript{26} Real-time detection of the DENV2 PCR product was correlated with the input cDNA copy number and the results were plotted.

### 2.18.5 Guanidine Resistance Assay

Resistance to 2 mM guanidine is conferred by a single C to U transition mutation in the 2C protein in poliovirus genome. HeLa S3 cells were plated one day before the experiment at 25% confluence in 10-cm dishes. Cells were infected with 1 × 10\textsuperscript{6} PFU poliovirus from the appropriate viral stock previously obtained in the presence or absence of the nucleosides at different concentrations. Cells were covered with 20-mL DMEM/F12 media supplemented with 10% FBS, 1% agarose, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 2 mM guanidine hydrochloride. The guanidine resistant viral titer (Gua\textsuperscript{r} plaques) was determined.
2.18.6 Nucleotide Incorporation Assay

**Nucleotide incorporation by PV 3D<sup>pol</sup> in vitro.** Incorporation of NTPs (83, 84 or natural nucleotide) opposite each of the four templating bases (A, C, G and U) was examined using pre-assembled 3D<sup>pol</sup>-primer/template complexes.<sup>29</sup> As previously described, symmetrical substrate (sym/sub) served as the primer and template RNA. Annealing of 32P-end-labeled and unlabeled sym/sub oligos to form the primer/template duplex was preformed as previously described. 3D<sup>pol</sup> was allowed to preassemble with sym/sub duplex for 3 min at 30 °C. Incorporation assays with A, C, G and U as templating bases were performed at 30 °C in HEPES buffer (pH 7.5, 50 mM) containing 2-mercaptoethanol (10 mM), MgCl₂ (5 mM), 3D<sup>pol</sup> (1 μM), sym/sub duplex RNA (0.5 μM) and nucleotide (0.5 mM). All incorporation assays were initiated by addition of nucleotide, reaction products were separated by denaturing PAGE, gels were visualized using a phosphorimager, and radioactivity was quantified using ImageQuant software (Molecular Dynamics).

**Steady state incorporation assay.** Steady State incorporation of 83 and 84 by PV RdRP (3D<sup>pol</sup>) opposite templating bases U and C was examined. Incorporation of the correct nucleotide (ATP templated by U and GTP templated by C) and an incorrect nucleotide (GTP templated by U and ATP templated by C) served as comparative controls. Nucleotide incorporation reactions contained HEPES buffer (pH 7.5, 50 mM), 2-mercaptoethanol (10 mM), MgCl₂ (5 mM), ZnCl₂ (60 μM), NTP (1 mM), sym/sub duplex RNA (10 μM) and 3D<sup>pol</sup> (1 μM). All incorporation assays were initiated by addition of 3D<sup>pol</sup>, and stopped with 50 mM EDTA (final concentration). For steady state assays involving addition of more than one nucleotide, and where the total nucleotide concentration was greater than 1 mM, the concentration of MgCl₂ was increased to maintain the concentration of free Mg<sup>2+</sup> at 4 mM above the total NTP concentration. The reaction products were separated by denaturing PAGE, gels were visualized using a phosphorimager, and
radioactivity was quantified using ImageQuant software (Molecular Dynamics). Data were fit to a linear model using GraphPad Prism 5.0.

2.19 References


Chapter 3

Synthesis of a Antiviral Pyrazinecarboxamide Nucleoside Derivative

3.1 Overview

To combat drug resistance and emerging viruses that cause serious threats to human health, new broad-spectrum therapeutics are needed. Recently, the structurally related pyrazinecarboxamide compounds T-1105 (85), T-705 (86) and T-1106 (87) were discovered by Toyama Chemical Co., Ltd. to exhibit antiviral activity against several RNA viruses. However, their mechanism of action is not well understood. The broad-spectrum antiviral activity of T-1106 (87), coupled with its structural similarity with the clinically used antiviral drug ribavirin (29), attracted the Cameron lab at The Pennsylvania State University to study the activity of 87 against poliovirus. However, only one patent reported the synthesis of 87, and the coupling of its nucleobase with ribose led to a mixture of α and β anomers. Therefore, an improved stereoselective synthesis of 87 was desired to facilitate studies of this compound. Moreover, treatment of polioirus-infected HeLa S3 cells with 87 caused a dramatic decrease in viral titer. To probe its mechanism of action, the triphosphate of 87 was synthesized and evaluated in a primer-extension assay. Interestingly, the triphosphate can be incorporated by poliovirus RdRP across all four natural nucleotides, suggesting the mechanism of action of 87 against poliovirus is likely to be lethal mutagenesis.
3.2 Broad Spectrum Antiviral Activities of Pyrazinecarboxamide Derivatives

By screening a chemical library against influenza virus A, Toyama Chemical Co., Ltd. discovered T1105 (85) in 2000. Starting from this lead compound, its derivatives including T-705 (86) and T-1106 (87, Figure 3-1) were synthesized and evaluated against different viruses. T-705 inhibits influenza viruses both in cell-based assays and infected mice, and is currently in phase II clinical trials for the treatment of influenza infection. T-705 is also effective against West Nile virus, yellow fever virus, and several bunya and arenaviruses. T-1106 is effective against influenza virus, yellow fever virus, and foot-and-mouth disease virus. Mechanism of action studies show that T-705 is converted to T-705 ribomonophosphate (T-705RMP) and T-705 ribotriphosphate (T-705RTP), and T-705RTP inhibits influenza virus RNA polymerase in a GTP-competitive manner, suggesting that the ribophosphorylated product of T-705 functions as a purine nucleotide analogue that blocks viral RNA replication. The potency of inhibition of influenza virus by T-705RTP is nearly 10 times greater than ribavirin triphosphate. Moreover, T-705 is less toxic than ribavirin, because it does not inhibit cellular DNA and RNA synthesis, and T-705RMP inhibits cellular inosine monophosphate dehydrogenase (IMPDH) 150-fold less than ribavirin monophosphate. Similarly, phosphorylation of T-1106 was demonstrated in some mammalian cells and in the livers of animals treated with this compound. Considering the current biological results on T-1106, and the structural similarity between ribavirin and T-1106, the Cameron lab proposed that T-1106 may elicit its biological activity through lethal mutagenesis.
3.3 Stereoselective Synthesis of T-1106 Using the Vorbrüggen Method

The Vorbrüggen coupling method is commonly used to prepare pyrimidine nucleoside analogues. The general procedure for this method is to react a presilylated heterocyclic base with an acylated sugar in the presence of a Lewis acid catalyst. The synthesis of 87 through the Vorbrüggen method has been reported in a patent by silylating 3-hydroxypyrazine-2-carboxamide (89) with hexamethyldisilazane and condensing this silylated base with ribose derivative 88 catalyzed by tin (IV) chloride. This coupling product was generated as a mixture of α and β anomers. To provide a more stereoselective method, we used N,O-bis(trimethylsilyl)acetamide and trimethylsilyl trifluoromethanesulfonate to generate the anomerically pure β anomer in high yield. After deprotection in ammonia methanol, T-1106 was obtained as the final product (Figure 3-2). The mechanism of the glycosylation step is shown in Figure 3-3. It generates the carbocation through neighboring group participation, and the reaction undergoes a concerted SN₂ reaction process, so a single β anomer can be generated. In the reported procedure, when tin (IV) chloride is used as the Lewis acid, tin is chelated to the oxygen atom on the 1’ acetyl group, and the acetyl group falls off to generate a positive charge at the 1’-position. The incoming
nucleobase can attack from both faces, and the result of getting a mixture of α and β anomers is expected.

![Synthesis of T-1106](image)

Reagents and conditions: (a) N,O-bis(trimethylsilyl)acetamide, TMSOTf, CH$_3$CN; (b) NH$_3$, MeOH.

Figure 3-2: Synthesis of T-1106 (87).

![Mechanism of the glycosylation](image)

Figure 3-3: Mechanism of the glycosylation.

### 3.4 Toxicity and Antiviral Evaluation of T-1106

The cytotoxicity of T-1106 against HeLa S3 cells was evaluated in a crystal violet assay. HeLa S3 cells were treated with T-1106 at varying concentrations from 10 µM to 1000 µM for 7 h, and crystal violet was used to stain the HeLa S3 cell monolayer to evaluate toxicity. As shown in Figure 3-4, no reduction in viable cells was observed up to 1000 µM under these conditions.
The antiviral activity of T-1106 was tested using poliovirus infected HeLa S3 cells as described in section 2.7. As shown in Figure 3-5, the viral titer decreased in a dose-dependent manner in the presence of T-1106, and at a concentration of 500 µM, viral titer was reduced by approximately 8-fold. Using the same assay, the decrease in viral titer when cells were treated with 500 µM ribavirin was only 4-fold.\textsuperscript{12}

![Figure 3-4: Cytotoxicity of T-1106 to HeLa S3 cells after 7 h treatment. Data obtained by Dr. Hyung Suk Oh.](image1)

![Figure 3-5: Antiviral Activity of T-1106 against Poliovirus. HeLa S3 cells were incubated with T-1106 for 1 h at the concentrations shown, and subsequently infected with $10^6$ PFU of PV. After infection for 15 min, fresh media containing compounds was added and the infection continued for 6 h. Cell-associated virus was titrated by plaque assay. Data obtained by Dr. Hyung Suk Oh.](image2)
3.5 Plaque Assay of T-1106

To evaluate the mechanism of antiviral activity of T-1106, a plaque assay was performed. After treating poliovirus-infected HeLa S3 cells with 1 mM T-1106, the viral plaques that remained (passage 2 poliovirus) were used to infect HeLa S3 cells. The cells infected with passage 2 poliovirus were treated with 1 mM T-1106 for a second round, aiming to generate T-1106 resistant poliovirus after several passages. Sequencing of the T-1106 resistant strain will be used to further elucidate the mechanism of antiviral activity. Plaque numbers of the wild-type and passage 2 poliovirus (Figure 3-6) infected HeLa S3 cells with or without T-1106 treatment are shown, and the passage 2 virus plaques are smaller than the wild-type plaques, indicating alterations to viral structure.
3.6 Synthesis of T-1106-TP

3.6.1 Attempt to Synthesize T-1106-TP via the “One-Pot, Three-Step” Approach

To investigate the mechanism of action of T-1106, an efficient synthesis of the triphosphate was desired. The “one-pot, three-step” method is one of the most widely used approaches to synthesize nucleoside triphosphates (Figure 3-7). In this approach, the 5'-OH of an unprotected nucleoside is selectively converted to the phosphodichloridate using phosphorus oxytrichloride and trimethyl phosphate. This unstable phosphodichloridate compound is reacted immediately with pyrophosphate to form the cyclic phosphate 93, which is hydrolyzed to yield...
the final nucleoside triphosphate. However, reaction of T-1106 under these reaction conditions was not successful, presumably due to the incompatibility of phosphorus oxytrichloride and the carboxamide group of T-1106.

