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Title of Dissertation: Exploring Flexible Purine Nucleobase Recognition by Biological Targets

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ABSTRACT

Title of Document: EXPLORING FLEXIBLE NUCLEOBASE RECOGNITION BY BIOLOGICAL TARGETS.

Therese Chao-Yar Ku, Ph.D., 2018

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FDA approved nucleos(t)ide analogue inhibitors are increasingly plagued by the rapid loss of efficacy due to the diseases' ability to develop resistance. Consequently, the development of novel drugs that work through alternative modes of action are needed. The Seley-Radtke laboratory is focused on synthesizing nucleos(t)ide analogues endowed with additional flexibility in the nucleobase that may aid in circumventing point mutations associated with resistance mechanisms. In addition to retaining the hydrogen bonding and aromatic character required for binding site recognition, flexible purine nucleosides, termed "fleximers," possess several key advantages over normal nucleosides, notably the ability to adapt to a flexible enzyme binding site and possible mutations. To test their effectiveness, we have synthetically coupled these modified bases to clinically relevant 2'-fluorinated sugars. In addition, we are working to enzymatically couple fleximer bases to ribose or deoxyribose.

EXPLORING FLEXIBLE PURINE NUCLEOBASE RECOGNITION BY BIOLOGICAL TARGETS

By

Therese Chao-Yar Ku

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore County, in partial fulfillment
of the requirements for the degree of
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Dedication

To my family. I cannot express adequately my gratitude for your unwavering support throughout, not only my graduate years, but my entire life.

To Mom and Dad: you worked so hard such that my brothers and I could pursue a level of education many are not privy to. You did not give up on me when I gave up on myself and pushed me to get back up. You showed me that humbleness, hard work and dedication do pay off in the long run. I am who and where I am now because of you and I thank God for providing me with such wonderful parents to guide me through life.

To Chip and Betty: you are the set of parents God knew I needed in addition to the ones I already have. You gave me shoulders to cry on when I was overwhelmed. You are my shelter when I need a break from responsibilities and adulting. You knew I could persevere when I doubted myself. Thank you for being part of this segment and the rest of my life.

To Patrick: you were/are here the entire way. You watched me rejoice and cry. You made sure I did not give up. You made me strong. Also… your kid is pretty cute, good job!

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I would also like to thank my committee members Drs. Marie-Christine Daniel-Onuta, Wuyuan Lu, Zeev Rosenzweig, Paul Smith and Michael Summers for their time, guidance and support throughout my graduate career.

To my past and current Seley-Radtke lab mates Drs. Sarah Zimmermann, Kartik Temburnikar, Orrette Wauchope, Hannah Peters, Mr. Brian Cawrse, Ms. Mary Yates, Ms. Joelle Cusic, Mrs Natalie Steenrod Lopresti. I have learned a lot from each and every one of you and I am so grateful to have met you. I thank you all for your fellowship and support throughout my graduate journey, and I hope I will be able to give back to current and future Seley-Radtke students.

To the Chemistry Department Staff, you have all been wonderfully supportive. You made sure my colleagues and I were equipped with the resources and information we needed to succeed. If I ever had any questions, you had answers and even solutions. Some of you have also provided me with previous life lessons. I thank you for everything you have done (a lot).

To the UMBC graduate fellows that I have had the privilege to get to know and respect, thank you and you got this.

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Abbreviations

3TC	Lamivudine
Ac	*Ac*etate
Ac_2O	Acetic anhydride
AcCl	Acetyl chloride
AcOH	Acetic acid
ACV	*Ac*clo*v*ir
ADA	*A*denosine *d*e*a*minase
AIBN	*A*zobis*i*so*b*utyro*n*itrile
AIDS	*A*cquired *i*mmuno*d*eficiency *s*yndrome
AMBER	*A*ssisted *m*odel *b*uilding with *e*nergy *r*efinement
APCI	*A*tmospheric-*p*ressure *c*hemical *i*onization
Ari	*Ari*steromycin
ATP	*A*denosine *t*ri*p*hosphate
AZT	*Az*ido*t*hymidine
BME	*β*-*M*ercapto*e*thanol
Bn	*B*e*n*zyl
Boc	*tert*-*B*utyl*o*xy*c*arbonyl
BSA	Bis(trimethylsilyl)acetamide
t-Bu	*tert*-Butyl
n-BuLi	*n*-butyllithium
n-BuOH	*n*-butanol
Bz	Benzoyl

CA	*Ca*psid
CDI	*C*arbonyl *di*imidazole
COSY	*Co*rrelation *s*pectroscop*y*
cTAR	*C*omplementary *t*rans*a*ctivation *r*esponse
CuAAC	*C*opper-*c*atalyzed *a*lkyne-*a*zide *c*ycloaddition
CuTC	*Cu*prous *t*hiophene-2-*c*arboxylate
dba	*Di**b**e*nzylidene*a*cetone
DENV	*Den*gue *v*irus
dFdC	Gemcitabine or 2',2'-*di**f***luoro-2'-*d*eoxy*c*ytidine
DIAD	*Di**i***sopropyl *a*zo*d*icarboxylate
DIBA	2,2′-Dithiobisbenzamide
DIPEA	*N*,*N*-*Di**i***so*p*ropyl*e*thyl*a*mine
DMF-DMAc	*N*,*N*-*Di**m***ethyl*f*ormamide *di**m***ethyl *ac*etal
DMAP	4-*Di**m***ethyl*a*mino*p*yridine
DME	*Di**m***ethoxy*e*thane
DMEDA	1,2-*Di**m***ethyl*e*thylene*di**a***mine
DMF	*N*,*N*-*Di**m***ethyl*f*ormamide
DMSO	*Di**m***ethyl *s*ulf*o*xide
DNA	*D*eoxyribo*n*ucleic *a*cid
DPPE	Ethylenebis(diphenylphosphine)
dppf	1,1′-Bis(diphenylphosphino)ferrocene
DPPP	1,3-Bis(diphenylphosphino)propane
E. coli	*Escherichia coli*

EBOV	*Ebo*la *v*irus
EDTA	*E*thylene*d*iamine*t*etra*a*cetic acid
Et	*Et*hyl
EtOAc	Ethyl acetate
Env	*Env*elope glycoproteins
ESI	*E*lectro*s*pray *i*onization
FAB	*F*ast *a*tom *b*ombardment
FDA	*F*ood and *D*rug *A*dministration
Flex G	Fleximer guanosine
FTC	Emtricitabine
GOLD	*G*enetic *o*ptimization for *l*igand *d*ocking
GTP	*G*uanosine *tri**p**h*osphate
gRNA	*G*enomic *RNA*
HAART	*H*ighly *a*ctive *a*nti*r*etroviral *t*herapy
HBV	*H*epatitis *B* *v*irus
HCV	*H*epatitis *C* *v*irus
HIV	*H*uman *i*mmunodeficiency *v*irus
HSV	*H*erpes *s*implex *v*irus
IMPDH	*I*nosine-5′-*m*ono*p*hosphate *d*e*h*ydrogenase
INSTI	*In*tegrase *s*trand *t*ransfer *i*nhibitor
IPTG	*I*so*p*ropyl-β-D-*t*hio*g*alactopyranoside
Ll	*Lactobacillus leichmanii*
LRMS	*L*ow *r*esolution *m*ass *s*pectrometry

LTR	*L*ong *t*erminal *r*epeat
M	Molar
MA	*Ma*trix
MARV	*Mar*burg *v*irus
MERS	*M*iddle *E*ast *r*espiratory *s*yndrome
MOA	*M*echanism *o*f *a*ction
mRNA	*M*essenger *RNA*
MS	*M*ass *s*pectrometry
NA	*N*ucleic *a*cid
NBS	*N*-*B*romo*s*uccinimide
NC/NCp7	Nucleocapsid protein of HIV-1
NCI	*N*ational *C*ancer *I*nstitute
ND	*N*ot *d*etermined
NDT	*N*ucleoside *d*eoxyribosyl*t*ransferase
NIS	*N*-*I*odo*s*uccinimide
NMP	*N*-*M*ethyl-2-*p*yrrolidone
NMR	*N*uclear *m*agnetic *r*esonance
NNRTI	*N*on-*n*ucleoside *r*everse *t*ranscriptase *i*nhibitor
NaOAc	Sodium acetate
NaOEt	Sodium ethoxide
NOBA	3-nitrosobenzamide
NOESY	*N*uclear *O*verhauser *e*ffect *s*pectroscop*y*
NP	*N*ucleoside *p*hosphorylases

Npc	*Nep*lano*c*in
NRTI	*N*ucleoside *r*everse *t*ranscriptase *i*nhibitors
PATE	*P*yridino*a*lkanoyl *t*hio*e*ster
PBS	*P*rimer *b*inding *s*ite
PCC	*P*yridinium *c*hloro*c*hromate
Pd/C	Palladium on carbon
PE	*P*etroleum *e*ther
PIC	*P*re-*i*ntegration *c*omplex
PMB	*para*-*M*ethoxy*b*enzyl
PMSF	*P*henyl*m*ethyl*s*ulfonyl *f*luoride
PNP	*P*urine *n*ucleoside *p*hosphorylase
PPh_3	Triphenylphosphine
ppm	*P*arts *p*er *m*illion
PR	*Pr*otease
R-1-P	α-D-*R*ibose-*1*-*p*hosphate
RdRp	*R*NA-*d*ependent *R*NA *p*olymerase
RNA	*R*ibo*n*ucleic *a*cid
RNR	*R*ibo*n*ucleotide *r*eductase
rt	*R*oom *t*emperature
RT	*R*everse *t*ranscriptase
SAH	*S*-*A*denosyl*h*omocysteine
SAHase	*S*-*A*denosyl*h*omocysteine hydrol*ase*
SAM	*S*-*A*denosyl*m*ethionine

SAMT	*S*-*a*cyl-2-*m*ercaptobenzamide *t*hioester
SAR	*S*tructure *a*ctivity *r*elationship
SARS	*S*evere *a*cute *r*espiratory *s*yndrome
SD	*S*tandard *d*eviation
SI	*S*electivity *i*ndex
SL	*S*tem *l*oop
ssDNA	*S*trong *s*top *DNA*
SUDV	*Sud*an *v*irus
TAR	*T*rans*a*ctivation *r*esponse
TBAF	*T*etra*b*utyl*a*mmonium *f*luoride
TBAI	*T*etra*b*utyl*a*mmonium *i*odide
TBDMS	*tert*-*B*utyl*d*i*m*ethyl*s*ilyl
TEAA	*T*ri*e*thyl*a*mmonium *a*cetate
TFA	*T*ri*f*luoro*a*cetic acid
THF	*T*etra*h*ydro*f*uran
TIPDS	*T*etra*i*so*p*ropyl*d*i*s*iloxane
TK	*T*hymidine *k*inase
TLC	*T*hin *l*ayer *c*hromatography
TMEDA	*T*etra*m*ethyl*e*thylene*d*i*a*mine
TMS	*T*ri*m*ethyl*s*ilyl
TMSOTf	Trimethylsilyl trifluoromethanesulfonate
Tol	*Tol*uoyl
TPS	2,4,6-*T*riiso*p*ropylbenzene*s*ulfonyl

*t*RNA	*T*ransfer *RNA*
UNAIDS	*U*nited *N*ations Programme on HIV/*AIDS*
Val	*Val*ine
YFV	*Y*ellow *f*ever *v*irus
vDNA	*V*iral *DNA*
VZV	*V*aricella *z*oster *v*irus
ZF	*Z*inc *f*inger
ZIKV	*Zik*a *v*irus

Overall Goals of the Dissertation

As mentioned in the abstract, the Seley-Radtke laboratory is focused on synthesizing nucleos(t)ide analogues endowed with additional flexibility in the nucleobase, termed “fleximers,” that may aid in circumventing point mutations associated with resistance mechanisms. The aims of this dissertation follow a similar story as previous members of the Seley-Radtke lab, thereby furthering our understanding of fleximers mode of action and properties in biological systems.

This dissertation begins with two chapters that provide a background and history of nucleos(t)ides and their modifications, with Chapter 2 providing a full picture of the evolution of the synthetic methodologies used to achieve various fleximer analogues over the last two decades, including some of the author’s own work. Chapters 3-5 will detail the author’s work as a graduate student that include the synthesis of tricyclic and fleximer nucleosides with 2’-modifications on the sugar moiety and their biological relevance, the synthesis of fleximer bases through palladium-catalyzed cross-coupling and their affect on the nucleocapsid protein of HIV-1, and finally, an ongoing collaborative project that aims to simplify the synthesis of *distal* fleximer nucleosides. The projects discussed are varied in nature, thus, this section is meant to provide the reader with a cursory understanding of the overarching goals and specific aims of each of the author’s projects.

The goals of the project detailed in Chapter 3 entails the synthesis of the tricyclic and fleximer nucleosides with 2’-fluorinated sugars, carried out by the author and Dr. Zhe Chen, such as those found in Gemcitabine, an anticancer drug, and Sofosbuvir, an anti-HCV drug. Once the synthesis was complete, these analogues, as

well as their prodrugs were tested for their effectiveness against cancers and HCV by collaborators and the results are provided.

***Specific Aim 1*:** A series of 2'-fluorinated tricyclic and fleximer nucleoside analogues with sugars inspired by the scaffolds found in Gemcitabine and Sofosbuvir were synthesized, purified and characterized.

***Specific Aim 2*:** McGuigan based phosphoramidates of the nucleoside analogues synthesized in Aim 1 were synthesized, purified and characterized.

***Specific Aim 3*:** The nucleos(t)ide analogues possessing the sugar found in Sofosbuvir were tested for anti-HCV activity, whereas the nucleos(t)ide analogues possessing the sugar found in Gemcitabine were tested for anti-cancer activity through the NCI panel.

The goals of the project detailed in Chapter 4 describe the synthesis of fleximer bases using palladium-catalyzed cross-coupling. Once the synthesis was complete, the compounds were tested for their affinity to bind the nucleocapsid protein of HIV-1, NCp7, using ^{1}H NMR, and their effectiveness against HIV-1 replication.

***Specific Aim 1*:** A series of guanine analogues predicted to bind NCp7 through computational studies were synthesized, purified and characterized.

***Specific Aim 2*:** Using NMR spectroscopy (in collaboration with Dr. Michael Summers' group), the molecular interaction(s) between the small molecules synthesized in Aim 1 and the protein were studied.

***Specific Aim 3*:** The small molecules synthesized in Aim 1 were sent to Dr. Eric Freed (NCI) to determine biological activity of the small molecules against HIV-1.

The goals of of the project detailed in Chapter 5 entail the ongoing experiments by our collaborator Dr. Sylvie Pochet (Pasteur Institute, France) to determine whether the fleximer bases synthesized for the project detailed in Chapter 4 can be used for enzymatic transglycosylation so as to synthesize *distal* fleximer nucleosides through a shorter and more simplistic route.

***Specific Aim 1*:** A series of *distal* and *proximal* fleximer bases were synthesized by the author of this dissertation *via* palladium-catalyzed cross-coupling.

***Specific Aim 2*:** Dr. Sylvie Pochet (Pasteur Institute) will test the compounds from Aim 1 for their ability to act as substrates for enzymatic transglycosylation using *Ll*-NDT and PNP as catalysts.

***Specific Aim 3*:** Dr. Pochet will determine the optimum conditions to achieve the nucleoside isomers in the desired conformation.

While the direction of the author's projects did not follow the original and intended path, as unpredictable obstacles are sure to occur in novel research, exciting new ideas and projects were conceived from the unintended results of the author's work, and will be implemented by current and future Seley-Radtke graduate students.

Chapter 1: Background and Significance of Nucleos(t)ides and Nucleos(t)ide Therapeutic Agents.

Nucleos(t)ides (Figure 1 and Figure 2) are naturally occurring compounds that play a critical role in almost all parts of metabolism in biological systems, including storage and transfer of genetic information, inter- and intracellular communication, regulation of biological pathways, and energy storage.[1, 2]

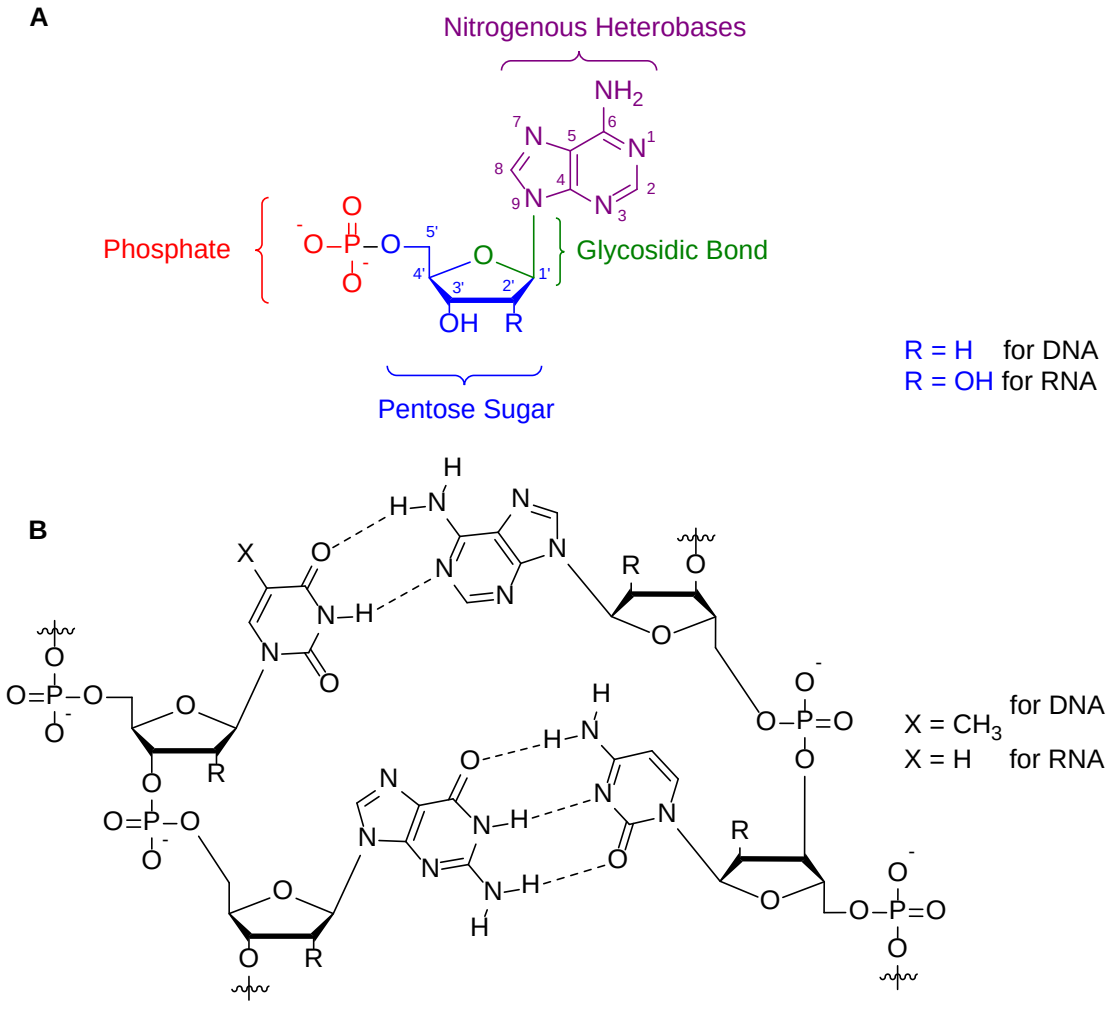


Figure 1. A) Example of a nucleotide. B) Watson-Crick base pairs.[2]

A nucleoside consists of the nitrogenous heterobase and furanose sugar, connected *via* a glycosidic bond (Figure 1A). There are two main categories of bases: purines and pyrimidines.[1, 3] Purines are bicyclic and include adenine and guanine, whereas pyrimidines are monocyclic and include cytosine, thymine and uracil (Figure 2). Nomenclature of the nucleobases and nucleosides, as well as numbering, are included in Figure 2. A nucleotide is a nucleoside with a 5'-phosphate on the furanose sugar and is the monomer of nucleic acids (Figure 1A, RNA, DNA). The furanose sugar moiety found in a ribonucleotide, the monomer of RNA, consists a β-D-ribofuranose (Figure 1A, R=OH), and the furanose sugar of a 2'-deoxyribonucleotide, the monomer of DNA, consists of a 2-deoxy-β-D-ribofuranose (Figure 1A, R=H).

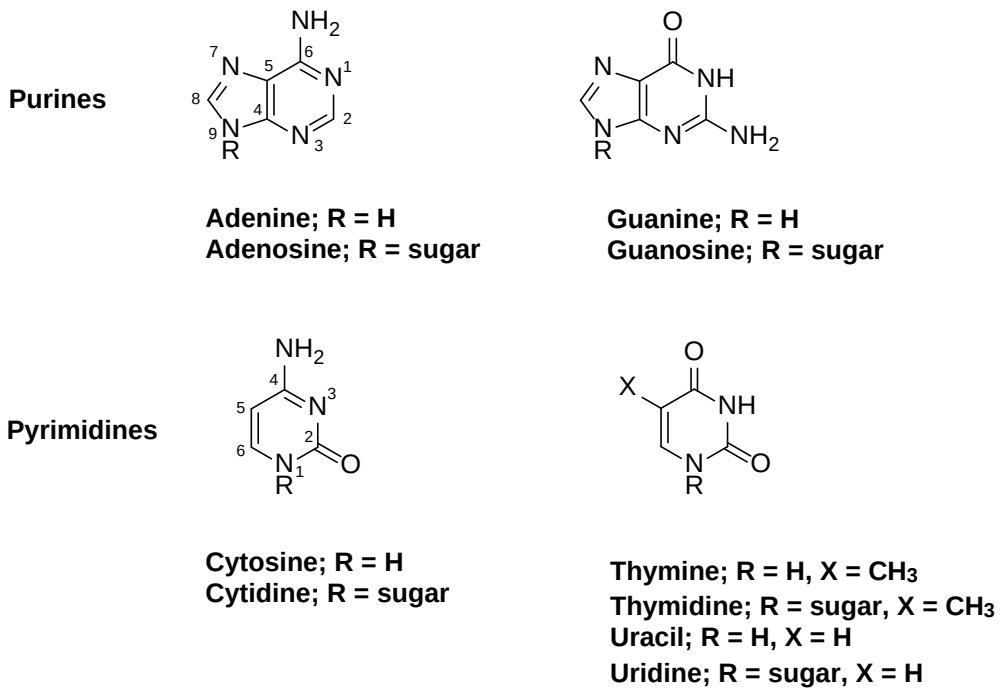


Figure 2. Nucleobase and nucleoside monomers of RNA/DNA.

As seen in Figure 1B, nucleotides can form phosphodiester bonds between the 3'-carbon of one nucleotide and the 5'-carbon of another, and a series of such attachments form a nucleic acid (NA) chain.[4] The heterobases of NA's, in turn, can form Watson-Crick base pairs through hydrogen bonding (H-bonding). In the presence of a single-stranded NA chain (typically RNA), this results in intramolecular base pairing and stacking that form short helices and loops.[1-5] In the presence of two NA chains (typically DNA), a double-stranded NA chain is established that may adopt various motifs, a double-helix motif being the most common.[1-5]

Tangential to the focus of this dissertation (but a fascinating topic), there has been great interest in understanding how nucleos(t)ides could have originated in the prebiotic era.[6-9] Findings have shown that precursors of ribonucleotides can be synthesized simply through reductive homologation of hydrogen cyanide and its derivatives, although with much serendipitous assistance from various environmental factors such as metals, photon sources, heat, amongst others (Figure 9).[6-9]

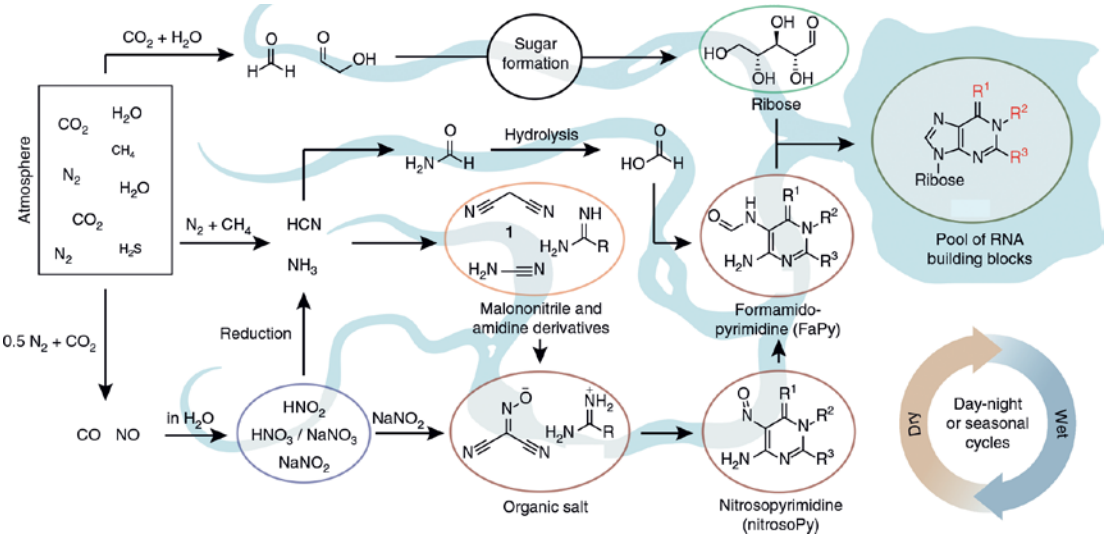


Figure 3. RNA nucleoside synthetic pathway in prebiotic conditions.[9]

Another area of interest involving nucles(t)ides is in drug discovery and design. Considering the roles nucleos(t)ides play in biology, any unnatural modifications imparted on the molecule should have a dramatic biological effect. This has proven successful in many anticancer, antiviral and antiparasitic therapeutics.[10-16] Examples of modified nucleos(t)ides, also known as nucleos(t)ide analogues, and their associated biological/biochemical impact will be discussed throughout this document as they pertain to the projects presented herein as well as those that are ongoing in the Seley-Radtke laboratory.

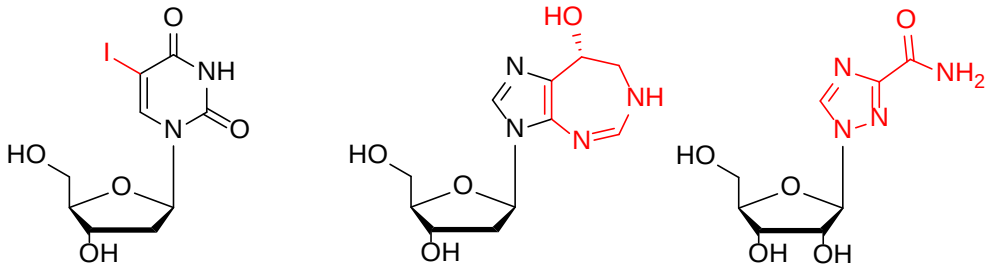
Nucleoside Modifications

There are various sites on a nucleos(t)ide that can be naturally and chemically modified (Figure 1): the heterobase, glycosidic bond, sugar, phosphate handle, and enantiomeric orientation. A few examples of each modification will be discussed in this section to explain some of the motivations for these changes, and the biological results they imparted.

I. Heterobase Modifications

The nitrogenous heterocyclic base is the recognition element of a nucleos(t)ide substrate for its target enzyme, and also participates in base pairing once incorporated into a NA chain.[17] Thus modifications at this site should theoretically disrupt the interaction between the analogue and its target nucleos(t)ide recognizing enzyme, thereby preventing the normal interactions. However, many base modified analogues have proven highly effective against various diseases, especially when

paired with other modifications (discussed later). Figure 4 shows Idoxuridine, Pentostatin and Ribavirin, three examples of base modified nucleoside analogues that have remained clinically relevant.



Idoxuridine **Pentostatin** **Ribavirin**

Figure 4. Examples of base modified nucleoside analogues.

Idoxuridine (Figure 4) was the first antiviral nucleoside drug to be approved in the U.S. in 1973 (discovered in 1959).[11, 18-21] It is a 2'-deoxyuridine analogue with an iodide atom installed at the C-5 of the pyrimidine. It was initially synthesized as an antitumor agent but was subsequently found to be more effective as a topical treatment for herpes simplex virus (HSV) infections.[11, 18] As is the case for most nucleoside analogues, Idoxuridine must first be phosphorylated to the active 5′-triphosphate form by cellular kinases, and subsequent inhibition of viral and cellular DNA synthesis occurs through incorrect base pair formation.[11, 18] Due to its high toxicity and low selectivity, other anti-HSV drugs such as Brivudine and Acyclovir have become the preferred therapies.[11, 18]

Pentostatin (Figure 4) is an anticancer natural product that was isolated from *Streptomyces antibioticus*.[22, 23] Pentostatin is a slow, tight-binding inhibitor of adenosine deaminase (ADA).[22, 23] This compound is able to mimic the transition state

structure of the adenosine and 2'-deoxyadenosine while being deaminated by ADA.[23, 24] Pentostatin is also selectively lymphotoxic.[23, 25] One theory for this selectivity is that accumulation of extracellular adenosine has been shown to suppress various lymphocytic functions.[23, 25] In addition, the accumulation of adenosine and 2'-deoxyadenosine, in turn, inhibits *r*ibo*n*ucleotide *r*eductase (RNR) and *S*-*a*denosyl*h*omocysteine hydrol*ase* (SAHase).[26] Inhibition of RNR, the enzyme that catalyzes the conversion of ribonucleotides to the corresponding 2'deoxyribonucleotides, would inhibit DNA biosynthesis. Inhibition of SAHase (Figure 5), the enzyme that degrades *S*-*a*denosyl*h*omocysteine (SAH), results in an accumulation of SAH, which competitively inhibits the methyltransferases that use *S*-*a*denosyl*m*ethionine (SAM) as the methyl donor. This in turn, inhibits the growth and replication of various tumors (and viruses), particularly those requiring a methylated 5'-cap structure of their mRNA. [27]

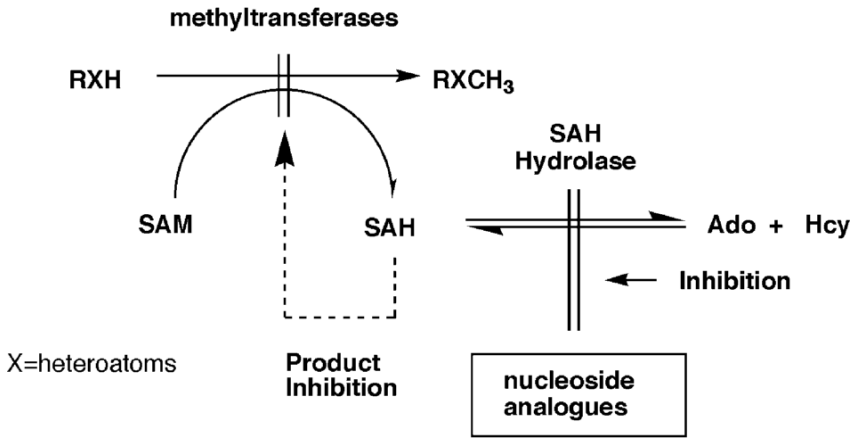


Figure 5. Inhibition of SAM mediated biomethylation via inhibition of SAHase.[27]

Ribavirin (Figure 4) is categorized as a guanosine analogue and is active against many DNA and RNA viruses, however, its exact *m*echanisms *o*f *a*ction (MOA) is still under debate.[28-30] The most common hypothesis is nucleic acid chain termination after incorporation of the nucleotide analogue into the growing chain, which has been confirmed *in vitro*.[29, 31, 32] Another theory is that since Ribavirin 5'-monophosphate inhibits *i*nosine *m*ono*p*hosphate *d*e*h*ydrogenase (IMPDH), it disrupts the *de novo* synthesis of guanosine triphosphate (GTP) and 2'-deoxyGTP (dGTP), and in turn disrupts the synthesis of viral RNA.[29, 33] The next hypothesis, dubbed the "error catastrophe" hypothesis, posits that Ribavirin induces mutations in the viral genome such that the resulting viral genetic material is non-viable.[29, 30] The last MOA postulates that Ribavirin acts as an immunomodulator, enhancing the response of interferon-stimulated genes, making cells more sensitive to exogenous interferon, thus increasing the production of endogenous interferon.[34] As all these theories have been proven plausible in experimental settings, Ribavirin could ultimately be acting in a polypharmacological fashion.

II. Glycosidic Bond Modifications

In most cases, the impetus behind modifications to the glycosidic bond lie in the labile nature of this bond. This bond is easily cleaved in acidic environments and through enzymatic digestions.[35, 36] As the intention is for all components of the nucleos(t)ide to reach its target, keeping this bond intact is highly desirable.

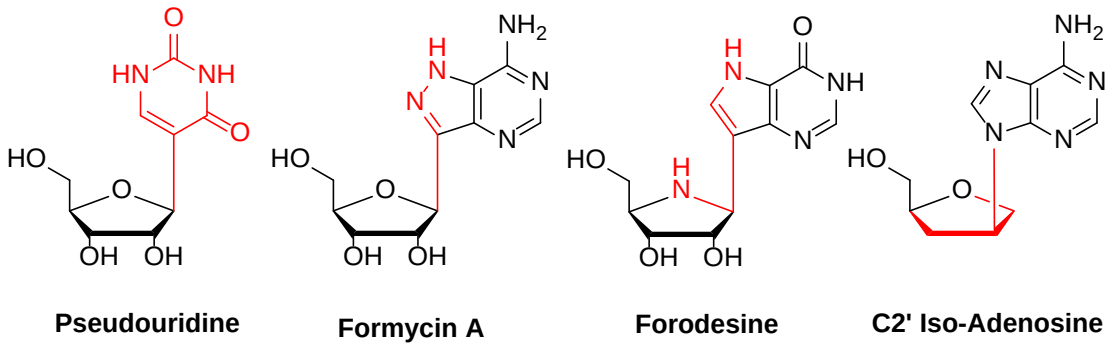


Figure 6. Examples of nucleoside analogues with modified glycosidic bonds.

Pseudouridine (Figure 6) is a naturally occuring C-nucleoside. A C-nucleoside does not possess the hemiaminal glycosidic bond on a normal nucleoside as the heterobase connecting nitrogen is replaced with a carbon (Figure 6). This transformation strengthens the nucleoside analogue against the aforementioned bond cleavage.[37] Pseudouridine is a ubiquitous molecule known as the "fifth nucleoside" in RNA due to its abundance.[38] As the N1 is “freed” from the glycosidic bond, Pseudouridine has an extra hydrogen bond donor at its non-Watson-Crick face, and when incorporated into RNA, it can alter the RNA secondary structure by increasing base stacking, improving base pairing and rigidifying the sugar-phosphate backbone.[39] Pseudouridine results from post-transcriptional modifications of uridine *via* pseudouridine synthases and is part of various structural RNAs (transfer, ribosomal, small nuclear, and small nucleolar).[38, 39] Although Pseudouridine was the first modified nucleoside to be discovered in RNA, and is the most abundant, its biosynthesis and biological roles have remained poorly understood, however, development of new techniques and improved instrumentation has revitalized the field’s interest.[38-43]

Formycin A (8-aza-9-deazaadenosine, Figure 6) is also a natural product C-nucleoside. It was isolated from the bacteria *Nocardia interforma* and *Streptomyces kaniharaensis*, and has demonstrated, anticancer, antiviral and antibiotic activities.[44-52] Due to its structural similarity to adenosine, Formycin A has shown to be a potent inhibitor of various adenosine recognizing enzymes including adenosine kinase from *Mycobacterium tuberculosis*, which is involved in the purine salvage pathway.[44] Likewise, Formycin A and its analogues are also known to inhibit bacterial *p*urine *n*ucleoside *p*hosphorylase (PNP), the enzyme that catalyzes the phosphate-dependent conversion of purine nucleosides or deoxynucleosides to α-D-(deoxy)ribose-1-phosphate and the corresponding base.[53, 54]

Forodesine (Immucillin-H, Figure 6) is another example of a unnatural C-nucleoside, but also possesses an imino sugar.[55, 56] Similar to Pentostatin, Forodesine is a transition state analogue inhibitor (Figure 7), in this case, against bovine PNP.[55, 56] Inhibition of PNP causes elevated levels of 2'-deoxyguanosine, leading to the accumulation of dGTP after 2'-deoxyguanosine is triphosphorylated in lymphocytes.[55, 57] Buildup of dGTP is known to be toxic to T-cells and will subsequently induce apoptosis.[58, 59] Through this cascade, inhibition of PNP could possibly treat T-cell autoimmune diseases, such as psoriasis, inflammatory bowel disease, rheumatoid arthritis and insulin-dependent juvenile diabetes.[23, 60] Through kinetic isotope effect studies using inosine as the substrate for PNP, the transition state structure was elucidated (Figure 7), and Forodesine was designed to mimic this transition state.[55, 56]

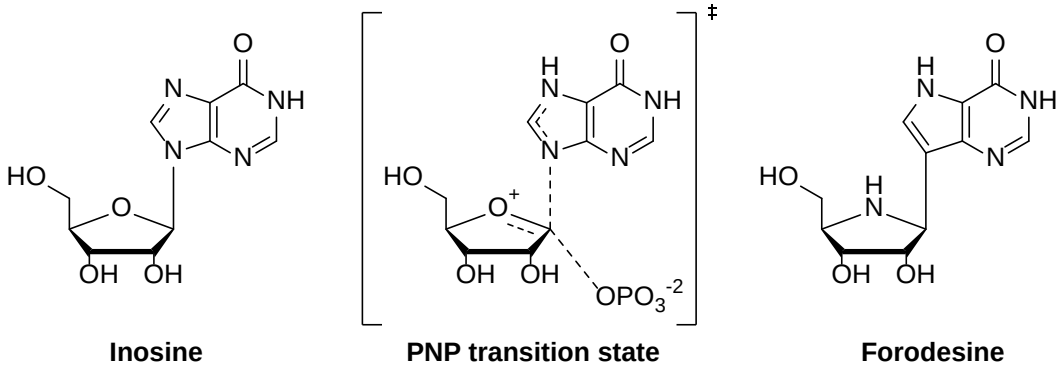
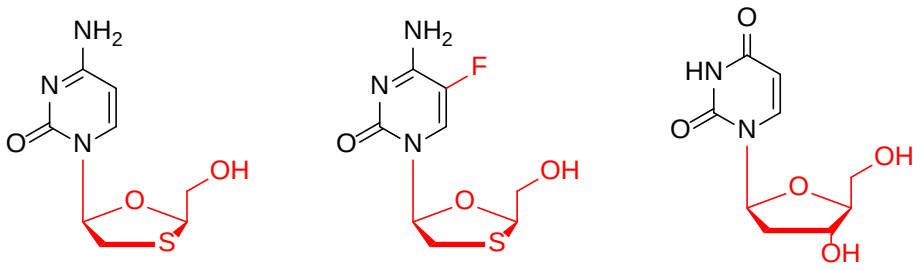


Figure 7. Inosine, hypothesized transition state structure for the reaction catalyzed by bovine PNP, and Forodesine.[55, 61]

The C2' iso-nucleosides' glycosidic bond is connected at the 2'-carbon of a 2,3-dideoxyribose sugar (Figure 6), which consequently stabilized the bond in acidic conditions.[62-64] Promisingly, the adenosine analogue (Figure 6) was active against the *human immunodeficiency virus* (HIV) *in vitro*, but further studies of these molecules have been limited due to their synthetic difficulties and complexities.[62-64] Alternative routes are being pursued that may re-encourage interest in these nucleoside analogues.[65]

III. Orientation

The examples discussed to this point have been exclusively D-nucleosides, and the majority of available nucleoside therapeutics do retain the natural D-orientation. Interestingly, L-nucleoside analogues, the enantiomers of D-nucleosides, have also shown interesting biological activities even though they do not possess the natural stereochemistry for recognition.[66]



Lamivudine **Emtricitabine** **Telbivudine**

Figure 8. Examples of modified L-nucleoside analogues.