3.6.2 Synthesis of Nucleoside Triphosphate from Nucleoside 5'-H-Phosphonate

Our research group recently reported an approach for the synthesis of nucleoside triphosphates from nucleoside 5'-H-phosphonate monoesters (Figure 3-8). In this procedure, the fully deprotected nucleoside 5'-H-phosphonate monoester is converted to silyl phosphate using TMSCl, and oxidized by iodine to yield the pyridinium phosphoramidate 96. This intermediate is electrophilic, and reacts with nucleophilic tris(tetra-n-butylammonium) hydrogen pyrophosphate to generate the corresponding nucleoside triphosphate. Ribavirin triphosphate was successfully obtained in 40% yield using this procedure. Since ribavirin also has carboxamide functionality,
this approach was explored with T-1106 to generate T-1106TP. To this end, an effective method
to synthesize the 5'-H-phosphonate of T-1106 was needed.

3.6.3 Overview of Methods for the Synthesis of Nucleoside 5'-H-Phosphonate

In nucleotide chemistry, 5'-H-phosphonates are useful tools to generate nucleotide prodrugs,\textsuperscript{15} phosphoamidate dinucleosides,\textsuperscript{16} dinucleotide phosphates,\textsuperscript{17} and oligonucleotides.\textsuperscript{18} An advantage of H-phosphonates as intermediates is that they are relatively easy to generate and stable during prolonged storage.\textsuperscript{15a} There are four general methods to access this intermediate (Figure 3-9). In all cases, masking of 2'- and 3'-OH of nucleoside is necessary to selectively phosphonylate the 5'-OH group. Isopropylidene and benzylidene acetal are commonly used to protect 1,2-diols, and they can be easily removed by treating with aqueous trifluoroacetic acid or hydrogenation, respectively. The protected nucleoside can react with excess phosphorus trichloride to generate the monosubstituted product, leading to the H-phosphonate monoester after hydrolysis.\textsuperscript{19} However, this method can only be used for nucleosides lacking hydroxy or amino groups in the nucleobase, because phosphorus trichloride is not inert to these functionalities. Salicyl phosphorochloridate can also be used to generate H-phosphonate, but this procedure exhibits difficulties during work-up and purification, probably due to the similar chromatographic mobility of the product and side product.\textsuperscript{19} The reagents used in the remaining two procedures are
completely inert to exocyclic amino groups of natural nucleosides.\textsuperscript{15a} Excess diphenyl phosphite and pyridine are used to treat the protected nucleoside to yield the corresponding $H$-phosphonate after deprotection.\textsuperscript{15b} The last phosphorylating agent is in situ generated $H$-pyrophosphonate ($H_4P_2O_5$) by reacting pivaloyl chloride with phosphorous acid in pyridine.\textsuperscript{15a} A near-quantitative yield was claimed using this procedure for guanidine derivatives.\textsuperscript{20}

![Diagram of nucleoside synthesis](image)

Figure 3-9: Methods for synthesis of nucleoside 5'-$H$-phosphonates.
3.6.4 Synthesis of T-1106-5’-H-Phosphonate and T-1106-TP

Protection of the 2’- and 3’-OH of T-1106 with isopropyldene, followed by reacting the 5’-OH with the phoshonylating agent diphenyl phosphite and deprotection yielded the corresponding H-phosphonate in good yield. Conversion of this compound to the triphosphate via the pyridinium phosphoramidate intermediate\textsuperscript{19} afforded T-1106-TP in moderate yield (Figure 3-10).

![Diagram of the synthesis process]

Reagents and conditions: (a) 2,2-dimethoxypropane, p-TsOH, acetone; (b) (1) Diphenyl phosphite, pyridine; (2) Et\textsubscript{3}N, H\textsubscript{2}O; (3) TFA, H\textsubscript{2}O; (c) TMSCl, pyridine, DMF, I\textsubscript{2}, (NBu\textsubscript{4})\textsubscript{3}HP\textsubscript{2}O\textsubscript{7}.

Figure 3-10: Synthesis of T-1106-TP.

3.7 Incorporation of T-1106-TP by PV RdRP across Natural Nucleotides

To investigate the mechanism of antiviral activity of T-1106, the incorporation of T-1106-TP by PV RdRP was evaluated using the primer-extension assay described in section 2.12. After incorporation for 10 min, T-1106-TP (101) functioned as a universal nucleotide, and was
incorporated opposite all four natural nucleotides by viral RdRP (Figure 3-11). The templating properties of T-1106-TP are more ambiguous than ribavirin triphosphate, which can only be incorporated against UMP and CMP as a purine analogue.21

**Figure 3-11**: Incorporation of T-1106-TP (101) opposite all four natural nucleotides mediated by PV RdRP. Complexes of RNA template and PV RdRP were incubated for 90 s, 101 or the natural NTP (0.1 mM) was added respectively, and the reaction was allowed to proceed at 30 °C for 10 min. Data obtained by Dr. Hyung Suk Oh.

3.8 Conclusion

A stereoselective synthesis of a broad spectrum antiviral agent T-1106 (87) was developed using Vorbrüggen coupling method. Treatment of poliovirus-infected HeLa S3 cells with this compound caused a dramatic decrease in viral titer. The corresponding nucleoside triphosphate was synthesized from the hydrogen phosphonate monoester, and it was incorporated opposite all four natural nucleotides by viral RdRP *in vitro*, suggesting lethal mutagenesis as a possible mechanism of action. Evaluation of T-1106 as an antiviral agent and the mechanistic
studies revealed that nucleosides containing carboxamide substitutions on the nucleobase represent a promising class of antiviral compounds that may function as lethal mutagens. Future work should investigate nucleosides containing this functionality in the nucleobase.

3.9 Experimental Section

3.9.1 Chemical Synthesis

*General Synthesis Information.* Chemical reagents and solvents were obtained from Acros, Aldrich, TCI or Fisher without further purification unless otherwise noted. 3-Hydroxypyrazine-2-carboxamide was ordered from Alfa Aesar. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). Reactions were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EMD Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with 20% sulfuric acid in ethanol. Nuclear magnetic resonance (NMR) spectroscopy employed a Bruker DRX 400 spectrometer. NMR peaks are reported as parts per million (ppm) referenced to internal CHCl₃, MeOH or H₂O peaks. Mass spectral data was obtained from The University of Kansas Mass Spectrometry Facility. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Column chromatography employed silica gel (ICN SiliTech, 32-63 µm) or Sephadex LH-20 (GE Healthcare, column dimensions: 2.5 cm × 40 cm). Purification by preparative HPLC employed an Agilent 1100 series instrument equipped with an Atlantis C18 preparative column (19 × 150 mm, 5 µm; Waters Corporation). The HPLC flow rate was maintained at 25 mL/min for the duration of the run. Determination of purity of compound 87 by analytical HPLC used a PRP-1 (polystyrene-
divinylbenzene) reverse-phase column (4.1 × 250 mm, 7 µm; Hamilton) and a gradient of 1% to 99% CH$_3$CN (containing 0.1% TFA) in nanopure water (containing 0.1% TFA, 0 to 30 min) followed by 99% CH$_3$CN (containing 0.1% TFA) from 30-40 min at a flow rate of 0.8 mL/min.

Analysis of purity of compounds 101 by HPLC used an Aquasil column (4.6 × 250 mm, 5 µm; Thermoelectron Corporation) at a flow rate of 1.0 mL/min with a gradient of 1% to 50% CH$_3$CN in aqueous KH$_2$PO$_4$ (100 mM) over 30 min followed by 50% CH$_3$CN in aqueous KH$_2$PO$_4$ from 30-40 min.

3,4-Dihydro-4-(2',3',5'-tri-O-benzoyl-β-D-ribofuranosyl)-3-oxopyrazine-2-carboxamide (90)

3-Hydroxypyrazine-2-carboxamide (89, Alfa Aesar, 50.0 mg, 0.359 mmol) was suspended in dry CH$_3$CN (1.5 mL), and N,O-bis(trimethylsilyl)acetamide (98.0 µL, 0.395 mmol) was added dropwise. The mixture was stirred at room temperature (22 °C) for 1 h, before adding β-D-ribofuranose 1-acetate-2,3,5-tribenzoate (Aldrich, 181 mg, 0.359 mmol) and trimethylsilyl trifluoromethanesulfonate (72.0 µL, 0.395 mmol). The solution was stirred at room temperature (22 °C) for 18 h. EtOAc (100 mL) was added to dilute the solution, and the organic phase was washed with saturated aqueous NaHCO$_3$ solution (40 mL × 2) and brine (40 mL × 2). The solution was dried over anhydrous sodium sulfate, and the solvent was removed in vacuo. The crude product was purified by flash column chromatography (eluent: CH$_2$Cl$_2$, MeOH, 20:1) to yield 90 (171 mg, 81%) as a white foam. $^1$H NMR (400 MHz, CDCl$_3$) δ 8.90 (s, 1H), 8.06-8.04 (m, 2H), 7.96-7.90 (m, 4H), 7.84 (d, 1H, J = 4.2 Hz), 7.67 (d, 1H, J = 4.2 Hz), 7.61-7.52 (m,
96

3H), 7.47-7.33 (m, 6H), 6.84 (s, 1H), 6.36 (d, 1H, \( J = 2.8 \) Hz), 5.93-5.87 (m, 2H), 4.91-4.70 (m, 3H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) δ 166.0, 165.2, 165.1, 163.5, 163.1, 155.3, 144.1, 133.9, 133.8, 129.9 (×2), 129.8 (×2), 129.6 (×2), 129.1, 128.8 (×2), 128.6 (×2), 128.5 (×2), 128.4, 128.0, 125.0, 90.7, 80.8, 74.6, 70.2, 62.8.

\[\text{\chemdraw image}\]

**3,4-Dihydro-4-\(\beta\)-D-ribofuranosyl-3-oxopyrazine-2-carboxamide (T-1106, 87)**

Compound 90 (171 mg, 0.293 mmol) was treated with ammonia in methanol (7 N, 10 mL), and stirred at room temperature (22 °C) for 5 h. The solvent was removed in vacuo and the residue was purified by flash column chromatography (eluent: CH\(_2\)Cl\(_2\), MeOH, 5:1) to yield T-1106 (87, 70.0 mg, 88%) as a white solid. mp 189-191 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) δ 8.35 (s, 1H), 8.28 (d, 1H, \( J = 4.3 \) Hz), 7.31 (s, 1H), 7.54 (d, 1H, \( J = 4.3 \) Hz), 5.92 (d, 1H, \( J = 2.2 \) Hz), 5.62 (d, 1H, \( J = 4.8 \) Hz), 5.26 (t, 1H, \( J = 4.9 \) Hz), 5.10 (d, 1H, \( J = 5.3 \) Hz), 4.02-3.95 (m, 3H), 3.82-3.62 (m, 4H); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) δ 164.6, 154.6, 147.6, 127.6, 123.3, 90.1, 84.8, 75.2, 68.7, 59.9; IR (film) ν max 3363, 2918, 2846, 1673, 1558, 1490, 1451, 1237, 1182, 1100, 1056, cm\(^{-1}\); HRMS (ESI+) \(m/z\) 294.0709 (M+Na\(^+\), \(\text{C}_{10}\text{H}_{13}\text{N}_3\text{O}_6\)Na requires 294.0702).
Analytical reverse-phase HPLC profile of T-1106 (87). Retention time = 7.6 min. Purity = 97%. Absorbance wavelength = 254 nm.

3,4-Dihydro-4-(2',3'-O-isopropylidene-β-D-ribofuranosyl)-3-oxopyrazine-2-carboxamide (99)

Compound 87 (50.0 mg, 0.184 mmol) was dissolved in acetone (2 mL). 1,2-Dimethoxy propane (2 mL) and p-toluenesulfonic acid (87.5 mg, 0.460 mmol) were added into the reaction solution. The mixture was stirred at room temperature (22 °C) for 20 min. Acetone (10 mL) was added to dilute the solution, and Amberlite IRA-400 (OH) resin (from Supelco) was used to modify the pH value to 7. The resin was removed by filtration and washed with acetone (20 mL), and the filtrate was evaporated in vacuo. The product was purified by flash chromatography (eluent: CH₂Cl₂, MeOH, 20:1) to afford 36.7 mg 99 as a white solid (0.118 mmol, 64%). mp 126-127 °C; ¹H NMR (400 MHz, CD₃OD) δ 8.22 (d, 1H, J = 4.2 Hz), 7.70 (d, 1H, J = 4.2 Hz), 6.07 (d, 1H, J = 2.0 Hz), 4.98-4.83 (m, 2H), 4.51-4.49 (m, 1H), 3.90-3.51 (m, 2H), 1.60 (s, 3H), 1.39 (s, 3H); ¹³C NMR (100 MHz, CD₃OD) δ 164.7, 155.8, 143.1, 129.4, 123.5, 113.2, 94.9, 88.8,
86.1, 80.9, 61.4, 26.0, 24.0; IR (film) v max 3390, 2984, 2928, 1677, 1558, 1487, 1454, 1383, 1215, 1114, 1067, 848, 804 cm⁻¹; HRMS (ESI+) m/z 334.1009 (M+Na⁺, C₁₃H₁₇N₃O₆Na requires 334.1015).

3,4-Dihydro-4-β-D-ribofuranosyl-3-oxopyrazine-2-carboxamide 5'-H-phosphonate (100)

Diphenyl phosphite (Aldrich, 0.260 mL, 1.35 mmol) was dissolved in dry pyridine (1.5 mL). Compound 99 (156 mg, 0.500 mmol) was dissolved in dry pyridine (1.5 mL), and added dropwise to the solution of diphenyl phosphite. The solution was stirred at room temperature (22 °C) for 30 min, and pyridine was removed in vacuo. The crude product was purified by flash chromatography (eluent: CH₂Cl₂, MeOH, 5:1). This fully protected H-phosphonate was treated with aqueous triethylamine solution (50%, 1 mL) for 30 min, dried in vacuo, treated with aqueous trifluoroacetic acid solution (25%, 1 mL) for 30 min, and dried in vacuo. The crude product was dissolved in aqueous triethylammonium bicarbonate (TEAB) buffer (1 mL, 10 mM, pH = 8), and the product was purified by Sephadex LH-20 column (2.5 cm × 40 cm). Elution with 10 mM aqueous TEAB buffer and lyophilization yielded compound 100 (155 mg, 71%) as a glassy solid.

¹H NMR (400 MHz, D₂O) δ 8.23 (s, 1H), 7.72 (s, 1H), 6.74 (d, 1H, Jₚ,₇ = 639 Hz), 6.02 (s, 1H), 4.24-4.17 (m, 4H), 4.08-4.04 (m, 1H), 3.11 (q, 6H, J = 7.3 Hz, N(CH₂CH₃)₃), 1.19 (t, 9H, J = 7.3 Hz, N(CH₂CH₃)₃); ¹³C NMR (100 MHz, D₂O) δ 165.8, 155.9, 142.5, 129.2, 125.0, 91.0, 82.5 (d, Jₚ,₇ = 8 Hz), 74.7, 68.1, 61.4 (d, Jₚ,₇ = 4 Hz), 46.6, 82.2; ³¹P NMR (162 MHz, D₂O), 6.61 (s); HRMS (ESI-) m/z 334.0431 (M⁻, C₁₀H₁₃N₃O₆P requires 334.0440).
Prior to the reaction, tris(tetra-n-butylammonium) hydrogen pyrophosphate (90.0 mg, 0.100 mmol) and hydrogen phosphonate 100 (43.6 mg, 0.100 mmol) were dried overnight under vacuum at room temperature (22 °C) in two separate round bottom flasks. Anhydrous DMF (1.5 mL) and pyridine (200 µL, 1.60 mmol) were added to the round bottom flask containing the hydrogen phosphonate. After the solid was dissolved, TMSCl (100 µL, 0.803 mmol) was added by gas tight microsyringe. After 5 min, a solution of I₂ in DMF (0.2 M, 700 µL) was added dropwise. The mixture was stirred for 5 min at 22 °C before a solution of tris(tetra-n-butylammonium) hydrogen pyrophosphate in DMF (0.5 mL) was added. The reaction mixture was stirred at room temperature for 30 min and concentrated in vacuo. The residue was dissolved in cold deionized water (1 mL) by sonication for 1 min. The precipitated iodine was removed by filtration through a small plug of cotton inserted in the bottom of a 12 cm glass Pasteur pipet. The product was purified by Sephadex LH-20 column with aqueous ice-cold TEAB buffer (10 mM, pH = 8) as the eluent. The fractions containing the triphosphate were identified by mass spectrometry, combined and lyophilized to yield the crude product, which was further purified by preparative reverse-phase HPLC with a linear gradient of 0% to 10% CH₃CN in TEAB buffer (10 mM, pH = 7.5, adjusted with acetic acid) over 30 min. Repeated lyophilization and resuspension
in deionized water (3 mL × 3) yielded the corresponding triphosphate 101 as the triethylammonium salt (11.0 mg, 14%). $^1$H NMR (400 MHz, D$_2$O) $\delta$ 8.22 (s, 1H), 7.69 (s, 1H), 6.68 (d, 1H, $J = 1.9$ Hz), 4.27-4.17 (m, 4H), 4.08-4.04 (m, 1H), 3.06 (q, 1H, $J = 7.3$ Hz), 1.19 (t, 27H, $J = 7.3$ Hz); $^{31}$P NMR (162 MHz, D$_2$O) $\delta$ -10.3 (d, $J_{p,p} = 19.9$ Hz), -11.2 (d, $J_{p,p} = 19.9$ Hz), -22.9 (t, $J_{p,p} = 19.9$ Hz); HRMS (ESI-) $m/z$ 509.9582 (M⁻, C$_{10}$H$_{15}$N$_3$O$_{15}$P$_3$ requires 509.9576).

Analytical reverse-phase HPLC profile of compound 101. Retention time = 5.3 min. Purity = 94%. Absorbance wavelength = 254 nm.

### 3.9.2 Cytotoxicity Assays

HeLa S3 cells were maintained in DMEM/F-12 media supplemented with 2% dialyzed fetal bovine serum and 1% penicillin/streptomycin (1×, Invitrogen). HeLa S3 cells ($1 \times 10^5$) in 500 μL media were loaded on a 24-well plate one day before use. Cells were incubated with ribonucleosides at various concentrations for 7 h at 37 °C. All wells were adjusted to a final concentration of 1% DMSO. Media was removed and cells were washed with PBS (500 μL). Cells were allowed to recover for 24 h in media without the compound. Media was removed, and the cell monolayer was stained by crystal violet.
3.9.3 Antiviral Assay against Poliovirus

HeLa S3 cells ($1 \times 10^5$) were plated one day prior to treatment in a 24-well plate. Cells were pretreated with different concentrations of nucleosides in fresh media containing 1% DMSO. After 1 h incubation at 37 °C, media was removed and cells were infected with PV ($1 \times 10^6$ PFU) in PBS (100 μL). Plates were incubated for 15 min at room temperature (22 °C), PBS was removed by aspiration, and fresh media containing the specified amount of nucleoside was added. The infection was allowed to proceed at 37 °C for 6 h. Cells were washed with PBS and collected by treatment with trypsin. Cells were pelleted by centrifugation, resuspended in PBS (500 μL), and subjected to 3 freeze-thaw cycles. Cell debris was removed by centrifugation and the supernatant containing the cell-associated virus was saved. Viral titer was determined by applying serial dilutions of supernatant to HeLa S3 monolayers (plated in 6-well plates 1 day before at $5 \times 10^5$ cells/well) and overlaying with growth media containing low melting point agarose (1%). Plates were incubated for 2 days at 37 °C. The agar was removed and plaques were visualized by staining with crystal violet (1%) in aqueous ethanol (20%).

3.9.4 Plaque Assay

HeLa S3 cells ($1 \times 10^5$) were plated one day prior to treatment in a 24-well plate. Cells infected with wild type poliovirus were treated with 1 mM T-1106, and the surviving virus plaques (passage 2 poliovirus), as well as wild type poliovirus, were used for infection. HeLa S3 cells ($1 \times 10^5$) in a 24-well plate were treated with 1 mM T-1106 in media containing 1% DMSO for 1 h, and subsequently infected with 50 or 25 PFU of wild type poliovirus or passage 2 poliovirus for 15 min at room temperature (22 °C). Cells with no drug treatment were infected
with poliovirus and served as controls. After infection for 15 min, cells were washed with PBS and overlayed with 1% agarose and incubated at 37 °C for 2 days (without T-1106) or 4 days (with T-1106). Viral plaques were counted after staining with crystal violet (1%) in aqueous ethanol (20%).

3.10 References


Chapter 4

Hydrophobic Rhodamine Analogues as Novel Cell-Permeable Fluorophores for Detection of Small Molecule-Protein Interactions in Living Cells

4.1 Overview

Rhodamines are a xanthenes class of dyes that have relatively red-shifted absorption and emission spectra comparing with fluorescein. Because of their high fluorescence quantum yield, pH-insensitivity, excellent photostability, and photophysical properties, rhodamines are widely used as probes for labeling of biomolecules. These long-wavelength fluorophores are advantageous as molecular probes because they minimize interference from natural chromophores, and allow multicolor labeling experiments when used with other spectrally orthogonal fluorescent probes. However, rhodamine analogues have some limitations. The high polarity of many rhodamines limits their cellular permeability. Some rhodamines carrying fixed positive charges accumulate in mitochondria, causing increased background fluorescence. Novel rhodamines 115-117 lacking fixed positive charges were designed and synthesized. Compounds 115 and 117 had red-shifted absorption/emission spectra, and all of these compounds had high quantum yields. The utility of 115 as molecular probes was explored by studies of the biotinylated fluorophores 144 that specifically bind SA expressed in yeast, and 114 represents a potentially useful tool for cellular imaging.