Lamivudine (3TC) and Emtricitabine (FTC) are L-nucleosides (Figure 8) whose ribofuranose 3'-carbon is replaced with a sulfur.[67, 68] The initial draw to these analogues came from the interest in the D-oxathiolane analogues and their antiviral effects.[69] Remarkably, 3TC not only showed equipotent activity against HIV compared to the D-enantiomer, it was also significantly less cytotoxic.[69-71] FTC was discovered while performing structure activity relationship (SAR) studies on 5-substituted pyrimidine oxathiolanes for their efficacy against HIV.[69] In this case, neither enantiomers of FTC were cytotoxic, however, FTC was 100 times more potent than the D-enantiomer.[69, 72] Both were eventually approved for the use against HIV and hepatitis B virus (HBV) after demonstrating low levels of toxicity and diminished side effects compared to azidothymidine (AZT), another clinically relevant nucleoside analogue.[16, 67, 68, 73]

Telbivudine (Figure 8) also exhibited potent anti-HBV activity, but in addition, its activity was specific.[74, 75] Once Telbivudine is triphosphorylated through cellular kinases, it inhibits HBV DNA polymerase as a chain terminator, and consequently inhibits HBV replication.[76] More importantly, the Telbivudine nucleotide does not inhibit human DNA polymerases α, β or γ, thereby avoiding the

mitochondrial toxicity associated with numerous nucleoside analogues used in antiviral therapy.[74-76]

IV. Sugar Modifications

A wide array of therapeutic activities can be imparted to a nucleos(t)ide analogue through modifications to the sugar moiety. As seen in Figure 9, there are various types of sugar changes than can be performed with each providing a different therapeutic profile. To illustrate their differences, Aristeromycin, Acyclovir and Gemcitabine, three examples of sugar modified nucleos(t)ides will be discussed.

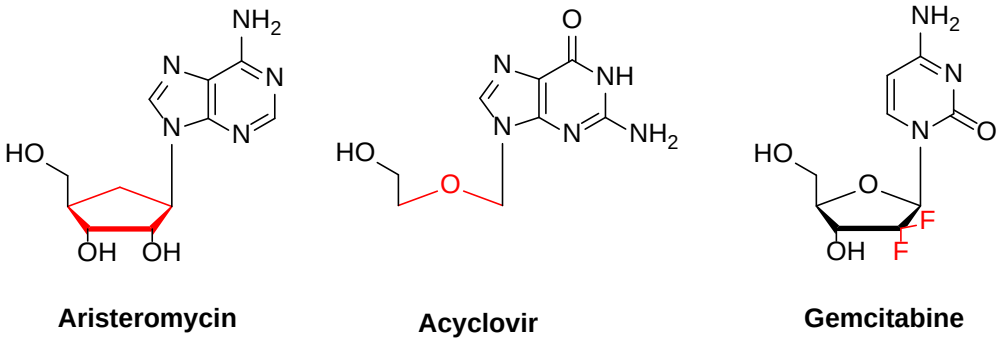


Figure 9. Examples of sugar modified nucleoside analogues.

Aristeromycin (Ari, Figure 9) is a carbocyclic analogue of adenosine which was first synthesized in racemic form in 1966, and was later isolated from the marine sponge *Streptomyces citricolor*.[77, 78] The sugar of a carbocyclic nucleosides is essentially a cyclopentane ring, where the furanose oxygen is replaced with a methylene group.[12] Similar to C-nucleosides, carbocyclic nucleosides are immune to enzymes that cleave the glycosidic bond.[12] These properties, as well as their similarity

to endogenous nucleosides, allows Ari to display various biological activities including inhibition of SAHase (Figure 5).[79]

Acyclovir (ACV, Figure 9) is a guanosine analogue that possesses an acyclic sugar and this additional flexibility grants optimized interactions in target enzyme binding sites.[80-82] ACV targets herpes simplex viruses with high specificity (Figure 10) as it is initially preferentially recognized and monophosphorylated by viral thymidine kinase (TK) over cellular TK.[83-88] Once further phosphorylated to the di- and triphosphate versions by cellular kinases, the viral DNA polymerase recognizes and incorporates ACV triphosphate more efficiently compared to cellular polymerases.[83-88] Once incorporated, ACV terminates the growing viral DNA as it lacks the 3'-OH that is required to elongate the nucleic acid chain, hence it is classified as an obligate chain terminator.[80-82]

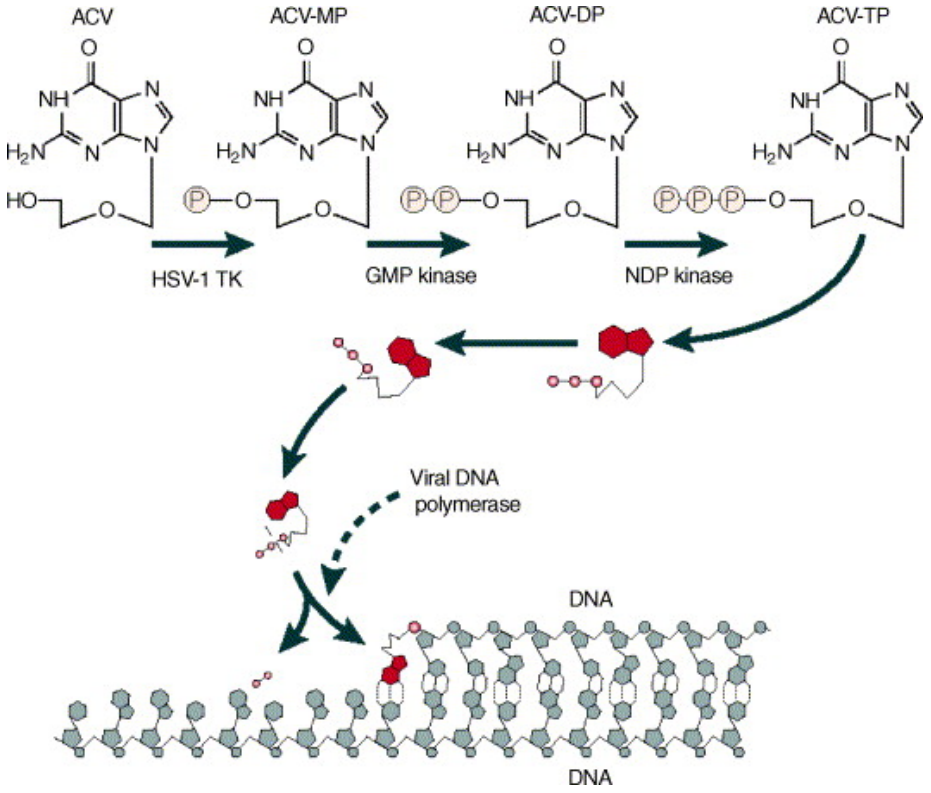


Figure 10. Obligate chain termination of HSV DNA synthesis by ACV.[89]

Gemcitabine (2',2'-difluoro-2'deoxycytidine, dFdC, Figure 9) is a 2'-deoxycytidine analogue with two fluorines installed at the 2'-carbon of the sugar. It was first approved in 1996 as a first-line treatment for advanced stage pancreatic cancer patients, and gained approval for other cancers in later years.[90] Gemcitabine's mechanism of action is two-fold (Figure 11); not only does Gemcitabine inhibit DNA synthesis through chain termination mechanisms, but it also inhibits RNR, further inhibiting DNA synthesis, and eventually induces the cell to undergo apoptosis.[91-93]

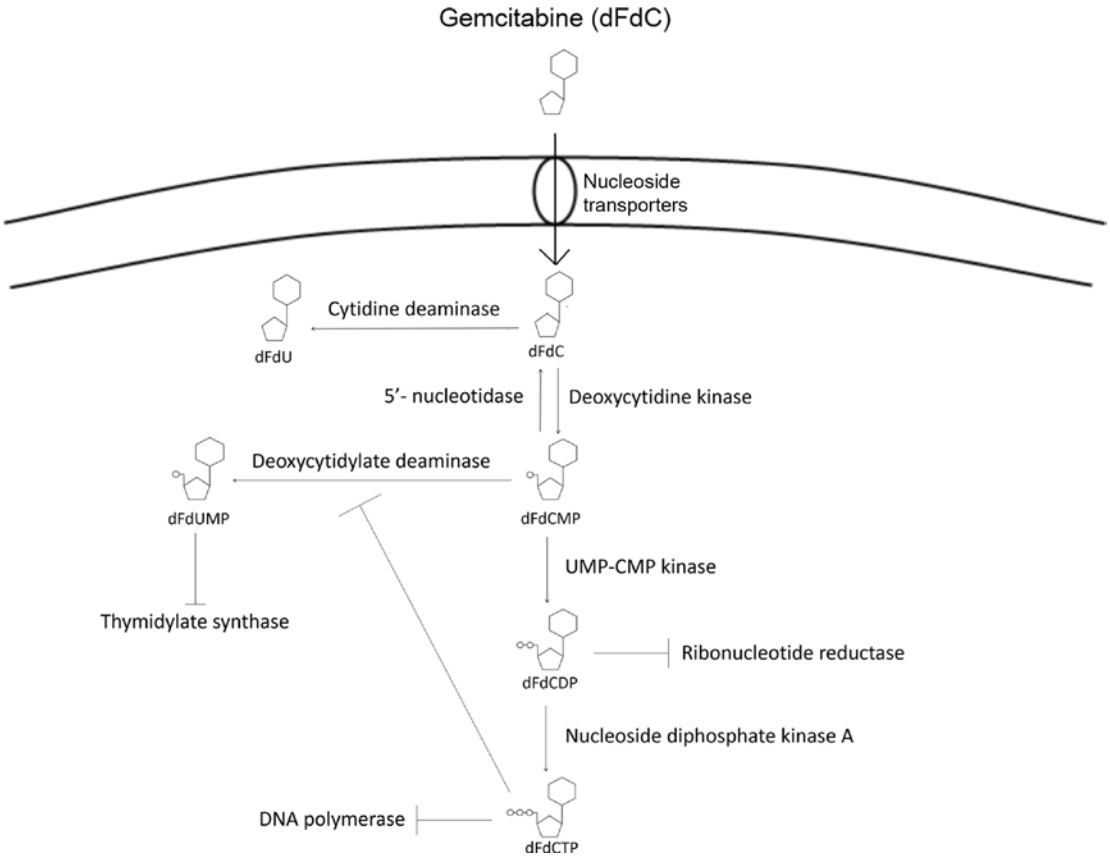


Figure 11. Metabolism of Gemcitabine and its MOA.[94, 95]

Gemcitabine is considered to be a non-obligate delayed chain terminator as it retains the 3'-OH, but, once phosphorylated to the triphosphate, becomes incorporated into to the growing DNA chain and subsequently allows for only one additional nucleotide to be inserted, thus halting DNA synthesis (Figure 12) .[95-97] Furthermore, proofreading and repair mechanisms are unable to remove the Gemcitabine nucleotide from this position, securing its place within the defunct DNA strand.[95, 96] Hence, it has also been dubbed a "masked chain terminator."[95-97]

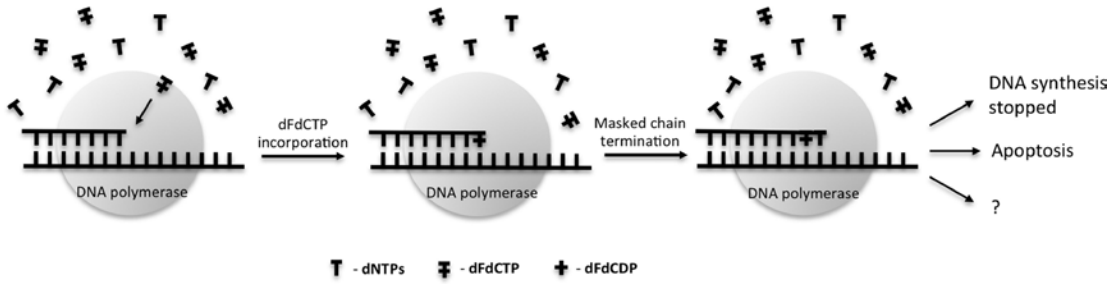


Figure 12. Gemcitabine chain termination mechanism.[95]

As the 2'-fluorinated sugars are a scaffold explored as part of this dissertation, this class of nucleos(t)ides will be discussed in more detail in Chapter 3.

V. Phosphate Group Modifications and Prodrugs

A significant challenge in nucleos(t)ide drug design is the degree of phosphorylation of the drug. As mentioned in several previous examples, many nucleoside drugs must be activated through phosphorylation and the first phosphorylation step is usually the rate-limiting step as the kinases that catalyze this step are usually rather discriminating.[98-100] To overcome this problem, the nucleotide monophosphate can be administered, however, the phosphate bond is highly labile and the monophosphorylated drug is highly polar, preventing cell penetration.[98-100] The examples below (Figure 13) elucidate some of the approaches that have been taken to solve the aforementioned problems.

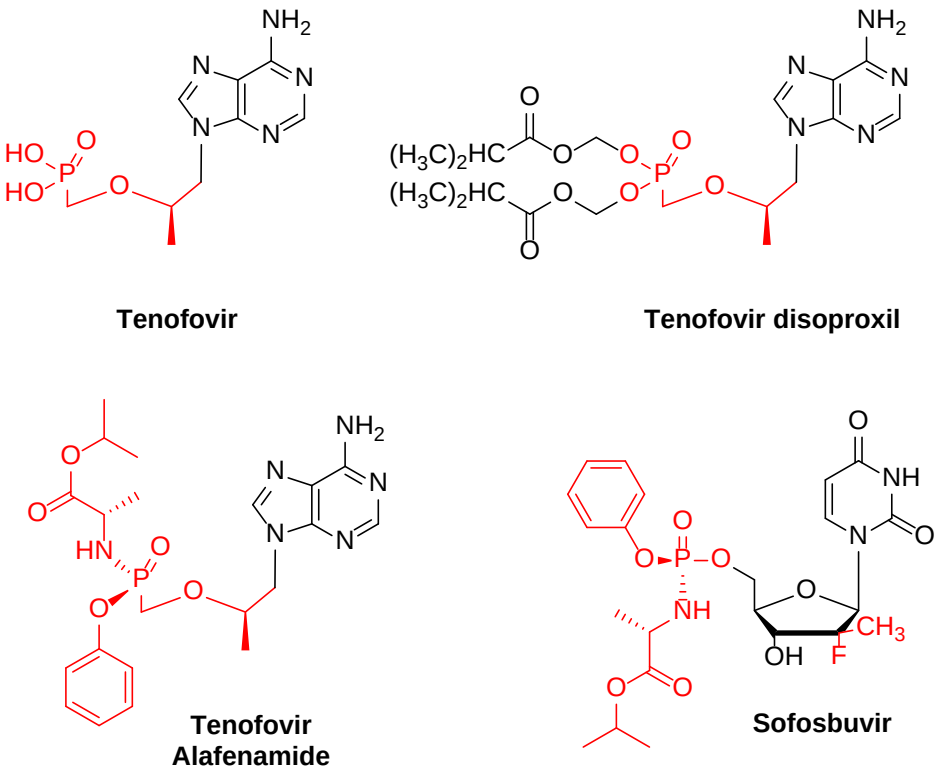


Figure 13. Examples of nucleotide analogues and prodrugs.

Similar to ACV, Tenofovir (Figure 13) possesses an acyclic sugar, however, it also includes a phosphonate group (Figure 14).[101] Like a phosphate group, a phosphonate serves as the "monophosphorylated" intermediate for subsequent phosphorylation.[101] However, unlike a phosphate group, a phosphonate is immune to esterase cleavage as it lacks the phosphoester bond connection to the main moiety.[101]

Figure 14. Phosphate versus phosphonate.

Once Tenofovir is phosphorylated to the active Tenofovir diphosphate, it acts as an obligate chain terminator and inhibits HIV *r*everse *t*ranscriptase (RT).[101, 102] At physiological pH, Tenofovir is deprotonated and does not readily undergo passive diffusion, resulting in low bioavailability after oral administration. [101, 102] As a results, Tenofovir disoproxil, an ester prodrug of Tenofovir, was developed to bypass this problem, however, renal and bone toxic effects were observed.[102] The second generation prodrug is Tenofovir alafenamide (Figure 13), which employs a McGuigan ProTide strategy (Figure 15).[103]

McGuigan's ProTides have had a major impact on the nucleoside field since many inactive nucleosides that were subsequently converted to their corresponding ProTide form were shown to exhibit potent activity due to the ability to overcome the rate limiting step of monophosphorylation and also to cross the cell membrane (Figure 15).[104-108] Through a series of trials, Professor Chris McGuigan and his group discovered that by masking the monophosphate (or phosphonate) of the nucleotide analogue with an aryl ester and amino acid phosphoramidate, the lipophilic ProTide can cross the cell membrane (Figure 15).[109, 110]

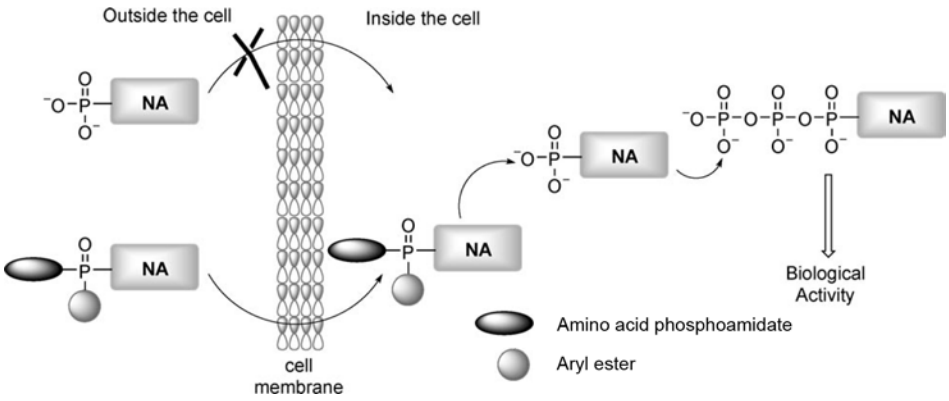


Figure 15. Delivery of McGuigan ProTides.[109]

Once inside the cell, the masking moieties are removed *via* two intracellular esterases (Figure 16).[109, 110] The first esterase (typically cathepsin A) catalyzes the hydrolysis of the ester, which, in turn, catalyzes the intramolecular displacement of the phenoxy group to form a cyclic anhydride intermediate (Figure 16, metabolite B).[109, 110] Non-enzymatic hydrolysis of the cyclic anhydride followed by cleavage of the P-N bond through a phosphoramidase results in the nucleotide monophosphate (Figure 16), which is subsequently phosphorylated to its active triphosphate form by cellular kinases (Figure 15).[109, 110]

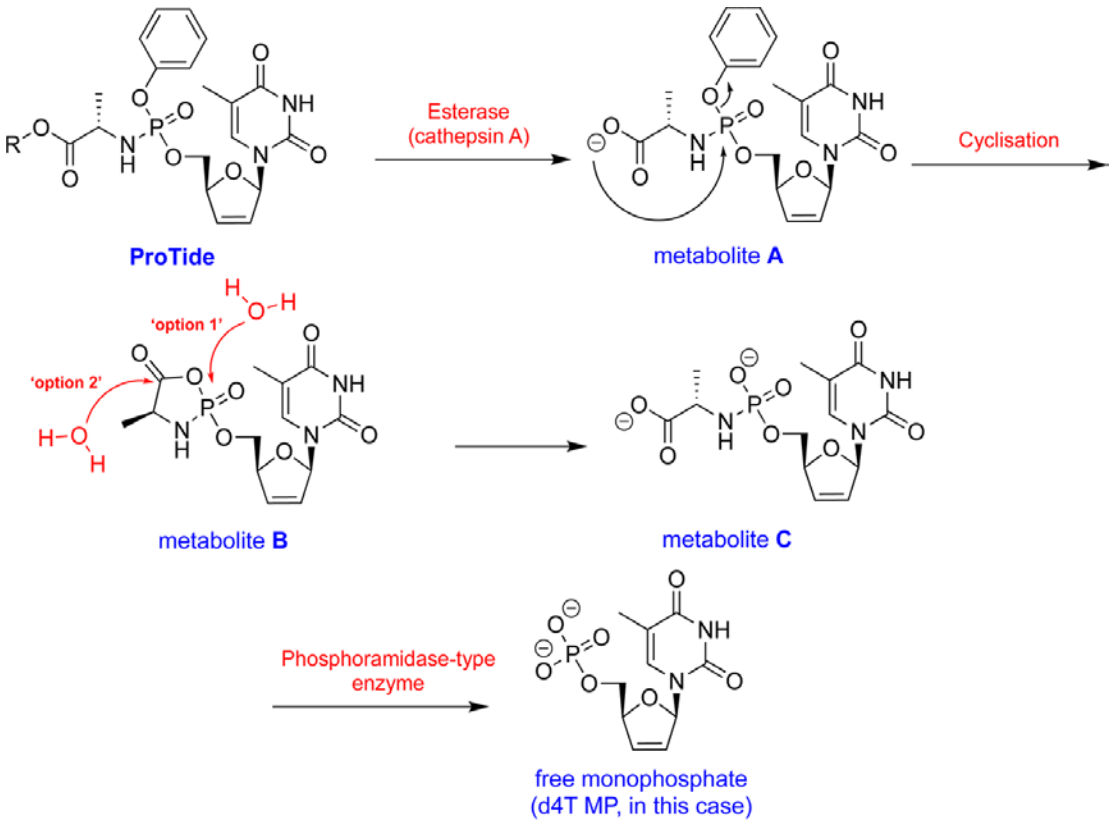


Figure 16. Hypothetical d4T ProTide metabolism mechanism.[109, 110]

Sofosbuvir (Figure 13) is another example of a sugar modified nucleotide analogue with a ProTide moiety installed. It was approved in 2013 as treatment for chronic *h*epatitis *C* *v*irus (HCV), and its impact is unprecedented; given that just a few years ago, there was no available treatment that was effective for many patients, much less a cure, thereby pointing to the importance of this drug.[111, 112] In combination with other therapies, Sofosbuvir can effectively cure hepatitis in 90% of patients.[111, 112] Sofosbuvir halts viral replication by inhibiting the HCV *R*NA-*d*ependent *R*NA *p*olymerase (RdRp), an essential enzyme in HCV replication.[113-116] Again, the 2'-fluorinated nucleos(t)ides will be further discussed in Chapter 4.

As discussed, significant therapeutic effects can be elicited *via* simple nucleos(t)ide modifications. However, a considerable problem has confronted medicinal chemists for a number of years: the rapid development of drug resistance.

Drug Resistance Development Mechanisms

Advancements in drug design efforts have indeed improved the quality of life for individuals who can afford the therapeutics available, but major drawbacks have also developed in the advent of these advancements, most significantly, the rapid development of anti-therapeutic resistance.[23] Alexander Fleming warned at the end of his Nobel Prize lecture in 1945, that although his discovery of penicillin had, and would continue to save many lives, there will be a time when resistance against penicillin, or any other therapeutic drug, would occur, especially if the antibiotic was not adequately dosed and the microbes are "educated.. to resist" the drug.[117] Unfortunately, his warning came to fruition soon after, and with the widespread use of penicillin during World War II came antibiotic resistance development a decade

after it became commonly prescribed.[118] Nowadays, there are only a limited number of antibiotics that have not yet seen widespread resistance (Figure 17).[118]

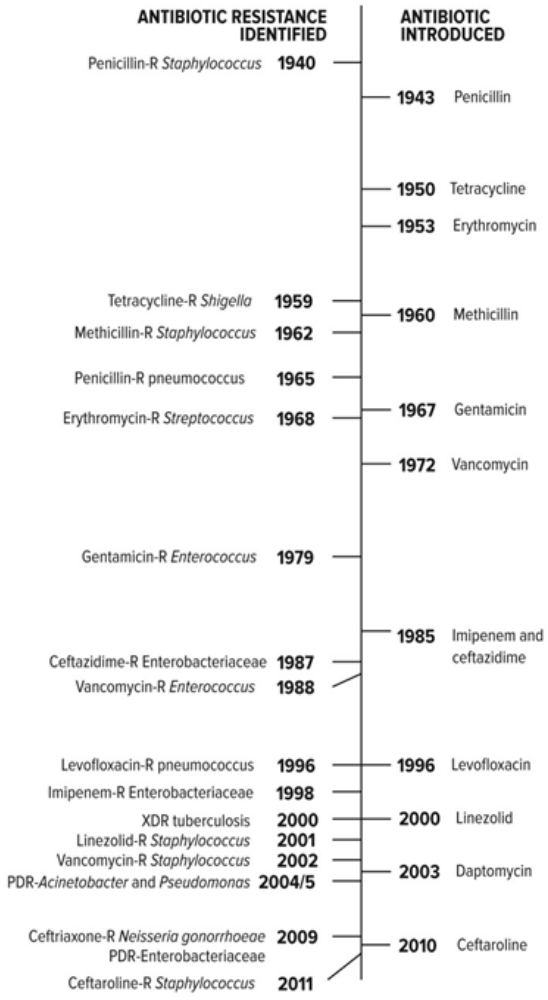


Figure 17. Key antibiotic resistance development over time.

PDR = pan-drug-resistant; R = resistant; XDR = extensively drug-resistant

Listed below are some of the more well-known mechanisms through which drug resistance can occur:[23]

1. Altered Target Enzyme or Receptor
2. Overproduction of the Target Enzyme or Receptor
3. Overproduction of the Substrate or Ligand for the Target Protein
4. Increased Drug-Destroying Mechanism
5. Decreased Prodrug-Activating Mechanism
6. Activation of New Pathway Circumventing the Drug Effect
7. Reversal of Drug Action
8. Altered Drug Distribution to the Site of Action

Mechanism #1 is the most common and involves the mutation of amino acid residues in the active site of the target protein and this can result in decreased affinity of the drug to the active site. Similarly, mutations elsewhere in the target protein can cause a conformational change so that the interaction with the drug is altered.[23] This mechanism has rendered many nucleos(t)ide therapeutics ineffective against their target diseases, especially in viruses. As mentioned previously, the triphosphate nucleotide of AZT and 3TC inhibit HIV-RT and are used in the treatment of *A*cquired *I*mmuno*d*eficiency *S*yndrome, or AIDS, however, resistance emerged relatively quickly for both antivirals when used as monotherapies.[69, 119, 120] Resistance to AZT arises from mutation of several residues in HIV-RT whereas resistance to 3TC is induced by a single mutation (different from the ones arising from AZT administration) in which a valine (typically Val184) or isoleucine is substituted for methionine.[120, 121] Interestingly, when both AZT and 3TC are administered in

combination, a much longer delay in resistance development occurs, even though Val184 mutants rapidly emerge.[69, 120] Serendipitously, Val184 mutant that emerges from 3TC treatment is sensitive to AZT, which consequently suppresses resistance to the latter.[69, 120] This example perfectly demonstrates the value and necessity of combination therapies.

Although combination therapies have been fairly successful in keeping resistance development at bay, occurrences remain, mostly due to poor patient compliance. Hence, new strategies have been studied to look for more conserved targets or multitargeting therapies, or drugs that may adapt to the conformational changes of its target. The Seley-Radtke “fleximers” aim to do just that.

Chapter 2: A History of Fleximers

For almost two decades, the Seley-Radtke group has designed and synthesized various classes of flexible purine nucleos(t)ides, or "fleximers."[27, 122-133] These novel nucleosides were designed to better understand how flexibility in the nucleobase, one of the primary recognition sites of a nucleoside, affects receptor-ligand recognition and function. They were also developed for their potential to overcome the challenges of binding site mutations. This chapter explores the various types of fleximers (Figure 18, **A**-**D**) that have been designed and synthesized by previous and current Seley-Radtke PhD graduate students (including the author), as well as other groups, and the synthetic methodologies that have been developed and improved over the years.

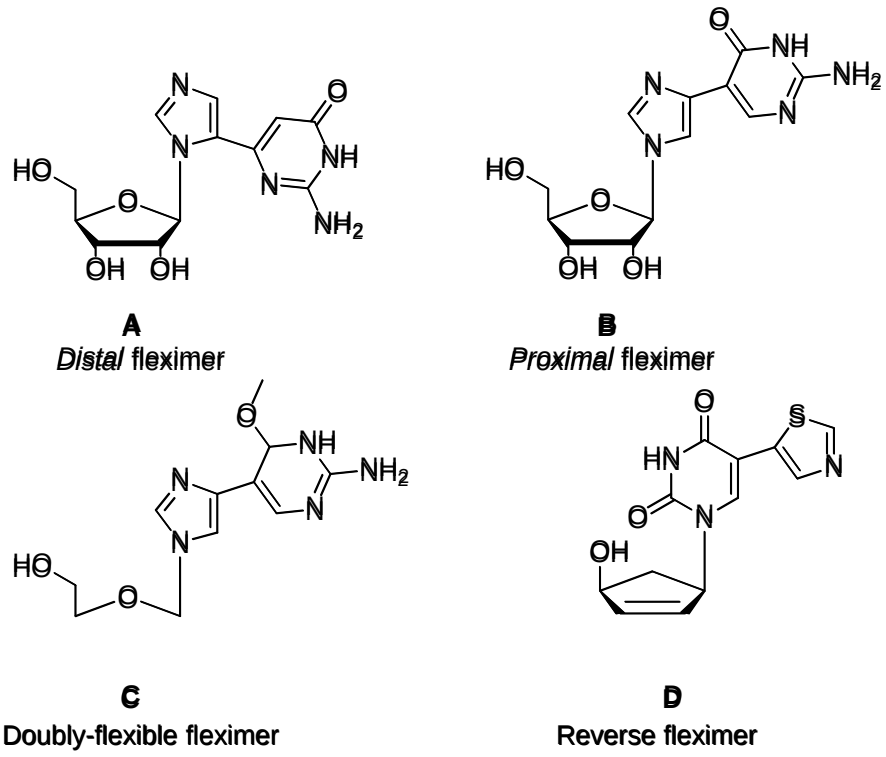


Figure 18. Examples of Seley-Radtke's Fleximers.

Fleximers have demonstrated several key advantages over their corresponding natural rigid purine nucleosides. For example, the distal guanosine fleximer (Flex-G, Figure 18, **A**) proved to be an inhibitor of *S*-adenosyl-L-homocysteine hydrolase (SAHase), an adenosine-metabolizing enzyme.[27] By rotating the hemiaminal bond into a *syn*-like conformation (Figure 19, A), as opposed to the thermodynamically favored *anti*-conformation (Figure 19, B), the guanosine flex-base model was able to position the amino group in such a way that it mimicked the adenosine nucleobase.[27, 124] Moreover, the flex-guanosine triphosphate (Flex-GTP) was shown to be a superior substrate of human GDP-L-fucose pyrophosphorylase compared to the natural substrate guanosine triphosphate (GTP),[125] likely due to the fleximer's ability to interact with amino acids in the active site not accessible by the natural substrate (Figure 19, C).[126] This also allowed Flex-GTP to retain all activity when essential catalytic residues needed for GTP binding were mutated.[125, 126]

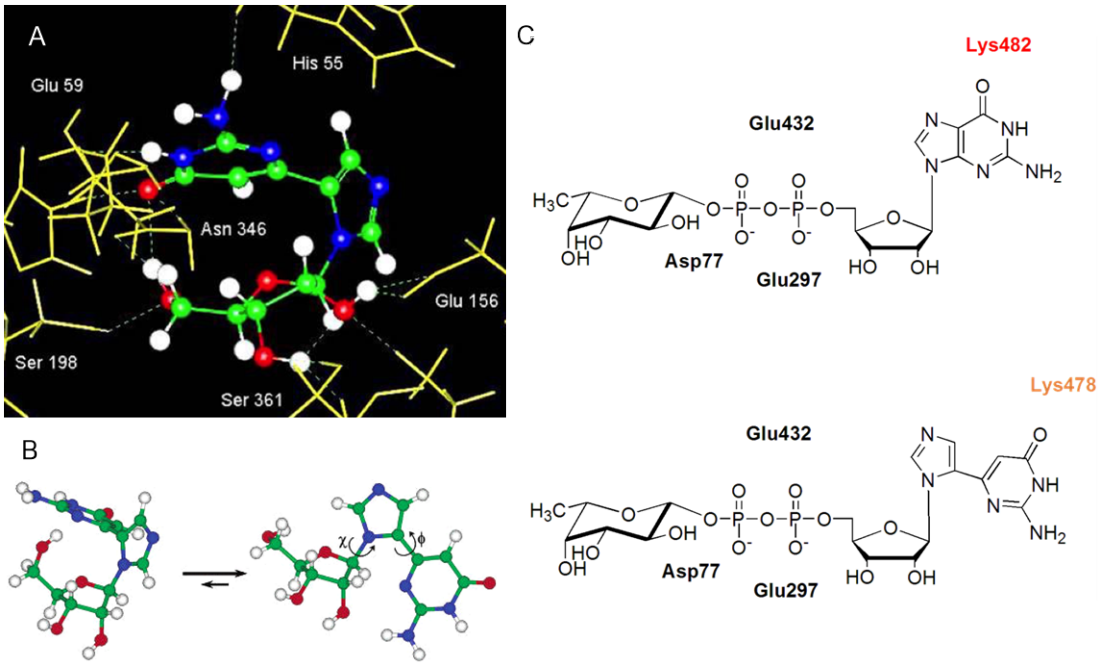


Figure 19. Biological advantages of Fleximers.[27, 124-126]

More recently, a series of doubly-flexible fleximers (Figure 18, **C**) based on the FDA approved acyclic nucleoside Acyclovir (ACV) were shown to inhibit hard-to-treat viruses such as the coronaviruses[130] and filoviruses,[131] while ACV itself was completely inactive. Even more notable, these compounds inhibit several flaviviruses, including Dengue and Zika. These biological results, as well as others obtained more recently, were described in a recently published review covering the biological impact of fleximers,[134] provide strong impetus to further explore these structurally interesting molecules.

While the biological results for these compounds have been groundbreaking in some cases, their syntheses have proven nontrivial. An examination of the methodology of fleximer synthesis in a historic fashion is presented herein, displaying

both published and unpublished works, as well as the most current and successful synthetic approaches used today.

The "fleximer concept" originally arose from a separate study focused on a series of thieno-expanded nucleoside analogues, or tricyclics (Figure 20, **A**), which, in turn, was inspired by Nelson Leonard's benzene expanded analogues (Figure 20, **B**-**D**).[135-139] These molecules, especially the *lin*-benzo derivatives, were among the first examples of expanded nucleobases designed to investigate the structural limitations of enzyme binding sites,[135-138, 140-144] As an extension of Leonard's work, the Seley-Radtke group designed and synthesized a series of tricyclic nucleosides with five-membered spacer rings rather than the benzene rings employed by Leonard.[122, 123, 139, 145-147] These were shown via *ab initio* calculations to be able to form the same hydrogen bonding patterns as the natural nucleoside base pairs, but exhibited increased polarizability and decreased the distance between base pairs, a problem that the benzene analogues encountered.[148]

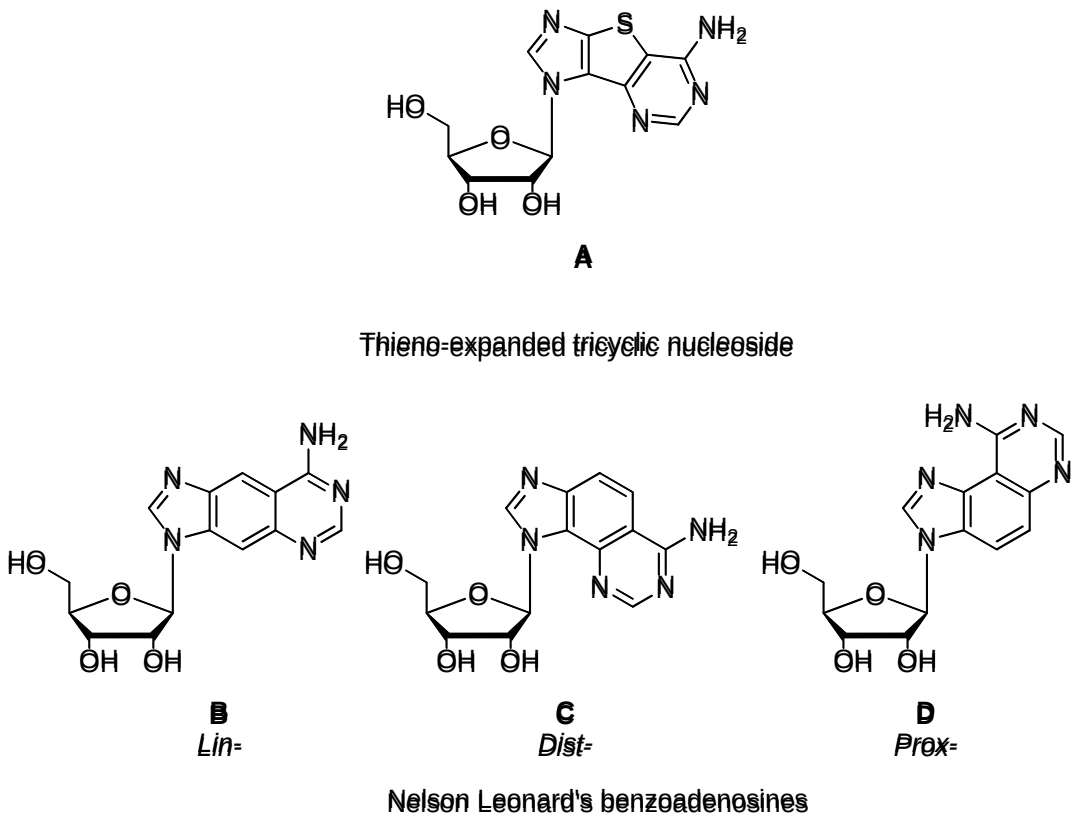


Figure 20. Expanded base nucleosides.[122, 123, 135-139, 145-147]

During the efforts to synthesize the tricyclic nucleosides, it was recognized that treating the tricyclics with Raney nickel would remove the bridging sulfur (Figure 21).[122, 123] This would leave a carbon-carbon bond between the imidazole and pyrimidine moieties of the purine base, thereby producing a "flexible" purine nucleoside. Introducing flexibility into the nucleobase was a groundbreaking concept, especially in the field of nucleoside drug design, thus the fleximers have been of interest to many.