4.2 Introduction

Numerous biological and medicinal studies depend on fluorescence-based methods. Fluorescent molecules are used as molecular reporters or molecular probes, because a fluorescent
signal can contain information about both neighboring molecules and the environment. Fluorescence occurs in three stages that include excitation, the excited state lifetime, and fluorescence emission (Figure 4-1). Excitation occurs when a molecule absorbs a photon from an external source, creating an excited electronic singlet state. The excited singlet state usually lasts for nanoseconds, and it can return to the ground state through different routes. Fluorescence emission happens if the photon emission is between the same spin states (e.g. $S_1$ to $S_0$), while phosphorescence occurs if the initial and final energy levels have different spin states (e.g. $T_1$ to $S_0$). The photon generated by fluorescence emission is lower in energy than the photon absorbed during excitation, and therefore of longer wavelength. The difference in wavelength between the maximum of the absorption spectra and the maximum of the emission spectra is termed the Stoke’s shift, which is characteristic of all fluorescent molecules. The excited state can also return to the ground state through some nonradiative processes, such as internal conversion (IC), intersystem crossing (ISC) and vibrational relaxation. Internal conversion is between the same spin state, and intersystem crossing is between different spin states. Vibrational relaxation is the most frequently occurred nonradiative process for many molecules, and it happens due to interactions with other intra- or intermolecular degrees of freedom.
4.3 Common Fluorophores for Bioimaging Applications

Compared with other technologies, such as radioisotope labeling, MRI, and ESR, fluorescence imaging has many advantages for reporting the behavior of biomolecules of interest. Using widely available instruments, fluorescence imaging enables non-invasive, very sensitive, and non-radioactive detection of a wide variety of molecules.\(^2\)

Common organic fluorophores used for bioimaging range from blue fluorescent to red fluorescent and are diverse in structure. Fluorophores that are excited by UV irradiation typically have limited applications in biological systems, because UV can damage nucleic acids and other biomolecules, and some cells and tissues exhibit autofluorescence when excited by UV light. Therefore, organic fluorophores with absorption and emission at longer wavelengths can be more useful for biological applications.
4.3.1 Green Fluorescent Fluorescein and Fluorescein Derivatives

Fluorescein (102) is one of the most widely used fluorophores. Its absorption and emission maxima lie in the visible region (λ_{abs} = 490 nm, λ_{em} = 512 nm, in water). The absorption maximum of fluorescein closely matches the 488 nm spectral line of the argon laser, and this organic fluorophore has high molar absorptivity, excellent quantum yield and good water solubility, making it useful in confocal laser-scanning microscopy and flow cytometry applications. However, fluorescein has some limitations. For example, it is highly susceptible to photobleaching, it is pH sensitive (pKa = 6.5), and its fluorescence tends to quench upon conjugation with other molecules. To improve the properties of fluorescein, fluorine substitution was introduced to lower the pKa and therefore decrease the pH sensitivity. Oregon Green (103) bearing two fluorines in the xanthone moiety is a dye with absorption and emission spectra similar to fluorescein, less pH-sensitivity, and less susceptibility to photobleaching. Rhodamine Green (104), replacing oxygens of fluorescein with primary amines, is another photostable green fluorescent dye. This fluorophore forms better conjugates than fluorescein. Moreover, it is more photostable than Oregon Green and is pH insensitive between pH 4 and 9. As a more hydrophobic, photostable, and less pH-sensitive dye than fluorescein, Pennsylvania Green (105) was developed by our research group to provide a new tool to image living cells. This fluorophore replaces the carboxylate of Oregon Green with a methyl group (Figure 4-2).
4.3.2 Red-shifted Rhodamine Derivatives

Rhodamines belong to the xanthene class of dyes, replacing oxygens of fluorescein with nitrogens on the xanthene moiety. The absorption and emission spectra of rhodamines can be tuned to be red-shifted by substituting the nitrogens with alkyl groups. Tetramethylrhodamine (TAMRA, 106), rhodamine 19 (Rho 19, 107), rhodamine B (Rho B, 108) and Rhodamine 101 (Rho 101, 109) are commercially available red-shifted rhodamines (Figure 4-3).\(^8\) Rho B (108) bearing ethyl substitutions on nitrogens has absorption and emission maxima at 553 nm and 572 nm respectively in ethanol.\(^8\) With the introduction of rigid rings, Rho 101 (109) is further red-shifted, with absorption maximum at 574 nm and emission maximum at 599 nm in ethanol.\(^8\) Rhodamines with only one alkyl substituent on each nitrogen (eg, Rho 19, 107) or rigid amino groups (eg, Rho 101, 109) tend to show high quantum yields,\(^8\) while rhodamines with two alkyl substituents at each nitrogen have activated internal conversion, and thus a lower quantum yield, and a temperature-dependent fluorescence lifetime.\(^10\)

Figure 4-2: Structures of fluorescein and its analogues.
Because of their high fluorescence quantum yield, pH-insensitivity, excellent photostability, and photophysical properties, rhodamines are widely used as probes for labeling of biomolecules. In addition, these compounds are used as chemosensors for the detection of Hg (II), Cu (II), Fe (III), Cr (III) and thiols among other analytes. These long-wavelength fluorophores are advantageous as molecular probes because they minimize interference from natural chromophores such as hemoglobin, and they allow multicolor labeling experiments when used with other spectrally orthogonal fluorescent probes. Recently, fluorinated rhodamine analogues with good photophysical properties were made by the Hell group and used for labeling biomolecules in optical microscopy and nanoscopy.

Although rhodamine dyes have a variety of applications as fluorescent probes, the use of rhodamine derived molecular probes to detect proteins expressed in the cytoplasm or nucleus of living cells is challenging. The high polarity of many rhodamines limits their cellular
permeability. Moreover, some rhodamines accumulate in mitochondria, causing increased background fluorescence.\textsuperscript{14} For coupling to biomolecules without affecting photophysical properties, rhodamines are typically modified as activated esters at the 5'-position. Although several of the activated dyes are commercially available, they are either sold as a mixture of 4'- and 5'-activated isomers or as the expensive isomerically pure compound. Using a mixture of the two isomers can cause problems because of differences in bioactivity.\textsuperscript{15} The typical synthesis of rhodamines by condensing aminophenol with trimellitic anhydride leads to a mixture of the 5'-isomer (112) and 4'-isomer (113) (Figure 4-4). The separation of the isomers is laborious because of the similarities between the two structures and the cationic nature of rhodamine dyes. Ideal rhodamine fluorophores for cellular studies would be cell permeable, devoid of non-specific binding to intracellular proteins or organelles, and easy to synthesize and purify.

![Figure 4-4: General synthesis route for rhodamine dyes modified at the 4' and 5' positions.](image)

**Figure 4-4:** General synthesis route for rhodamine dyes modified at the 4' and 5' positions.

### 4.4 Design of Novel Rhodamine Dyes

To improve the cellular properties of rhodamines, we designed novel rhodamine analogues (115-117). These compounds bear either a mono-substituted amino group or a rigid amino group to maximize the quantum yield, and they lack fixed positive charges on the amino
group, designed to improve cell permeability. Because rhodamine 19 (107) is known to bind mitochondria in living cells, the methyl groups in the xanthene ring were substituted with fluorines (compounds 116 and 117) to reduce the basicity of the nitrogen atoms, a strategy for minimizing binding to endogenous cellular targets. The carboxylate in the benzene ring was replaced with a more hydrophobic methyl group in 117 to further improve the cellular permeability. Rhodamine dye 114 bearing rigid amino groups and its more hydrophobic analogue 115 were also synthesized.

Figure 4-5: Structures of known (107, 114) and novel rhodamine dyes (115-117).

4.5 Synthesis of Novel Rhodamine Dyes

To obtain compound 114, an established synthetic method was used (Figure 4-6). Starting from the commercially available 1,2,3,4-tetrahydroquinoline 118, nitration, reduction of the amino group to amine, and hydrolysis of the amino group with 85% H₃PO₄ provided
aminophenol 121. This aminophenol was condensed with phthalic anhydride (122) or trimellitic anhydride (111) to afford the parent fluorophore or its 5'-carboxylic acid derivative.

![Chemical diagram]

Reagents and conditions: (a) HNO₃, H₂SO₄; (b) Ra-Ni, hydrazine, EtOH; (c) H₃PO₄; (d) ZnCl₂.

Figure 4-6: Synthesis of compound 114 and the 5'-carboxy derivative 123.

The synthesis of compound 115 was based on the synthesis of Pennsylvania Green reported by our research group (Figure 4-7). The aminophenol was condensed with o-tolualdehyde 124 or 3-methyl-4-formyl benzoic acid methyl ester 125 in propionic acid with p-TsOH as the catalyst to yield triarylmethanes 126 and 127. Oxidative cyclization of triarylmethanes in the presence of quinone oxidant DDQ provided fluorophores 115 and 128 in moderate yield. Compound 128 was hydrolyzed in basic solution to yield 129.
Condensation with an anhydride was also used to accomplish the synthesis of compound 116 (Figure 4-8). Reductive amination of commercially available 3-amino-4-fluorophenol 130 (Ark Pharm, Inc.) yielded the mono-substituted aminophenol 131, which was condensed with phthalic anhydride (122) or trimellitic anhydride (111) to afford the parent fluorophore or a mixture of 4'-carboxy and 5'-carboxy derivatives. The desired 5'-carboxy isomer was isolated by HPLC.

Figure 4-7: Synthesis of compound 115 and the 4'-carboxy derivative 129.

Figure 4-8: Synthesis of compound 116 and the 5'-carboxy derivative 132.
The widely known Buchwald-Hartwig amination reaction was used by the Yang group to couple fluorescein triflate with different amines to afford rhodol fluorophores. This chemistry was used here to synthesize compound 117 (Figure 4-9). Ditriflate 133, derived from a key intermediate to make Pennsylvania Green in large scale, was synthesized in four steps and high yield by Dr. Zachary Woydziak in our group. Addition of the Grignard derived from 134 or 135 to the xanthene ketone yielded the tertiary alcohol 136 or 137, which was subjected to Buchwald-Hartwig cross coupling to afford the corresponding fluorophore. Ester hydrolysis of 138 with KOH afforded the carboxylic acid 139.

Reagents and conditions: (a) iPrMgCl LiCl, THF; (b) Pd(OAc)$_2$, BINAP, Cs$_2$CO$_3$, toluene, i-PrNH$_2$; (c) KOH, H$_2$O, MeOH.

Figure 4-9: Synthesis of compound 117 and the 4'-carboxy derivative 139.

4.6 Absorption and Emission Spectra of Rhodamine Dyes

The absorption and fluorescence emission spectra of fluorophores are important criteria when choosing fluorophores for molecular probes. The absorption and fluorescence emission
spectra for compounds 114-117 were compared to rhodamine 19 (107) using ethanol as the solvent (Figure 4-10). The absorption and emission maxima of 114, 115 and 117 are red-shifted compared to 107, and the maximum wavelengths are summarized in Table 4-1.

Figure 4-10: Normalized absorbance spectra (Panel A) and fluorescence emission spectra (Panel B) of compounds 114-117 compared with rhodamine 19 (107).

Table 4-1: Wavelengths of maximum absorption and emission of rhodamine analogues.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Absorption (nm)</th>
<th>Emission (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine 19 (107)</td>
<td>522</td>
<td>544</td>
</tr>
<tr>
<td>114</td>
<td>538</td>
<td>558</td>
</tr>
<tr>
<td>115</td>
<td>546</td>
<td>559</td>
</tr>
<tr>
<td>116</td>
<td>518</td>
<td>540</td>
</tr>
<tr>
<td>117</td>
<td>533</td>
<td>553</td>
</tr>
</tbody>
</table>
4.7 Determination of Quantum Yields of Rhodamine Dyes

The fluorescence quantum yield is a term used in fluorescence spectroscopy to define the efficiency with which a compound converts light absorbed to light emitted. In other words, the quantum yield indicates the probability of the excited state being deactivated by fluorescence rather than another non-radiative mechanism. If a compound absorbs photons and emits nothing, the quantum yield is zero. If every photon absorbed leads to emission of a photon, the quantum yield is one.

As shown in Figure 4-11, the fluorescence quantum yields of fluorophores 114-117 were determined using the method of Williams. This method relies on the use of fluorescent standards with known quantum yields. Solutions of the standard and the sample with the same absorbance at the same excitation wavelength can be assumed to be absorbing the same amount of photons. Therefore, the ratio of the integrated fluorescence intensities for the standard and the sample is equal to the ratio of the quantum yields (detailed experimental procedure shown in Section 4.11.2). Rhodamine 19 and rhodamine 6G were used as standards in these experiments. The quantum yields of rhodamine analogues 114-117 range from 0.79 to 0.83. Substitution of the methyl group for carboxylate in rhodamine analogues did not affect the quantum yield.
4.8 Studies of Binding to Intracellular Streptavidin with Biotinylated Fluorophores

To study the utility of the novel rhodamine analogues, biotinylated conjugates were prepared to examine binding to streptavidin expressed in yeast. Streptavidin is a biotin-binding
protein that has versatile applications in the biosciences. Fluorimetric assays for streptavidin have been developed based on the stoichiometric binding of fluorescent biotin derivatives to streptavidin. Biotin-4-fluorescein (140), an alternative to radioactive biotin, was used in vitro to detect and quantitate biotin-binding sites. The Tsien group used a membrane permeable biotin-fluorescein diester Flubida-2 (141), an analogue of cell impermeable compound Flubi-2 (142), to probe an avidin-chimera protein expressed in the endoplasmic reticulum and Golgi of Hela cells. The ester modification was used to improve cellular permeability of the probe, and the ester group was removed by intracellular esterases to unmask the fluorophore of 142. These results illustrate the utility of cell permeable fluorophores in studies of cellular biology.

We aimed to design better fluorescent biotin derivatives as probes of intracellular streptavidin and other proteins using cell permeable fluorophores. Biotinylated analogues of rhodamines 114 and 115 (Figure 4-13) were prepared by coupling of biotin-ethylene diamine (Sigma-Aldrich) with activated N-hydroxysuccinimidyl ester derivatives of fluorophores.
Figure 4-12: Structures of previously reported biotinylated fluoresceins.

Biotin-4-fluorescein (140)

Flubida-2 (141)

Flubi-2 (142)
4.9 Imaging of Streptavidin Fusion Proteins

To study the interaction between biotinylated fluorophores and streptavidin (SA) expressed in the cytoplasm of cells, FY250 yeast was transformed with the streptavidin (SA) expression vector pSA1 SA yEGFP. In this construct, SA is expressed fused to yeast enhanced green fluorescent protein to allow visualization of SA. As a control, FY250 yeast was transformed with the empty vector pSA1. The yeast culture was treated with compounds 143 or 144 (10 μM) at 30 °C for 4 h, washed and visualized in confocal microscopy. Compound 143 was not observed to colocalize with SA-yEGFP (Panel B, Figure 4-14), but the hydrophobic mimic 144 was more cell permeable, and the red fluorescence of 144 was observed to colocalize with the green fluorescent SA-yEGFP (Panel D, Figure 4-14). In the control experiment using the empty vector pSA1, no fluorescence was observed (Panels A and C, Figure 4-14). When 1 mM
biotin was added, biotin was observed to compete with the fluorescent probe for the SA binding sites and thereby blocks the fluorescence colocalization (Panel C, Figure 4-14).

Figure 4-14: Differential interference contrast (DIC) and confocal laser scanning micrographs of FY250 yeast. 488 nm and 532 nm indicate the excitation wavelength. Images acquired by Dr. Kalai Selvi Shanmugam.
4.10 Conclusion

Novel rhodamines 115-117 and rhodamine 114 lacking fixed positive charges were synthesized. Substitution of a carboxylate with a methyl group did not affect their photophysical properties. Compounds 114, 115 and 117 had red-shifted absorption/emission spectra, and all of these compounds had high quantum yields. The utility of 114 and 115 as molecular probes was explored by studies of the biotinylated fluorophores 143 and 144 that specifically bind SA expressed in yeast. The more hydrophobic analogue 144 was more cell permeable compared to 143, and it represents a potentially useful tool for cellular imaging. Fluorine substitutions in rhodamines 116 and 117 were predicted to lower the basicity of the adjacent anilines, making these two fluorophores compatible with peptide coupling protocols and are likely to reduce non-specific binding to endogenous cellular targets. Future study may include synthesis of hydrophobic rhodamine analogues with different substitutions on the nitrogen atoms or the benzene ring to make the fluorophore red-shifted further.

4.11 Experimental Section

4.11.1 Chemical Synthesis

*General Synthesis Information.* Chemical reagents and solvents were obtained from Acros, Aldrich, TCI or Fisher without further purification unless otherwise noted. Anhydrous solvents were obtained after passage through a drying column of a solvent purification system from GlassContour (Laguna Beach, CA). Reactions were performed under an atmosphere of dry nitrogen. Reactions were monitored by analytical thin-layer chromatography on plates coated with 0.25 mm silica gel 60 F254 (EMD Chemicals). TLC plates were visualized by UV irradiation (254 nm) or stained with 20% sulfuric acid in ethanol. Nuclear magnetic resonance
(NMR) spectroscopy employed a Bruker DRX 400 spectrometer. NMR peaks are reported as parts per million (ppm) referenced to internal CHCl$_3$, MeOH or H$_2$O peaks. Mass spectral data was obtained from The University of Kansas Mass Spectrometry Facility. Melting points were determined on a Thomas Hoover apparatus and are uncorrected. Column chromatography employed silica gel (ICN SiliTech, 32-63 µm). Purification by preparative HPLC employed an Agilent 1100 series instrument equipped with a Hamilton PRP-1 (polystyrene-divinylbenzene) column (21.5 × 250 mm, 7 µm). The HPLC flow rate was maintained at 25 mL/min for the duration of the run. Determination of purity of compounds 143 and 144 by analytical HPLC used a PRP-1 (polystyrene-divinylbenzene) reverse-phase column (4.1 × 250 mm, 7 µm; Hamilton) and a gradient of 1% to 99% CH$_3$CN (containing 0.1% TFA) in nanopure water (containing 0.1% TFA, 0 to 30 min) followed by 99% CH$_3$CN (containing 0.1% TFA) from 30-40 min at a flow rate of 0.8 mL/min.

1,2,3,4-Tetrahydro-7-nitroquinoline (119)

1,2,3,4-Tetrahydroquinoline (118, 12.3 mL, 0.100 mol) was slowly added to concentrated sulfuric acid (45 mL) with stirring and cooling with an ice water bath (4 °C). After 15 min, a precooled mixture of concentrated sulfuric acid (20 mL) and concentrated nitric acid (4.4 mL) was added slowly into the reaction mixture with vigorous stirring and intensive cooling in an ice water bath. The mixture was stirred at 4 °C for 3 h, and poured into a beaker with crushed ice (500 g). The resulted solution was carefully neutralized to pH 7-8 with cold concentrated aqueous NaOH solution. The precipitate was filtered and recrystallized in MeOH to yield the product as a
red solid (11.2 g, 63%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 8.06 (dd, 1H, $J_1 = 2.3$ Hz, $J_2 = 8.2$ Hz), 7.93 (d, 1H, $J = 2.3$ Hz), 7.68 (d, 1H, $J = 8.2$ Hz), 4.02 (t, 2H, $J = 5.6$ Hz), 3.48 (t, 2H, $J = 6.4$ Hz), 2.65-2.59 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 147.2, 145.2, 129.7, 128.3, 111.2, 107.8, 41.5, 27.3, 21.1.

**1,2,3,4-Tetrahydroquinolin-7-amine (120)**

1,2,3,4-tetrahydro-7-nitroquinoline (119, 4.50 g, 25.0 mmol) was dissolved in MeOH (20 mL), and raney nickel was added into the solution. The reaction mixture was warmed to 60 °C, and a solution of hydrazine monohydrate in MeOH (5 mL) was added slowly over 1 h. After reacting at 60 °C for 3 h, the reaction solution was cooled and filtered through celite. The filtrate was evaporated in vacuo and the crude product was purified by flash chromatography (eluent: hexane, EtOAc, 1:1) to yield 3.50 g of an off-white solid 120 (94%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.77 (d, 1H, $J = 7.9$ Hz), 6.04 (dd, 1H, $J_1 = 2.3$ Hz, $J_2 = 7.9$ Hz), 5.86 (d, 1H, $J = 2.3$ Hz), 3.55 (br, 3H), 3.29-3.27 (m, 2H), 2.69 (t, 2H, $J = 6.4$ Hz), 1.96-1.90 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 145.4, 145.3, 130.2, 112.3, 105.0, 100.8, 42.1, 26.3, 22.7.

**1,2,3,4-Tetrahydroquinolin-7-ol (121)**

1,2,3,4-Tetrahydroquinolin-7-amine (120, 2.00 g, 13.5 mmol) was dissolved in 85% aqueous H$_3$PO$_4$ solution, and the solution was refluxed under N$_2$ at 150 °C for 14 h. The reaction
mixture was poured into crushed ice (160 g) containing NaOH (30 g). The pH value of the mixture obtained was ~12. After cooling to room temperature (22 °C), the precipitated salt was filtered off. The filtrate was washed with CH$_2$Cl$_2$ (3 × 100 mL), and carefully neutralized to pH 7-8 with cold aqueous HCl solution (18%). The aqueous phase was extracted with CH$_2$Cl$_2$ (3 × 150 mL), and the combined organic layers were dried over anhydrous sodium sulfate and evaporated in vacuo. The crude product was recrystallized in MeOH to afford 1.30 g 121 as an off-white solid (65%). $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 6.55 (d, 1H, $J$ = 7.8 Hz), 5.85-5.82 (m, 2H), 5.44 (s, 1H), 3.08 (t, 2H, $J$ = 4.4 Hz), 2.52-2.48 (m, 2H), 1.74-1.68 (m, 2H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta$ 156.4, 146.5, 129.8, 111.3, 103.4, 100.3, 41.2, 26.5, 22.5.