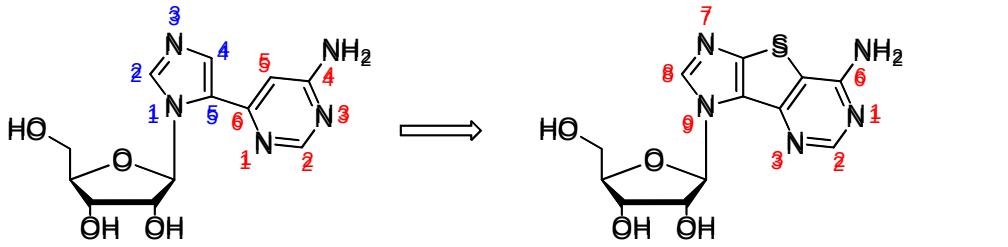


Figure 21. Retrosynthetic method for fleximer adenosine (numbering included for clarity).

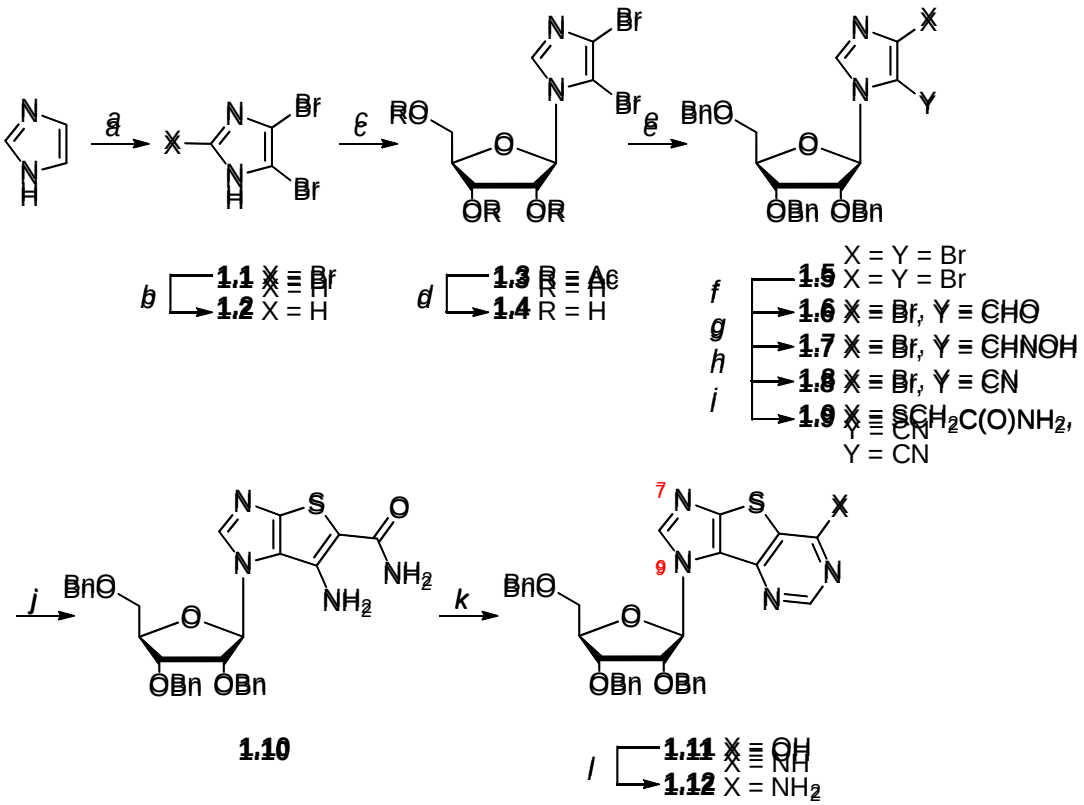
Distal *Fleximers*

I. Ribose *Distal* Fleximers

As mentioned, the first approach to realize the fleximers began with construction of the tricyclic nucleoside (Scheme 1).[122, 123, 139] This initially involved synthesis of the tricyclic bases and then coupling the base to the sugar to avoid the tedious sugar protection and deprotection procedures that are typical in nucleoside syntheses.[139] Although a mixture of the N7 and N9 coupled products was anticipated, only the undesired N7 isomer was isolated.[123, 139] Needing a new approach, the Vorbrüggen coupling between 4,5-dibromoimidazole (**1.2**) and β-D-ribofuranose-1,2,3,5-tetraacetate was pursued as the former is symmetrical, and ultimately, the correct product (**1.3**) was selectively obtained.[123] The acetate groups on the sugar were then replaced with more robust benzyl protecting groups to withstand the harsh reaction conditions for base synthesis. Treatment of **1.3** with ethylmagnesium bromide (EtMgBr), then *N*,*N*-dimethylformamide (DMF) afforded the carbaldehyde **1.6** (*N*-formylpiperidine was initially used instead of DMF, however the yields were consistently low). Carbaldehyde **1.6** was converted to the aldoxime **1.7** using hydroxylamine hydrochloride and sodium bicarbonate, and subsequent dehydration in

acetic anhydride (Ac_2O) yielded the nitrile **1.8**. The thieno-bicyclic intermediate **1.10** was achieved by treating the nitrile with excess potassium carbonate and freshly made mercaptoacetamide, followed by cyclization under basic conditions. Finally, the thieno-bicycle was refluxed with triethyl orthoformate in Ac_2O to form the inosine intermediate **1.11**. Since chlorination of the exocyclic enol was unsuccessful using phosphorus oxychloride,[149] the oxo moiety was converted to a thioketone intermediate that was methylated using methyliodide and finally subjected to high pressure aminolysis to produce the benzyl protected tricyclic adenosine **1.12**.[122, 123]

Scheme 1. Synthesis of thieno-expanded tricyclic adenosine and inosine.[122, 123]

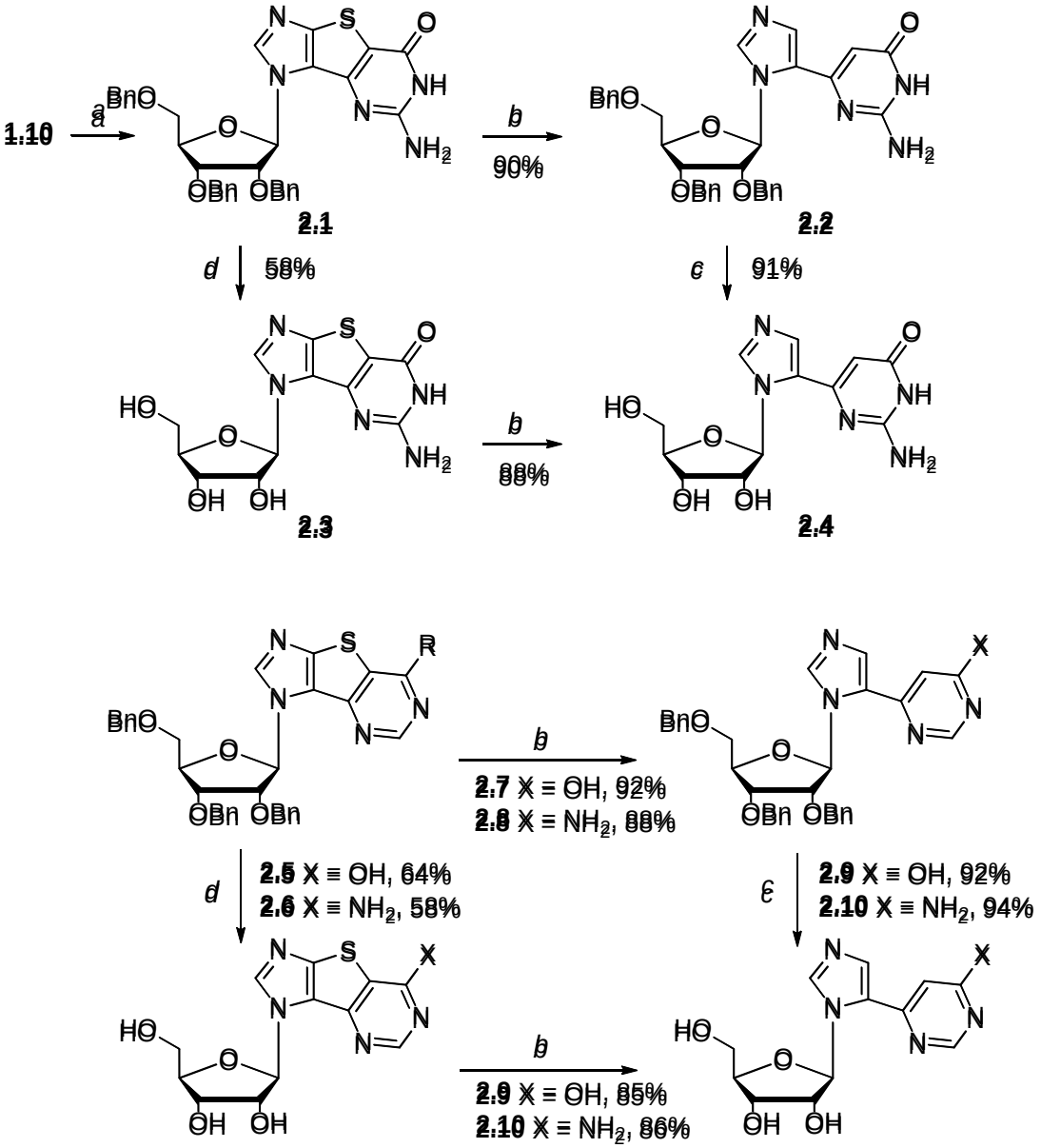


Reagents and conditions: a. Br_2, AcOH, NaOAc, rt, 3 h (71%); b. EtMgBr, Et_2O, rt, 3 h (90%); c. (i) β-D-ribofuranose-1,2,3,5-tetraacetate, BSA, CH_3CN, rt, 6 h, (ii) TMSOTf, 60°C,

18 h (66%); d. NH_3, CH_3OH, 100°C, 3 h (quant.); e. (i) NaH, THF, rt, 3 h, (ii) BnBr, TBAI, rt, 6 h (87%); f. i) EtMgBr, Et_2O, rt, 4 h ii) DMF, rt, 3 h; g. hydroxylamine HCl, $NaHCO_3$, EtOH, H_2O, rt, 18 h; h. Ac_2O, reflux, 2 h; i. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 55°C, 12 h; j. NaOEt, EtOH, reflux, 1.5 h (26%, 5 steps); k. triethyl orthoformate, Ac_2O, reflux, 3 h (82%); l. (i) P_2S_5, pyridine, reflux, 24 h, (ii) K_2CO_3, CH_3I, CH_3OH, rt, 10 min, (iii) NH_3, n-BuOH, 160°C, 90 h (60%, three steps).

In addition, the original method to achieve the tricyclic guanine base using chloroformamidine hydrochloride in dimethyl sulfone was too acidic and readily cleaved the glycosidic bond.[139] Instead, the desired tricyclic guanosine **2.1** was constructed in basic conditions starting from the bicyclic intermediate **1.10** (Scheme 2).[122, 123] Deprotection of the tricyclic nucleosides also proved challenging as using the common palladium catalyzed deprotection methods failed,[150] since sulfur is a well-known palladium catalyst poison.[151] Fortunately, the benzyl groups were ultimately able to be removed using boron trifluoride diethyl etherate (BF_3•OEt_2), although not in good yields.[122, 123] Deprotection of the fleximers, however, was much more facile since removing the sulfur first using Raney nickel allowed for use of standard palladium deprotection methods, which subsequently went in good yields.[123]

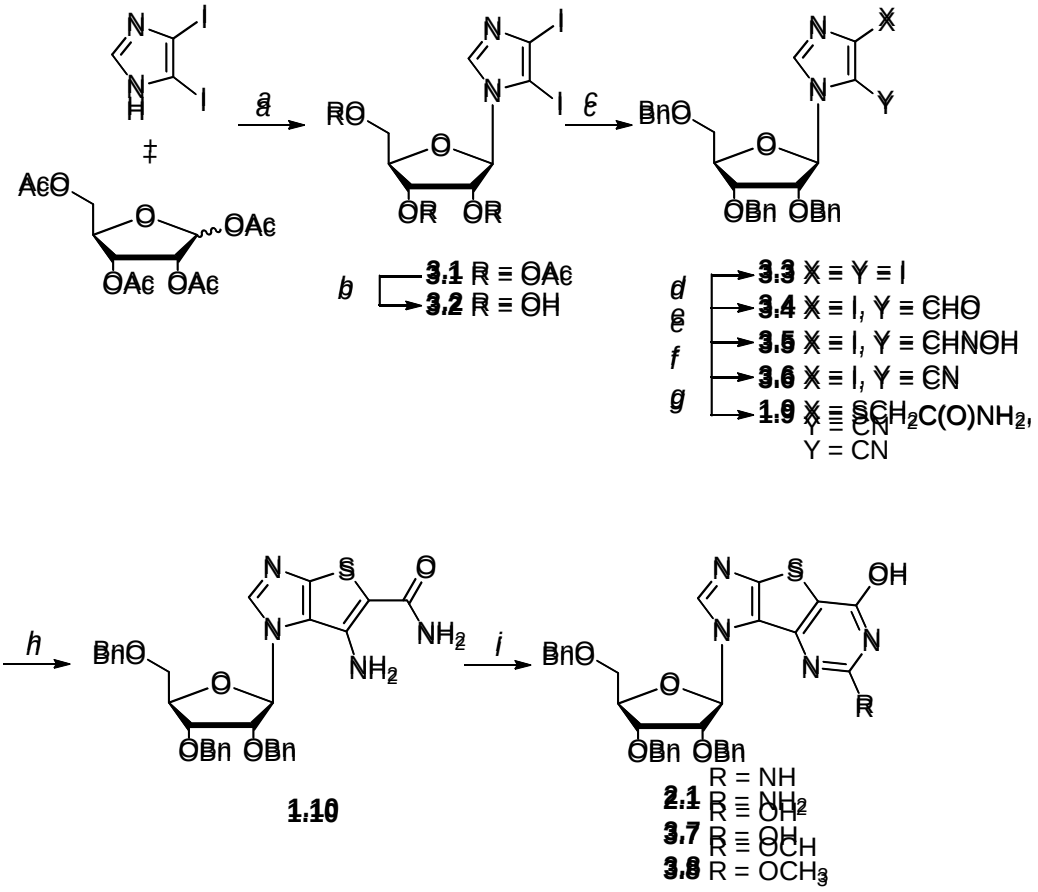
Scheme 2. Synthesis of thieno-expanded and fleximers guanosine, adenosine and inosine.[122, 123]



Reagents and conditions: a. (i) NaOH, CS_2, CH_3OH, 150°C, 18 h, (ii) H_2O_2, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 120°C,12 h (57%); b. Raney Nickel, CH_3OH, H_2O, reflux, 18 h; c. Pd/C, ammonium formate, CH_3OH, reflux, 18 h; d. BF_3•OEt_2, EtSH, CH_2Cl_2.

Due to the tedious and low yielding nature of the methodologies required to achieve these complex modified nucleosides, additional optimizations were necessary to improve the yields and reduce the reaction times. A simple, yet significant, replacement of 4,5-dibromodoimidazole with 4,5-diiodoimidazole improved yields dramatically in subsequent steps (using the same reaction conditions, Scheme 3).[128, 145]

Scheme 3. Optimized synthesis of thieno-expanded purine nucleosides.8,25



Reagents and conditions: a. (i) BSA, CH_3CN, 4 h, (ii) TMSOTf, 60°C, 18 h; b. NH_4OH, EtOH, rt, 18 h (75%, two steps); c. (i) NaH, THF, rt, 3 h, (ii) BnBr, TBAI, rt, 18 h (80%); d. (i) EtMgBr, THF, 5 h; (ii) DMF, 18 h (60%); e. NH_2OH•HCl, $NaHCO_3$ or pyridine, EtOH,

reflux, 2.5 h (quant.); f. Ac_2O, reflux, 3 h (85%); g. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 60°C, 40 h (50%); h. EtONa, EtOH, 80°C, 1 h (80%); i. (i) NaOH, CS_2, CH_3OH, 145°C, 18 h, (ii) H_2O_2, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 120°C,18 h.

As discussed earlier, the approach to the third ring closure to realize the tricyclic guanosine (**2.1**) had to be modified to avoid cleavage of the glycosidic bond. Additional studies of this step proved that in addition to the tricyclic guanosine (**2.1**) being formed, tricyclic xanthosine (**3.7**) as well as 2-*O*-methylxanthosine (**3.8**) were also products of this reaction, however, the yield ratios of the three products differed greatly depending on reaction conditions.[145] As shown in Table 1, a mechanistic study to determine the conditions to control specific outcomes was performed.[145] In this study, the ratio of sodium hydroxide (NaOH) to starting material (**1.10**), as well as the reaction temperature, reaction time and volume of methanol varied. Ultimately, a 1:1 ratio of **2.1**/**3.7** was able to be achieved (entry 7, Table 1) and the undesired product **3.8** was not observed.[145] Since the tricyclic xanthosine **3.7** was also useful, these proved to be the optimal conditions for this reaction.

Table 1. Mechanistic study of the terminal cyclization step of tricyclic guanosine/xanthosine.[145]

Entry	**1.10**/NaOH ratio	Temperature (°C)	Reactions time (h)	Volume of CH_3OH (mL)	**2.1**/**3.7**/**3.8** ratio
1	1:5	145	18	80	3:1:0
2	1:6	145	18	80	3:1:1

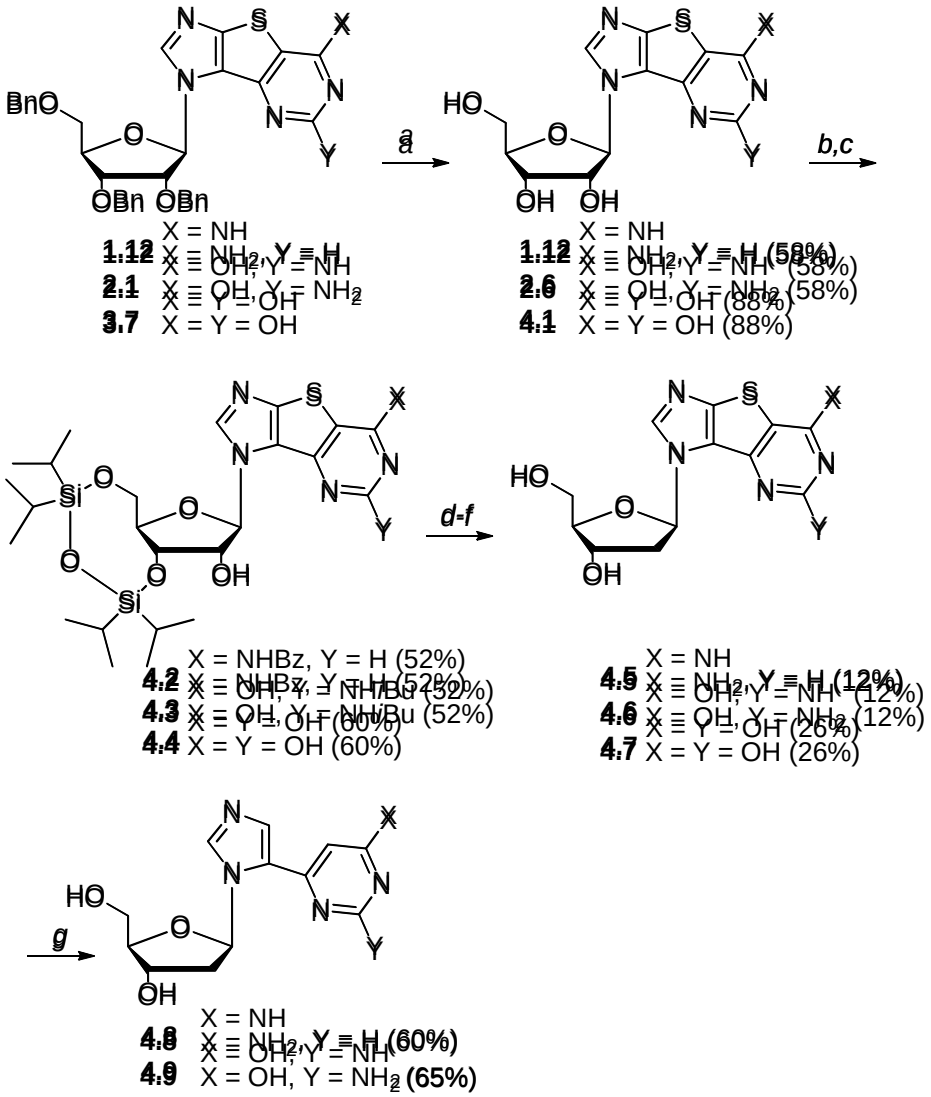
3	1:10	145	18	80	2:3:4
4	1:40	145	18	80	2:3:7
5	1:5	180	6	80	3:1:2
6	1:5	145	18	160	2:2:1
7	**1:5**	**145**	**18**	**250**	**1:1:0**
8	1:6	145	18	250	1:1:trace
9	1:8	145	18	250	2:2:1

II. 2’-Deoxyribose *Distal* Fleximers

Following completion of the ribose fleximer series, the 2’-deoxyribose series of tricyclics and distal fleximers were then pursued.[129, 147] The initial approach sought to modify the ribose analogues by chemically removing the 2’-OH (Scheme 4) since they were already in hand.[145, 147] To accomplish this, the functional groups on the bases were first protected using an *in situ* trimethylsilyl (TMS) protection, followed by simultaneous protection of the 3’- and 5’-OH using the Markiewicz tetraisopropyldisiloxane (TIPDS) bis-protecting group.[145, 147, 152] This TIPDS protection leaves the 2’-OH unblocked, allowing for modifications.[147] Subsequent removal of the 2’-OH through Barton deoxygenation procedures,[145, 146] and complete deprotection of the nucleoside provided the final 2’-deoxy tricyclic analogues **4.5**-**4.7**. These 2′-deoxy tricyclics were then treated with Raney nickel to yield the distal fleximers **4.8** and **4.9**. Ultimately, this route proved tedious as the overall yield starting from 4,5-diiodoimidazole was unacceptably low (0.5% overall yield for the

2’-deoxy tricyclic guanosine) thus a different route was designed starting with a 2’-deoxyribose sugar (Scheme 5 and Scheme 6).[129, 146, 147, 153]

Scheme 4. Synthesis of 2'-deoxy fleximers starting with the ribose analogue.[145, 147]

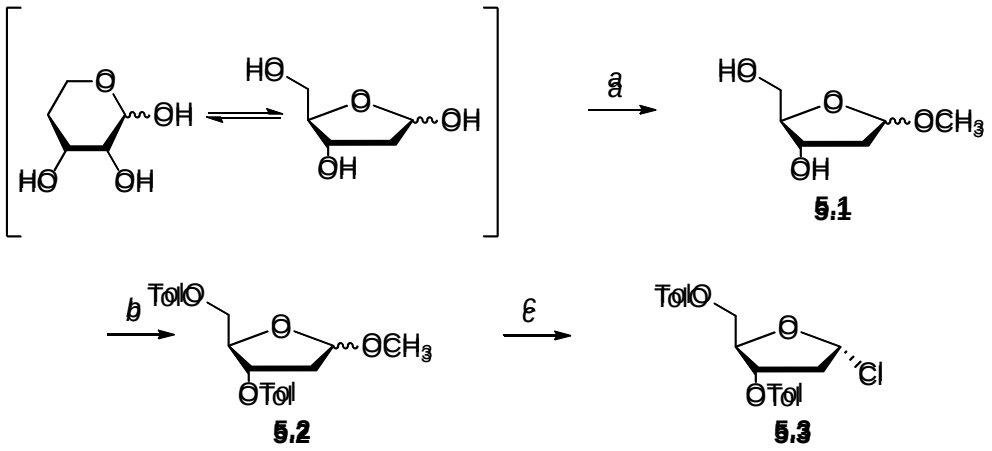


Reagents and conditions: a. BF_3•OEt_2, EtSH, CH_2Cl_2, 24 h; b. i) TMSCl, pyridine, 0°C to rt, 2.5 h, ii) *for adenosine*, BzCl, 0°C to rt, 24 h, *for guanosine*, isobutyryl chloride, 0°C to rt, 24 h; c. TIPDSCl, pyridine, rt, 24 h; d. phenyl chlorothionoformate, DMAP, rt, 24 h; e. AIBN,

Bu_3SnH, toluene, reflux, 6 h; f. i) 1M TBAF, THF, rt, 4 h, ii) NH_3, CH_3OH, 40°C, 18 h; g. Raney nickel, CH_3OH, reflux, 18 h.

The well known Hoffer's chlorosugar **5.3** was chosen due to the presence of the stereospecific anomeric chlorine.[154, 155] However, Hoffer's methodology (Pathway A, Scheme 5) proved to be quite sensitive and resulted in low yields overall, thus another more efficient method was pursued (Pathway B, Scheme 5).[156, 157]

Scheme 5. Synthesis of Hoffer's chloro sugar. [154-157]

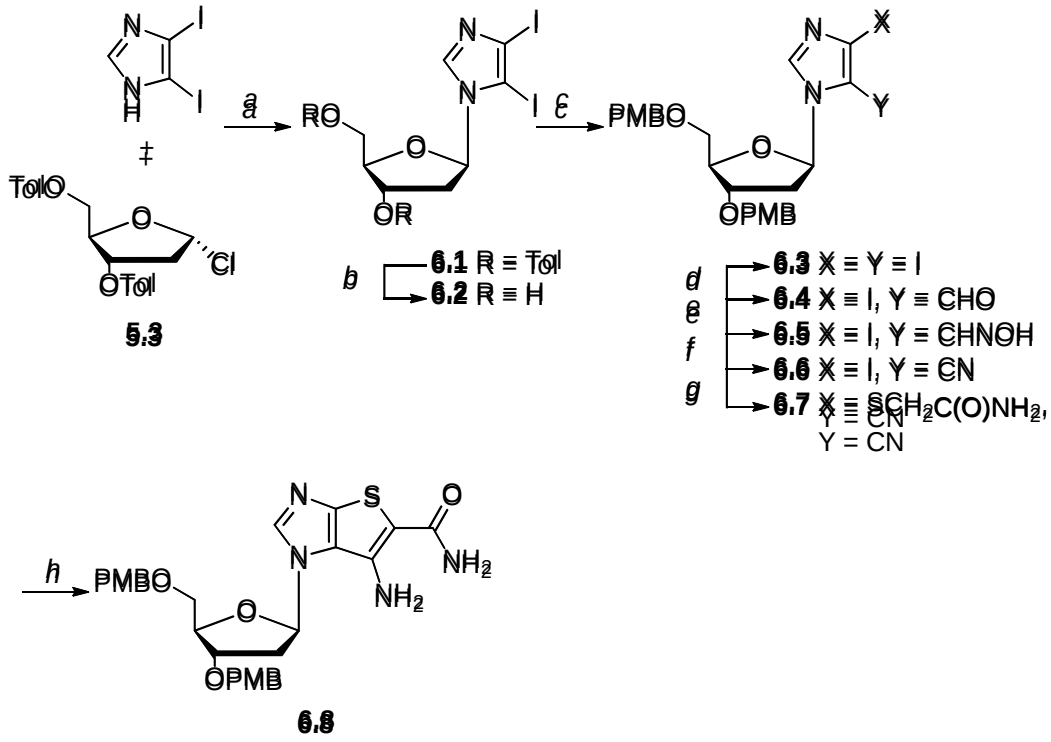


Reagents and conditions: Pathway A (Hoffer way): a. HCl(g) in CH_3OH; b. *p*-TolCl, pyridine, 0°C; c. HCl(g), CH_3OH. (65%, 3 steps). Pathway B: a. AcCl, CH_3OH; b. *p*-TolCl, pyridine, 0°C; c. AcCl, AcOH. (73%, 3 steps).

After coupling of Hoffer's chlorosugar to 4,5-diiodoimidazole, the toluoyl groups had to be replaced with more robust group to withstand the harsh conditions used throughout tricyclic synthesis (Scheme 6). As a result, the *p*-methoxybenzyl (PMB) group was chosen as it is typically more facile to remove compared to the

previously used benzyl groups. However, once again, BF_3•OEt_2 was ultimately determined to be best for final deprotection as ceric ammonium nitrate, the deprotection reagent initially chosen (and commonly used for this deprotection), resulted in cleavage of the glycosidic bond.[129, 147] Construction of the tricyclic base remained similar to the ribose analogues with a few changes: instead of using Ac_2O to obtain the nitrile **6.6**, the aldoxime **6.5** was refluxed with carbonyl diimidazole (CDI) in THF to prevent glycosidic bond cleavage as acetic acid (AcOH) was a byproduct using the previous conditions.[122, 123, 139]

Scheme 6. Synthesis of 2'-deoxy thieno-bicyclic nucleoside.[129, 147]

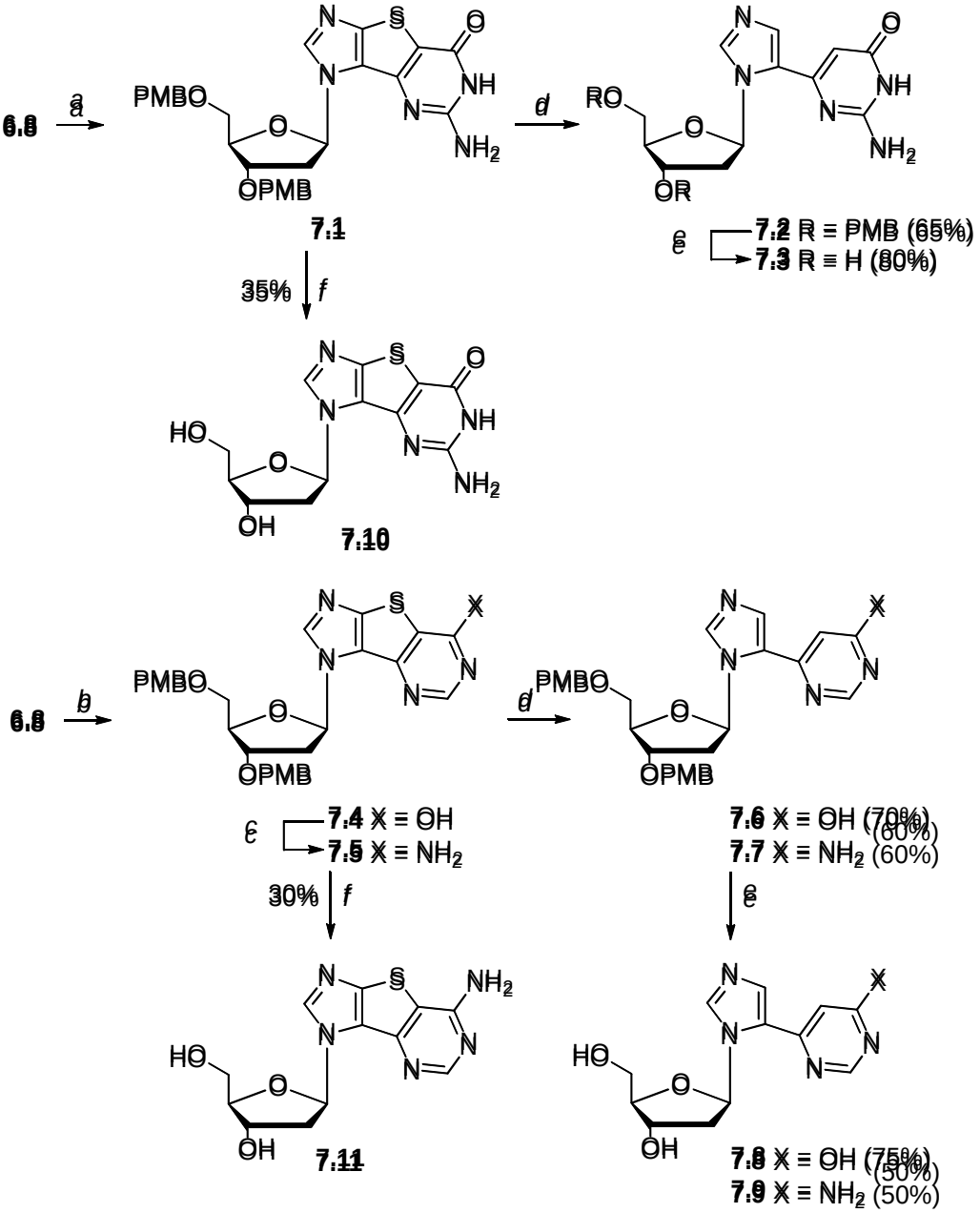


Reagents and conditions: a. (i) NaH, CH_3CN, rt, 30 min, (ii) 4,5-diiodoimidazole, rt, 18 h (84%); b. $NaOCH_3$, CH_3OH, rt, 4 h (87%); c. (i) NaH, THF, rt, 3 h, (ii) TBAI, PMBCl, rt, 18 h (64%); d. (i) EtMgBr, THF, 0°C, 15 min; (ii) DMF, 0°C, 20 min (90%); e. NH_2OH•HCl,

$NaHCO_3$, H_2O, EtOH, rt, 18 h (79%); f. CDI, THF, 60°C, 18 h (60%); g. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 60°C, 19 h; h. EtONa, EtOH, 80°C, 6-9 h (62% in two steps).

Unfortunately, the previous methodology for the final cyclization step of the tricyclic guanosine proved ineffective in the presence of the 2'-deoxy sugar, thus another route was developed (Scheme 7). The bicyclic intermediate **6.8** was treated with potassium ethyl xanthate instead of carbon disulfide for the initial cyclization. *t*-Butylhydroperoxide was used instead of hydrogen peroxide to decrease the water content. This change gave the guanosine product **7.1** with only a trace amount of the xanthosine analogue.[129, 146, 147] The route to the inosine derivative utilized molecular sieves instead of Ac_2O to prevent production of AcOH while refluxing in triethyl orthoformate. Also, instead of the three-step modification of the exocyclic enol to obtain the adenosine tricyclic from the inosine (Scheme 1), a two-step reaction starting by treating **7.4** with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) followed by aminolysis, shortened the overall reaction time by two days.[145, 147] Finally, treatment of the thieno-expanded guanosine, adenosine and inosine analogues with Raney nickel followed by palladium assisted deprotection gave the desired fleximers (**9.3**, **9.8** and **9.9**).[129]

Scheme 7. Optimized synthesis of the tricyclic and fleximer nucleosides.[129, 147]



Reagents and conditions: a. (i) KSC(S)OEt, DMF, reflux, 4 h, (ii) *t*-butylhydroperoxide, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 125°C, 18 h. (53%); b. $CH(OEt)_3$, 4Å molecular sieves, 150°C, 6 h (88%); c. (i) TPSCl, DMAP, NEt_3, CH_3CN, 3 h, (ii) NH_3, CH_3OH, 24 h (65%); d. Raney Ni, CH_3OH, 65°C, 72 h; e. Pd/C, ammonium formate, EtOH, reflux, 18 h; f. BF_3•OEt_2, EtSH, CH_2Cl_2.

As described above, the guanosine, adenosine and inosine fleximer analogues were readily achieved following treatment with Raney nickel, however, to our surprise, the xanthosine fleximer was recalcitrant to removal with Raney nickel despite many different attempts using various reaction times and temperatures.[129] Ultimately, the xanthosine fleximer was achieved through a different approach, using palladium catalyzed coupling of the two heterocyclic moieties (Figure 22).[129]

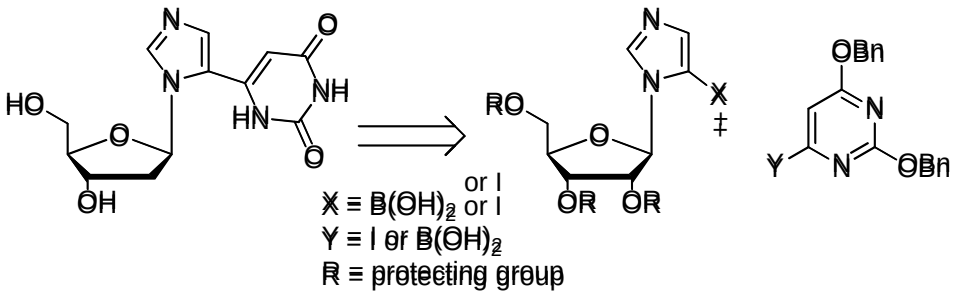
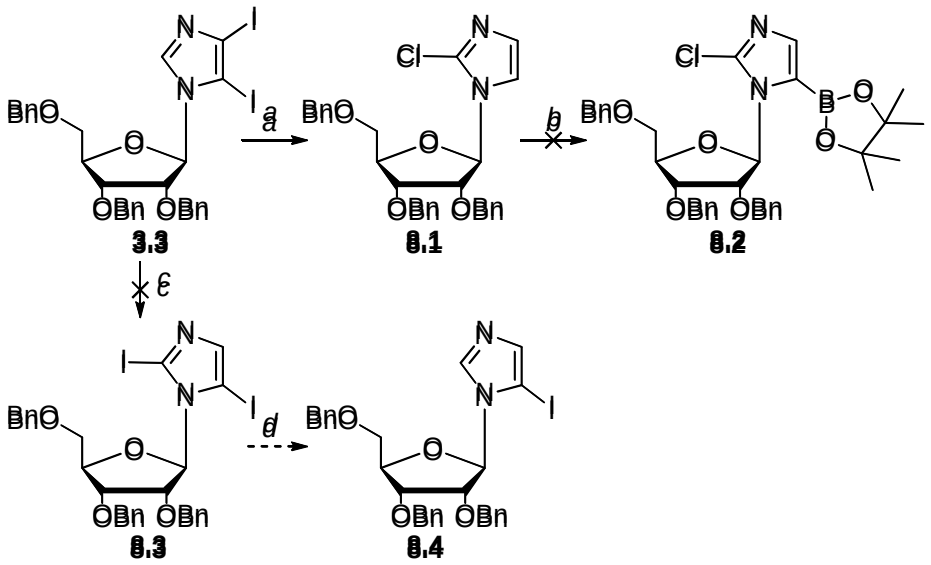


Figure 22. Retrosynthetic outline to constructing the xanthosine fleximer.

To realize the distal xanthosine fleximer, a 5-iodoimidazole nucleoside needed to be constructed to be used in subsequent palladium catalyzed coupling reactions (Figure 22). As the reactivities of the different positions of the imidazole ring are well known,[158] strategic manipulation of the halogens on the 4,5-diiodoimidazole analogue **3.3** could be carried out (Scheme 8). The protected diiodoimidazole nucleoside **3.3** was first deiodinated followed by lithiation and electrophilic trapping using hexachloroethane to produce the 2-chloro intermediate **8.1**.[159] Chlorine was employed to occupy the most reactive C-2 position so that treatment of **8.1** with *n*-butyllithium (*n*-BuLi)/tetramethylethylenediamine would produce the transient 5-lithioimidazole, which could then be slowly quenched with bis(pinacolato)diboron to generate the

boronic ester **8.2**. Unfortunately, the reaction mixture proved too difficult to purification, thus **8.2** was never isolated. Alternatively, once both iodines were removed, reiodination of the imidazole would theoretically provide the 2,5-diiodoimidazole intermediate **8.3**. This intermediate could then be selectively deiodinated at the C-2 position to give the requisite C-5 substituted imidazole **8.4**.[160] However, this methodology was also unsuccessful and was abandoned.