**Compound 114**

A mixture of 1,2,3,4-tetrahydroquinolin-7-ol (121, 100 mg, 0.671 mmol), phthalic anhydride (122, 50.0 mg, 0.336 mmol) and zinc chloride (274 mg, 2.01 mmol) was heated to 170-180 °C for 1 h and cooled to room temperature (22 °C). Aqueous HCl (1.5 mL 1 M) was added, and the mixture was heated to 110 °C for 1 h. The reaction mixture was cooled to room temperature (22 °C), and further cooled to 0 °C, and the product crashed out of the solution as a purple solid. The crude product was filtered and purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 10:1) to yield the final product as a purple solid (114, 23.4 mg, 17%). mp 248-250 °C; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.30 (dd, 1H, $J_1$ = 1.0 Hz, $J_2$ = 7.6 Hz), 7.83-7.74 (m,
2H), 7.33 (dd, 1H, J₁ = 1.2 Hz, J₂ = 7.4 Hz), 6.71 (s, 2H), 6.60 (s, 2H), 3.42 (t, 4H, J = 5.5 Hz), 2.66 (t, 4H, J = 6.0 Hz), 1.90-1.84 (m, 4H); ¹³C NMR (100 MHz, CD₃OD) δ 157.9, 156.3, 155.3, 134.3, 132.4, 131.0, 130.6, 130.1, 129.8, 128.6, 128.5, 123.6, 113.4, 96.3, 41.2, 26.4, 20.0; IR (film) v max 3324, 2940, 1687, 1646, 1620, 1572, 1510, 1407, 1314, 1178, 1130, 1070, 827 cm⁻¹; HRMS (ESI⁺) m/z 411.1705 (M+H⁺, C₂₆H₂₃N₂O₃ requires 411.1709).

**Compound 123**

A mixture of 1,2,3,4-tetrahydroquinolin-7-ol (121, 200 mg, 1.34 mmol), trimellitic anhydride (111, 129 mg, 0.670 mmol) and zinc chloride (549 mg, 4.03 mmol) was heated up to 170-180 °C for 1 h and cooled down to room temperature (22 °C). Aqueous HCl (2.5 mL 1 M) was added, and the mixture was heated to 110 °C for 1 h. The reaction mixture was cooled to room temperature (22 °C), and further cooled to 0 °C, and the product crashed out of the solution as a purple solid. The crude product was filtered out and purified by prep HPLC to yield the final product as a purple solid (123, 45.7 mg, 15%). mp 259-260 °C; ¹H NMR (400 MHz, CD₃OD) δ 8.89 (d, 1H, J = 1.6 Hz), 8.41 (dd, 1H, J₁ = 1.6 Hz, J₂ = 7.8 Hz), 7.48 (d, 1H, J = 7.8 Hz), 6.71 (s, 2H), 6.64 (s, 2H), 3.44 (t, 4H, J = 5.4 Hz), 2.69 (t, 4H, J = 5.6 Hz), 1.90-1.84 (m, 4H); ¹³C NMR (100 MHz, CD₃OD) δ 166.6, 166.0, 156.6, 156.2, 155.4, 153.0, 132.6, 132.1, 131.4, 130.7, 128.6, 128.3, 123.8, 113.0, 96.4, 41.2, 26.4, 20.0; IR (film) v max 3270, 3154, 2923, 2857, 1706, 1646, 1610, 1505, 1411, 1342, 1306, 1227, 1174, 1078, 990, 840, 763 cm⁻¹; HRMS (ESI⁺) m/z 455.1612 (M+H⁺, C₂₇H₂₃N₂O₅ requires 455.1607).
Compound 143

123 (20.0 mg, 0.0440 mmol), DCC (18.2 mg, 0.0880 mmol) and N-hydroxysuccinimide (5.60 mg, 0.0484 mmol) were dissolved in DMF (2 mL) and allowed to stir at room temperature (22 °C) for 14 h. After 14 h, the urea was removed by filtration, the filtrate was concentrated in vacuo, and purified by flash chromatography (eluent: CH₂Cl₂, MeOH, 15:1) to yield the NHS ester. The NHS ester and biotin ethylene diamine (Sigma-Aldrich) (16.2 mg, 0.0440 mmol) were dissolved in DMF (1 mL), and DIEA (40 µL) was added. The reaction was stirred at room temperature (22 °C) for 16 h, and purified by preparative reverse phase HPLC (gradient: 1% to 99% CH₃CN containing 0.1% TFA in nanopure water containing 0.1% TFA, 0 to 20 min) to afford 143 (20.0 mg, 63%) as a purple solid. mp 261-263 °C; ¹H NMR (400 MHz, CD₃OD) δ 8.48 (d, 1H, J = 1.4 Hz), 8.35 (dd, 1H, J₁ = 1.5 Hz, J₂ = 7.9 Hz), 7.52 (d, 1H, J = 7.9 Hz), 6.82 (d, 2H, J = 6.1 Hz), 6.63 (d, 2H, J = 3.2 Hz), 4.49-4.46 (m, 1H), 4.29-4.26 (m, 1H), 3.44 (t, 4H, J = 5.4 Hz), 3.31-3.11 (m, 5H), 2.90 (dd, 1H, J₁ = 5.0 Hz, J₂ = 12.8 Hz), 2.68 (m, 5H), 2.10 (t, 2H, J = 7.4 Hz), 1.93-1.84 (m, 4H), 1.59-1.28 (m, 6H); ¹³C NMR (100 MHz, CD₃OD) δ 174.8, 168.2, 156.2, 155.5, 155.3, 155.2, 136.8, 136.3, 132.5, 131.4, 130.8, 128.8, 128.6, 128.4, 123.8, 123.6, 113.1, 113.0, 96.5, 96.4, 61.8, 60.3, 55.5, 41.2, 39.6, 38.9, 35.4, 29.3, 28.3, 28.0, 26.4, 25.3, 23.8, 20.0; IR (film) ν max 3291, 2928, 2846, 1677, 1643, 1610, 1503, 1306, 1200, 1139, 990, 837, 798 cm⁻¹; HRMS (ESI+) m/z 723.2955 (M+H⁺, C₃₀H₃₃N₆O₆S requires 723.2965).
Analytical reverse-phase HPLC profile of 143. Retention time = 15.7 min. Purity = 95%. Absorbance wavelength = 254 nm.

1,2,3,4-Tetrahydro-6-((1,2,3,4-tetrahydro-7-hydroxyquinolin-6-yl)(o-tolyl)methyl)quinolin-7-ol (126)

1,2,3,4-tetrahydroquinolin-7-ol (121, 100 mg, 0.671 mmol) and o-tolualdehyde (39 μL, 0.335 mmol) were dissolved in propionic acid (2 mL), and p-TsOH (3 mg, 0.0158 mmol) was added. The reaction mixture was heated to 80-85 °C under an atmosphere of N₂ for 2 h. The solution was cooled to room temperature (22 °C), further cooled to 0 °C, and poured into excess 3 M aqueous sodium acetate solution (12 mL). The precipitate was collected by filtration, washed with cold water, and purified by flash chromatography (eluent: hexane, EtOAc, 1:1) to yield 126 (106 mg, 79%) as a white solid. mp 195-197 °C; ¹H NMR (400 MHz, CD₃OD) δ 7.04-6.96 (m, 3H), 6.78 (d, 1H, J = 7.0 Hz), 6.21 (s, 2H), 6.04 (s, 2H), 5.87 (s, 1H), 3.13 (t, 4H, J = 4.6 Hz), 2.48 (t, 4H, J = 5.8 Hz), 2.13 (s, 3H), 1.85-1.81 (m, 4H); ¹³C NMR (100 MHz, CD₃OD) δ 153.1,
143.8, 143.3, 136.5, 130.5, 128.0, 125.0, 124.6, 120.6, 113.0, 101.8, 41.6, 39.0, 26.0, 22.5, 18.3; IR (film) ν max 3401, 3330, 2923, 2841, 1620, 1518, 1328, 1235, 1184, 1111, 834 cm⁻¹; HRMS (ESI⁺) m/z 401.2237 (M+H⁺, C₂₆H₂₉N₂O₂ requires 401.2229).

**Compound 115**

1,2,3,4-Tetrahydro-6-((1,2,3,4-tetrahydro-7-hydroxyquinolin-6-yl)(o-tolyl)methyl)quinolin-7-ol (126, 53.0 mg, 0.132 mmol) was dissolved in AcOH/benzene (6 mL, 1:1), and a solution of DDQ (60.0 mg, 0.264 mmol) in AcOH/benzene (3 mL, 1:1) was added dropwise. The mixture was stirred at room temperature (22 °C) for 15 min, and the solvent was removed in vacuo. The product was purified by flash chromatography (eluent: CH₂Cl₂, MeOH, 20:1) and further purified by prep HPLC to yield 115 (15.0 mg, 31%) as a purple solid. mp 127-128 °C; ¹H NMR (400 MHz, CD₃OD) δ 7.55-7.41 (m, 3H), 7.19 (d, 1H, J = 6.9 Hz), 6.77 (s, 2H), 6.65 (s, 2H), 3.45 (t, 4H, J = 5.6 Hz), 2.70 (t, 4H, J = 6.1 Hz), 2.03 (s, 3H), 1.92-1.87 (m, 4H); ¹³C NMR (100 MHz, CD₃OD) δ 157.9, 157.4, 157.0, 137.2, 133.7, 131.8, 131.0, 130.1, 127.3, 125.4, 114.5, 97.9, 42.7, 27.8, 21.4, 19.6; IR (film) ν max 3253, 3137, 2923, 2857, 1676, 1849, 1611, 1507, 1412, 1342, 1308, 1196, 1174, 1130, 990, 831, 716 cm⁻¹; HRMS (ESI⁺) m/z 381.1960 (M+H⁺, C₂₆H₂₃N₂O requires 381.1967).
Methyl 4-(bis(1,2,3,4-tetrahydro-7-hydroxyquinolin-6-yl)methyl)-3-methylbenzoate (127)

1,2,3,4-tetrahydroquinolin-7-ol (121, 300 mg, 2.01 mmol) and 4-formyl-3-methylmethylbenzoate (125, 180 mg, 1.01 mmol) were dissolved in propionic acid (8 mL), and p-TsOH (12.0 mg, 0.0631 mmol) was added. The reaction mixture was heated to 80-85 °C under an atmosphere of N₂ for 2 h. The solution was cooled to room temperature (22 °C), further cooled to 0 °C, and poured into excess 3 M aqueous sodium acetate solution (48 mL). The precipitate was collected by filtration, washed with cold water, and purified by flash chromatography (eluent: hexane, EtOAc, 1:1) to yield 127 (340 mg, 73%) as a white solid. mp 132-134 °C; 

$^1$H NMR (400 MHz, DMSO-$d_6$) δ 8.51 (s, 1H), 7.65-7.62 (m, 2H), 6.91 (d, 1H, $J = 7.9$ Hz), 6.09 (s, 2H), 5.92 (s, 2H), 5.76 (s, 1H), 5.35 (s, 2H), 3.80 (s, 3H), 3.08 (t, 4H, $J = 5.0$ Hz), 2.40 (t, 4H, $J = 5.8$ Hz), 2.16 (s, 3H), 1.72-1.67 (m, 4H); 

$^{13}$C NMR (100 MHz, DMSO-$d_6$) δ 167.0, 153.7, 151.9, 144.4, 136.8, 130.8, 130.4, 128.6, 126.8, 126.5, 117.3, 110.6, 100.7, 55.4, 52.2, 41.3, 26.7, 22.7, 19.4; IR (film) ν max 3385, 2918, 2841, 1702, 1619, 1518, 1432, 1296, 1188, 1114, 905, 842 cm$^{-1}$; HRMS (ESI+) $m/z$ 459.2277 (M+H+, C$_{28}$H$_{31}$N$_2$O$_4$ requires 459.2284).
**Compound 129**

Methyl 4-(bis(1,2,3,4-tetrahydro-7-hydroxyquinolin-6-yl)methyl)-3-methylbenzoate (127, 175 mg, 0.382 mmol) was dissolved in AcOH/benzene (6 mL, 1:1), and a solution of DDQ (170 mg, 0.764 mmol) in AcOH/benzene (3 mL, 1:1) was added dropwise. The mixture was stirred at room temperature (22 °C) for 15 min, and the solvent was removed in vacuo. The product was purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 10:1) and further purified by preparative HPLC to yield 128 (45.3 mg, 28%) as a purple solid. mp 242-244 °C; $^1$H NMR (400 MHz, CD$_3$OD) δ 8.08 (s, 1H), 8.02 (d, 1H, $J = 7.6$ Hz), 7.20 (d, 1H, $J = 7.6$ Hz), 6.74 (s, 2H), 6.64 (s, 2H), 3.44 (t, 4H, $J = 5.8$ Hz), 2.66 (t, 4H, $J = 5.8$ Hz), 2.05 (s, 3H), 1.90-1.84 (m, 4H); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 156.4, 155.6, 155.4, 137.3, 135.7, 134.9, 131.3, 128.6, 128.5, 126.8, 124.0, 112.9, 96.6, 41.3, 25.6, 20.0, 18.3; IR (film) ν max 3242, 3137, 2923, 2852, 1684, 1649, 1611, 1506, 1413, 1308, 1224, 1174, 1130, 990, 952, 834 cm$^{-1}$; HRMS (ESI+) m/z 425.1858 (M+H$, C_{27}H_{25}N_{2}O_{3}$ requires 425.1865).

**Compound 144**

129 (15.0 mg, 0.0354 mmol), DCC (14.6 mg, 0.0708 mmol) and $N$-hydroxysuccinimide (8.20 mg, 0.0712 mmol) were dissolved in DMF (2 mL) and allowed to stir at room temperature (22 °C) for 14 h. After 14 h, the urea was removed by filtration, and the filtrate was concentrated in vacuo and purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 15:1) to yield the NHS...
ester. The NHS ester and biotin ethylene diamine (Sigma-Aldrich) (13.0 mg, 0.0354 mmol) were dissolved in DMF (1 mL), and DIEA (30 µL) was added. The reaction was stirred at room temperature (22 °C) for 16 h, and purified by preparative reverse phase HPLC (gradient: 1% to 99% CH₃CN containing 0.1% TFA in nanopure water containing 0.1% TFA, 0 to 20 min) to afford 144 (16.0 mg, 65%) as a purple solid. mp 256-258 °C; ¹H NMR (400 MHz, CD₃OD) δ 7.92 (s, 1H), 7.86 (d, 1H, J = 7.8 Hz), 7.32 (d, 1H, J = 7.8 Hz), 6.73 (s, 2H), 6.66 (s, 2H), 4.48-4.44 (m, 1H), 4.30-4.26 (m, 1H), 3.57-3.44 (m, 8H), 3.19-3.15 (m, 1H), 2.88 (dd, 1H, J₁ = 4.9 Hz, J₂ = 12.7 Hz), 2.70-2.67 (m, 5H), 2.23 (t, 2H, J = 7.0 Hz), 2.10 (s, 3H), 1.90-1.87 (m, 4H), 1.70-1.42 (m, 6H); ¹³C NMR (100 MHz, CD₃OD) δ 175.4, 168.2, 156.4, 155.7, 154.6, 136.6, 135.7, 135.6, 129.2, 129.1, 128.4, 124.8, 124.2, 112.8, 96.6, 61.9, 60.2, 55.5, 41.3, 39.8, 39.6, 38.6, 35.4, 28.3, 28.0, 26.4, 25.4, 23.8, 20.0, 18.3; IR (film) ν max 3258, 2923, 2852, 1676, 1646, 1611, 1505, 1413, 1309, 1199, 1174, 1133, 991, 836, 719 cm⁻¹; HRMS (ESI+) m/z 693.3221 (M+H⁺, C₃₉H₄₅N₆O₄S requires 693.3223).

Analytical reverse-phase HPLC profile of 144. Retention time = 16.3 min. Purity = 97%. Absorbance wavelength = 254 nm.
3-(Ethylamino)-4-fluorophenol (131)

3-Amino-4-fluorophenol (130, 127 mg, 1.00 mmol) in ethanol (8 mL) containing acetaldehyde (40% wt% in iPrOH, 1.00 mmol, 0.140 mL) and palladium on carbon (10%, 10.6 mg, 0.01 mmol) was shaken on a Parr apparatus for 3 h under H₂ at 40 psi. The catalyst was removed by filtration through celite, and the filtrate was concentrated in vacuo. The crude product was purified by flash chromatography (eluent: hexane, EtOAc, 10:1) to afford 131 (113 mg, 73%) as a white solid. mp 62-63 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.02 (dd, 1H, J₁ = 2.9 Hz, J₂ = 8.6 Hz), 7.41 (dd, 1H, J₁ = 2.9 Hz, J₂ = 7.2 Hz), 7.28-7.24 (m, 1H), 4.32 (q, 2H, J = 7.2 Hz), 2.48 (t, 3H, J = 7.2 Hz); ¹³C NMR (100 MHz, CDCl₃) δ 152.4, 147.6, 145.3, 137.6, 114.6, 114.4, 102.1, 99.9; IR (film) ν max 3350, 2974, 2868, 1633, 1602, 1524, 1456, 1376, 1292, 1197, 1141, 983, 769 cm⁻¹; HRMS (ESI+) m/z 156.0820 (M+H⁺, C₈H₁₁FNO requires 156.0825).

2-(3-(Ethylamino)-6-(ethylimino)-2,7-difluoro-6H-xanthen-9-yl)benzoic acid (116)

A mixture of 3-(ethylamino)-4-fluorophenol (131, 61.1 mg, 0.394 mmol), phthalic anhydride (122, 29.2 mg, 0.197 mmol) and zinc chloride (161 mg, 1.18 mmol) was heated to 170-180 °C for 1 h and cooled to room temperature (22 °C). Aqueous HCl (1.3 mL, 1 M) was added, and the mixture was heated to 110 °C for 1 h. The reaction mixture was cooled to room temperature (22 °C), further cooled to 0 °C, and the product crashed out of the solution as a
purple solid. The crude product was filtered out and purified by flash chromatography (eluent: CH$_2$Cl$_2$, MeOH, 10:1) to yield the final product as a purple solid (131, 14.0 mg, 17%). mp 151-152 °C; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.36 (d, 1H, $J = 7.3$ Hz), 7.88-7.80 (m, 2H), 7.41 (d, 1H, $J = 7.0$ Hz), 7.06 (d, 2H, $J = 6.7$ Hz), 6.82 (d, 2H, $J = 11.6$ Hz), 3.49 (q, 4H, $J = 7.0$ Hz), 1.34 (t, 6H, $J = 7.0$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 160.7, 155.8, 151.3, 148.8, 148.0, 147.8, 133.4, 132.7, 131.4, 130.5, 129.8, 112.8, 112.7, 110.9, 110.7, 95.4, 95.3, 37.8, 12.4; IR (film) $\nu$ max 3306, 2923, 2852, 1657, 1621, 1512, 1314, 1194, 1136, 971, 744, 719 cm$^{-1}$; HRMS (ESI+) $m/z$ 423.1514 (M+H$^+$, C$_{24}$H$_{21}$F$_2$N$_2$O$_3$ requires 423.1520).

4-(3-(Ethylamino)-6-(ethylimino)-2,7-difluoro-6H-xanthen-9-yl)benzene-1,3-dioic acid (132)

A mixture of 3-(ethylamino)-4-fluorophenol (131, 136 mg, 0.872 mmol), trimellitic anhydride (111, 84.0 mg, 0.436 mmol) and zinc chloride (356 mg, 2.62 mmol) was heated to 170-180 °C for 1 h and cooled to room temperature (22 °C). Aqueous HCl (2.5 mL, 1 M) was added, and the mixture was heated to 110 °C for 1 h. The reaction mixture was cooled to room temperature (22 °C), further cooled to 0 °C, and the product crashed out of the solution as a purple solid. The crude product was filtered out and purified by preparative HPLC to yield the final product as a purple solid (132, 28.5 mg, 14%). mp 148-150 °C; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 8.94 (s, 1H), 8.45 (d, 1H, $J = 7.8$ Hz), 7.53 (d, 1H, $J = 7.8$ Hz), 7.08 (d, 1H, $J = 6.7$ Hz), 6.84 (d, 2H, $J = 11.6$ Hz), 3.50 (q, 4H, $J = 7.2$ Hz), 1.35 (t, 6H, $J = 7.2$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 166.4, 165.8, 155.8, 151.4, 148.9, 148.1, 147.9, 137.3, 133.4, 133.2, 132.4, 131.1, 130.4, 112.6, 112.5, 110.8, 110.6, 95.4, 37.9, 12.4; IR (film) $\nu$ max 3363, 2923, 2852,
2,7-Difluoro-9-hydroxy-9-(o-tolyl)-9H-xanthene-3,6-diyl bis(trifluoromethanesulfonate) (136)

2-Iodo-toluene (134, Aldrich, 530 µL, 2.00 mmol) was dissolved in dry THF (15 mL) in a flame dried flask. The solution was cooled to -78 °C, and isopropylmagnesium chloride lithium chloride complex solution (Aldrich, 1.3 M in THF, 1.50 mL, 2.00 mmol) was added dropwise. The mixture was warmed to 4 °C, stirred at 4 °C for 1 h, and cooled to -78 °C. A solution of 133 (840 mg, 1.60 mmol) in dry THF was added dropwise. The solution was stirred at -78 °C for 15 min, warmed up to 4 °C, allowed to warm up to room temperature (22 °C), and stirred at room temperature for 14 h. The reaction was quenched by adding saturated NaHCO₃ solution (50 mL), and the mixture was extracted with CH₂Cl₂ (100 mL × 2). The organic layers were combined, washed with saturated NaCl solution (100 mL), dried over anhydrous sodium sulfate and evaporated in vacuo. The crude product was purified by flash chromatography (hexane, EtOAc, 20:1) to yield 136 as a white foam (734 mg, 74%). mp 80-82 °C; ¹H NMR (400 MHz, CDCl₃) δ 7.39-7.37 (m, 1H), 7.29-6.95 (m, 3H), 7.08 (d, 2H, J = 8.1 Hz), 6.95 (d, 2H, J = 6.1 Hz), 2.91 (s, 1H), 2.53 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 152.4, 149.9, 145.6, 145.0, 144.5, 137.0, 135.1, 134.6, 130.5, 127.1, 125.6, 125.1, 125.0, 120.2, 116.9, 116.7, 112.0, 70.9, 20.3; IR (film) v max 3402, 1726, 1708, 1491, 1430, 1322, 1223, 1215, 1140, 1069, 1003, 923, 832, 792 748 cm⁻¹; HRMS (ESI+) m/z 602.9853 (M-OH⁺, C₂₂H₁₁F₈O₇S₂ requires 602.9846).
2,7-Difluoro-N-isopropyl-3-(isopropylimino)-9-(o-tolyl)-3H-xanthen-6-amine (117)