Scheme 8. Failed attempts to achieve the xanthosine fleximer.

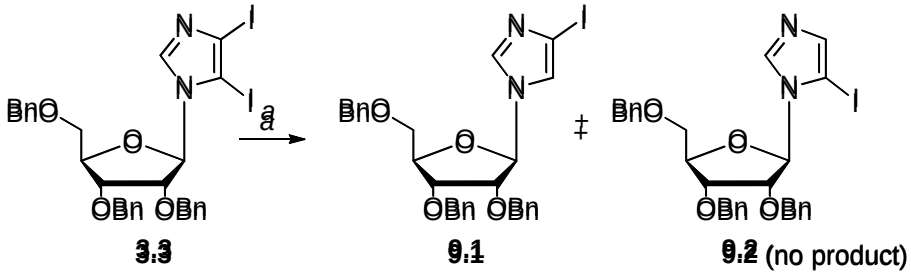


Reagents and conditions: a. (i) EtMgBr, THF, rt, 3 h (ii) *n*-BuLi, C_2Cl_6, -78°C to rt, 2.5 h (50%); b. (i) TMEDA, *n*-BuLi, bis(pinacolato)diboron, -78°C to rt, 18 h; c. (i) EtMgBr, THF, rt, 3 h (ii) TMEDA, *n*-BuLi, I2, -78°C to rt, 18 h; d. *n*-BuLi or EtMgBr, THF, rt, 3 h.

Next, selective deiodination of the 4,5-diiodoimidazole nucleoside **3.3** was tried using sodium sulfite (Na_2SO_3) in an attempt to obtain both C-4 and C-5 mono-

substituted imidazole nucleosides (Scheme 9), however only the unwanted C-4 iodo isomer **9.1** was obtained as confirmed *via* NMR using a NOESY experiment.

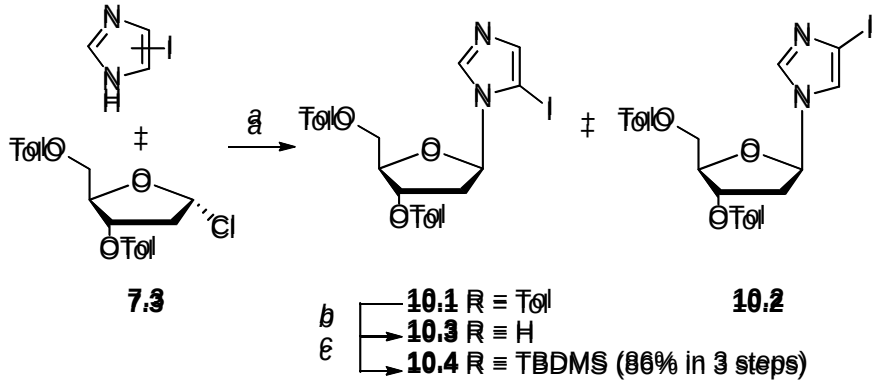
Scheme 9. Deiodination of **3.3** using sodium sulfite.



Reagents and conditions: a. Na_2SO_3, DMF, H_2O, reflux, 18 h.

Since attempting to deiodinate to the correct isomer failed while diiodoimidazole was coupled to the protected ribose ring (Scheme 9), Hoffer's chlorosugar **7.3** was coupled to 4(5)-iodoimidazole to yield the two isomeric compounds **10.1** and **10.2**.[129] The structure of each isomer was confirmed via COSY and NOESY experiments (Scheme 10).[129] Since the distal xanthosine was the desired compound, the toluoyl groups of the 5-iodo imidazole nucleoside **10.1** were replaced with *t*-butyldimethylsilane (TBDMS).[129] This deprotection and reprotection strategy was necessary since the subsequent coupling was unsuccessful with the toluoyl groups present.[129] Through previous attempts, it was already known that the imidazole moiety would not form a stable organometallic complex,[127, 128] therefore the pyrimidine was chosen as the most suitable organometallic coupling partner.

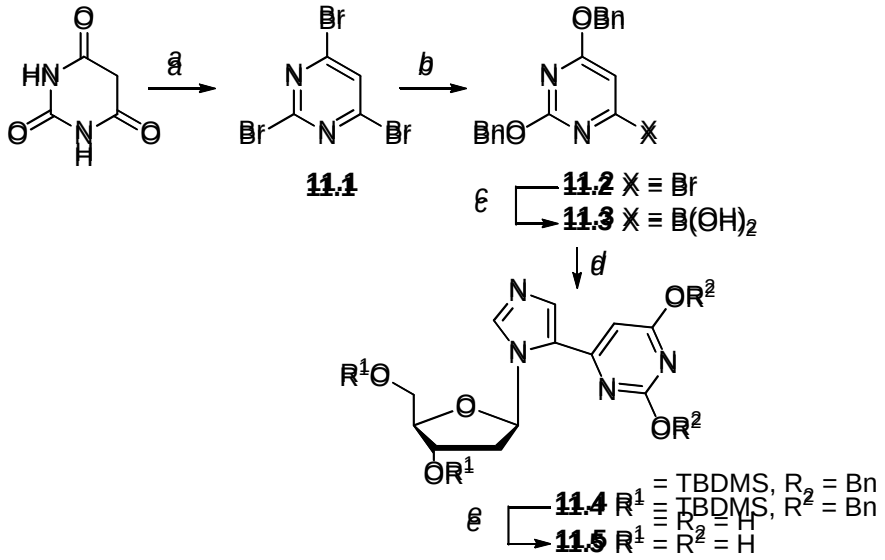
Scheme 10. Coupling of 4(5)-iodoimidazole to Hoffer's chlorosugar.[129]



Reagents and conditions: a. (i) NaH, CH_3CN, 30 min, (ii) 4(5)-iodoimidazole, 24 h, rt.; b. $NaOCH_3$, CH_3OH, 4 h, rt; c. TBDMSCl, imidazole, DMF, 18 h.

The xanthosine fleximer **11.5** was finally achieved *via* Suzuki-Miyaura coupling of **10.4** and **11.3** (Scheme 11), the latter of which was constructed in three steps from barbituric acid.[129]

Scheme 11. Synthesis of 2'-deoxyxanthosine distal fleximer.[129]



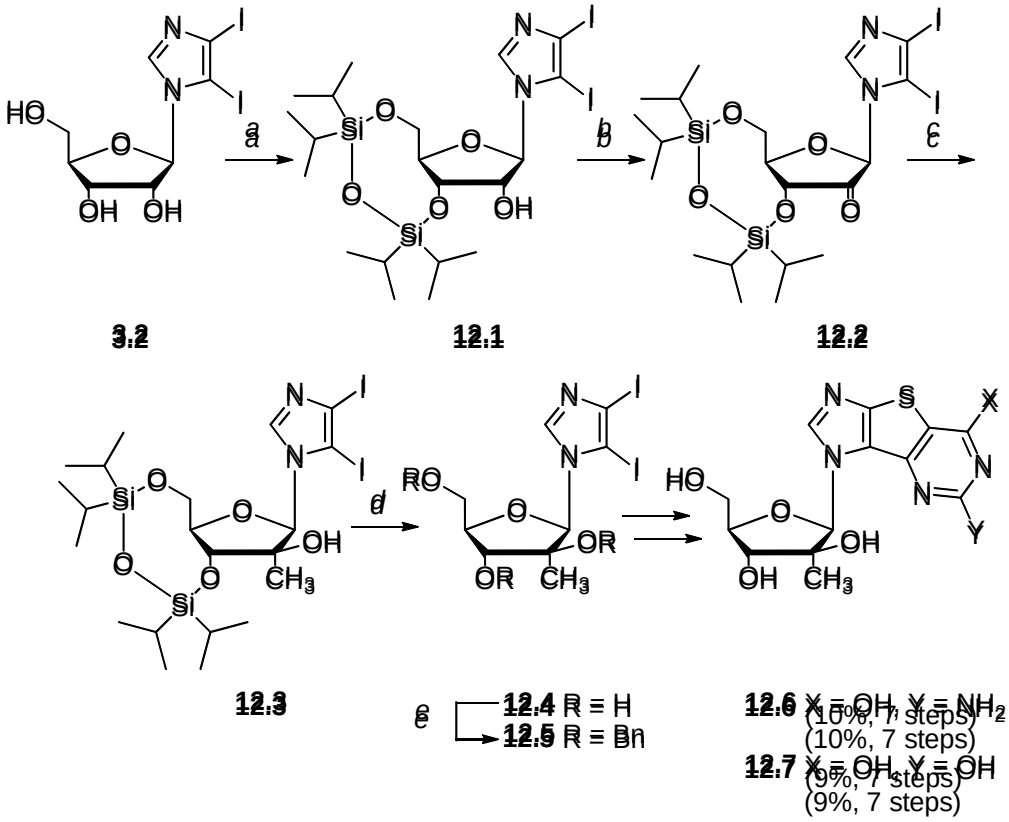
Reagents and conditions: a. $POBr_3$, *N*,*N*-dimethylaniline, toluene, reflux, 3 h (97%); b. BnOH, NaOBn, benzene, rt, 18 h (79%); c. (i) triisopropylborate, THF/toluene (1:4), -78°C, (ii) *n*-BuLi, 18 h, (iii) HCl; d. (i) 10.4, $Pd(PPh_3)_4$, DME, 10 min, (ii) $NaHCO_3$, reflux, 4 h (75%); e. (i) ammonium formate, Pd/C, EtOH, reflux, 18 h, (ii) TBAF, THF (53%).

III. 2'-Modified *Distal* Fleximers

In addition to the 2'-deoxy series, substitutions at the 2'-position have become a popular modification given the success of FDA approved therapeutics such Sofosbuvir.[11, 146, 161-163] As such, the guanosine fleximers of Sofosbuvir (CH_3 "up," F "down") and its precursor (OH "up," CH_3 "down") were pursued. The initial attempt took the route previously tried with the 2'-deoxy Barton deoxygenation reaction presented in

Scheme **4**.[146, 163] The TIPDS protecting group was installed onto the diiodoimidazole nucleoside intermediate **3.2** such that the 2'-OH was left unprotected and could then be oxidized using pyridinium chlorochromate (PCC). Methylation using trimethylaluminum yielded the arabinose analogue **12.3** (confirmed through NOESY). Construction of the tricyclic base utilized the same reagents and conditions found in Scheme 6 and Scheme 7 to generate the tricyclic guanosine (**12.6**) and xanthosine (**12.7**) derivatives. Unfortunately, both compounds were subsequently determined to be inactive against HCV, and due to the extremely low yields, the project was temporarily abandoned.[146]

Scheme 12. Synthesis of 2'-OH/CH_3 tricyclic nucleosides.[146]

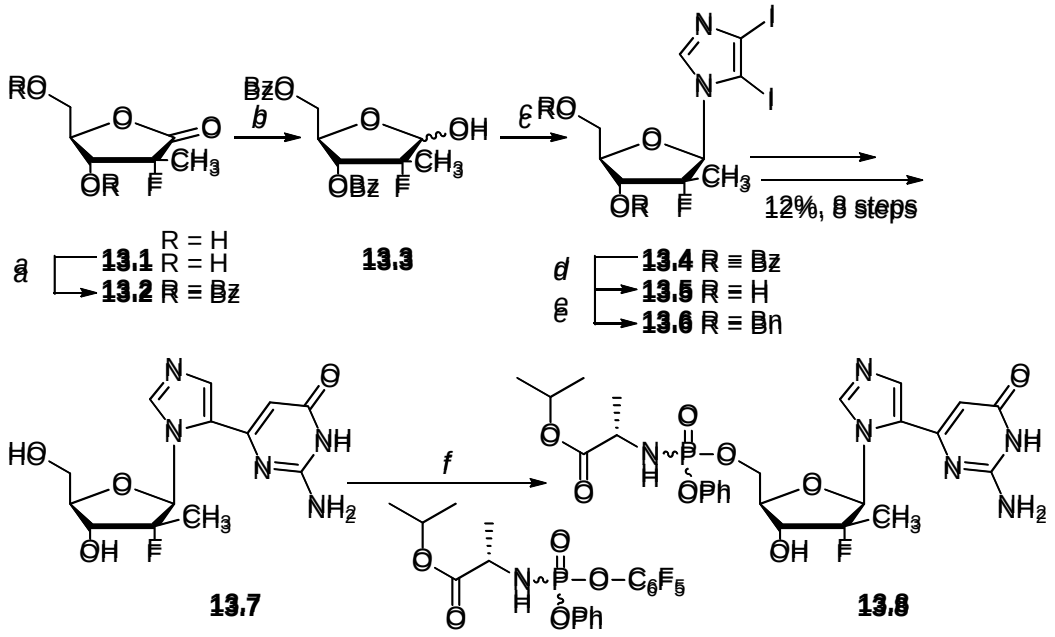


Reagents and conditions: a. TIPDSCl, pyridine, rt, 14 h (81%); b. PCC, CH_2Cl_2, 4Å molecular sieves, rt, 12 h (67%); c.$(CH_3)_3Al$, CH_2Cl_2, 0°C to rt, 2 h (73%); d. TBAF, THF, rt, 5 h (76%); e. (i) NaH, THF, rt, 3 h, (ii) TBAI, BnBr, rt, 18 h (65%).

An attempt to construct a fleximer with the same modified sugar as Sofosbuvir (CH_3 "up," F "down") was also pursued.[161, 162, 164] The author of this dissertation and Dr. Zhe Chen spearheaded this aspect of the 2'-modified project, which will be discussed further in depth in Chapter 3. As the previous procedure was not facile or useful (Scheme 12), a route starting with D-mannitose was tried (Scheme 13).[164] Commercially available D-mannitol was converted to the ribolactone **13.1** in nine steps, which was then benzoyl protected at the 3' and 5' positions prior to

reduction of the anomeric oxygen.[161, 164] Unfortunately, stereospecific orientation of the anomeric OH could not be controlled via various reducing reagents, and subsequent Mitsunobu coupling produced mainly the α-isomer instead of the desired β-isomer (**13.4**), leading to significantly low yields.[161] The guanosine fleximer **13.7** was finally obtained using reagents and conditions used in Scheme 3 and Scheme 7.[161] The McGuigan ProTide of **13.7** was also pursued (**13.8**) as a comparison to Sofosbuvir (mentioned in Chapter 1). Unfortunately, these analogues showed only minor activity against HCV (EC_{50} = 63.1 μM and 50.6 μM for the nucleoside and prodrug, respectively, unpublished) and the yields were once again extremely low (0.09% overall yield).

Scheme 13. Synthesis of 2'-CH_3/F Flex G and its McGuigan Protide.[161]



Reagents and conditions: a. BzCl, pyridine, 0°C, 10 min, rt, 30 min (80%); b. LiAl(O*t*Bu)$_3$H, THF, -20°C, 6 h (85%); c. 4,5-diiodoimidazole, Ph_3P, DIAD, THF, 0°C to rt,

24 h (20%); d. MeONa, CH_3OH, 0°C, 1 h (85%); e. (i) NaH, DMF, 0°C, 1 h, (ii) TBAI, BnBr, rt, 4 h (81%); f. *t*-BuMgCl, DMF, 0°C to rt, 18 h..

Proximal *Fleximers*

I. Ribose *Proximal* Fleximers

The ribose proximal fleximer derivatives were obtained by a much more facile route using organometallic coupling methodologies since the desired substitution patterns on the heterocycles were either commercially available or easily achievable. The desired C-C bond between the two aromatic moieties of the fleximer base could be constructed through palladium catalyzed cross-coupling methodologies (Stille, Suzuki-Miyaura, Kumada or Negishi), avoiding the multistep linear synthesis observed in the distal fleximer section. Initial attempts were surprisingly unproductive however, so extensive studies were undertaken using a number of those approaches before suitable coupling conditions were ultimately found.

Since the 4-iodoimidazole nucleoside could be obtained easily through various dehalogenation techniques mentioned previously, that aspect was straightforward. From there, however, a decision had to be made as to which aromatic moiety would be the organometallic partner and which would be the halogenated coupling partner (Figure 23). Initially, attempts focused on installing the organometallic moieties onto the imidazole ring, which proved highly ineffective. The best yields occurred after forming the organostannane complex with the imidazole (<5%) using *n*-BuLi in the presence of tributyltin chloride, however, the

major product was the completely deiodinated imidazole nucleoside. Forming the organoborate, Grignard and organozinc reagents were also attempted, however, the former two were never generated and while the latter was shown to have formed *in situ*, it did not yield the correct product during the coupling step with the halopyrimidine. Because of these challenges, the pyrimidine was then selected as the organometallic coupling partner.

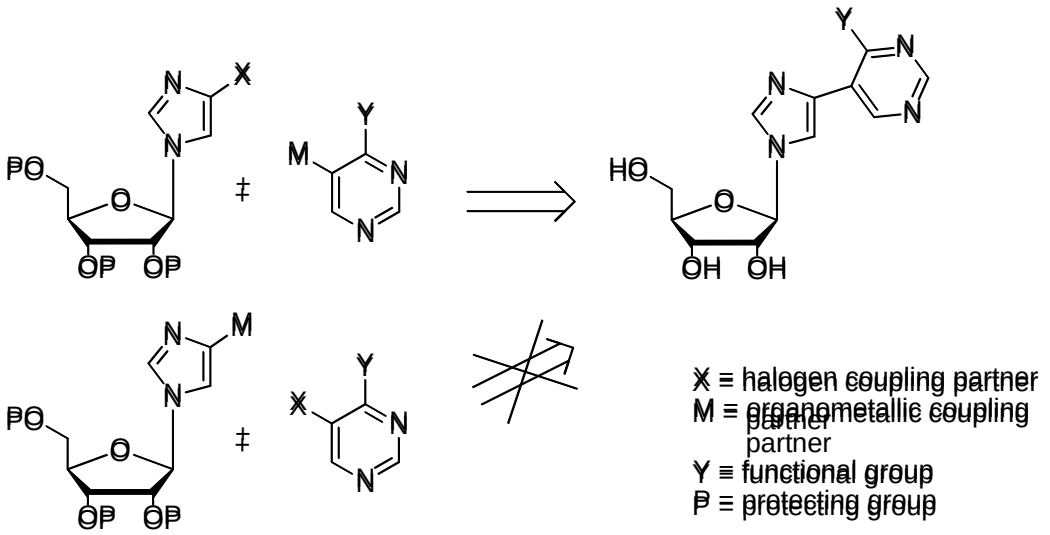
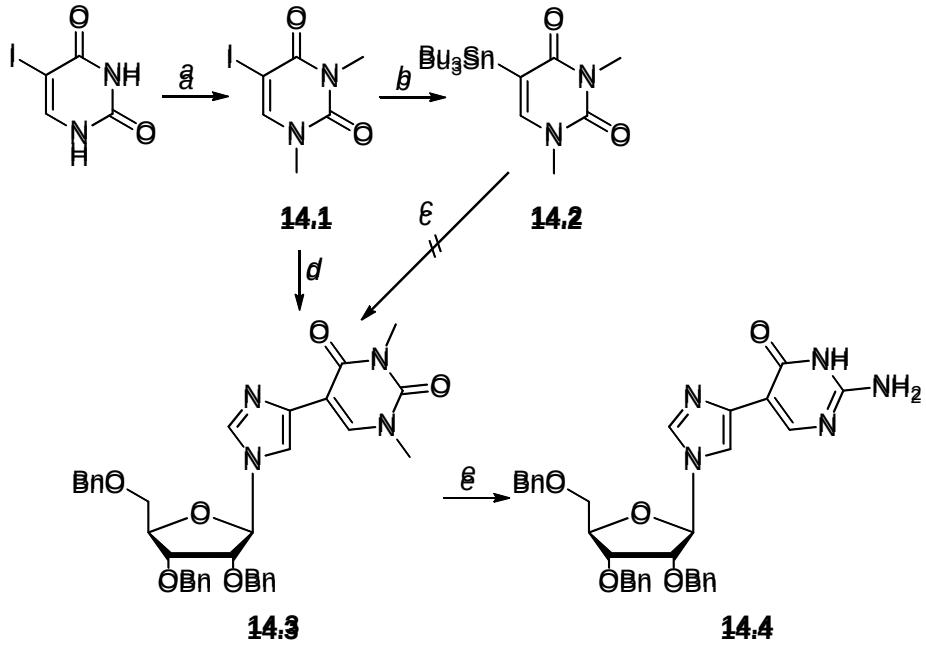


Figure 23. Retrosynthetic outline for synthesizing proximal analogues.

The first attempt to synthesize the guanosine proximal fleximer started with bis-1,3-methyl protected 5-iodouracil (**14.1**, Scheme 14). The 5-tributylstannane was then installed using palladium catalysis to give 5-tri-*n*-butylstannyl-1,3-dimethylpyrimidine **14.2**. It is important to note that the synthesis of the corresponding Kumada and Negishi metalated pyrimidines were also attempted, however, only the stannyl pyrimidine was successfully made.[128]

Unfortunately, all attempts at using palladium catalyzed coupling proved ineffective despite using various palladium catalysts and conditions.[128] Instead, adding excess cuprous thiophene-2-carboxylate (CuTC) ultimately produced **14.3** in a 20% yield.[128] Synthesis of the desired protected guanosine proximal fleximer **14.4** was then attempted by refluxing **14.3** with guanidine and sodium ethoxide in ethanol, however, only a trace amount of the product was found (identified through mass spectrometry).[128]

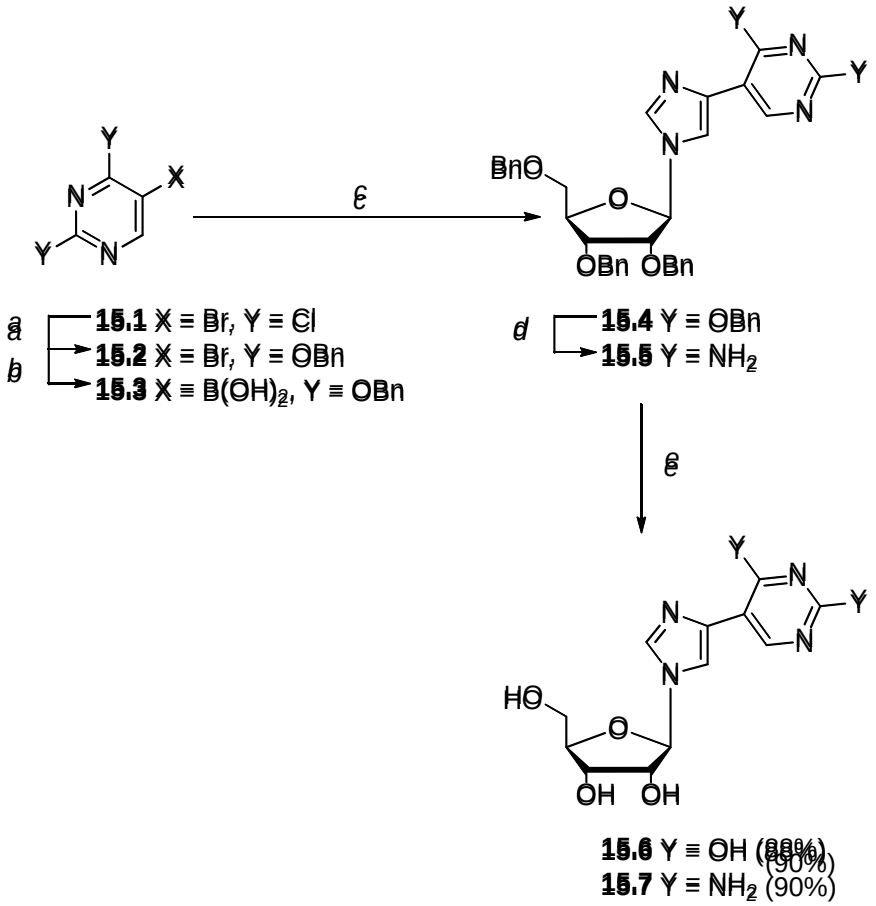
Scheme 14. First synthesis of the guanosine proximal fleximer.[128]



Reagents and conditions: a. $(CH_3)_2SO_4$, H_2O, reflux, 2 h (80%); b. $(SnBu_3)_2$, $PdCl_2(PPh_3)_2$, toluene, 90°C, 4 h (62%); c. **9.1**, Pd catalysts; d. **9.1**, CuTC, NMP, rt, 18 h (20%); e. guanidine, NaOEt, EtOH, reflux, 24 h (trace).

The guanosine proximal fleximer was finally achieved via Suzuki-Miyaura coupling with 5-(dihydroxyboryl)-2,4-bis(benzyloxy)pyrimidine **15.3** and **13.1** (Scheme 15);[128] a procedure described by Schinazi and Prusoff showed that slow, dropwise addition of *n*-BuLi to an aryl halide and alkyl borate provided excellent yield of the boronic acids.[165] The di-OBn intermediate **15.4** was converted to the diamino intermediate **15.5**, and deprotection of **15.4** and **15.5** yielded the xanthosine and 2,6-diaminopurine proximal fleximers **15.6** and **15.7**, respectively.[128]

Scheme 15. Synthesis of xanthosine and 2,6-diaminopurine proximal fleximers.[128]

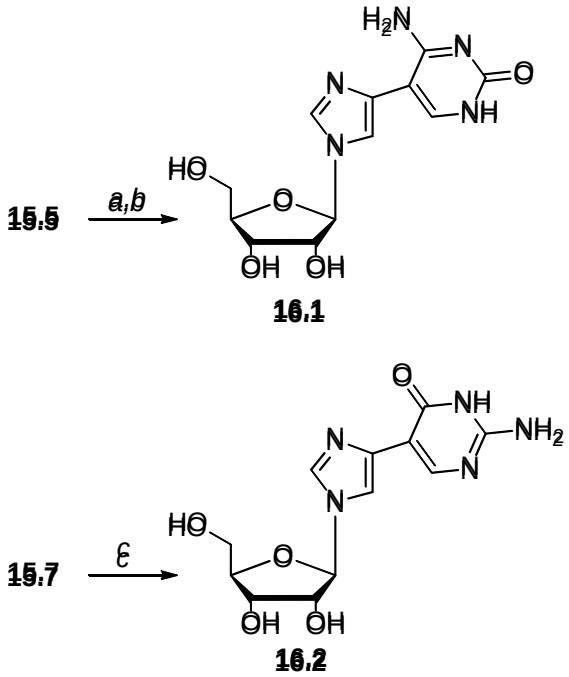


Reagents and conditions: a. NaH, BnOH, toluene, rt, 18 h (91%); b. (i) triisopropylborate, THF/toluene (1:4), -78°C, (ii) *n*-BuLi, rt, 18 h, (iii) HCl (95%); c. (i) **13.1**, $Pd(PPh_3)_4$, DME,

10 min, (ii) $NaHCO_3$, reflux, 4 h (67%); d. NH_3, CH_3OH, 170°C, 96 h (40%); e. Pd/C, ammonium formate, EtOH, reflux, 2h.

As seen in Scheme 16, the isoguanosine fleximer **16.1** was obtained by treating **15.5** with sodium nitrite which selectively converted the C-2 pyrimidine amine to a ketone.[128] The guanosine fleximer **16.2** was then obtained by refluxing aqueous sodium bisulfite with **15.7**.[128]

Scheme 16. Synthesis of isoguanosine and guanosine proximal fleximers.[128]

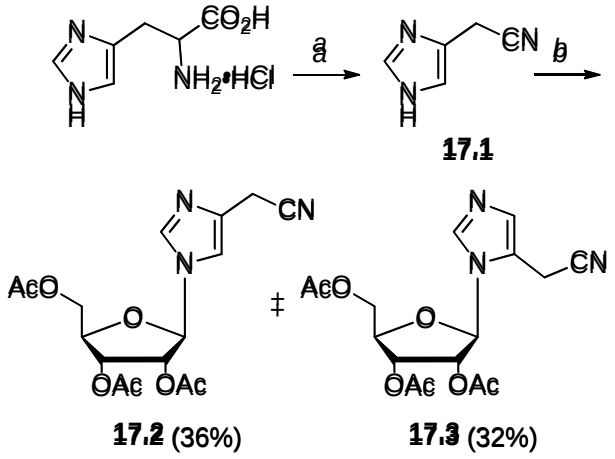


Reagents and conditions: a. $NaNO_2$, AcOH, H_2O/THF (1:1), 60°C, 2 h; b. Pd/C, ammonium formate, EtOH, reflux, 2 h (41%, two steps); c. $NaHSO_3$, H_2O, 60°C, 10 h (88%).

Since the proximal adenosine and inosine fleximer was unable to be realized through palladium catalyzed coupling, the pyrimidine moiety was generated through

a cyclization methodology (Scheme 17).[127] Commercially available histidine monohydrate was treated with sodium hypochlorite to give 4(5)-cyanomethylimidazole **17.1**.[127] Interestingly, only Clorox® bleach consistently produced the desired product whereas solutions of sodium hypochlorite obtained from commercially available chemical suppliers did not, nor did various "house brand" bleaches. Those reactions led to poor yields or no reaction at all. Speculation was that the house brands might be more dilute thereby leading to the lack of reaction or poor yields, although this does not explain the lack of reaction with the commercially available solutions. Finally, Vorbrüggen coupling with β-D-ribofuranose-1,2,3,5-*O*-tetraacetate gave a mixture of the 4- and 5-cyanomethylimidazole nucleosides (**17.2** and **17.3**, respectively) with a nearly 1:1 ratio.[127]

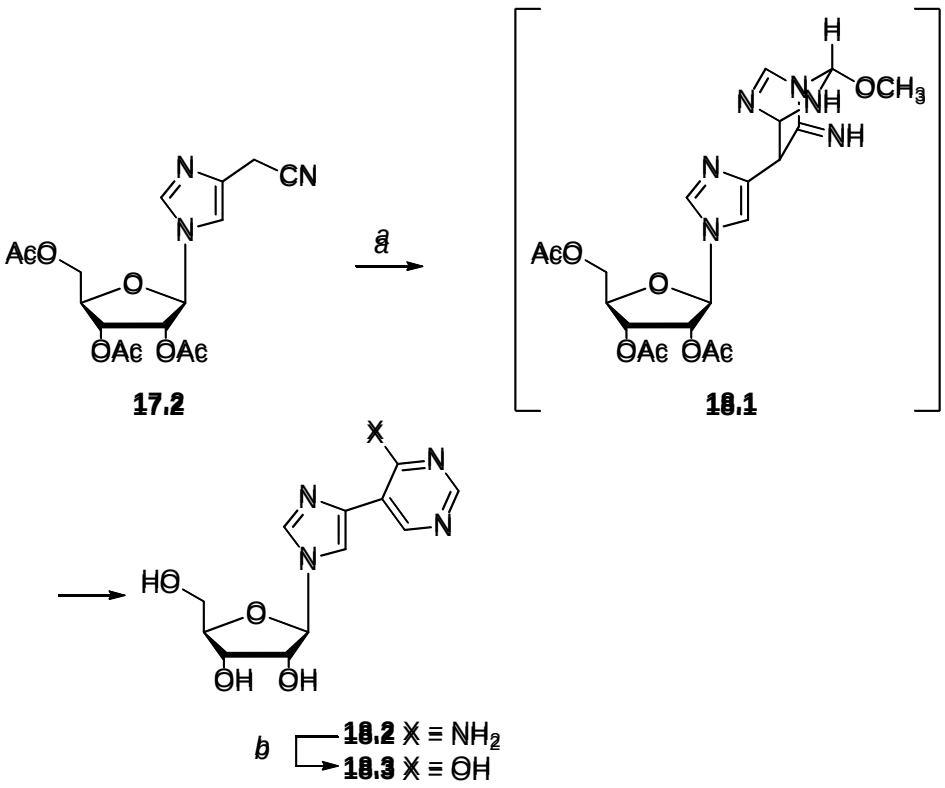
Scheme 17. Synthesis of 4- or 5-cyanomethylimidazole nucleoside from histidine.[127]



Reagents and conditions: a. NaOCl, H_2O, 10-20°C, 3 h, then rt, 18 h (70%); b. (i) β-D-ribofuranose-1,2,3,5-*O*-tetraacetate, BSA, CH_3CN, 3 h, (ii) TMSOTf, 60°C, 18 h.

In situ formation of the proximal adenosine fleximer from **17.2** (Scheme 18) was accomplished via a [4+2] Diels-Alders cycloaddition using sodium methoxide and 1,3,5-triazine, which was followed by a retro-Diels-Alders fragmentation of the intermediate **18.1** that then formed the desired product **18.2**.[127] The acetate protecting groups were also removed during this reaction, however, this particular reaction also went in poor yield (15%, 3.8% overall from histidine).[127] The inosine fleximer **18.3** was then synthesized from the adenosine analogues using standard diazotization conditions, followed by hydrolysis.[127, 166]

Scheme 18. Synthesis of the proximal adenosine fleximer.[127]

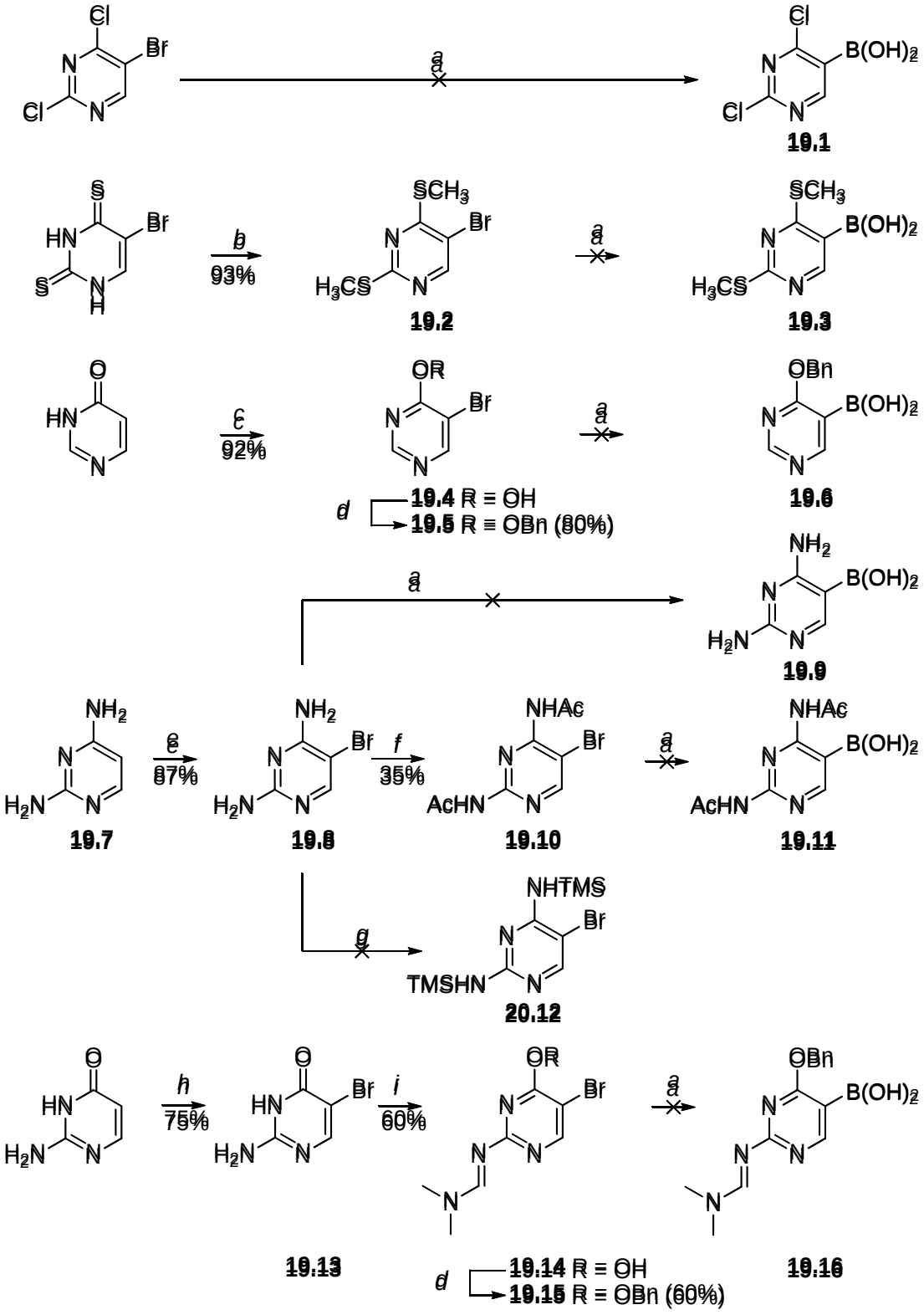


Reagents and conditions: a. 1,3,5-triazine, $NaOCH_3$, CH_3OH, 40°C, 18 h (15%); b. $NaNO_2$, AcOH, H_2O/THF (1:1), 80°C, 5 h (68%).

II. 2’-Deoxyribose *Proximal* Fleximers

Scheme 20 and Scheme 21 outline the attempts at synthesizing various pyrimidine organometallic complexes for use in either Suzuki-Miyaura or Stille coupling.[129] Prior to constructing the organoborane or stannyl pyrimidines, halogens were installed at the C-5 position of the pyrimidines if starting materials could not be purchased commercially. The halogenation reactions proved to be straightforward as the C-5 position is the least electron deficient,[167] which was also synergistically more reactive due to by the presence of electron donating substituents at the C-2 and C-4.[166] Organoborane synthesis similar to Scheme 11 was then attempted on several 2,4-subsitituted 5-halopyrimidnes (Scheme 19), however, unlike **15.3**, all efforts to isolate and characterize these boronic acids failed.[129] The purification step may have degraded the boronic acids as using the crude material did ultimately produce the correct products, although in low yields and with significant purification difficulties.

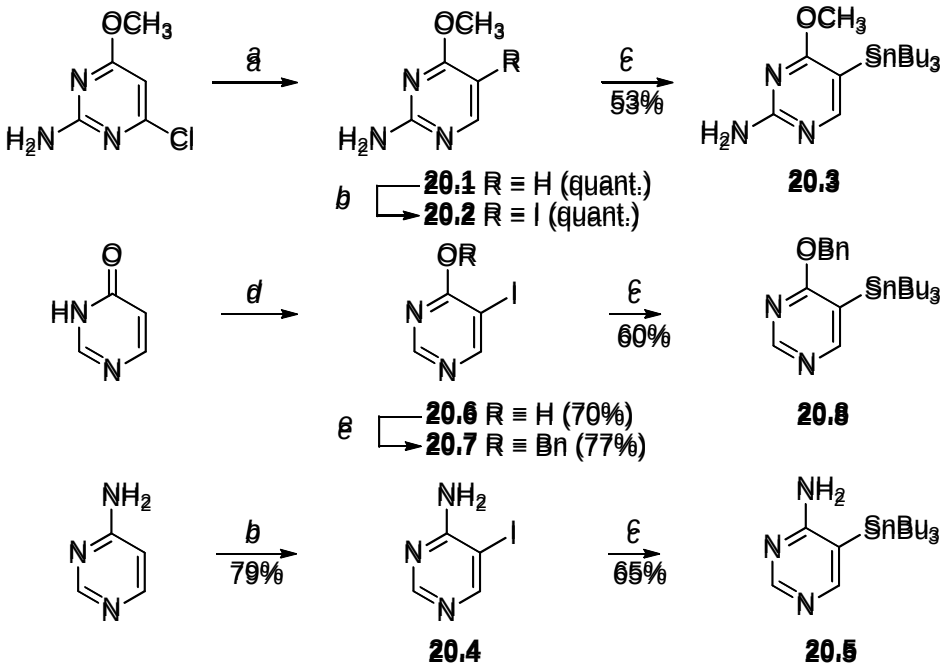
Scheme 19. Synthesis of boronic acid pyrimidine precursors.[129]



Reagents and conditions: a. (i) triisopropylborate, THF/toluene (1:4), -78°C, (ii) *n*-BuLi, rt, 18 h, (iii) HCl; b. NaOH, CH_3I, H_2O, 0°C, 4 h; c. Br_2, $CHCl_3$, CH_3OH, 12 h; d. DIAD, PPh_3, BnOH, DMF, 1.5 h; e. (i) NBS, $CHCl_3$, rt, 12 h (ii) CH_2Cl_2, 1M NaOH; f. AcCl, pyridine, 0°C, 2 h; g. TMSCl, pyridine; h. (i) Br_2, H_2O, rt, 2 h (ii) 0°C, 18 h; i. DMF-DMAc, DMF, rt, 18 h.

Synthesis of the 5-stannyl pyrimidines proved more facile (Scheme 20); once the halogens were installed, the stannyl was substituted *via* palladium catalysis.[129, 168]

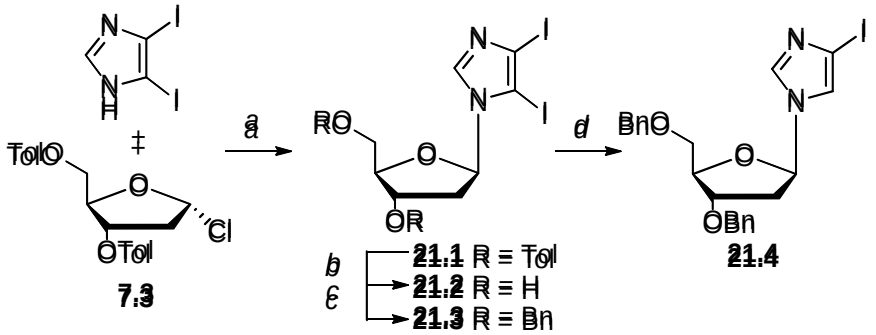
Scheme 20. Synthesis of tributylstannyl pyrimidine precursors.[129]



Reagents and conditions: a. H_2, Pd/C, DIPEA, EtOAc, EtOH, rt, 3 h; b. NIS, AcOH, 80°C, 2 h; c. $(SnBu_3)_2$, $Pd_2dba_3 \cdot CHCl_3$, 65°C, 3 h; d. NaOH, I_2, 80°C, 18 h; e. DIAD, PPh_3, BnOH, DMF, 1.5 h.

Palladium catalyzed cross-coupling was then possible once the pyrimidine organometal complexes were achieved.[129] In that regard, 4,5-diiodoimidazole was coupled to the Hoffer's chlorosugar **7.3** in the same fashion as was used in Scheme 6 (Scheme 21).[129, 147] Once again, the toluoyl protecting groups of the 2'-deoxyribose imidazole nucleoside **21.1** were replaced with benzyl protecting groups prior to removal of the C-5 iodine using Grignard conditions (**21.4**) such that palladium catalyzed coupling could then occur to achieve the proximal analogues.[129]

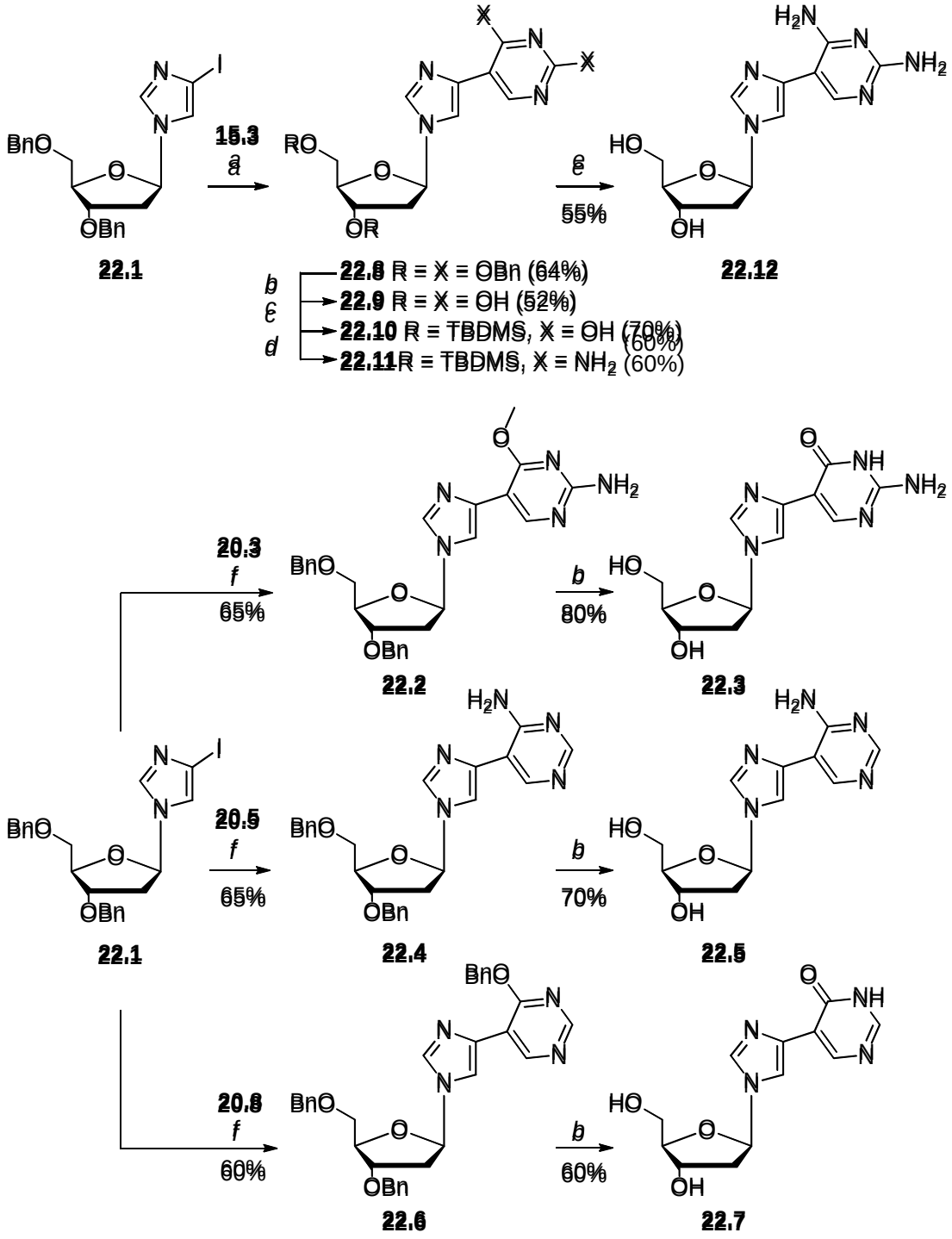
Scheme 21. Synthesis of 4-iodoimidazole 2'-deoxynucleoside precursor.[129, 147]



Reagents and conditions: a. (i) NaH, CH_3CN, rt, 30 min, (ii) 4,5-diiodoimidazole, rt, 24 h, rt.; b. $NaOCH_3$, CH_3OH, 4 h, rt; c. (i) NaH, THF, rt, 3 h, (ii) TBAI, BnBr, rt, 18 h (85%); d. EtMgBr, THF, 0°C, 3 h (80%).

Through conventional Suzuki and Stille heteroaryl cross-coupling methods (Scheme 22), followed by the deprotection steps, the guanosine (**22.3**), adenosine (**22.5**), inosine (**22.7**), xanthosine (**22.9**), and 2,6-diaminopurine (**22.12**) proximal fleximers were then obtained.[129]

Scheme 22. Stille couplings to achieve proximal fleximers guanosine, adenosine and inosine.[129]



Reagents and conditions: a. **15.3**, $Pd(PPh_3)_4$, DME, $NaHCO_3$, reflux, 4 h; b. ammonium formate, Pd/C, EtOH, reflux, 18 h; c. TBDMSCl, imidazole, DMF, rt, 18 h; d. (i) TPSCl,

DMAP, TEA, CH_3CN, rt, 18 h (ii) NH_3, THF, 110°C, 72 h; e. TBAF, THF, rt, 18 h; f. Pd_2dba_3•$CHCl_3$, DMF, 100°C, 18 h.

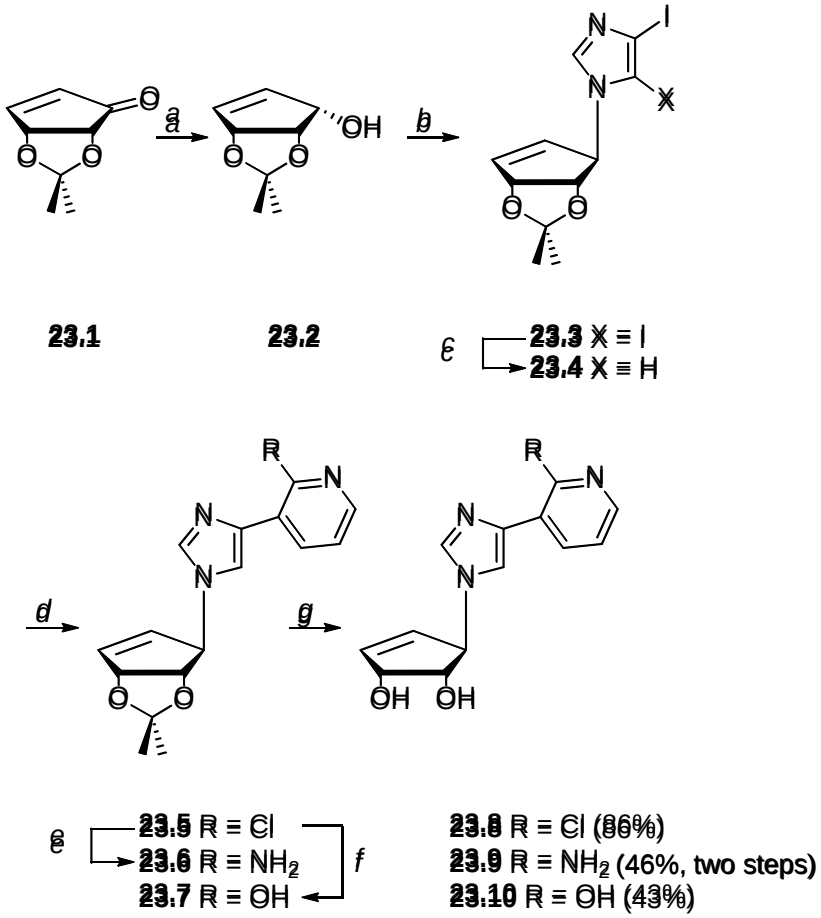
III. Carbocyclic *Proximal* Fleximers

Carbocyclic nucleosides are a naturally occurring type of sugar modified nucleoside wherein the furanose oxygen is replaced with a carbon.[12] For example Neplanocin A (NpcA) and Ari are both naturally occurring carbocyclic adenosine analogues that have exhibited potent antiviral, antiparasitic and anticancer properties, although both are associated with significant cytotoxicity due to their ability to be converted to their corresponding triphosphate nucleotides by adenosine kinase.[12] The carbocyclic triphosphates closely mimic ATP in ATP-recognizing enzymes, and are also metabolized by adenosine deaminase, leading to toxic metabolites.[169-175] Because of the potential of this class of nucleosides however, particularly given their activity against viruses such as Ebola, the Seley-Radtke group was interested in exploring carbocyclic fleximers. Thus, a series of proximal carbocyclic 5'-truncated deazaneplanocin fleximers were constructed (Scheme 23).[176] The truncated forms of both compounds, wherein the 4'-CH_2OH is removed, drastically lowers the cytotoxicity because these analogues cannot be phosphorylated.[177] In addition, it was found that the truncated 3-deaza analogues of NpcA and Ari were more effective inhibitors than their parent compounds.[175, 178-180]

To construct the Npc sugar, cyclopentanone **23.1** was synthesized from D-ribose[181], and using a stereospecific reduction of the ketone *via* Luche conditions, provided the "α" oriented allyl alcohol **23.2**.[176] Mitsunobu coupling of 4,5-

diiodoimidazole with the sugar gave the desired β-product as inversion at the anomeric site takes place.[176] The C-5 iodine was removed through Grignard conditions, and subsequent Stille coupling using 2-chloro-3-stannylpyridine produced the 6-chloro-3-deazapurine analogue **23.5**, which was then converted to the 3-deazaadenosine and 3-deazainosine derivatives.[176] Products **23.8**-**23.10** were achieved following isopropylidene deprotection.[150, 176]

Scheme 23. Synthesis of 5'-truncated 1-deazaneplanocin fleximer derivatives.[176]



Reagents and conditions: a. $NaBH_4$, CH_3OH, $CeCl_3 \cdot 7H_2O$, rt, 1 h (quant.); b. 4,5-diiodoimidazole, DPPE, DIAD, THF, rt, 18 h (41%); c. EtMgBr, THF, 0°C, 1 h (85%); d. 2-

chloro-3-stannylpyridine, $Pd(PPh_3)_4$, CuBr, 1,4-dioxane, 120°C, 12 h (64%); e. (i) hydrazine, 80°C, 1 h, (ii) $TiCl_3$, THF, 70°C, 4 h; f. AcOH, 120°C (56%); g. TFA/H_2O/THF (1:1:5), 18 h

.

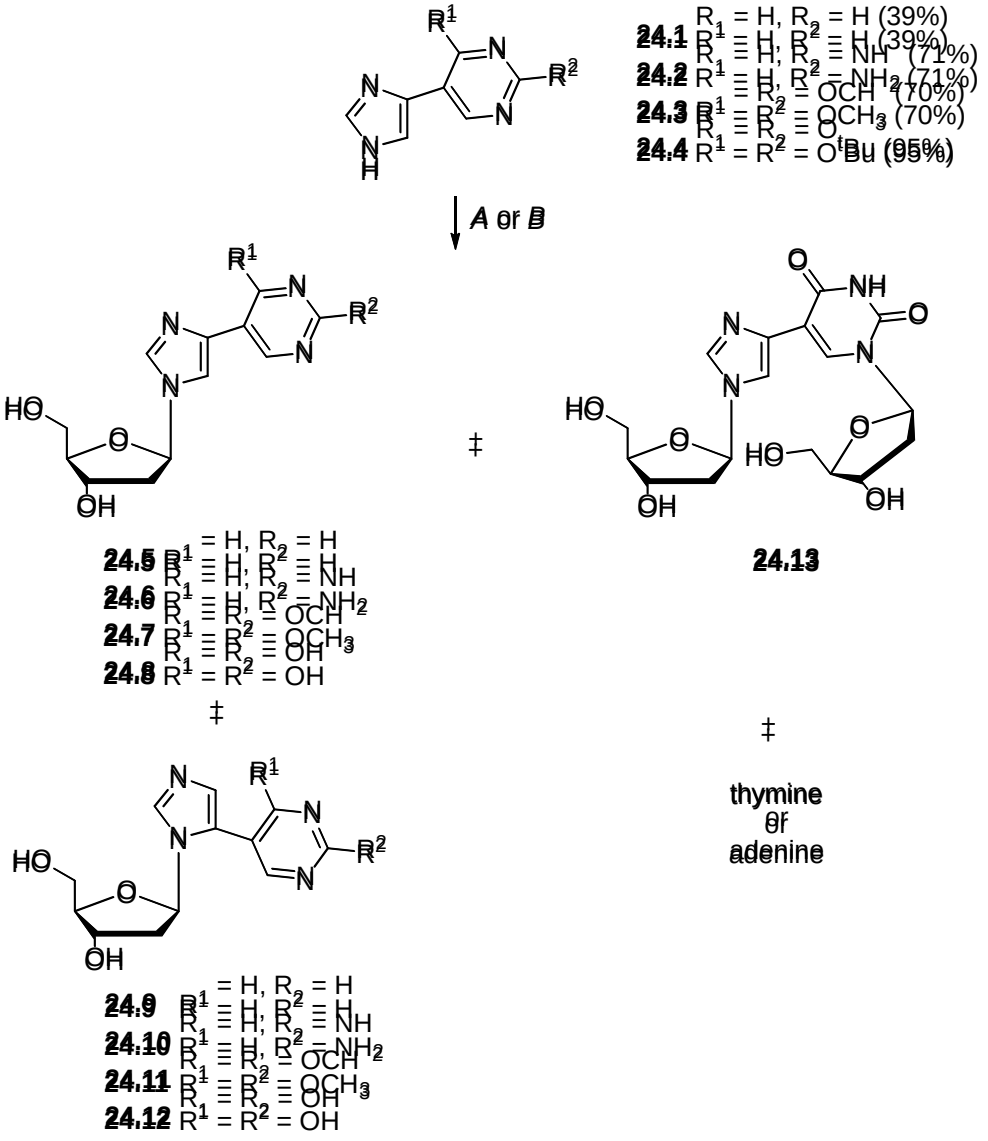
IV. *Proximal* Fleximers from other groups

In addition to our efforts, other groups have begun to explore the fleximer scaffold given their biological potential. For example, Pochet's group has found that the fleximer bases could be coupled to ribose and 2'-deoxyribose through enzymatic transglycosylation rather than synthetic coupling methodologies.[182, 183] Through a microwave assisted Suzuki-Miyaura cross-coupling procedure,[184] various 4-(hetero)aryl-1*H*-imidazoles were first constructed, four of which resemble the Seley-Radtke proximal fleximers (Scheme 24).

Enzymatic transglycoslation was then accomplished using *N*-deoxyribosyltransferase II of *L. leichmanii (Ll*-NDT*)* and *E. coli* purine nucleoside phosphorylase (PNP). They determined the relative substrate selectivity, and transglycosylation efficiency and regioselectivity of N1 versus N3 substitution (imidazole numbering).[182, 183] Results in Table 2 show that both enzymes could utilize all four fleximer bases as substrates however *Ll*-NDT proved to be the more efficient enzyme with better yields. Surprisingly, products from both N1 (**24.7**, the thermodynamic product) or N3 (**24.11**, the kinetic product) substitution were observed for *Ll*-NDT with the dimethoxy analogue **24.3** at 2 h, however only the proximal fleximer **24.7** was observed at the conclusion of the reaction. Interestingly, the *t*-butyl group of compound **24.4** was removed after 16 h, and the intended nucleoside analogues **24.8** and **24.12**, and a double-glycosylated product **24.16** were

observed.[182-184] A collaboration with Dr. Pochet's laboratory has been established using targets from Chapter 4 as the fleximer bases to be transglycosylated, more detail of the impetus and results we expect will be detailed in Chapter 5.

Scheme 24. Fleximer Transglycosylation products.[182-184]



Reagents and conditions: A. thymidine, *Ll*-NDT, citrate buffer pH 6.5, 50°C; B. adenosine, *E. coli* PNP, phosphate buffer pH 7.4, 50°C.

Table 2. Fleximer products *via* transglycosylation through *Ll*-NDT or PNP and their percent yields.[182, 184]

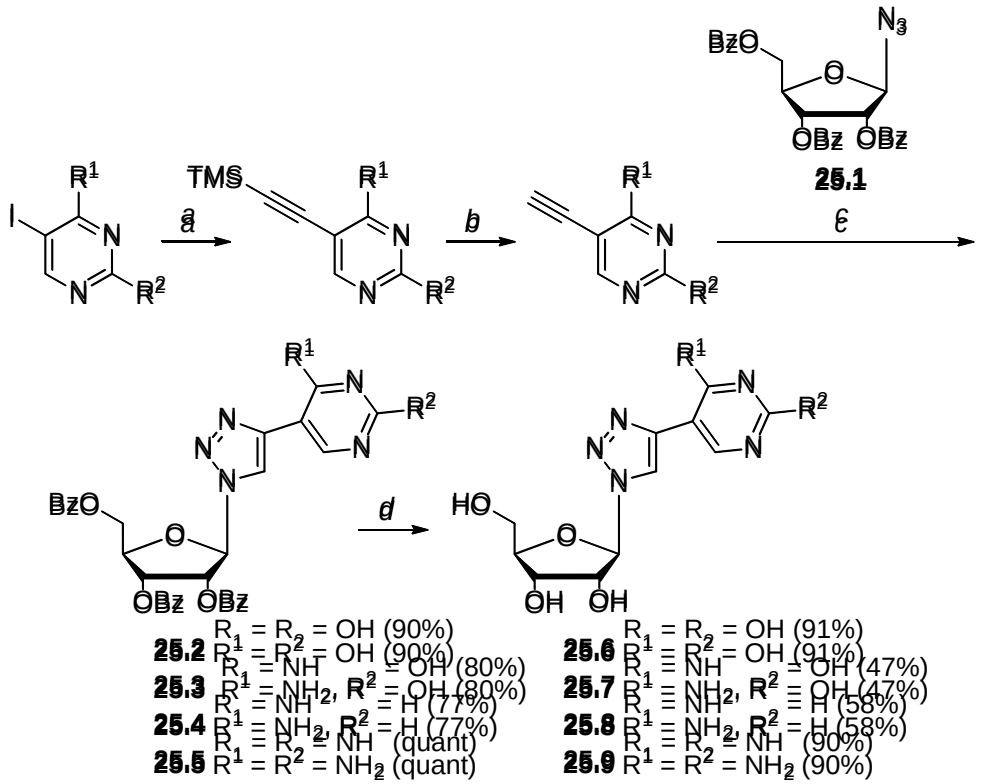
Entry	Base	Enzyme	Product	% conversion after 2/18 h[a]	% Final conversion[a]	Yield (%)[b]
1	**24.1**	*Ll*-NDT	**24.5**	31/nd	95	60
2	**24.2**	*Ll*-NDT	**24.6**	30/nd	91	50
3	**24.3**	*Ll*-NDT	**24.7/24.11**	20/24/nd/nd	0/90	0/82
4	**24.4**	*Ll*-NDT	**24.8/24.13**	7/nd	70	17/37
5	**24.1**	PNP	**24.5**	nd/8	76	43
6	**24.2**	PNP	**24.6**	nd/nd	nd	51
7	**24.3**	PNP	**24.7**	nd/40	67	51
8	**24.4**	PNP	**24.8**	nd/nd	nd	12

[a]% Conversions were determined by RP-HPLC analysis of an aliquot of the incubation mixtures monitored at 254 nm with a diode array detector. [b]Values refer to isolated yields after RP-HPLC purification of the incubation mixtures for specified times and amounts of enzyme. nd: not determined because of the partial solubility of acceptors in the media.

Hudson's group has also pursued several fleximers (Scheme 25).[185] They were attempting to derivatize 5-ethynyl-2'-deoxycytidines with various azides *via* a copper catalyzed azide-alkyne Huisgen cycloaddition (CuAAC) to form 5-triazoylcytidine analogues.[186] A similar approach using β-azidoribose[187] and 5-ethynyl-pyrimidines would yield "click" fleximers (**25.6**-**25.9**), where the imidazole was replaced with a triazole. The desired 5-(alkyn-1-yl)pyrimidine analogues were obtained in reasonable yields *via* Sonogashira coupling of 5-iodopyrimidines and

TMS-acetylene, followed by TMS removal.[188-191] The β-azidoribose was achieved through a Vorbrüggen coupling between azidotrimethylsilane and 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribofuranose.[187] CuAAC between the azide and alkyne produced the desired benzoyl protected click-fleximers, however, the conditions required to produce these analogues were somewhat harsh; lengthy reflux was required whereas normal CuAAC conditions typically require only room temperature stirring.[192]

Scheme 25. Robert Hudson's "click" fleximers.[185]



Reagents and conditions: a. TMS-acetylene, $Pd(PPh_3)_4$, CuI, DMF or THF, NEt_3; b. (i) 1M NaOH, (ii) HCl; c. CuI, DMEDA, THF, NEt_3, reflux; d. NH_3, CH_3OH, 0°C to rt.

“Reverse” Fleximers

Another series of fleximers that the Seley-Radtke group pursued was the “reverse” fleximer series. These 5-aryl substituted uracil analogues can also be viewed as N-3 substituted purine analogues. While Herdewijn *et al.* focused on the modified ribose derivatives, and many of them showed anti-HSV and varicella zoster virus (VZV) activities,[193-196] the Seley-Radtke group pursued the corresponding carbocyclic uracil-substituted, or fleximer isoxanthosine, analogues (Fig. 7).[132, 133] Previous studies on these isoanalogues determined that substituting a thiazole for the imidazole moiety of the purine scaffold imparted excellent SAHase inhibition,[197] and because interesting activity was observed for Herdewijn’s ribose derivatives, a similar reverse-fleximer scaffold was coupled to several carbocyclic sugars that have been previously studied by the Seley-Radtke group and others (Scheme 23).[132, 133, 176, 197, 198]

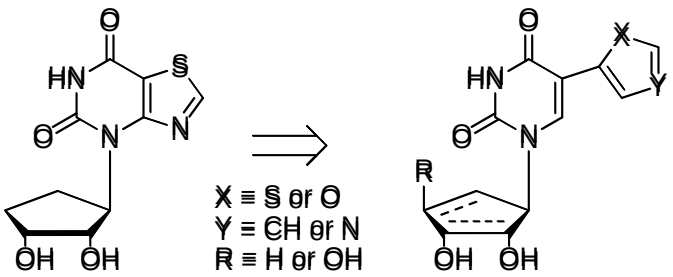
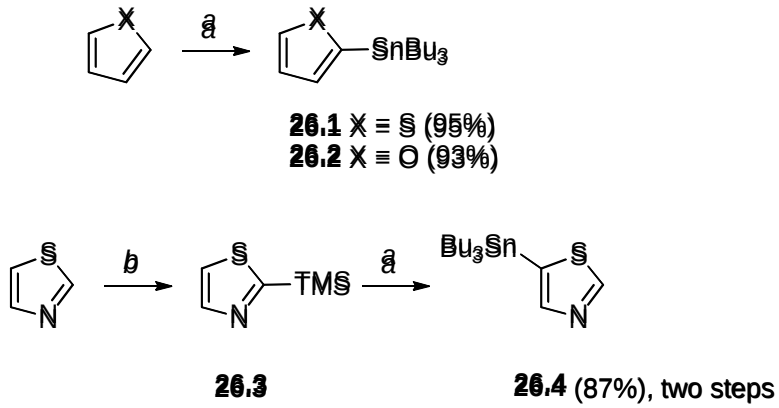


Figure 24. Retrosynthetic outline for synthesizing reverse fleximer analogues.

In contrast to imidazole, other 5-membered heteroaryl groups were able to act as the organometallic partner. As shown in Scheme 26, facile construction of 2-

(tributylstannyl)thiophene **26.1** and furan **26.2** was accomplished by treating either thiophene or furan with *n*-BuLi and quenching with tributyltin chloride.[199] The thiazole required more manipulation, thus the C-2 was first protected with TMS (**26.3**) so the stannyl could be installed at the C-5 position (**26.4**).[200]

Scheme 26. Synthesis of thiophene, furan and thiazole organostannanes.[132, 133]

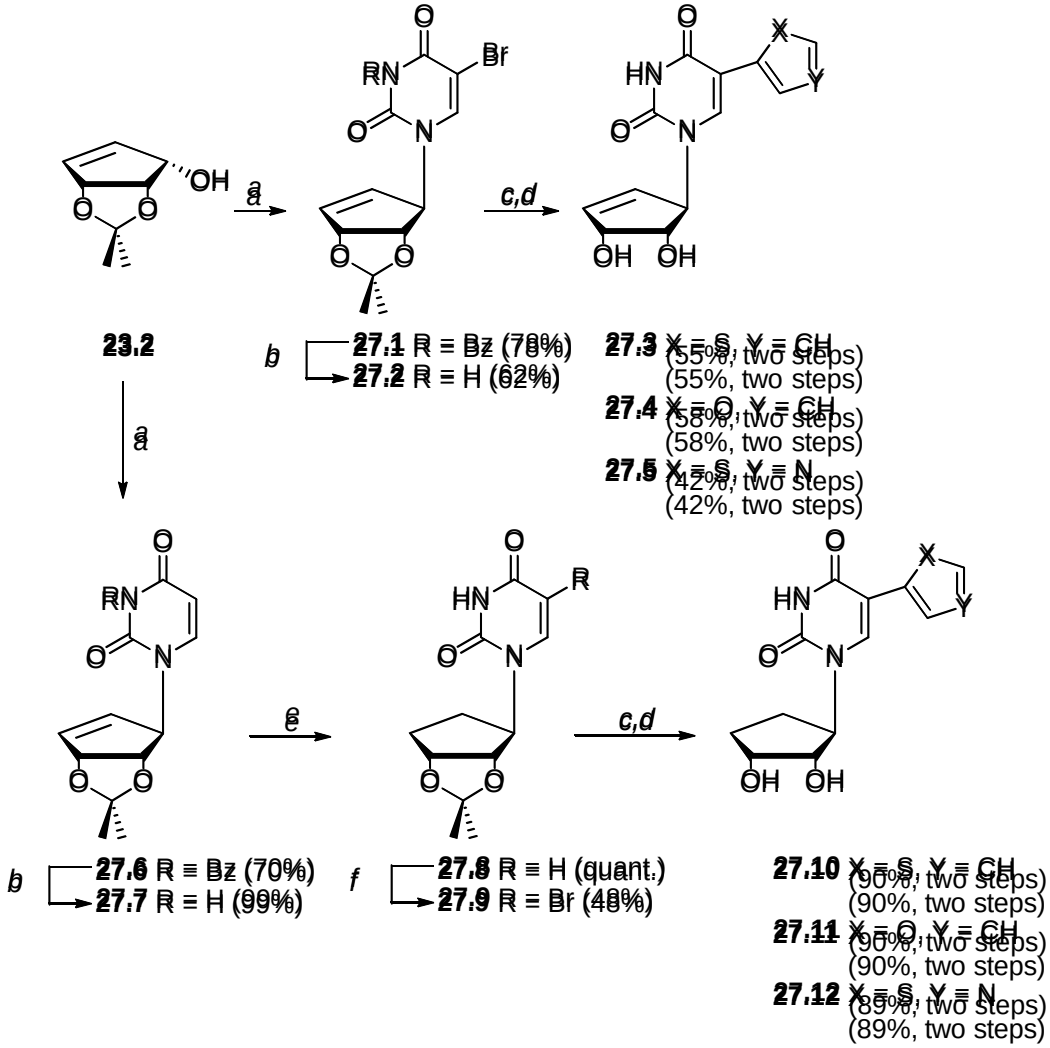


Reagents and conditions: a. *n*-BuLi, THF, Bu_3SnCl, -78°C; b. *n*-BuLi, THF, TMSCl, -78°C.

The truncated Npc analogues were achieved through a Mitsunobu reaction (Scheme 27) using **23.2** and N^3-benzoyl-5-bromouracil in the presence of DIAD and DPPE, instead of the traditional PPh_3.[133, 201] Due to the orientation of the alcohol, the pyrimidine was able to couple in the desired β-orientation (**27.1**).[181] Stille coupling with the thiophene, furan and thiazole (**26.1**, **26.2**, **26.4**), followed by deprotection in acidic conditions gave the carbocyclic reverse fleximers **27.3**-**27.5**. The Ari analogues were achieved in an analogous manner, however using N^3-benzoyluracil since the subsequent hydrogenation reduction of the double bond using palladium on carbon on the carbocycle would also remove the halogens. Therefore, the C-5 bromine of the pyrimidine was installed through *in situ* formation of azido bromide (**27.9**), and Stille

coupling occurred the same fashion as the truncated Npc analogues. Deprotection of the isopropylidene group occurred through standard aqueous acid hydrolysis.[150]

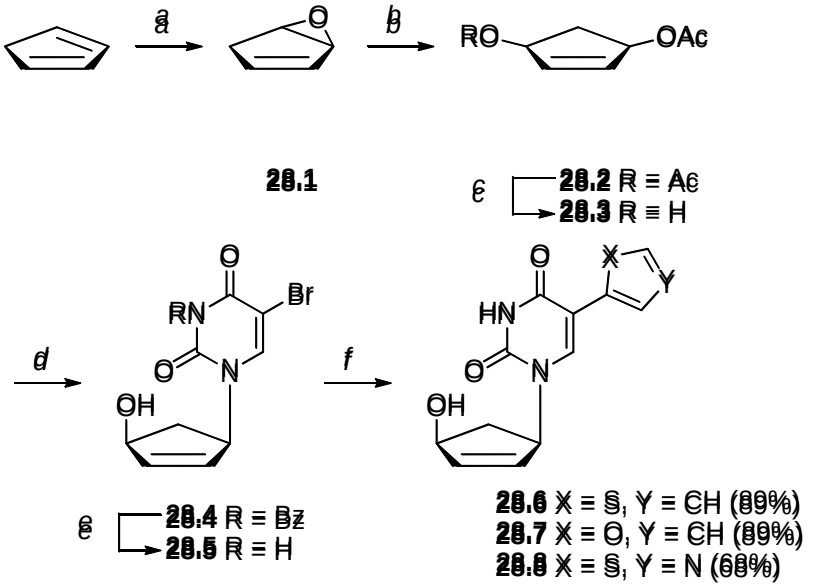
Scheme 27. Synthesis of Npc and Ari reverse fleximers.[133]



Reagents and conditions: a. N^3-benzoyluracil or N^3-benzoyl-5-bromouracil, DPPE, DIAD, CH_3CN, rt, 18 h; b. NH_3, CH_3OH, rt, 12 h; c. for thiophene, **26.1**, 1,4-dioxane, $PdCl_2(PPh_3)_2$, 90°C, 18 h; for furan, **26.2**, 1,4-dioxane, $PdCl_2(PPh_3)_2$, 90°C, 18 h; for thiazole, **26.4**, THF, $Pd(PPh_3)_4$, reflux, 72 h; d. TFA/H_2O (2:1), rt, 6 h; e. Pd/C, CH_3OH, H_2, 25 psi; f. NBS, NaN_3, DME, H_2O, rt, 24 h.

Another potent group of carbocyclic nucleosides that feature an OH group directly attached at the 4'-carbon of the carbocyclic ring are known as 5'-nor carbocyclic nucleosides.[132, 201-212] This modification has been shown to overcome the toxicity seen with Npc and Ari as the OH group cannot reach the appropriate residue in the kinase binding site to be phosphorylated.[12, 213] Thus, the 5'-nor carbocyclic sugar **28.3**[214] was synthesized by first cracking dicyclopentadiene to obtain cyclopentadiene using previously reported methods.[132] Treatment with peracetic acid in the presence of sodium acetate and sodium bicarbonate yielded epoxide **28.1**.[132, 214] Reaction of the epoxide with acetic acid and triphenylphosphine yielded *meso*-diacetate **28.2** and subsequent resolution with *Pseudomonas cepacia* lipase stereospecifically yielded **28.3**.[132] Tsuji-Trost coupling with the uracil derivative[215] yielded the protected halogenated carbocyclic uridine **28.4**.[132] Deprotection of the N^3-benzoyl followed by Stille coupling in a manner identical to Scheme 28 yielded the desired products **28.6**-**28.8**.[132, 150]

Scheme 28. Carbocyclic 5'-nor "reverse" fleximers.[132]



Reagents and conditions: a. peracetic acid, NaOAc, $NaHCO_3$, CH_2Cl_2, 4 h; b. AcOH, $Pd(PPh_3)_4$, THF, 0°C-rt, 18 h (39%, two steps); c. *Pseudomonas cepacia* lipase, potassium phosphate buffer pH 7.4, acetone, 1N NaOH, 4 h, (49%); d. NaH, DMF, Pd_2dba_3, DPPP, N^3-benzoylated-5-bromouracil, 55°C, 72 h (31%); e. NH_3, CH_3OH, rt, 3 h (77%); f. for thiophene, **26.1**, 1,4-dioxane, $PdCl_2(PPh_3)_2$, 120°C, 18 h; for furan, **26.2**, 1,4-dioxane, $PdCl_2(PPh_3)_2$, 120°C, 18 h; for thiazole, **26.4**, THF, $Pd(PPh_3)_4$, reflux, 72 h.