A flame dried flask was charged with 136 (40.0 mg, 0.0645 mmol), Pd(OAc)$_2$ (4.00 mg, 0.0177 mmol), (±)-BINAP (11.0 mg, 0.0177 mmol), cesium carbonate (57.5 mg, 0.177 mmol), and the flask was degassed with argon twice. Toluene (2 mL) was flushed with argon for 20 min and added into the reaction flask. Isopropylamine (100 μL, 1.18 mmol) was added. The solution was stirred at room temperature (22 °C) for 20 min, and heated to 90 °C for 16 h. The reaction mixture was cooled to room temperature (22 °C), diluted with CH$_2$Cl$_2$ (30 mL), and filtered through a frit funnel. The filtrate was evaporated in vacuo, and the crude product was purified by flash chromatography (CH$_2$Cl$_2$, MeOH, 20:1) to yield 117 as a purple solid (5.4 mg, 20%). mp 248-250 °C; $^1$H NMR (400 MHz, CD$_3$OD) $\delta$ 7.38-7.36 (m, 1H), 7.31-6.94 (m, 3H), 7.06 (d, 2H, $J$ = 8.0 Hz), 6.92 (d, 2H, $J$ = 5.9 Hz), 3.57 (q, 4H, $J$ = 7.2 Hz), 2.73 (s, 3H), 1.39 (t, 6H, $J$ = 7.2 Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) $\delta$ 165.3, 155.2, 151.2, 148.9, 148.0, 147.5, 137.2, 133.7, 133.0, 131.4, 131.1, 130.2, 112.5, 112.2, 110.3, 110.2, 95.8, 12.6; IR (film) $\nu$ max 3263, 2911, 1710, 1673, 1526, 1413, 1328, 1185, 1132, 1054, 739 cm$^{-1}$; HRMS (ESI+) m/z 421.2020 (M+H$^+$, C$_{26}$H$_{27}$F$_2$N$_2$O$_1$ requires 421.2013).
Compound 137

Methyl 4-iodo-3-methylbenzoate (135, TCI America, 745 mg, 2.70 mmol) was dissolved in dry THF (15 mL) in a flame dried flask. The solution was cooled to -78 °C, and isopropylmagnesium chloride lithium chloride complex solution (Aldrich, 1.3 M in THF, 2.10 mL, 2.70 mmol) was added dropwise. The mixture was warmed to 4 °C, stirred at 4 °C for 1 h, and cooled to -78 °C. A solution of 133 (950 mg, 1.80 mmol) in dry THF was added dropwise. The solution was stirred at -78 °C for 15 min, warmed up to 4 °C, allowed to warm up to room temperature (22 °C), and stirred at room temperature for 14 h. The reaction was quenched by adding saturated NaHCO₃ solution (50 mL), and the mixture was extracted with CH₂Cl₂ (100 mL × 2). The organic layers were combined, washed with saturated NaCl solution (100 mL), dried over anhydrous sodium sulfate and evaporated in vacuo. The crude product was purified by flash chromatography (hexane, EtOAc, 20:1) to yield 137 as a white foam (867 mg, 71%). mp 86-88 °C; ¹H NMR (400 MHz, CDCl₃) δ 8.38 (d, 1H, J = 8.2 Hz), 8.08 (dd, 1H, J₁ = 1.4 Hz, J₂ = 8.2 Hz), 7.75 (s, 1H), 7.25 (d, 2H, J = 6.1 Hz), 6.94 (d, 2H, J = 9.6 Hz), 3.92 (s, 3H), 2.91 (s, 1H), 1.53 (s, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 166.6, 151.4, 148.9, 145.9, 145.3, 137.2, 137.1, 135.4, 133.6, 130.8, 127.4, 125.9, 125.1, 125.0, 120.2, 116.9, 116.7, 112.0, 69.0, 52.3, 20.6; IR (film) ν max 3422, 1723, 1700, 1490, 1429, 1303, 1246, 1215, 1138, 1067, 1008, 958, 844, 797, 765 cm⁻¹; HRMS (ESI+) m/z 660.9883 (M-OH⁺, C₂₅H₁₃F₆O₉S₂ requires 660.9873).
A flame dried flask was charged with 137 (50.0 mg, 0.0737 mmol), Pd(OAc)$_2$ (5.00 mg, 0.0221 mmol), (±)-BINAP (13.8 mg, 0.0221 mmol), cesium carbonate (72.0 mg, 0.221 mmol), and the flask was degassed with argon twice. Toluene (2 mL) was flushed with argon for 20 min and added into the reaction flask. Isopropylamine (125 μL, 1.47 mmol) was added. The solution was stirred at room temperature (22 °C) for 20 min, and heated to 90 °C for 16 h. The reaction mixture was cooled to room temperature (22 °C), diluted with CH$_2$Cl$_2$ (30 mL), and filtered through a frit funnel. The filtrate was evaporated in vacuo, and the crude product was purified by flash chromatography (CH$_2$Cl$_2$, MeOH, 20:1) to yield 138 as a purple solid (8 mg, 23%). mp 173-175 °C; $^1$H NMR (400 MHz, CD$_3$OD) δ 8.17 (s, 1H), 8.09 (dd, 1H, $J_1 = 1.0$ Hz, $J_2 = 7.9$ Hz), 7.40 (d, 1H, $J = 7.9$ Hz), 7.17 (d, 2H, $J = 6.8$ Hz), 6.84 (d, 2H, $J = 11.6$ Hz), 4.09-4.05 (m, 2H), 3.97 (s, 3H), 2.12 (s, 3H), 1.38 (d, 12 H, $J = 6.4$ Hz); $^{13}$C NMR (100 MHz, CD$_3$OD) δ 166.4, 157.0, 156.1, 151.6, 149.1, 147.6, 147.4, 136.7, 136.0, 132.0, 131.5, 129.0, 127.0, 112.4, 112.3, 110.9, 110.6, 96.0, 95.9, 51.6, 45.4, 20.5, 18.1; IR (film) ν max 3232, 1720, 1689, 1620, 1580, 1507, 1389, 1316, 1195, 1166, 1132, 985, 754 cm$^{-1}$; HRMS (ESI+) $m/z$ 479.2140 (M+H$^+$, C$_{28}$H$_{26}$F$_2$N$_2$O$_3$ requires 479.2146).
9-(4-Carboxy-2-methylphenyl)-6-(isopropylamino)-N-isopropyl-3H-xanthen-3-imine (139)

138 (8.00 mg, 0.0167 mmol) was dissolved in MeOH/H₂O (3:2, 2 mL), and treated with aqueous KOH solution (2 M, 0.2 mL). After complete deprotection of the methyl ester (4 h), methanol was removed in vacuo, and the resulting solution was acidified with concentrated HCl solution to pH 4. The aqueous solution was extracted with EtOAc (30 mL × 3), and the combined organic layers were washed with aqueous saturated NaCl solution (50 mL), and dried over anhydrous sodium sulfate. Flash chromatography (CH₂Cl₂, MeOH, 10:1) yielded 139 (6.6 mg, 85%) as a purple solid. mp 270-272 °C; ¹H NMR (400 MHz, CD₃OD) δ 8.90 (s, 1H), 8.42 (d, 1H, J = 7.7 Hz), 7.49 (d, 1H, J = 7.7 Hz), 7.09 (d, 1H, J = 6.7 Hz), 6.81 (d, 2H, J = 11.6 Hz), 3.57 (q, 4H, J = 7.2 Hz), 1.39 (t, 6H, J = 7.2 Hz); ¹³C NMR (100 MHz, CD₃OD) δ 166.4, 165.8, 155.8, 151.4, 148.9, 148.1, 147.9, 137.3, 133.4, 133.2, 132.4, 131.1, 130.4, 112.6, 112.5, 110.8, 110.6, 95.4, 37.9, 12.4; IR (film) ν max 3264, 2923, 1720, 1654, 1618, 1504, 1418, 1314, 1191, 1133, 1030, 754 cm⁻¹; HRMS (ESI+) m/z 465.1985 (M+H⁺, C₂₇H₂₇F₂N₂O₃ requires 465.1990).

4.11.2 Determination of Quantum Yield

Quantum yields for 114-117 were determined using a PTi MD-5020 fluorimeter. Rhodamine 19 (QY = 0.95) and rhodamine 6G (QY = 0.94) were used as the standards. Solutions of standards and samples in ethanol in a 1 cm path length quartz cuvette were excited at 540 nm and the integrated fluorescence emission (550 nm to 620 nm) was quantified. This was repeated
with increasing concentrations of sample (20 nM to 100 nM). The integrated fluorescence intensity (the area of the fluorescence emission spectrum) was plotted against the absorbance of the sample at the concentration studied as extrapolated from absorbance measurement at higher concentrations (2 μM). The slope was determined by linear least squares fitting of the data (including a zero intercept), and it would be proportional to the quantum yield of the samples. The two standards were cross-calibrated to make sure that the experimental quantum yields match literature precedence within 10%. The quantum yields were calculated according to the following equation:

\[
\Phi_x = \Phi_{st} \frac{\text{Grad}_x}{\text{Grad}_{st}} \left( \frac{\eta^2_x}{\eta^2_{st}} \right)
\]

where the \( \Phi_{st} \) is the quantum yield of the standard, and \( \Phi_x \) is the quantum yield of the sample, \( \text{Grad} \) represents the slope from the plot of integrated fluorescence intensity vs absorbance, and \( \eta \) stands for the refractive index of the solvent. In this case, the refractive index values for the standard and the sample are identical, because the same solvent was used.

### 4.11.3 Imaging of Expressed Streptavidin

Saccharomyces cerevisiae FY250 were transformed with the Streptavidin (SA) expression vector pSA1 T7 SA Y43A yEGFP. As control, S. cerevisiae FY250 were transformed with the empty vector pSA1 by the lithium acetate method. The yeast transformant colonies were inoculated in selection media (yeast nitrogen base (YNB) without amino acids, Brent Supplement Mixture (BSM) minus Tryptophan, 2% raffinose, Penicillin and Streptomycin (100 units/mL)) and grown overnight at 30 °C. On a sterile 96-well plate, the yeast culture was diluted in 1:5 ratio in selection media (YNB without amino acids, BSM minus tryptophan, 1% raffinose, 2% galactose). Compounds 143 or 144 (10 μM in media containing 1% DMSO) were added and
the plate was shaken at 30 °C for 4 h. As a control, in addition to the compound, 1 mM biotin was added. The plate was centrifuged at 4000 rpm for 10 min and the pellet was washed twice with fresh selection media. The pellet was resuspended in selection media and visualized by confocal microscopy, by excitation with 20% 488 nm, 100% 488 nm laser or 20% 532 nm laser.

4.12 References


VITA

Runzhi Wu

Education

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<tr>
<th>Year</th>
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<tr>
<td>2005-present</td>
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Research and Scientific Experience

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<th>Year</th>
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Publications