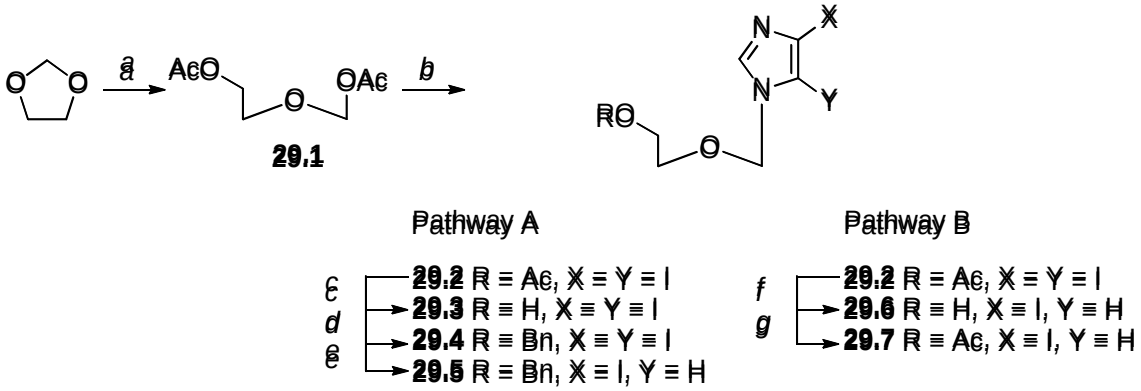
Although some antiviral activities against human cytomegalovirus and varicella zoster virus were seen for these analogues, they were also associated with cytoxicity against all tested cell lines, thus were not pursued further.[132]

Acyclic Fleximers

The most recent series of fleximers the Seley-Radtke group has pursued involves a doubly flexible scaffold obtained by combining the acyclic sugar of ACV

with the flex-base approach.[130, 131] As base flexibility had already demonstrated interesting results when coupled to ribose, we believed the effect would be even more pronounced when coupled to an acyclic sugar. The proximal acyclic fleximers were chosen as the first series of doubly flexible targets (Schemes 29 and 30). In that regard, 2-aceteoxyethyl acetoxymethylether (**29.1**)[216] was coupled to 4,5-diiodoimidazole through Vorbrüggen methods. From here, two paths were taken. Initially, the 5'-acetate protecting group was removed and substituted with a benzyl group such that the 4-iodo acyclic nucleoside (**29.5**) could be obtained using Grignard conditions.[130] Fortuitiously, however, it was found that removal of the iodine from the acyclic sugar coupled intermediate **29.2** using aqueous sodium sulfite also removed the acetate group, and subsequent reacetylation drastically improved the overall yield as the benzyl protection step could be bypassed.[131]

Scheme 29. Synthesis of acyclic halogenated imidazole nucleosides.[130, 131]

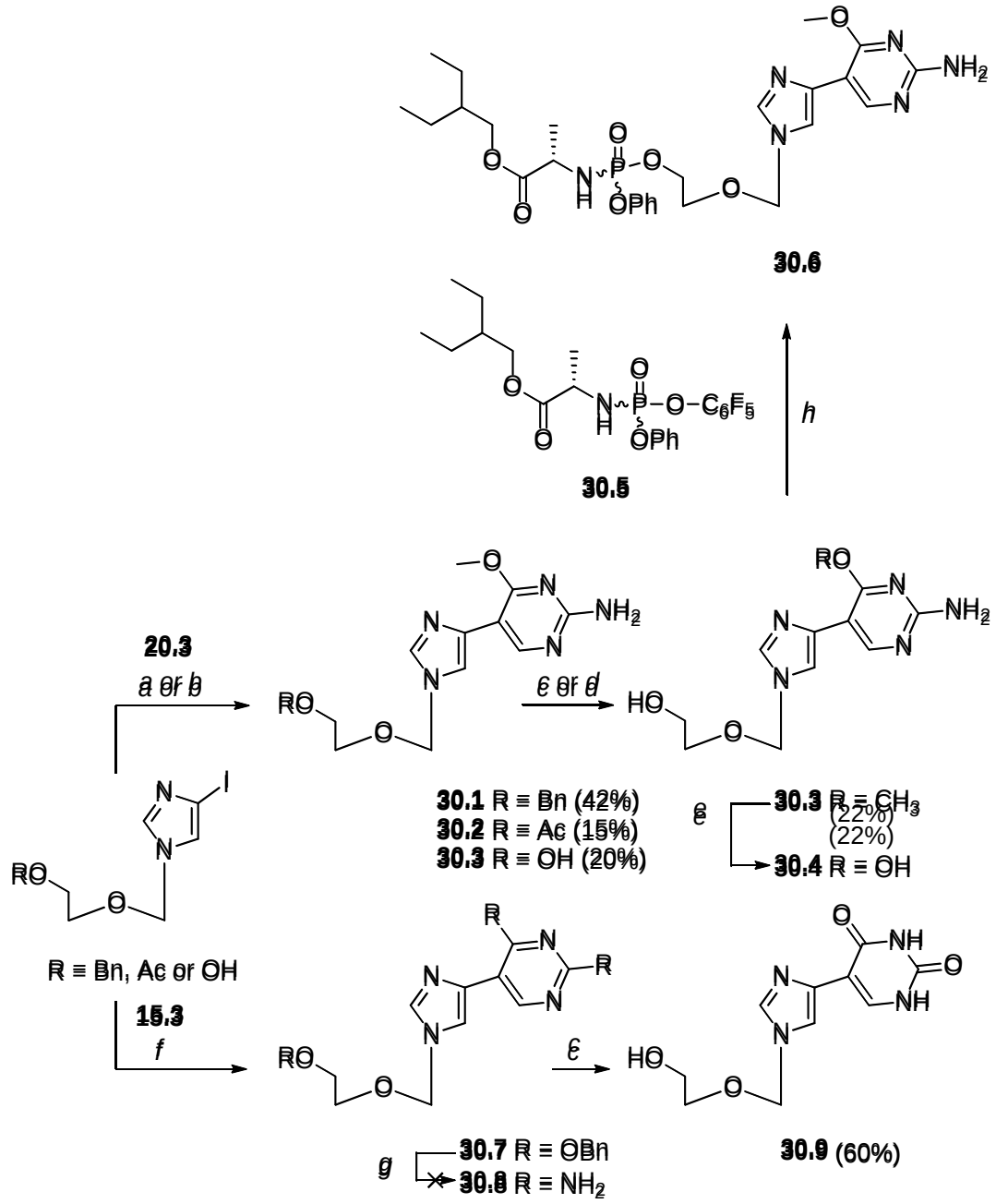


Reagents and conditions: a. Ac_2O, H_2SO_4, -15°C to rt, 18 h (52%); b. 4,5-diiodoimidazole, BSA, TMSOTf, CH_3CN, rt to 90°C, 18 h (47%); c. NH_4OH, EtOH, rt, 16 h (74%); d. (i) NaH, THF, rt, 3 h, (ii) TBAI, BnBr, rt, 12 h (70%); e. EtMgBr, THF, -15°C to rt, 4 h (78%);

f. Na_2SO_3, 30% $EtOH/H_2O$, 120°C, 18 h (84%); g. Ac_2O, NEt_3, DMAP, CH_2Cl_2, rt, 2 h (97%).

Stille and Suzuki heteroaryl coupling reactions (Scheme 30) were used analogous to those shown in Scheme 22.[130, 131] Conventional deprotections yielded the acyclic proximal guanosine and xanthosine fleximers (**30.4** and **30.9**, respectively). [130, 131, 150] As interesting biological activities against coronaviruses were observed for **30.3**, the McGuigan ProTide **30.6** was also constructed using conditions similar to Scheme 13.

Scheme 30. Synthesis of ACV fleximers.[130, 131]



Reagents and conditions: a. for Bn, $Pd(PPh_3)_4$, CuI, TBAF, DMF, 45°C, 18 h (42%); for Ac and OH, b. Pd_2dba_3•$CHCl_3$, PPh_3, CuI, TBAF, DMF, 50°C, 18 h (20%); c. for Bn, Pd/C, ammonium formate, EtOH, 120°C, 18 h; d. for Ac, NH_4OH, EtOH, rt, 18 h; e. BBr_3, CH_2Cl_2,

-15°C to rt, 18 h (49%); f. $Pd(PPh_3)_4$, $NaHCO_3$, DME, reflux, 4 h (21%); g. NH_3, CH_3OH, 210°C; h. **30.5**, *t*-BuMgCl, THF, rt, 2.5h.

As mentioned previously, these doubly flexible nucleos(t)ides have shown low micromolar activities (Table 3) against various families of viruses coronaviruses (Middle East Respiratory Syndrome = MERS, Severe Acute Respiratory Syndrome = SARS),[130] filoviruses (Ebola = EBOV, Sudan = SUDV, Marburg = MARV)[131] and flaviviruses (Dengue = DENV, Zika = ZIKV, yellow fever = YFV) (manuscript in preparation). The broad-spectrum activities of these compounds are especially impressive considering they are also non-toxic; toxicity is a common pitfall of nucleos(t)ide therapeutics due their lack of selectivity and inhibition of human polymerases. Because of these findings, we are currently carrying out additional structure activity relationship studies on the acyclic fleximers to enhance their therapeutic profiles against these and other hard to treat viruses.

Table 3. Antiviral activites of doubly-flexible fleximers (EC_{50} in μM).[130, 131]

#	MERS	SARS	SUDV	MARV	EBOV	DENV	ZIKV	YFV
30.2	5.3±0.7	11.9±0.2	20±10	70±27	ND[a]	>100	>100	68
30.3	10.1±1.2	28.1±0.2	>100	>100	2.2±0.3	32	>100	32
30.6	ND[a]	ND[a]	7 ± 2	62 ± 13	27.2±2.2	27	31	28

[a] ND = not determined

Conclusion

A structurally diverse group of fleximers has been synthesized and explored for their biological impact not only by the Seley-Radkte group, but by other groups as well, thus highlighting the importance of this unique class of nucleoside analogues. While the synthetic routes to some of the fleximers have been optimized and/or simplified over the years, in many cases their syntheses remain tedious. Furthermore, it is clear that modifications to the sugar moiety has an important influence on the approach used for constructing the base, thus there is no "one size fits all" approach that can be used for the synthetic strategies. The significant biological effects exhibited by the fleximers, particularly the most recent results for the doubly flexible fleximers however makes the synthetic challenges worth pursuing. Additional studies currently underway are focused on exploring new classes of fleximers based on other FDA approved nucleos(t)ide drugs to determine the effect of flexibility on activity compared to the parent compounds. In parallel, the ongoing mechanistic studies being carried out in our collaborators' laboratories with nucleoside recognizing enzymes involved in viral replication such as methyltransferases and viral polymerases will serve to better elucidate their mechanism(s) of action, particularly for the acyclic fleximers, given their apparent broad-spectrum activity. In summary, while the biological results to date have been, in some cases, groundbreaking, much remains to be done to fully understand the implications of flexibility on antiviral drug design, thus we look forward to exploring new challenges with these interesting (but often times difficult to obtain) nucleoside analogues.

Chapter 3: 2'-Fluorinated Tricyclic and Fleximer Nucleosides

Nucleoside/tide analogues mimic the body's natural nucleoside/tide(s) and impede replication processes that many diseases employ, while attempting to avoid harming the patient/host. The FDA has approved the use of numerous nucleoside/tide analogues as treatments for various diseases, particularly in the areas of viral and cancer diseases.[89, 101, 217] As mentioned in Chapter 1, Gemcitabine and Sofosbuvir (Figure 25) are two extremely successful FDA approved nucleos(t)ide therapeutics; the former is an anticancer cytidine nucleoside analogue, and the latter is an anti-HCV uridine nucleotide prodrug. The fluorine modifications at their 2'-carbon sugars are tied directly to their extraordinary biological effects, and those unique properties have been carefully studied and elucidated. [91, 218-229]

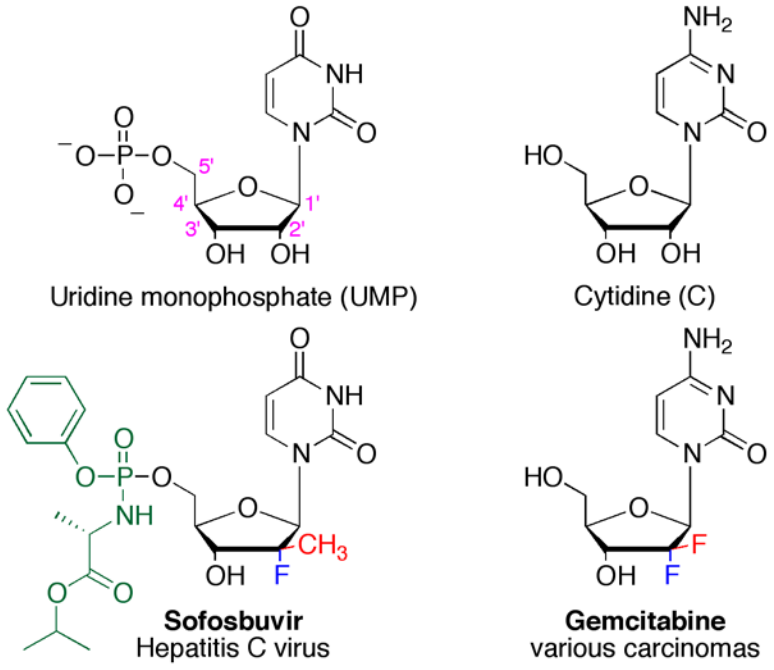


Figure 25. Gemcitabine and Sofosbuvir.

Specific Aims

***Specific Aim 1*:** A series of 2'-fluorinated tricyclic and fleximer nucleoside analogues with sugars inspired by the scaffolds found in Gemcitabine and Sofosbuvir were synthesized, purified and characterized.

***Specific Aim 2*:** McGuigan based phosphoramidates of the nucleoside analogues synthesized in Aim 1 were installed.

***Specific Aim 3*:** The nucleos(t)ide analogues possessing the sugar found in Sofosbuvir were tested for anti-HCV activity, whereas he nucleos(t)ide analogues possessing the sugar found in Gemcitabine were tested for anti-cancer activity through the NCI panel.

Biophysical Properties of 2'-Fluorinated Sugars

Fluorine substitutions on nucleosides were initially pursued due to fluorine's multi-faceted chemical properties; because of its high electronegativity and low polarizability, fluorine can imitate the size of a hydrogen atom, the polarity of a hydroxyl group, and can also act as a hydrogen bond acceptor.[91, 218-221] In addition, replacement of the 2'-OH with a fluorine increases the chemical and metabolic stability of the glycosidic bond.[91, 221-229] Acid-catalyzed hydrolysis of nucleosides, whether chemical or enzymatic, proceeds *via* an A1 mechanism in which the protonated nucleoside dissociates in the rate-limiting step to a oxocarbenium ion and the free nucleobase (Figure 26).[37] Fluorine, being the most electronegative element, is highly electron-withdrawing, thus by substituting the 2'-OH with the more electronegative fluorine, the oxocarbenium ion would be destabilized and the rate of hydrolysis reduced. [230]

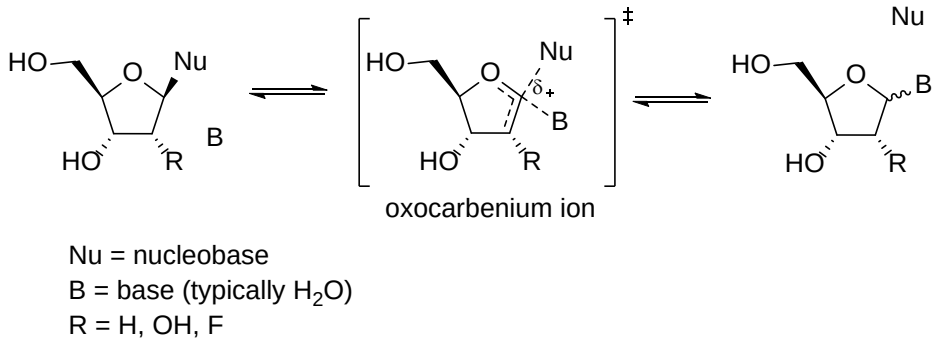


Figure 26. Mechanism of glycosidic bond cleavage.[37]

Furthermore, the orientation of the 2'-fluorine "locks" the sugar into a specific conformation (North or South, Figure 27), which in turn, affects excision mechanisms in some polymerases that may deactivate the inhibitor.[3, 91, 231] Nucleotides that favor a "North" sugar pucker (Figure 27), including nucleotides with a 2'-F "down", prefer to occupy the P-site (post-translocation site) of HIV-RT and consequently are poorly excised.[231] This may be because nucleotides that adopt a "North" conformation are the preferred substrate for DNA polymerization by RT.[232-234] The nucleotides that adopt a "South" conformation prefer to occupy the N-site (pre-translocation site) of HIV-RT and are rapidly excised.[3, 91, 231]

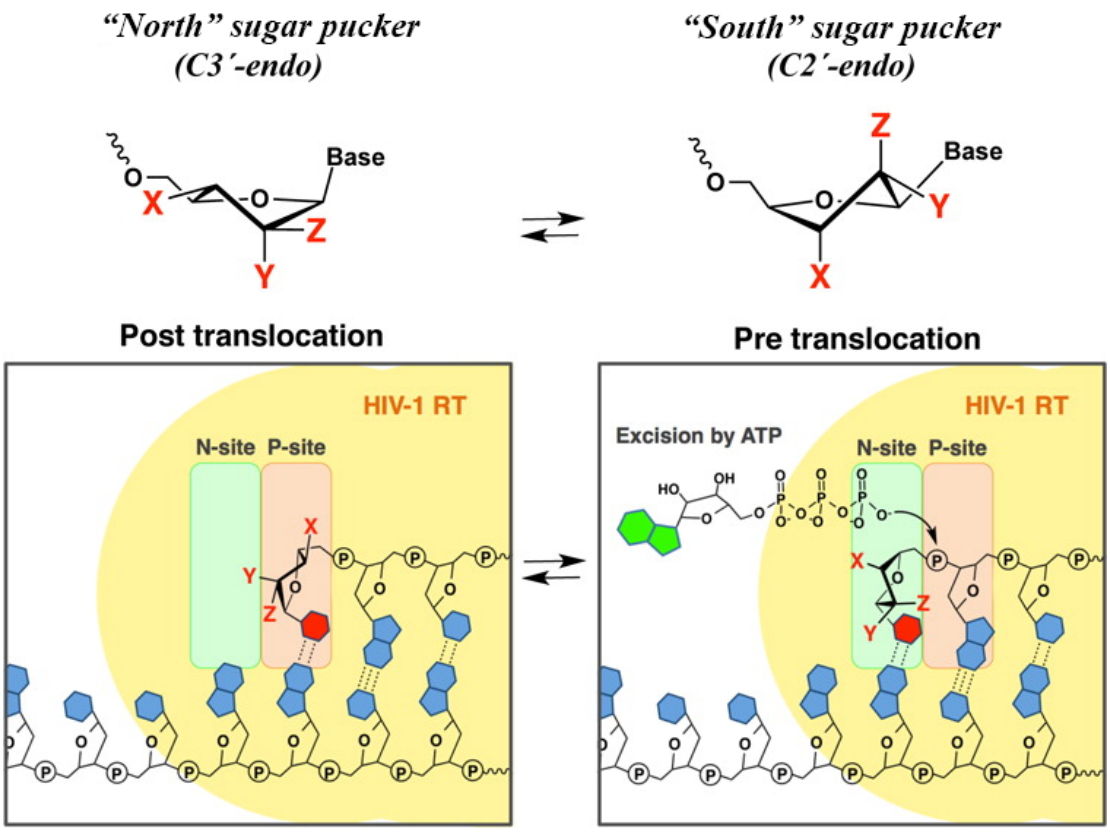


Figure 27. Sugar conformations of nucleosides (top), and NRTI excision strategy of HIV-RT (bottom).[231]

With the unique properties fluorine can endow to the nucleos(t)ide sugar and the remarkable biological activities imparted by these modifications, we were interested in determining how substituting modified bases to this scaffold could affect its therapeutic effects. As discussed in Chapter 2, our group developed a series of tricyclic "expanded" purines (Figure 28) related to Nelson Leonard's lin-benzoadenosine nucleosides that were designed to explore their effects on viral replication.[135, 137, 138, 143, 145, 146, 235-237] The parent tricyclic nucleosides (Figure 28) were previously tested against HCV, and the tricyclic guanosine and adenosine exhibited promising activities (Table 4).[146]

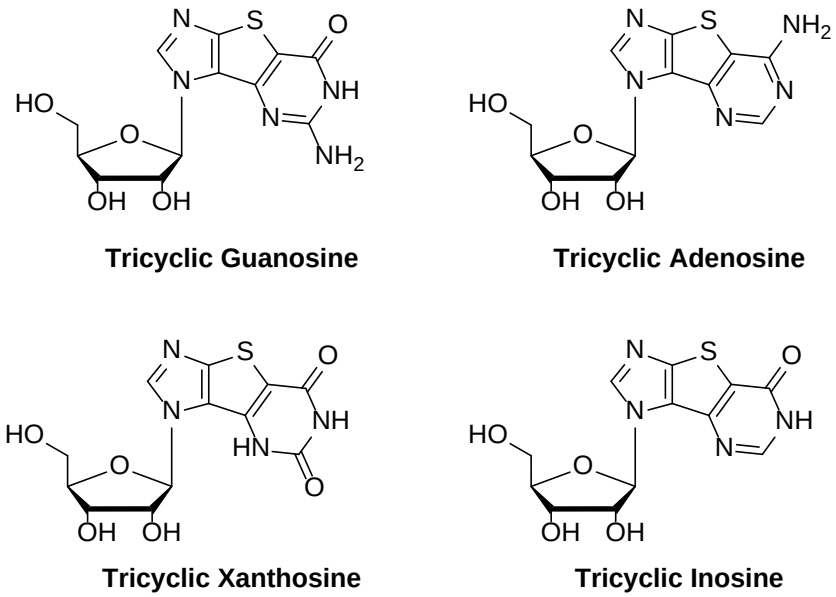


Figure 28. Seley-Radtke's tricyclic "expanded" purines.[146]

Table 4. Anti-HCV activity of expanded tricyclic nucleosides.[146]

Compound	Anti-HCV Activity EC_{50} (μM)[a]	MCC Huh7 cell CC_{50} (μM)[b]
Tricyclic Guanosine	87	250
Tricyclic Adenosine	74	190
Tricyclic Xanthosine	>250	ND
Tricyclic Inosine	>250	ND
Ribavirin	2	470

[a] EC_{50}: effective concentration of compound that inhibits the viral RNA replication by 50%. [b] CC_{50}: concentration of compound that reduces the overall cellular metabolic activity by 50%.

Thus, to further explore the potential of these interesting base and sugar scaffolds, the expanded and flexible nucleobases were combined with the 2'-modified

sugar found in Sofosbuvir and Gemcitabine to give the tricyclic and fleximer 2'-fluorinated analogues **1-4** as depicted in Figure 29.

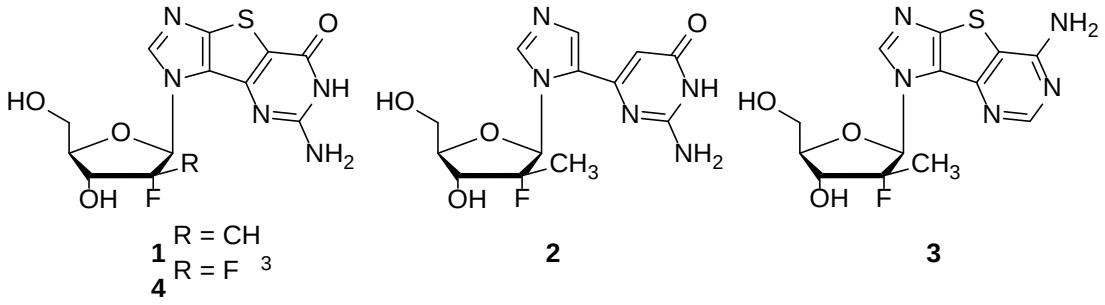
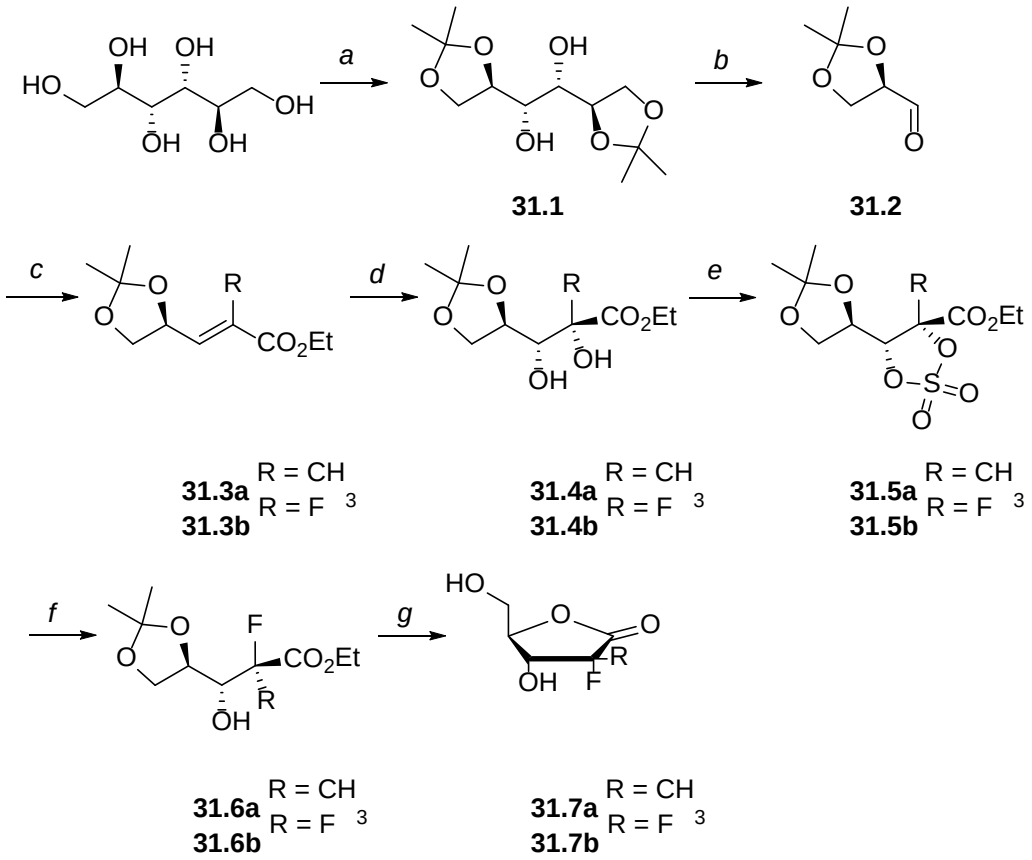


Figure 29. 2'-Fluoro project targets.

2'-Fluorinated Tricyclic and Fleximer Nucleos(t)ides

Two approaches were attempted to realize the target compounds – initially the more straightforward method of oxidizing the 2'-hydroxyl group and then stereospecifically functionalizing it as seen in Scheme 12 was chosen, however, the yields were low, so a linear approach was taken instead.[238] As shown in Scheme 31, the stereospecific synthesis began with the construction of the 2'-functionalized sugar **31.7a** and **31.7b**, which can be obtained from D-mannitol in 8 steps with an overall yield of 9.3% using previously reported literature procedures.[164, 239]

Scheme 31. Synthesis of 2'-fluorinated sugars starting from D-mannitol.[164, 239]



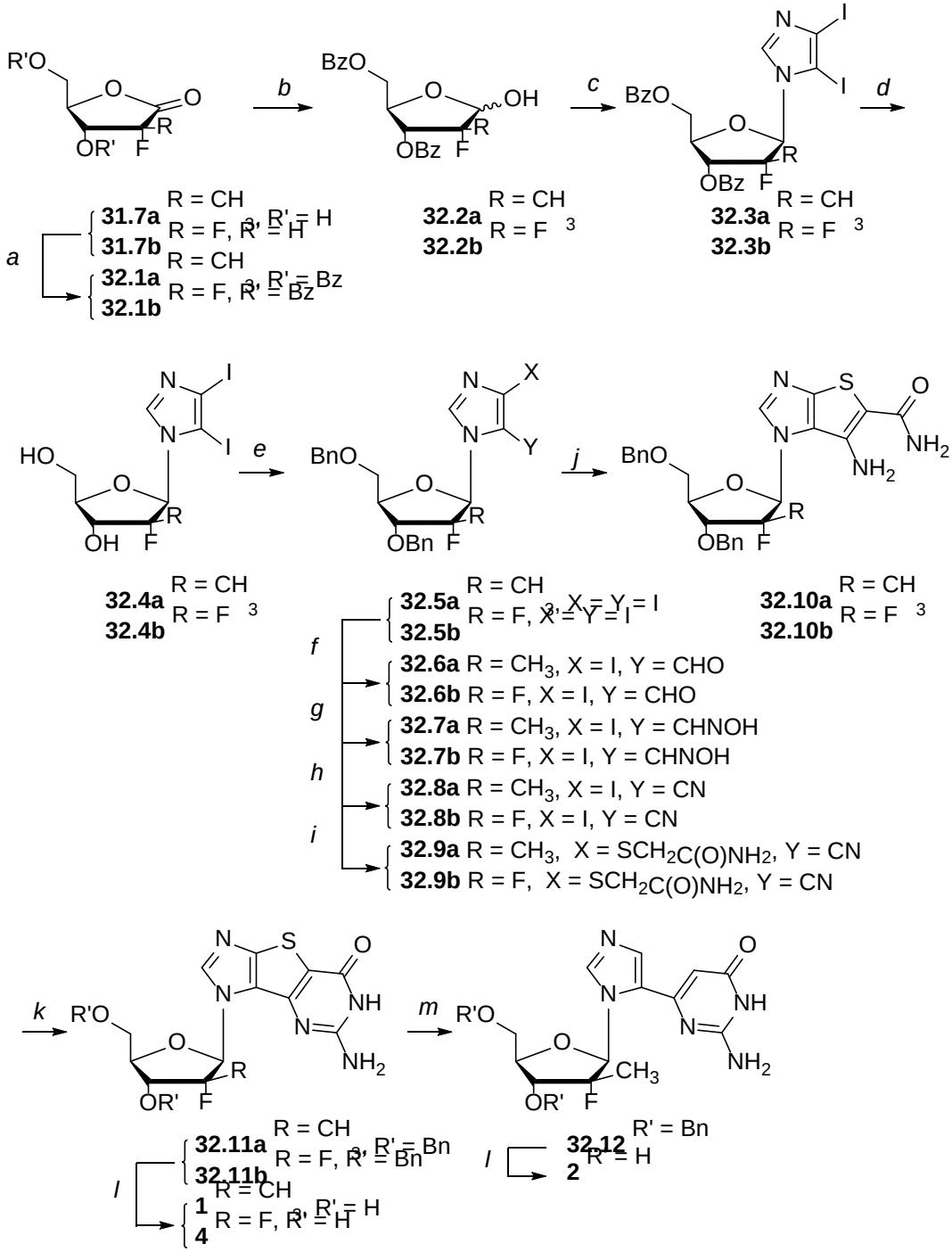
Reagents and conditions: *a.* 2,2-dimethoxypropane, $SnCl_2$, DME, 80°C, 2 h; *b*. $NaIO_4$, CH_2Cl_2, 20°C, 2 h; *c*. ethyl-2-(triphenylphosphoranylidene)propanoate or ethyl-2-fluoro-2-(triphenylphosphoranyli-dene)acetate, CH_2Cl_2, 0°C, 16 h; *d*. $KMnO_4$, $NaHCO_3$, ethylene glycol, H_2O, acetone, -20°C, 1 h; *e*. i) $SOCl_2$, NEt_3, CH_2Cl_2, 0°C, 1 h, ii) NaOCl, CH_3CN, 4°C, 48 h; *f*. i) TEAF•H_2O, 1,4-dioxane, 105°C, 1hr, ii) HCl, 2,2-dimethoxypropane, rt, 3 h; *g*. HCl, EtOH, rt, 16 h.

Once the lactone was in hand, the 3'- and 5'-hydroxyl groups were protected with benzoyl chloride using standard procedures to give **32.1a** and **32.1b** (Scheme 32). Non-stereospecific reduction of the carbonyl using lithium aluminum tert-butoxide hydride provided **32.2a** and **32.2b**. A Luche reduction was first attempted in

an effect to obtain the stereospecific α sugar, however, both α and β anomers were found, likely due to ring-chain tautomerism. Subsequent Mitsunobu coupling with 4,5-diiodoimidazole afforded **32.3a** and **32.3b**, with the undesired α anomer as the major product.[164, 240] Due to the use of Grignard conditions in subsequent steps, the benzoyl groups were removed and replaced with the more robust benzyl groups to provide **32.4a** and **32.4b**.[150] The tricyclic ring system of **32.11a** and **32.11b** was then constructed stepwise using our previously reported route.[145, 146] Desulfurization of **32.11a** using Raney nickel provided the fleximer nucleoside **32.12**. Final deprotection of the benzyl groups of **32.11a**, **32.11b** and **32.12** using boron trifluoride etherate afforded **1**, **2** and **4**.[150]

It should be noted that we initially approached this route by installing the benzyl protecting groups first, however, the yields proved to be significantly lower and the workups more tedious. Fortunately, the switch of the protecting group, using benzoyl then benzyl, increased the overall yields and rendered the workups quite facile.

Scheme 32. Synthesis of 2'-fluorinated tricyclic compounds.[161, 162]



Reagents and conditions: *a*. BzCl, pyridine, rt, 40 min-1h; *b*. $LiAl(O^tBu)_3H$, THF, -20°C, 6h; *c*. 4,5-diiodoimidazole, Ph_3P, DIAD, THF, rt, 24h; *d*. CH_3ONa, CH_3OH, 0°C, 1h; *e*. NaH (95%), BnBr, TBAI, DMF, rt, 6 h; *f*. (i) EtMgBr, THF, 0°C, 15-30 min; (ii) DMF, 18 h; *g*.

NH_2OH•HCl, $NaHCO_3$, EtOH, H_2O, rt, 18 h, or reflux, 5 h; *h*. CDI, THF, 60°C, 4-8 h; *i*. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 55-65°C, 24-40 h; *j*. EtONa, EtOH, 80°C, 1-2 h; *k*. (i) NaOH, CS_2, CH_3OH, 150°C, 18 h, (ii) H_2O_2, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 120-130°C, 12 h; *l*. BF_3•OEt_2, EtSH, CH_2Cl_2, 0°C-rt, 18-72 h; *m*. Raney nickel, CH_3OH, H_2O, 90°C, 48 h.

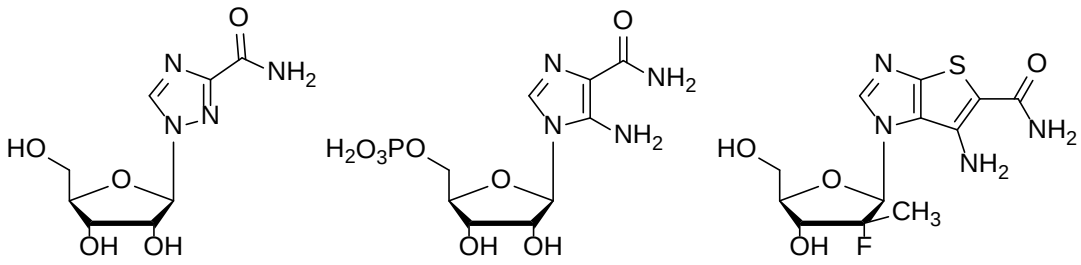
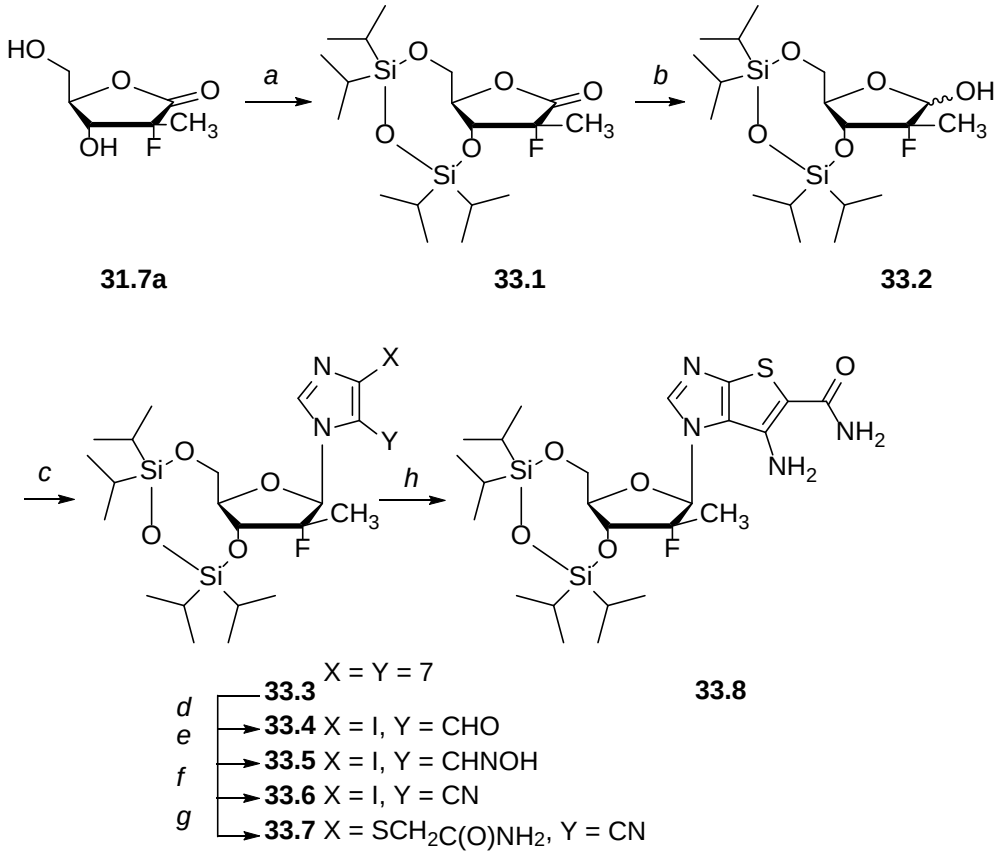


Figure 30. Ribavirin, AICAR and target bicyclic compound **9**.

Due to its resemblance to Ribavirin, AICAR (Figure 30) and other structurally related nucleosides that have shown promising antiviral activity, the bicyclic intermediate of the Sofosbuvir analogue (**5**) was also pursued, especially since it should theoretically be facile to obtain during the synthesis.[241, 242] While this was not as straightforward as initially envisioned, due to the presence of the exocyclic amine and amide functionalities attached to the thiophene ring, the desired bicyclic target **5** was finally obtained by manipulating the synthetic route to employ a different protecting group strategy as outlined in Scheme 33 and Scheme 34.

Scheme 33. Synthesis of 2'-CH_3/F bicyclic compound.

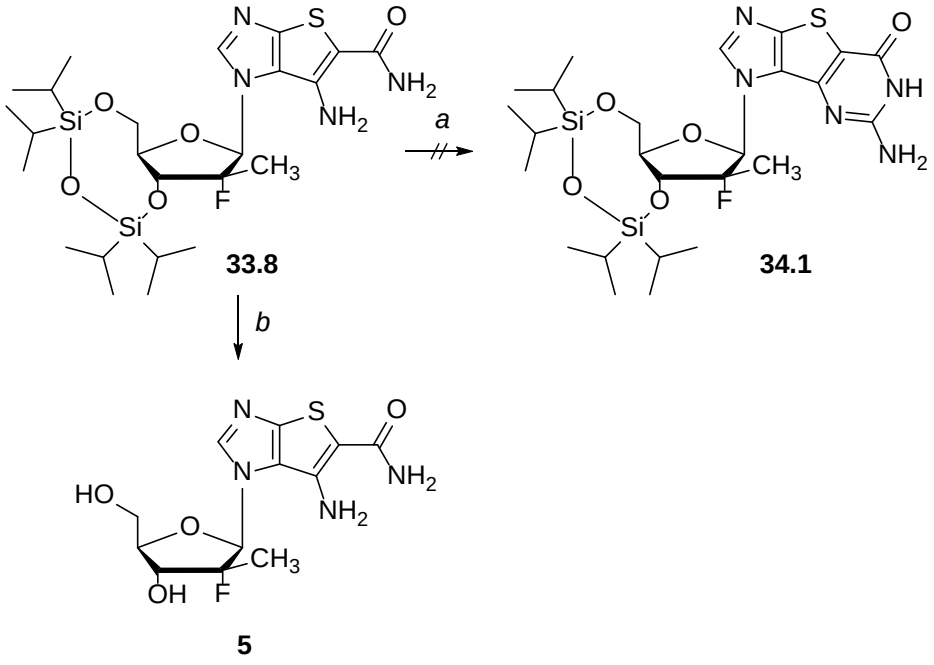


Reagents and conditions: a. $TIPDSCl_2$, pyridine, rt, 16 h; b. $LiAl(O^tBu)_3H$, THF, -20°C to 10°C, 3h; c. 4,5-diiodoimidazole, Ph_3P, DIAD, THF, 0°C to rt, 24h; d. (i) EtMgBr, THF, 0°C, 15 min; (ii) DMF, rt, 16 h; *e*. NH_2OH•HCl, $NaHCO_3$, EtOH, reflux, 5 h; *f*. CDI, THF, reflux, 16 h; *g*. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 65°C, 24 h; *h*. EtONa, EtOH, reflux, 2 h.

The key difference in this approach involved using the Markiewicz reagent to bis-protect the 3'- and 5'-hydroxyls instead of the previously employed benzyl groups.[150] This was necessary since the conditions for removal of the tetraisopropyldisilyl (TIPDS) group would be amenable to the sensitive amide and amine functionalities present. Interestingly, despite all efforts to the contrary, we were unable to cyclize the bicyclic to the tricyclic ring system of **34.1**. This was

unfortunate, as it would have also provided **1** following deblocking of the silyl groups.

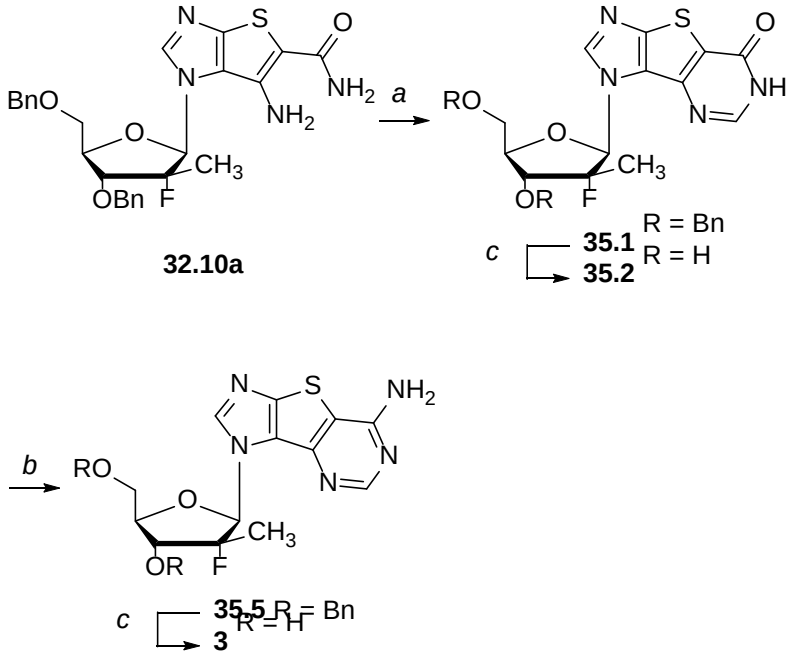
Scheme 34.



Reagents and conditions: *a*. (i) NaOH, CS_2, CH_3OH, 145°C, 18 h, (ii) H_2O_2, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 130°C,16 h; *b*. TBAF, THF, rt, 2h.

The tricylic Sofosbuvir inosine (**35.2**) and adenosine (**3**) analogues were also of interest and their syntheses proceeded in a similar manner as shown in Scheme 35.

Scheme 35. Synthesis of tricyclic inosine and adenosine Sofosbuvir analogues.

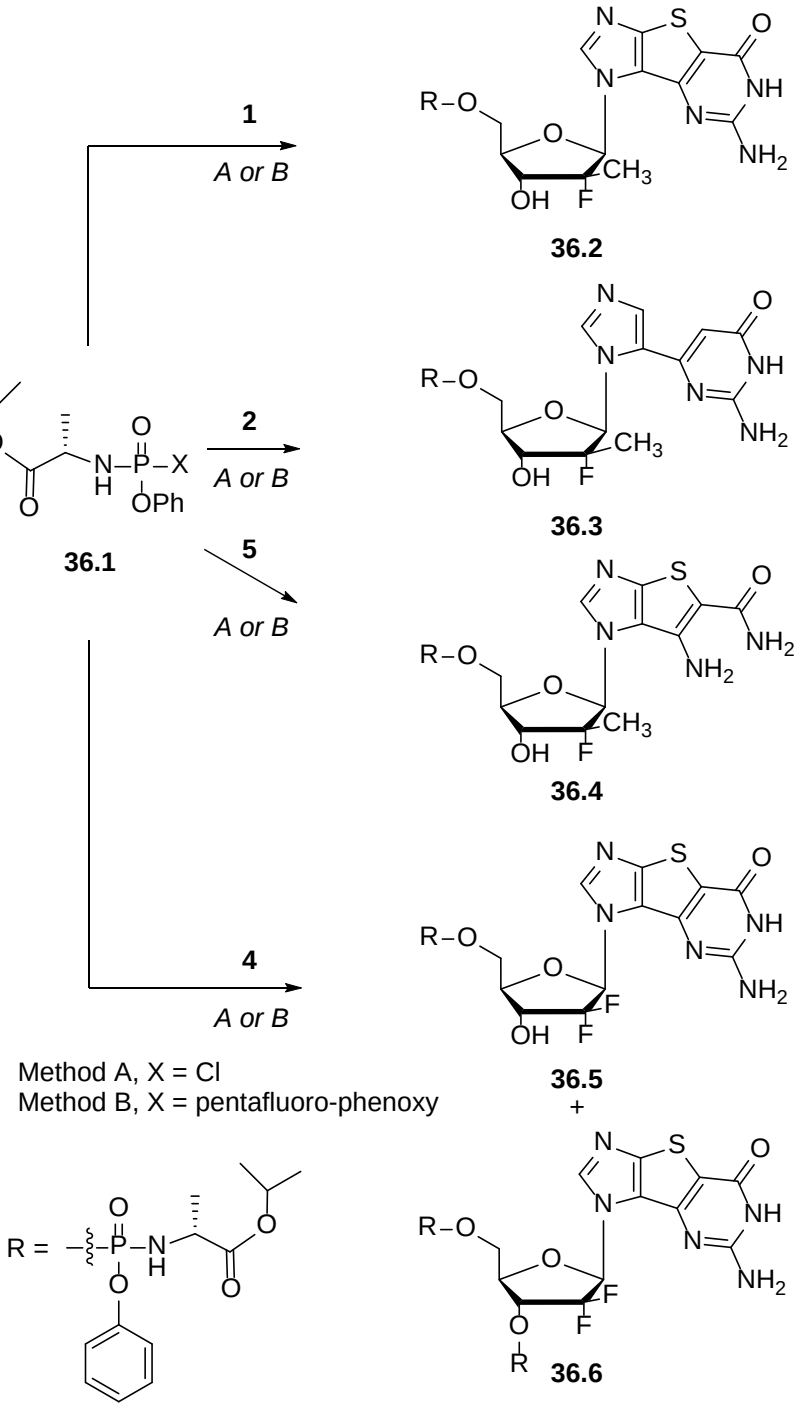


Reagents and conditions: *a*. $CH(OEt)_3$, 4Å molecular sieves, reflux, 6 h; *b*. (i) TPSCl, DMAP, NEt_3, CH_3CN, rt, 3 h, (ii) NH_4OH, rt, 24 h; *c*. BF_3•OEt_2, EtSH, CH_2Cl_2, rt, 72 h.

Once the parent compounds were in hand, the corresponding McGuigan ProTides (discussed in Chapter 1) of the tricyclic **1** and **4**, and the bicyclic **5** were sought.[107, 243]

Although there are numerous ProTides to choose from, the combination of the L-alanine (L-Ala) amino acid group, the phenyl aryl group, and the *i*PrO ester group (Scheme 36, **36.1**) was selected due to its success with similar nucleosides.[113] The two approaches to realize the prodrugs are outlined in Scheme 36 and followed literature procedures.[244-248] Although the yields were disappointingly low, enough compound was obtained to allow for limited biological testing. Interestingly, compound **4** yielded the mono- and bis-substituted ProTides.

Scheme 36. Synthesis of McGuigan ProTides of **1**, **4** and **5**.[161, 162]



Reagents and conditions: (A) ^{t}BuMgCl, DMF, -78 °C, 1h, then r.t., 4 h; (B) ^{t}BuMgCl, DMF, 0 °C, 1h, then rt, overnight.

Biological Results

The anti-HCV activity of the Sofosbuvir analogue target compounds was investigated in Huh 5.2 cells carrying a subgenomic genotype 1b replicon.[249] The 50% effective concentration (EC_{50}) is the compound concentration that inhibits vRNA replication by 50%. Similarly, the toxicity of the compounds were quantified in the same cell line, where the 50% cytotoxic concentration (CC_{50}) is the compound concentration that reduces cell viability by 50%. Obtaining both values allow for the calculation of a selectivity index ($SI = CC_{50}/EC_{50}$) which is a measure of the therapeutic potential of a compound. The values for the different compounds are shown below in Table 5.

Table 5. HCV Assay Results for Compounds **1**–**3**, **5**, **35.2**, **36.2-36.4**.[161]

Replicon 1b, Con 1 strain, Huh 5-2 cells (μM)			
Compound	EC_{50}[a] Replicon (μM)	CC_{50}[b] MTS (μM)	SI[c]
1[d]	32	54	1.7
2[d]	63.1	>100	>1.6
3[e]	20±6	161±5	8.1
5[e]	42±4	>100	>2.4
35.2[e]	76±28	>200	>2.6
36.2[e]	110±1	145±0.6	1.3
36.3[d]	50.6	>100	>2.0
36.4[e]	84±16	>100	>1.2

[a] EC_{50}: effective concentration of compound that inhibits the viral RNA replication by 50% respectively (luciferase-based assay). [b] CC_{50}: concentration of compound that reduces the

overall cellular metabolic activity by 50% (MTS-based assay). [c] SI: selectivity index, CC_{50}/EC_{50} ratio. [d] The data represent the measurement from a single experiment. [e] The data represent an average of at least two independent measurements in duplicate ± SD.

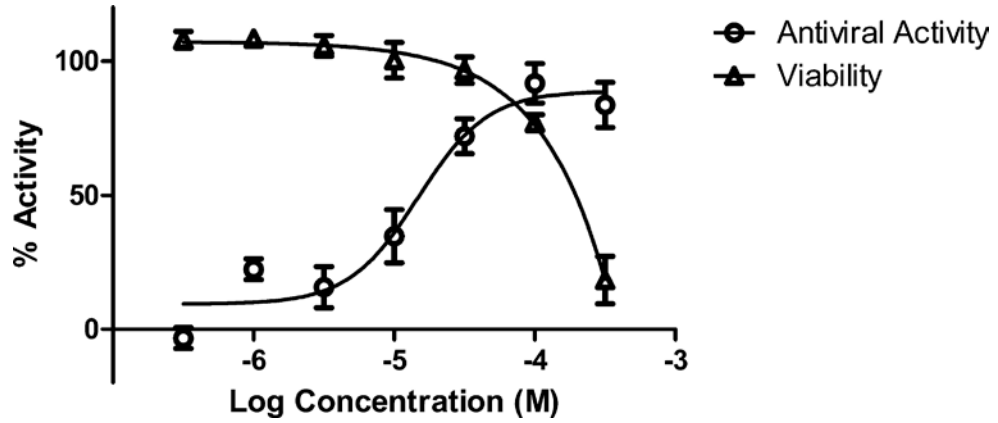


Figure 31. Dose–response curves of the effect of **3** on HCV viral RNA replication and cell viability.[161]

Only compounds that produce a significant inhibition of viral replication at concentrations where no antimetabolic effect (cell viability >90%) on the host cell can be observed are considered selective inhibitors of HCV replication. For most molecules shown in Table 5 the antiviral activity found was low (EC_{50} > 50 μM) and/or not significantly different from the antimetabolic activity (SI < 3). Regrettably, the fleximer nucleoside **2** showed the least activity compared to the rest of the nucleosides. For the tricyclic adenosine analogue **3**, we found selective antiviral activity with an SI of 8.1. A more detailed look at the dose−response curves (Figure 31) showed that at 30 μM, this molecule inhibited viral RNA replication by 70% without a significant antimetabolic effect. At 100 μM, 92% inhibition of viral RNA replication was observed and also cell viability was significantly reduced (cell

viability 77% as compared to that of an untreated control). Therefore, it can be concluded that this molecule can inhibit HCV viral RNA replication but only at high concentrations and with limited selectivity. Also, the ProTide approach has typically resulted in a significant increase in activity compared to the parent nucleosides,[244-247] as demonstrated by Sofosbuvir compared to its parent nucleoside;[113] however, this did not prove to be the case for our nucleoside. Although we have no immediate explanation for this, speculation that perhaps the ring systems may somehow be interfering with the cleavage of the prodrug is possible so perhaps the reaction is occurring more slowly, thus leading to the decreased or comparable activities.

The 2'-difluorinated compounds **4**, **36.5** and **36.6** were submitted to NCI for screening in their 60 cell line assay system. As shown below in Table 6, the compounds showed inhibitory activity against the MOLT-4 (leukemia), T-47D (breast cancer), LOX IMVI (melanoma) and NCI-H522 (non-small cell lung cancer) cell lines at 15μg/mL.

Table 6. Growth percent of cancer cell lines MOLT-4, T-47D, LOX IMVI and NCI-H522 at 15 μg/mL of **4**, **36.5** and **36.6**.[162]

Compound	Cell line (percent growth*)			
	MOLT-4	T-47D	LOX IMVI	NCI-H522
4	13.94	10.47	25.6	33.95
36.5	93.98	64.5	74.06	65.63
36.6	109.04	87.15	101.93	77.66

The results show that the best activity was observed for compound **4** against the breast cancer cell line T-47D (10.47%). Surprisingly, the McGuigan ProTides **36.4** and **36.5** did not demonstrate superior inhibition as compared to **4**, as would have been expected. In fact, the therapeutic activity worsened with a second ProTide moiety present. Subsequent to our synthesis, a report appeared in the literature that ultimately confirmed the biological results: Slusarczyk *et al.* found that the selected combination of the L-Ala amino acid group, the phenyl aryl group and the *i*PrO ester led to a decrease in the activity of Gemcitabine.[108] Thus, future efforts may explore alternative prodrug options.

Conclusions and Future Directions

The tricyclic thiophene-expanded and fleximer guanine bases were successfully coupled to the 2'-fluorinated sugars of Gemcitabine and Sofosbuvir. The synthetic approach was nontrivial as both the sugar and base had to be constructed in a linear fashion, thus the overall yield was too low to be practical. In addition, the corresponding McGuigan ProTides were also made and the compounds were screened against their designated disease targets. Although the compounds' activity against HCV proved less potent to that of Sofosbuvir, the activity observed should still be considered promising, particularly for tricyclic analogue **3**. And while the results from the NCI cancer screen also showed promising results against leukemia, melanoma and breast cancer, the McGuigan ProTides were less active as compared to the parent nucleoside. One hypothesis is that these analogues do not inhibit the target polymerases but rather other nucleoside metabolizing enzymes, hence, the additional

phosphate that is initially masked by the phosphoramidate moiety would hinder the inhibitory interaction that the parent nucleoside provides.

With recent exciting biological results from the *proximal* acyclic fleximers against certain coronaviruses, filoviruses and flaviviruses,[130, 131] one possible future goal include focusing on coupling the *proximal* base to 2'-modified sugars to determine their therapeutic potentials, as well as to compare the activity differences of *distal* to *proximal* connectivities.

In addition, results from Chapter 4 and 5 could lead to a more efficient route in achieving the fleximer analogues; a modular synthesis of fleximer nucleosides may be realized *via* enzymatic transglycosylation using fleximer bases and nucleosides with modified sugars.

Regardless of future of this project, it was unquestionably challenging, and the successful synthesis of the final products is itself an immense achievement. It was also a synthesis that allowed the author to develop a diverse synthetic organic chemistry skillset that has been, and will be, very useful towards her career development.

Chapter 4: HIV-1 Nucleocapsid Binding Fleximer Bases

Summary

Anti-HIV-1 drug design has been notably challenging due to the virus' ability to mutate and develop immunity against commercially available drugs. The aims of this project were to discover a new series of nucleobase analogues that not only possess inherent flexibility that could withstand active site mutations, but also target a non-canonical, more conserved target: the nucleocapsid protein of HIV (NCp7 or NC). Interestingly, these compounds are computationally predicted to not work by zinc ejection, which would endow them with significant advantages over non-specific zinc-ejectors. Several fleximer bases were synthesized (Figure 10) using palladium-catalyzed coupling techniques to test them against NCp7 specifically and HIV-1 as a whole. The background and results of this project are presented herein.

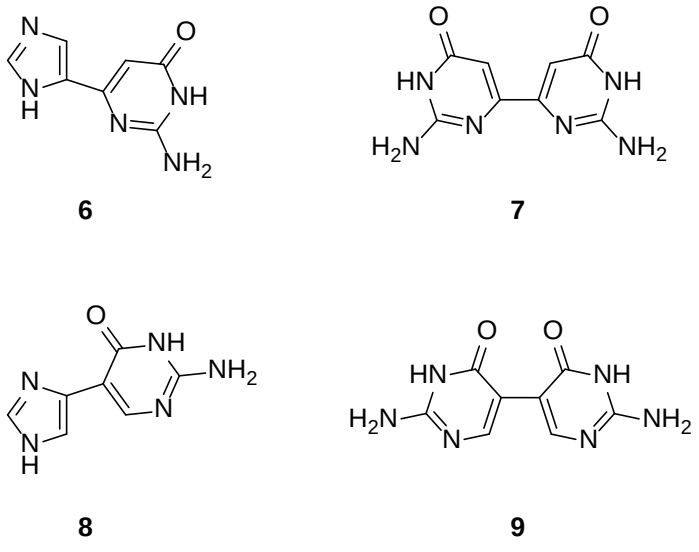


Figure 32. NCp7 project targets.

Specific Aims

***Specific Aim 1*:** A series of guanine analogues determined to bind NCp7 through computational studies were synthesized, purified and characterized.

***Specific Aim 2*:** Titration studies of the inhibitors synthesized in Aim 1 and NCp7 through NMR spectroscopy (in collaboration with Dr. Michael Summers' group) was utilized to elucidate the molecular interaction(s) between the small molecules and the protein.

***Specific Aim 3*:** The small molecules synthesized in Aim 1 were sent to Dr. Eric Freed (NCI) to determine biological activity of the small molecules against HIV-1.

HIV-1 and Current Therapeutics

HIV is a retrovirus that targets and infects human immune cells, mainly $CD4^+$ T helper lymphocytes, destroying or impairing their function.[250, 251] HIV-1 spreads mainly through sexual contact, however percutaneous and perinatal transmissions can also occur.[250] As the disease progresses, the patient's immune system weakens due to $CD4^+$ cell depletion, predisposing the patient to opportunistic infections.[251] The ultimate loss of immune control due to HIV infection has been termed AIDS.[252] The amount of time it takes for an HIV-infected person to develop AIDS varies upon the patient's condition, although antiretroviral drugs can slow down the process.[253]

According to the Joint *U*nited *N*ations Programme on HIV/*AIDS* (UNAIDS), death by HIV/AIDS-related illnesses reached 1 million in 2016.[254, 255] In the same year, the global estimate for people living with HIV was 36.7 million, with 2.1 million of those being children 15 years old or younger.[254] Fortunately, due to disease awareness as well as antiviral advancements, 20.9 million of those infected have

access to antiretroviral therapies.[254] Unfortunately however, there is still no cure.[254]

There are two types of HIV, HIV-1 and HIV-2.[256] HIV-2 is mostly isolated in West Africa while HIV-1 is prevalent throughout the world.[256] HIV-2 is more difficult to transmit, and the period between initial infection and illness is longer as compared to HIV-1 due to lower viral loads in HIV-2.[256] This may explain the lower transmission rates of HIV-2 and the near complete absence of mother-to-infant transmission.[256] Overall, prevalence rates are declining, and in most West African countries, HIV-2 is increasingly being replaced by HIV-1.[256] Hence, this study focused on HIV-1.

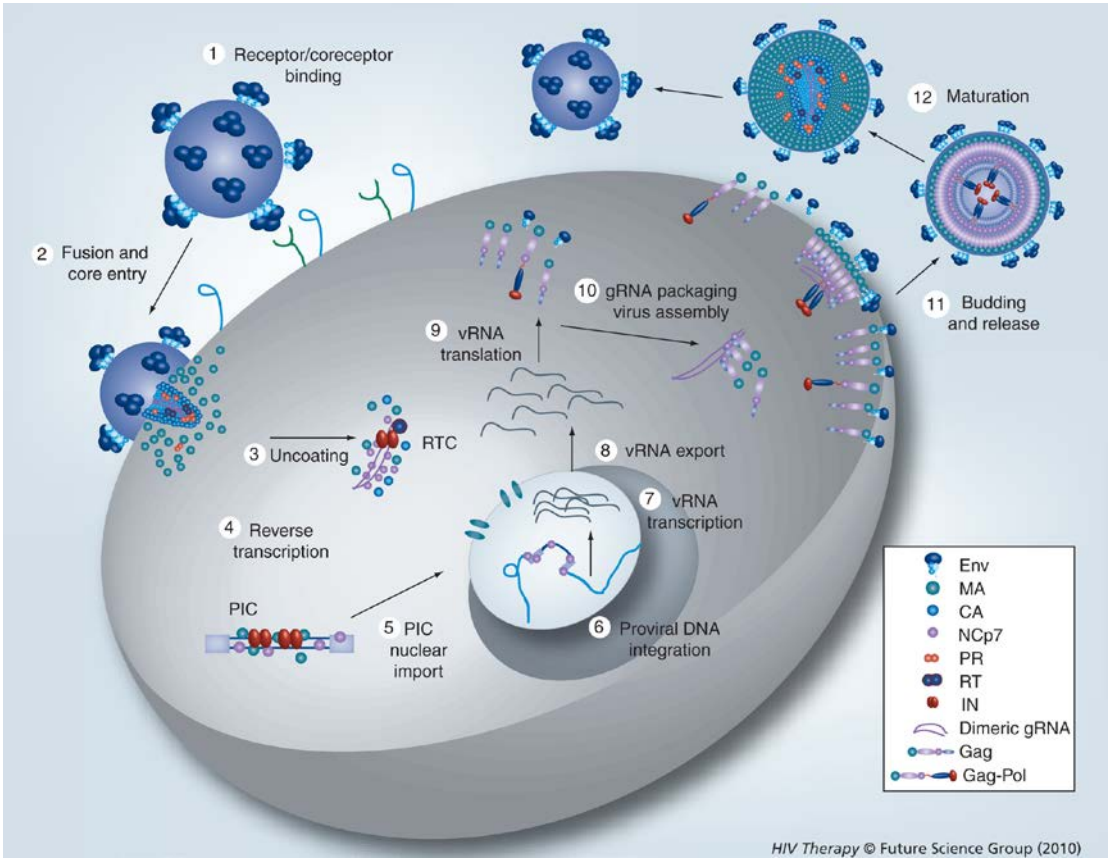


Figure 33. HIV replication pathway.[257]

Understanding the replication pathway of HIV-1 is vital to effectively combating the disease (Figure 33). To infect a host cell, the Envelope (Env) glycoproteins of the virus first targets and binds to receptors on the host immune cell membrane surface (i.e. CD4 and CCR5 or CXCR4).[258] Once bound, the viral and host cell membranes fuse and the viral components are injected into the host cell cytoplasm.[258] Reverse transcription by HIV-Reverse Transcriptase (RT) of the virion genomic RNA (gRNA) takes place after the viral Matrix (MA) and Capsid (CA) are uncoated, generating a linear double-stranded proviral DNA.[258] This proviral DNA and HIV-1 integrase form a *P*re-*I*ntegration *C*omplex (PIC) that is imported into the cell nucleus where the viral DNA (vDNA) is integrated into the host genome, establishing a life-long infection.[258] Viral messenger RNAs (mRNAs) and gRNA are subsequently produced through the host RNA polymerase II and, once complete, are shuttled out of the nucleus.[258] In the cytoplasm, the viral mRNAs are translated into viral polyproteins.[258] The polyproteins and gRNA are transported to the cytoplasmic surface of the host cell membrane where the assembly is pinched into a bud and is released from the cell as an immature and uninfectious viral particle.[258] To produce a mature virus, Protease (PR) cleaves the viral polyproteins at specific sites, resulting in reorganization of the viral proteins to form the infectious, mature construct.[258] As will be discussed later, NCp7 plays a significant role in these key events.

Initially, antiviral efforts were focused on inhibiting key enzymes in the HIV lifecycle (Figure 34).[13] Azidothymidine (Zidovudine), or AZT, a nucleoside RT inhibitor (NRTI), is a first-generation HIV-1 antiviral drug.[13, 259] NRTIs compete with

the natural substrates of RT (deoxyribonucleotides) and act as chain terminators.[13, 260] Most importantly, NRTIs have been designed to be specific towards viral RT over host polymerases.[13, 260] In contrast, non-nucleoside RT inhibitors (NNRTIs) bind to allosteric sites on the viral RT in order to inhibit enzyme activity.[13, 260] NRTIs and NNRTIs are the core components of the *H*ighly *A*ctive *A*nti*R*etroviral *T*herapy, also known as HAART.[13, 260] Fusion or entry inhibitors target either the HIV-1 glycoproteins, or the host $CD4^+$ cell receptors, preventing viral fusion.[13, 260] Integrase strand transfer inhibitors (INSTIs) block the insertion of vDNA into the host genome, preventing vDNA replication.[13, 260] PR inhibitors block the cleavage of the viral polyproteins within the immature virion, preventing maturation, and thus infectivity.[13, 260]

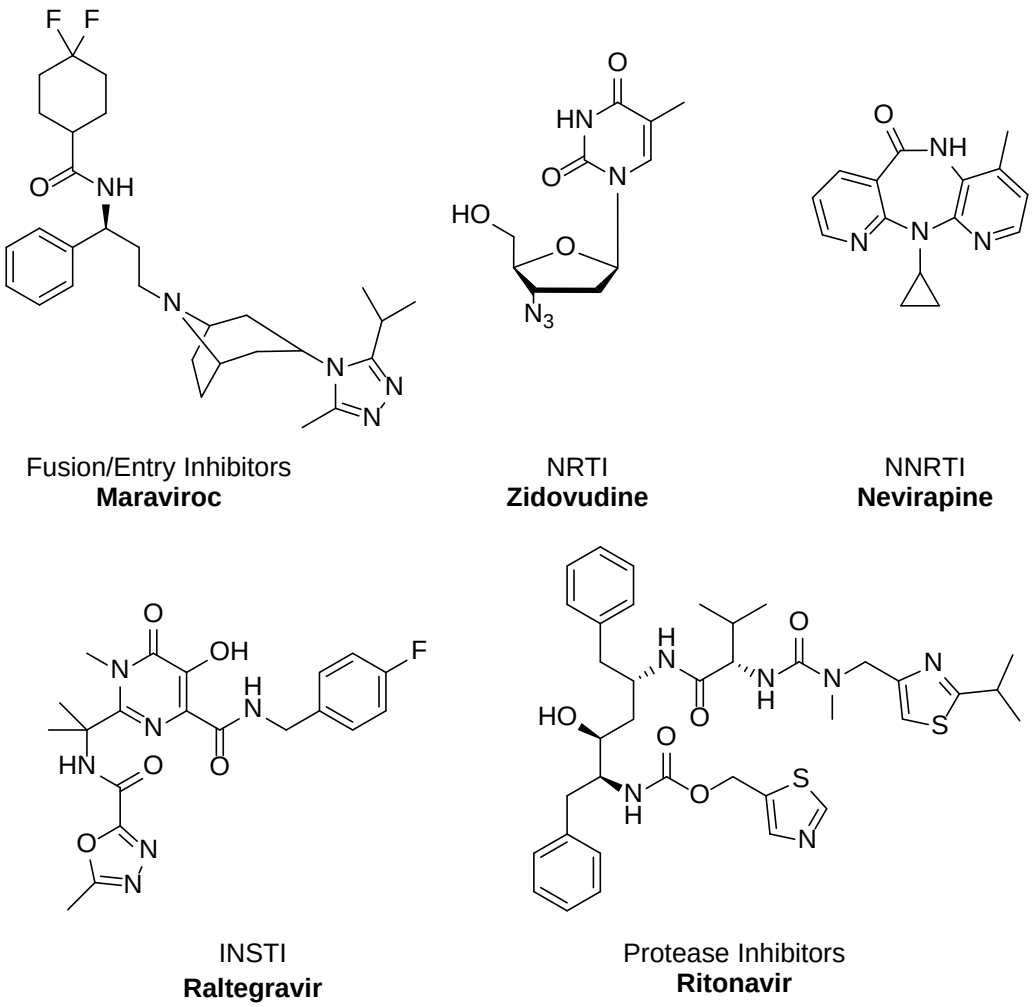


Figure 34. Examples of FDA-approved HIV-1 Antiretrovirals**.**

Promising activities were initially observed for these treatments, with each category having at least one FDA approved drug (Figure 34).[260, 261] Due to the developing structural variability of the viral enzymes caused by random mutations, most of these inhibitors are becoming ineffective as monotherapies.[261] For this reason, current standard first-line antiretroviral therapy for naïve antiretroviral HIV-1 patients in the US involves various combinations of antiretrovirals (Table 7) including mainly Emtricitabine (NRTI), Tenofovir Alafenamide (NRTI), Elvitegravir (INSTI) and

Rilpivirine (NNRTI).[262] Unfortunately, in addition to the ever-present development of antiviral resistances, HAARTs can be unaffordable to those in need (Table 7), thus it is necessary to develop therapeutics targeting more conserved components of HIV-1 to limit the number of drugs necessary to suppress or eliminate the virus.

Table 7. FDA-approved HIV combination therapies.[205]

Brand Name	Inhibitor 1	Inhibitor 2	Inhibitor 3	Inhibitor 4	Cost/year ($)
Genvoya	Tenofovir AF (NRTI)	Emtricitabine (NRTI)	Elvitegravir (INSTI)	Cobicistat (P450 3A)	37,118
Odefsey	Tenofovir (NRTI)	Emtricitabine (NRTI)	Rilpivirine (NNRTI)	-	33,780
Descovy	Tenofovir AF (NRTI)	Emtricitabine (NRTI)	-	-	21,117

HIV-1 Nucleocapsid

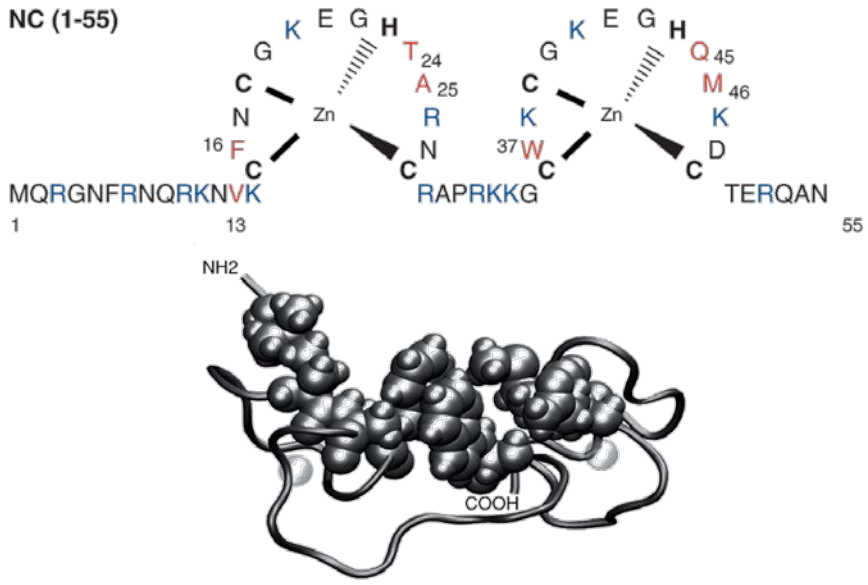


Figure 35. HIV-1 nucleocapsid 1° and 3° structure.[257]

Retroviral nucleocapsids (NCs) are small, basic proteins with one or two zinc binding domains, named zinc fingers (ZFs).[263, 264] Retroviral ZF proteins contain Zn^{2+} chelating amino acid segments $CX_2CX_4HX_4C$, dubbed the CCHC motif.[263, 264] The mature NC of HIV-1, NCp7 (Figure 35), is a 55 amino acid long, highly conserved structural viral protein that plays vital roles throughout the viral replication cycle.[257, 265] It has two ZFs (proximal ZF_1 and distal ZF_2) linked and flanked by highly basic sequences (Figure 35).[257, 266] The CCHC motifs, together with the basic linker, form the invariant central globular domain of NCp7; mutation inducing modifications in this domain led to significant defects in RNA packaging and virus core morphology.[263, 264] The folding of the ZF domain in the presence of Zn^{2+} forms a hydrophobic plateau with residues V13, F16, T24 and A25 of the proximal finger and residues W37, Q45 and M46 of the distal finger (Figure 35).[267, 268] This hydrophobic plateau dictates the nucleic acid (NA) binding properties of NCp7 through multiple contacts with the oligonucleotide bases and backbone.[267, 268] Additionally, the W37 residue always stacks with guanine bases (Figure 35 and Figure 36),[269] and has been extensively studied as a target for inhibition.[270] Moreover, NCp7 is flexible: it does not adopt a fixed shape until it is bound to its NA substrate, however, its binding conformation differs depending on whether it is bound to viral DNA or RNA (Figure 36).[271, 272] Many failures in drug design result from the flexibility of the target enzyme, enabling it to evade the inhibitor, however, as will be discussed later, this may work to our advantage, given the inherent flexibility of our potential drugs.

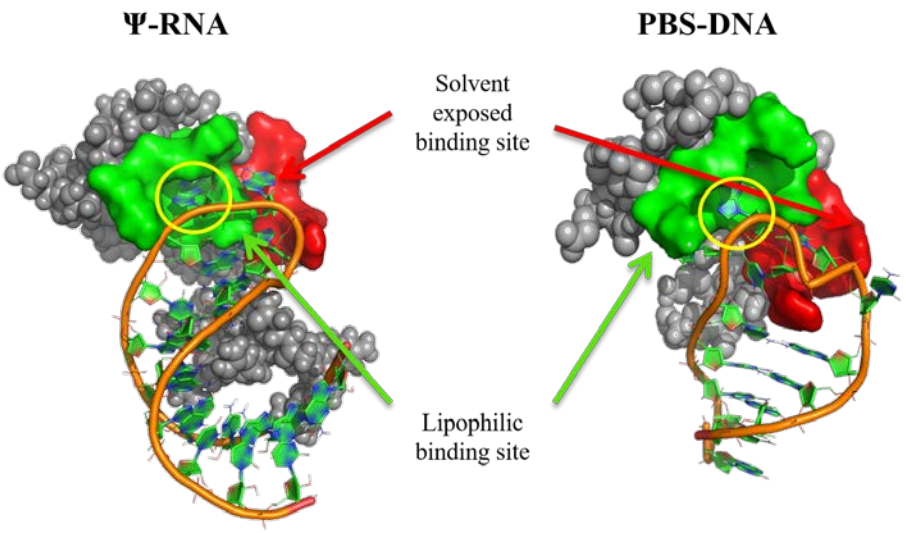


Figure 36. Computational model of NC Ψ-RNA/PBS-DNA complexes.[267, 273, Botta unpublished]

As mentioned previously, NCp7 plays several roles in HIV-1 replication (Figure 37). Through non-specific binding, it acts as a chaperone protein, similar to histones, by condensing, coating, and partially protecting the viral NAs.[274] During reverse transcription, NCp7 directs the annealing of cellular $tRNA^{(Lys,3)}$ primer to the HIV-1 primer binding site (PBS) in the 5'-long terminal repeat (LTR) sequence of the gRNA, initiating the synthesis of the (-)-strong stop DNA (ssDNA).[275, 276] NCp7 then facilitates the two strand transfers required for (-) and (+) strand synthesis. During the first strand transfer, NCp7 accelerates the annealing of the complementary trans-activation response (cTAR) to TAR located at the 3'- LTR of the gRNA.[277-279] During the second strand transfer, NCp7 facilitates the removal of the $tRNA^{(Lys,3)}$ primer from the 5'-end of the (-)-strand DNA, and promotes the annealing of the PBS DNA copy at the 3'-end of the (+) ssDNA with the complementary PBS located at the 5'-end of the (-)-strand DNA.[278, 280] NCp7 was also implicated to be a vital element in vDNA integration.[281, 282] Prior to encapsidation, NCp7 discriminates viral from host

NA by selectively binding to the GXG-containing loops of stem loop (SL) 2 and SL3 sequences of the HIV-1 Ψ-encapsidation signal RNA.[268, 271, 283, 284] Furthermore, *in vitro*, NCp7 was established to chaperone the dimerization of the two copies of HIV-1 viral gRNA by rearranging the kissing complex into an extended duplex, an important step prior to encapsidation.[263, 285]

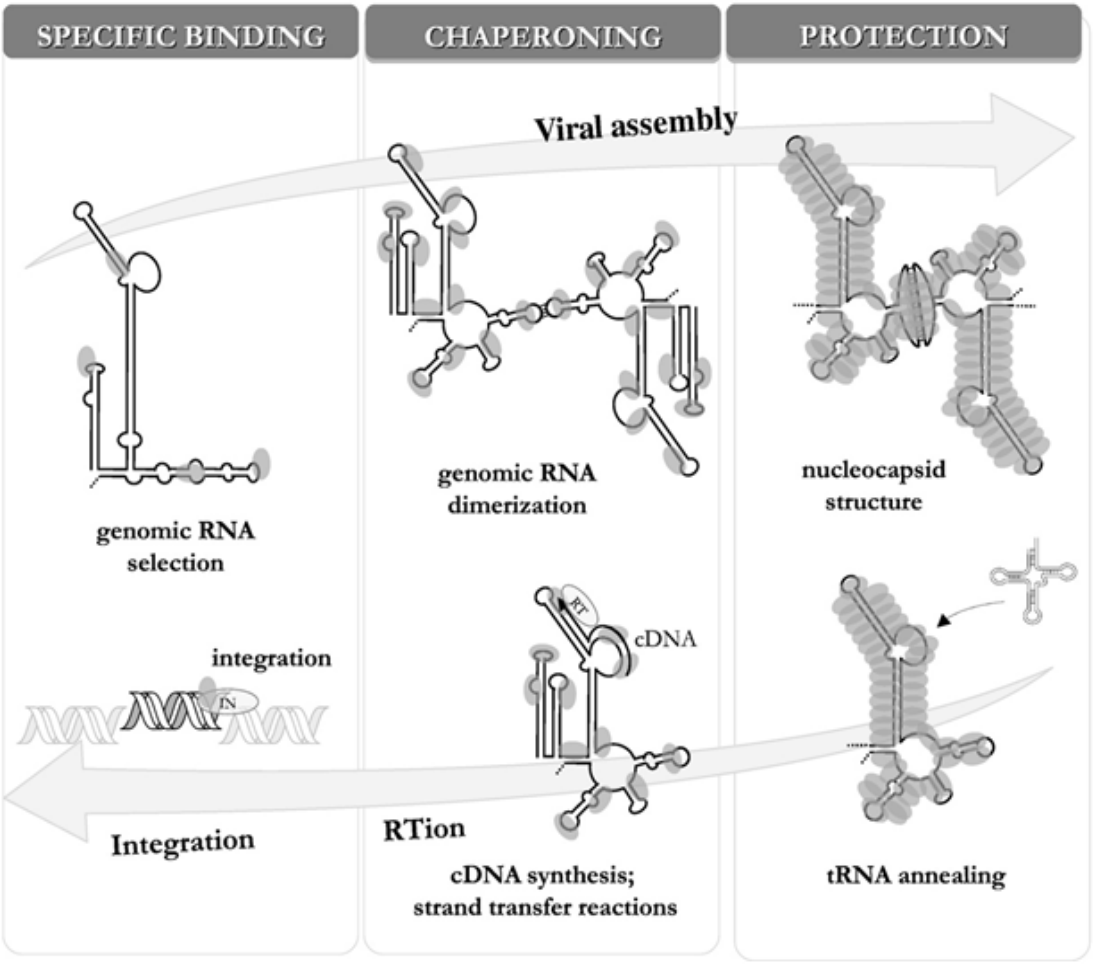


Figure 37. Functions of HIV-1 NC during HIV replication.[263]

Because of its interaction with highly conserved sequences of the HIV-1 genome found in all HIV-1 subtypes, NCp7 represents a powerful drug target for

developing novel antivirals.[286-288] More significantly, unlike other HIV-1 targets, it is believed to be highly resistant to mutation due to its multifunctional role, thus providing a significant advantage over other protein targets.[257, 263] As a result, inhibitors of the interaction between the NCp7 and Ψ-RNA or PBS-DNA could play a pivotal role in antiretroviral therapy.

Nucleocapsid Inhibitors

Having recognized the importance of NCp7, the first-generation of ZF protein inhibitors, also termed zinc ejectors, were developed (Figure 38).[289-294] They were named as such due to their mechanism of action; these compounds target the cysteine residues that are part of the zinc binding scaffold, "ejecting" the Zn^{2+}.[289-294] This prevents proper folding of the protein and subsequent loss of function.[289-294]

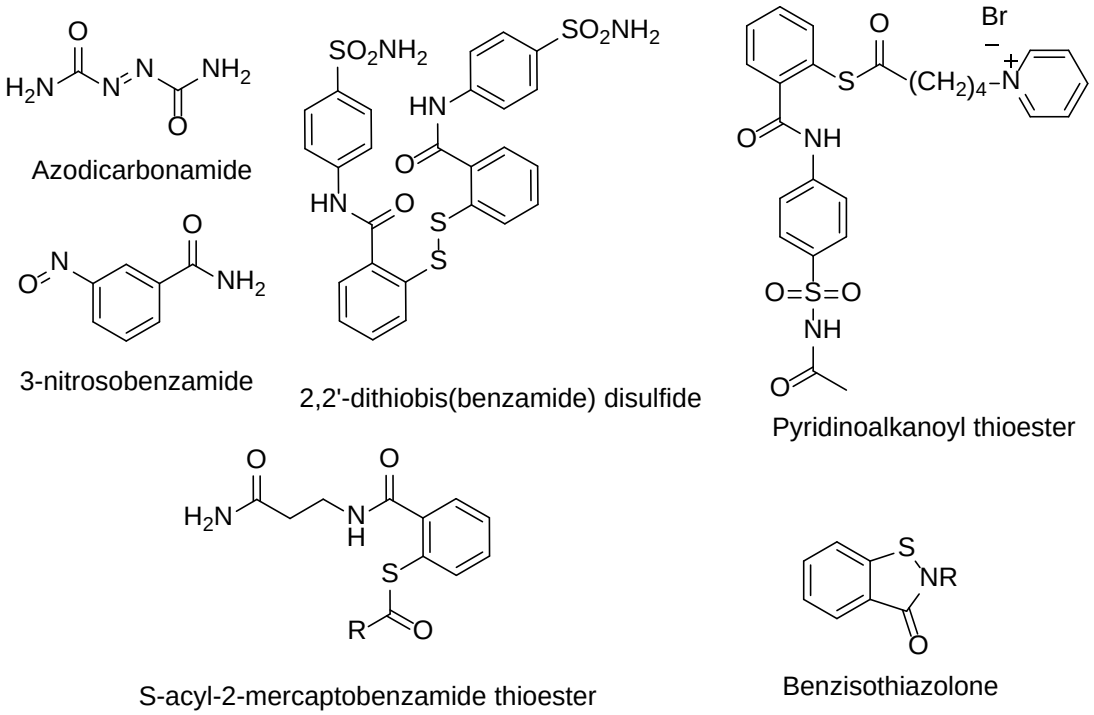


Figure 38. Covalent inhibitors of NCp7.[295]

There are two common mechanisms by which this can occur, both taking advantage of the nucleophilic character of the zinc coordinating cysteine residues.[289-294] In one mechanism, zinc ejectors such as azodicabonamide, 3-nitrosobenzamide (NOBA) and 2,2'-dithiobis(benzamide) disulfide (DIBA) (Figure 38) induce disulfide bond formation between the zinc coordinating cysteine residues, preventing proper Zn^{2+} binding (Figure 39).[289-291]

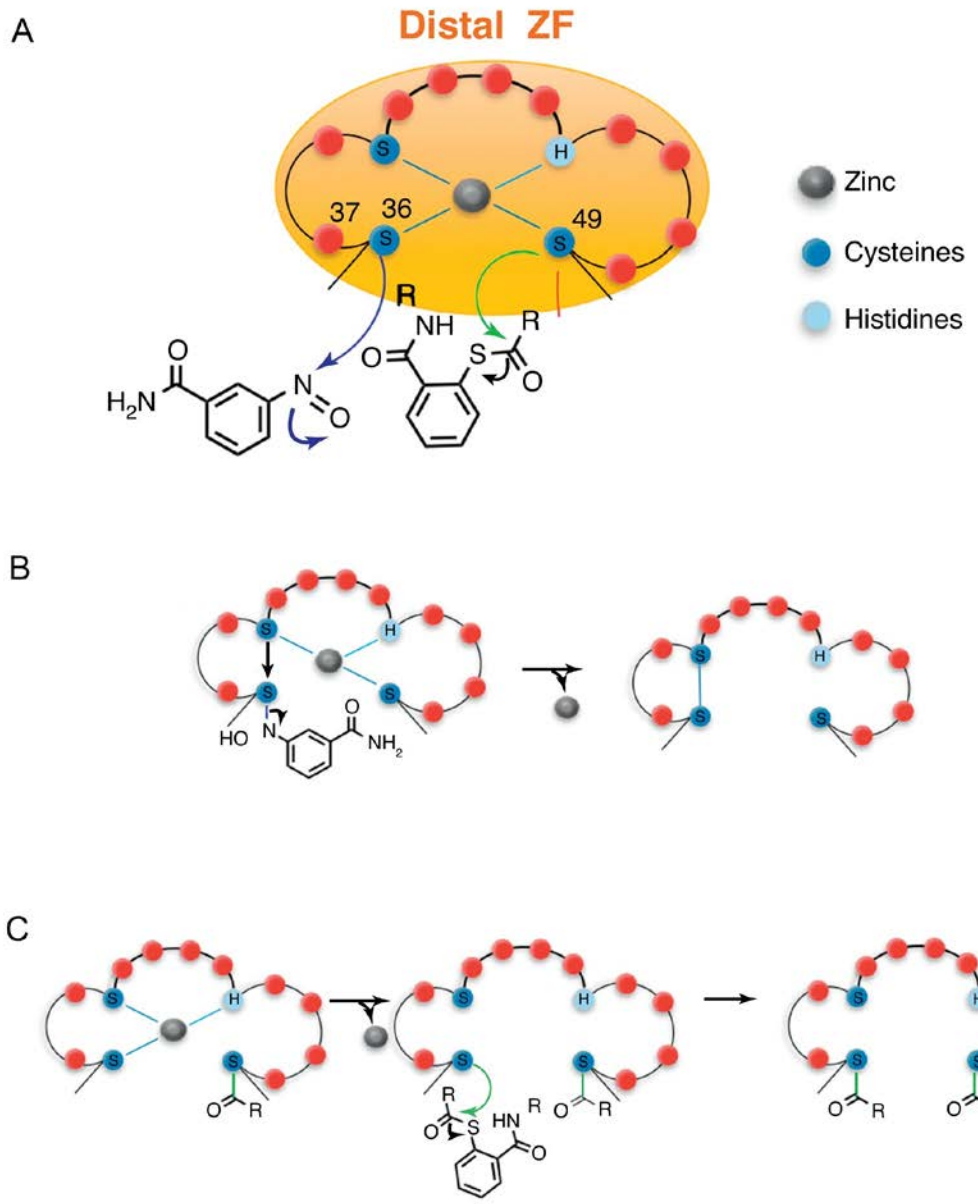


Figure 39. A) Proposed mechanisms of NCp7 covalent inhibition. B) NOBA compounds; and C) PATE compounds.[295]

Conversely, pyridinoalkanoyl thioesters (PATEs) and S-acyl-2-mercaptobenzamide thioesters (SAMTs) (Figure 38) disrupt zinc coordination by inducing a nucleophilic attack of the thioester carbonyl carbon by a zinc coordinating

cysteine, resulting in a covalent modification of the cysteine sulfur via an acyl transfer mechanism (Figure 39).[292-294] This modification weakens the zinc coordination at the site, which facilitates subsequent acyl transfer reactions with other cysteine residues, resulting in zinc ejection.[294] Due to their lack of specificity, most zinc ejectors also interact with host ZF proteins, of which there are many present in humans, resulting in major issues with toxicity.[296] Synthesis of more selective zinc ejectors are still being pursued, but because of reoccurring toxicity, other means of inhibiting NCp7 are needed that do not involve non-specific zinc ejection.

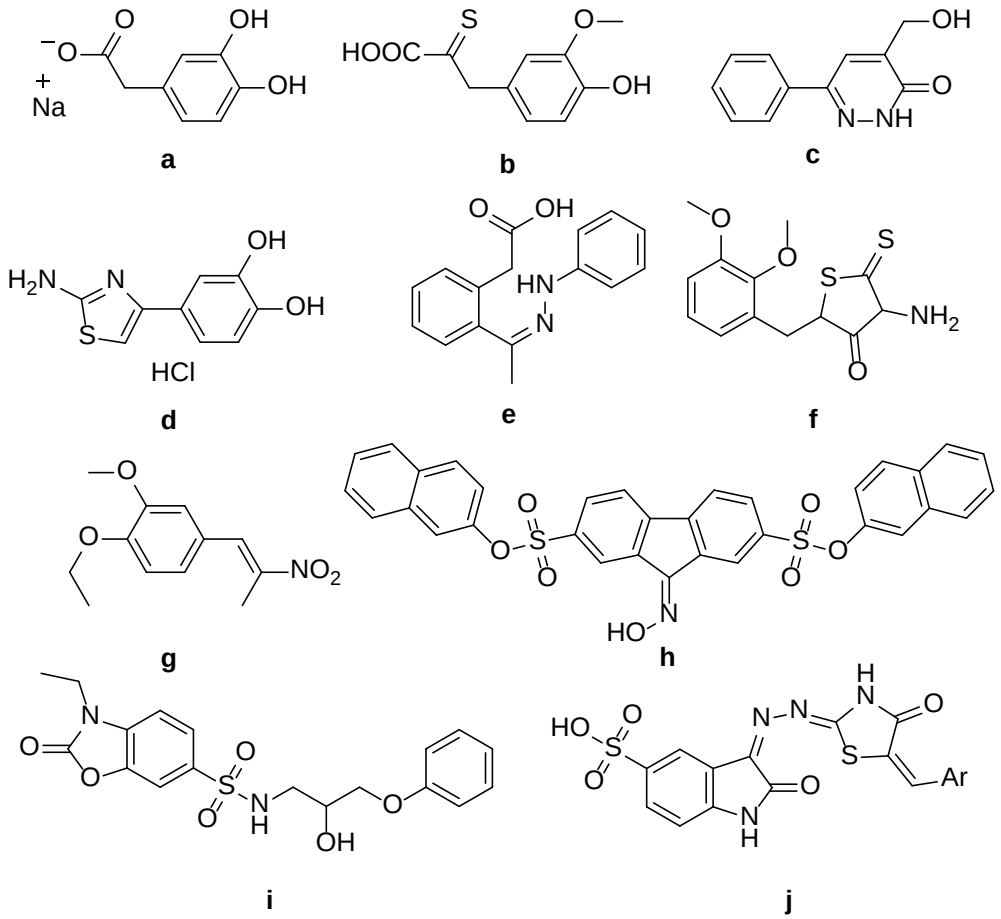


Figure 40. Non-covalent, non-zinc ejecting ZF binders. Ar = aryl group.[297-299]

An alternative strategy to inhibit NCp7 is to target its NA binding domain and avoid zinc ejection. Shvadchak *et al.* identified five small molecule compounds acting as non-zinc-ejecting NCp7 inhibitors (Figure 40, **a**-**e**).[300] These compounds exhibited K_i values ranging from 8.5 to 15 μM.[300] Breuer *et al.* also identified five non-zinc-ejecting compounds, including **f** and **g** (Figure 40), interestingly their K_i values were much lower, ranging from 14 to 73 nM.[287] From the data obtained from Shvadchak *et al.*, Mori *et al.* refined their computational NC module (discussed later) and found two compounds (Figure 40, **h** and **i**) that were active against HIV-1 *in vitro* at IC_{50} of 2 μM and around 100 μM.[297] Our goal is to synthesize molecules with similar inhibitory profiles to these non-zinc-ejecting compounds, but moreover, take advantage of the natural guanine substrate structure.

To screen our compounds' effect on NCp7, experimental procedures similar to those used by Goudreau *et al* were employed.[301] (Figure 41) Non-covalent NC binders (Figure 40, **j**) were titrated into a sample of NC and monitored via ^{1}H NMR. Both the aliphatic and aromatic regions of NC exhibited changes in peak intensities as well as chemical shift due to the protein's interaction with **j** (Figure 40). Similar patterns should be observed if our compounds interact with NC.

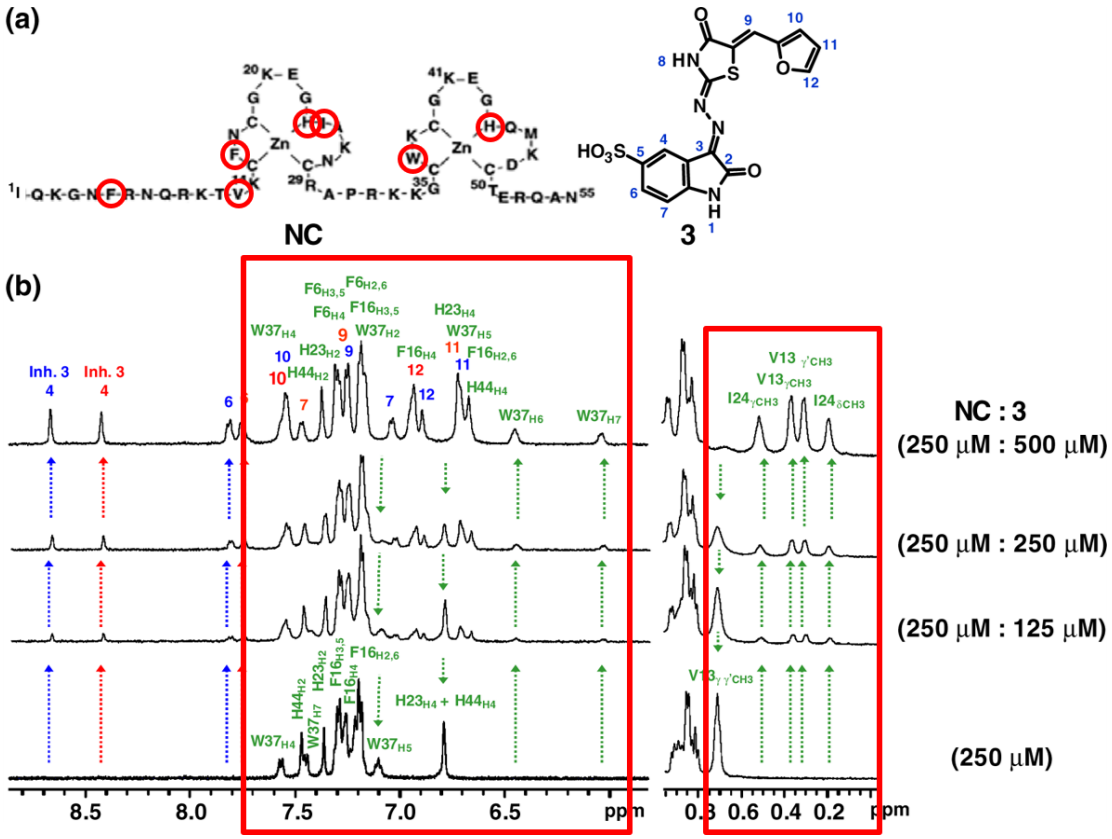


Figure 41. [1]H NMR titration experiment with NC by Goudreau *et al.*[301]

Computational modeling

Dr. Maurizio Botta (University of Siena) has developed computational models of NCp7 using previously established NMR data[267, 273] and an implemented version of the AMBER (*A*ssisted *M*odel *B*uilding with *E*nergy *R*efinement) force field to perform unrestricted molecular dynamics simulations.[270, 271] In addition, Dr. Botta validated his models against previously discovered NCp7 inhibitors (Figure 40, **a-e**).[270, 300] As has been previously established, a guanosine residue always stacks with the W37 residue of NCp7 whether bound to DNA or RNA (Figure 36), and, as such, we were interested in determining how the addition of flexibility to a guanosine analogue would affect binding and inhibition. Dr. Botta tested a wide range of our

flexible and expanded nucleoside analogues against his computational model and found that several of the Seley-Radtke compounds were predicted to be good binders of NC.

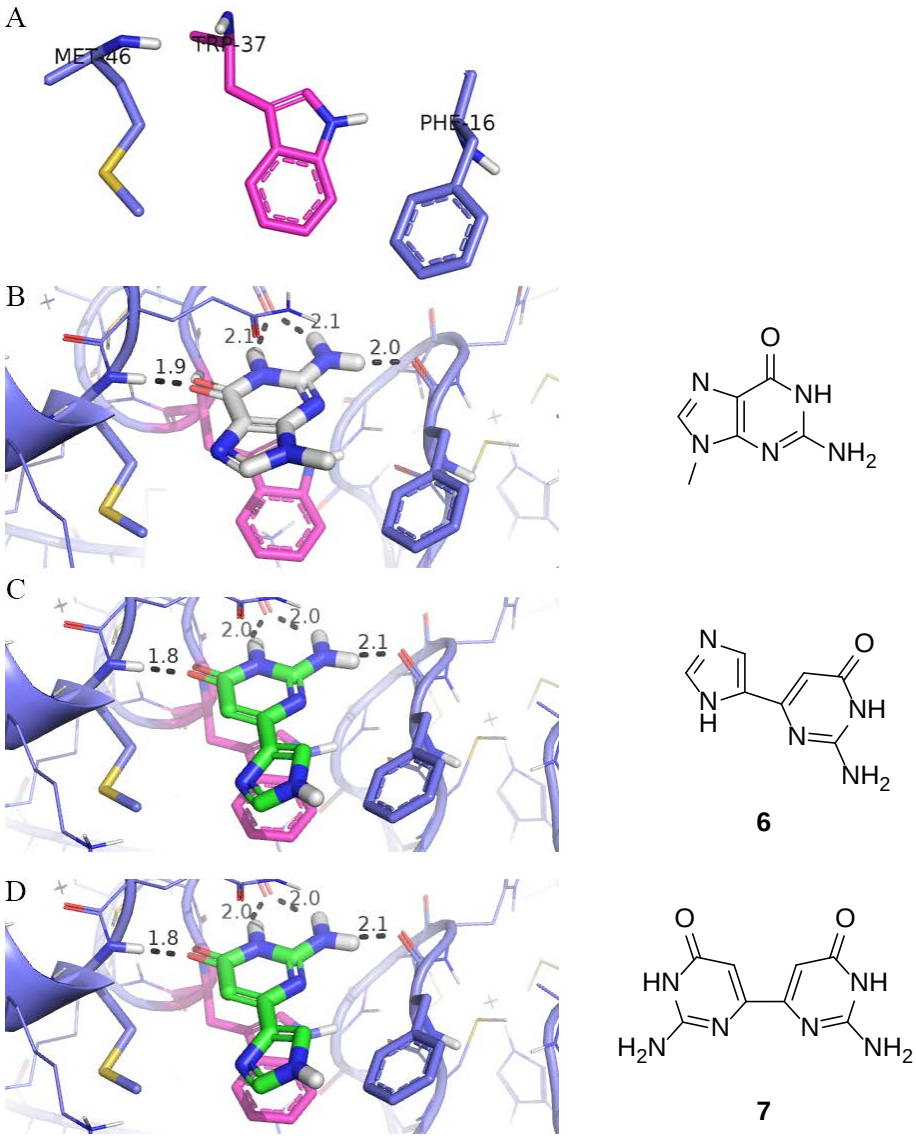


Figure 42. A) Key residues within NC/Ψ-RNA binding site, and B) 1-methyl guanine, C) **6**, D) **7** bound to NC/Ψ-RNA model.[Botta unpublished]

As seen in Figure 42, **6** and **7** adopt a similar stacking conformation to W37 as the natural guanine, however, the additional rotatable bond allows for the pyrimidine moiety to extend and interact with the neighboring F16. Interestingly, the fleximer nucleosides did not show enhanced binding over the fleximer bases, thus the nucleobases were chosen over the nucleosides to simplify the synthesis. In that regard, the fleximer nucleosides would require 10-12 steps, as opposed to the shorter synthetic scheme of the flex-bases (approximately 7 steps). In addition, the absence of the hemiaminal glycosidic bond in nucleobases makes the fleximer bases more robust compared to their parent nucleosides. Interestingly, the bipyridine base **7** was found to be a better inhibitor than the parent fleximer guanine base **6** through *G*enetic *O*ptimization for *L*igand *D*ocking (GOLD) fitness scoring (Table 8). GOLD scoring factors in hydrogen bonding energy, van der Waals energy for the protein-ligand interaction, and van der Waals energy for the internal energy of the ligand conformation.[302] Compounds **6-9** (Figure 32) were selected as the best candidates for this project. Initial synthesis focused on *distal* compounds **6** and **7**, as the *proximal* analogues **8** and **9** were determined to be less promising binders.

Table 8. GOLD Scores.

Compound	GOLD Score
Guanine	42.26
6	46.21
7	49.92

Note: The higher the score, the higher the affinity the receptor should have for the compound.

Fleximer Bases

The initial route to achieve both flexible purine and bipyrimidine bases employed palladium catalyzed coupling and installation of the pyrimidine as the organometallic coupling partner while using the imidazole as the halogenated coupling partner (Figure 43). The goal was to achieve two products from one reaction as the organometallic coupling moiety tended to go through homocoupling during the cross-coupling reaction.

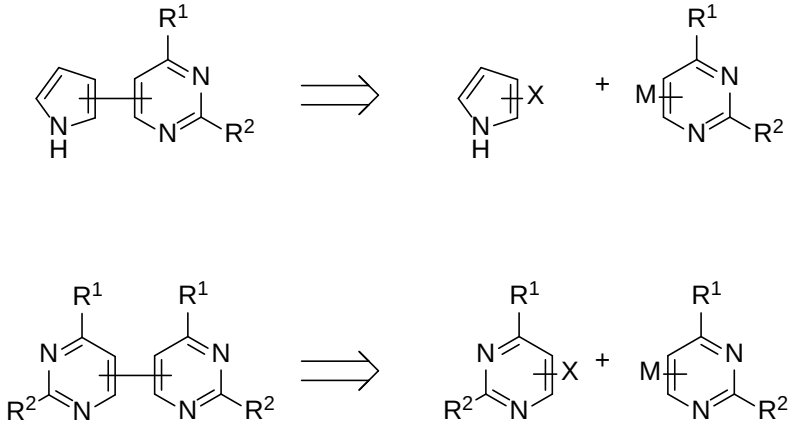
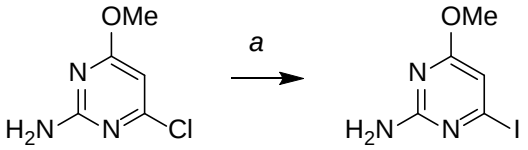


Figure 43. Initial strategy to attain fleximer bases.

As the *distal* compounds were determined to be the better binders of NC, the first goal was to install the organometal on the C-6 of 2-amino-6-iodo-4-methoxypyrimidine through palladium catalyzed cross-coupling to avoid having to protect the amino group. To obtain the iodinated intermediate, a commercially available 2-amino-6-chloro-4-methoxypyrimidine was iodinated using hydroiodic

acid (Scheme 37). The reaction was then neutralized and filtered, and the precipitate was recrystallized in ethanol to obtain **37.1**.

Scheme 37. Iodination of 2-amino-6-chloro-4-methoxypyrimidine.

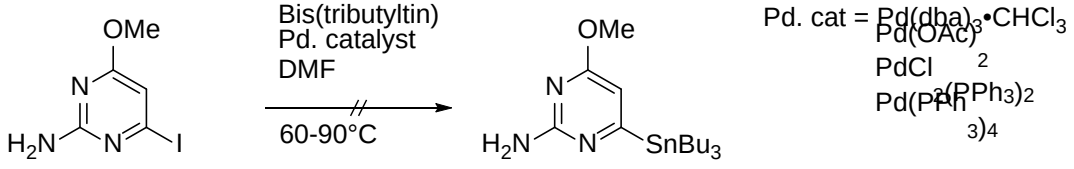


37.1

Reagents and conditions: a. HI (55%), 0°C to rt, 72 h.

Since the *proximal* intermediate 2-amino-4-methoxy-5-tributylstannylpyrimidine **20.2** was easily achievable starting with 2-amino-5-iodo-4-methoxypyrimidine (**20.1**),[129-131] it was believed that the same methodology could be applied to obtain the 2-amino-4-methoxy-6-tributylstannylpyrimidine **38.1**, which unfortunately proved to be untrue. To obtain the stannane intermediate, various palladium catalysts were used as well as a range of temperatures, but none of the conditions yielded the desired organostannane **38.1** (Scheme 38).

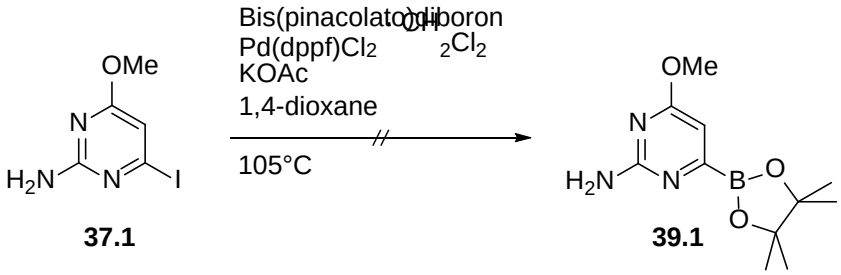
Scheme 38. Failed installation of tributyltin through palladium catalysis.



37.1 **38.1**

One of the biggest problems with using Stille couplings for products intended for biological studies is the possibility of tin associated toxicities.[208] The solution is to use other coupling procedures such as Suzuki coupling, thus the organoborane **39.1** was also pursued (Scheme 39). However, once again, the desired product was not observed.

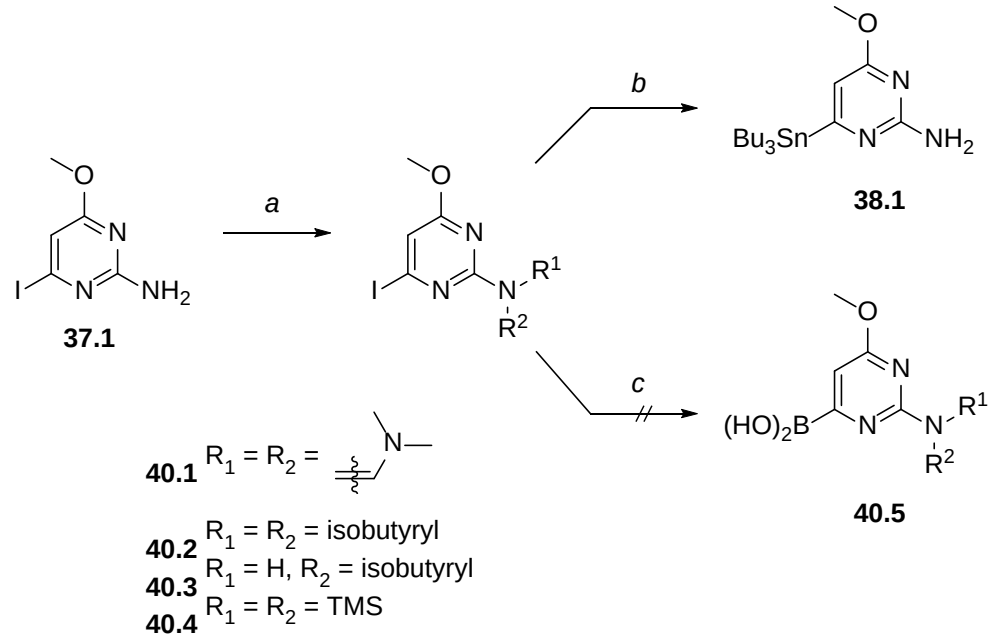
Scheme 39. Unsuccessful borane installation through palladium catalysis.



Because the cross-coupling intermediates could not be installed in the fashion shown, harsher conditions were used to achieve the desired organometallic coupling partners. In order to do so, the exocyclic amine group had to be protected (Scheme 40). A dimethyl acetal protecting group was first used due to its ability to protect both protons, however, it as well as the isobutyryl protecting groups were easily removed by the requisite *n*-butyllithium reagent needed to install the stannane and borane moieties.[150] Thus a TMS protecting group was employed. The reaction procedure to add the *in situ* TMS groups was more tedious as each TMS was added sequentially as opposed to simultaneously. Lithium halogen exchange using *n*-BuLi, followed by metallation with tributyltin chloride finally produced deprotected organostannane **38.1** in good yields (55%). Characterization through ^{1}H and ^{13}C NMR in addition to

LRMS confirmed the presence of the product. Regrettably, the stannane intermediate was unstable and the tributyltin moiety was cleaved within a day.

Scheme 40. Metallation of *N*-protected **37.1**.



Reagents and conditions: a. for **40.1**: DMF-DMAc, DMF, 60°C, 18 h; for **40.2** and **40.3**: isobutyryl chloride, pyridine, 0°C, 18 h; For **40.4**: (i) EtMgBr, TMSCl, THF, -78°C, (ii) *n*-BuLi, THF, -78°C, 10 min (ii) $SnBu_3Cl$, -78°C-rt, 18 h.

Considering the tedious nature of the procedures, the instability of the organometallic intermediates, as well as the toxicity associated with the byproducts, an alternative approach to achieve the distal fleximer base was pursued. From previous work done by our group, the distal fleximer guanine base could be obtained by subjecting the tricyclic guanine base to desulfurization via treatment with Raney nickel (Figure 44).

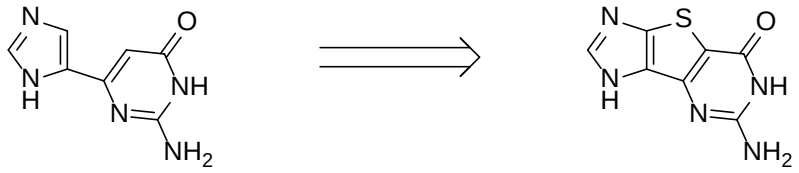
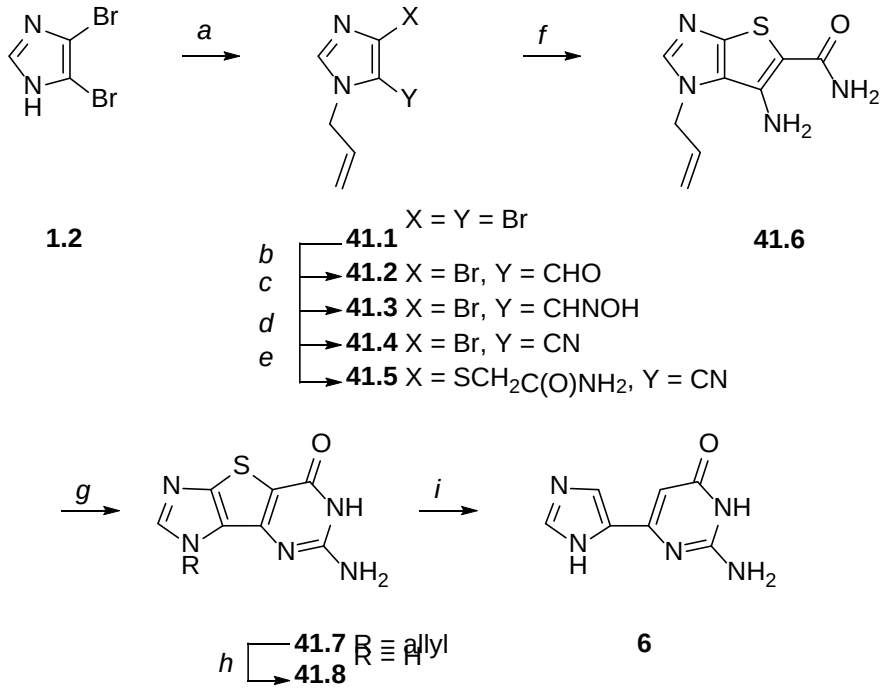


Figure 44. Retrosynthetic path to achieve the fleximer guanine from its tricyclic precursor.

Although the fleximer guanosine was routinely synthesized in our laboratory, an allyl protecting group was employed instead of using the ribose sugar to avoid the tedious protecting steps of nucleoside synthesis and the acid labile glycosidic bond. The tricyclic guanine was constructed using the methodologies as those described in Chapter 1 (Scheme 41).[139] 4,5-dibromoimidazole (**1.2**) was protected using allyl bromide and potassium carbonate as the base. The aldehyde intermediate **41.2** was obtained using ethylmagnesium bromide followed by addition of DMF. Subsequent transformation into the oxime using hydroxylamine hydrochloride, followed by dehydration using CDI yielded the nitrile **41.4**. Interestingly, the previous method of converting the oxime to nitrile using acetic anhydride yielded an unintended acetylated product. The thieno bicyclic intermediate **41.6** was obtained by first installing freshly made mercaptoacetamide onto the C-4 of the imidazole and inducing the cyclization using sodium ethoxide. The six-membered ring was formed using carbon disulfide, and aminolysis provided the correct functional group, and final deprotection yielded the tricyclic guanine **41.8**.

Scheme 41. Synthesis of tricyclic guanine.[139]



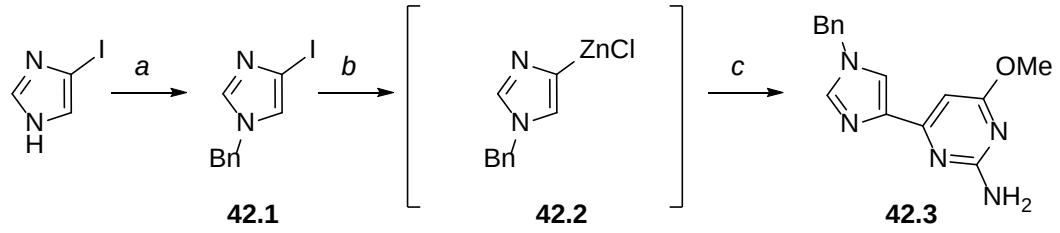
Reagents and conditions: a. allyl bromide, K_2CO_3, acetone, reflux, 18 h; b. (i) EtMgBr, THF, rt, 4 h, (ii) DMF, rt, 18 h; c. hydroxylamine HCl, $NaHCO_3$, EtOH, H_2O, rt, 18 h; d. CDI, THF, reflux, 18 h; e. $NH_2C(O)CH_2SH$, K_2CO_3, DMF, 55°C, 48 h; f. NaOEt, EtOH, reflux, 2 h; g. (i) NaOH, CS_2, CH_3OH, 150°C, 18 h, (ii) H_2O_2, CH_3OH, 0°C, 2 h, (iii) NH_3, CH_3OH, 120°C,12 h; h. phenylsilane, $Pd(PPh_3)_4$, AcOH/CH_2Cl_2 (1:1), 40°C, 48 h; i. Raney nickel, CH_3OH, reflux, 18 h.

In conjunction with building the tricyclic guanine, further literature research into palladium catalyzed cross-coupling showed that an organozinc intermediate could be generated *in situ* with the imidazole moiety for subsequent Negishi coupling,[303] a procedure that had not been previously successful in our laboratory as imidazole has been shown to be an inadequate organometallic coupling partner in the

past, potentially due to the instability of the intermediate, and the inability to be successfully isolated and characterized.

The benzyl protecting group was initially chosen for 4(5)-monoiodoimidazole as it has been proven to be robust against various harsh conditions in our lab. Using Grignard conditions, the organozinc was generated *in situ* and subjected to Negishi coupling with **37.1**. The protected distal fleximer **42.3** was achieved, although in poor yield (24%).

Scheme 42. Protected distal fleximer guanine synthesized through Negishi coupling.



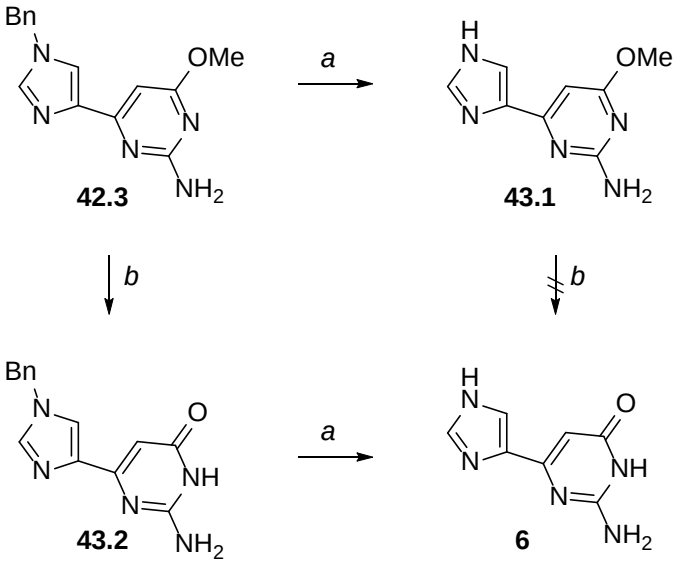
42.1 **42.2** **42.3**

Reagents and conditions: a. NaH (95%), BnBr, TBAI, THF, reflux, 18 h; b. (i) EtMgBr, THF, -78°C, (ii) $ZnCl_2$, 2 h, rt; c. **37.1**, $Pd(PPh_3)_4$, CuI, THF, reflux, 18 h.

Moreover, the benzyl and methyl groups were extremely difficult to deprotect (Scheme 43). Hydrogenation using Pd/C at room temperature was thought to be sufficient to remove both moieties, however no reaction occurred. Deprotection through palladium assisted hydrogenation using ammonium formate as the hydrogen donor in refluxing methanol did not debenzylate the compound. The Bn group was ultimately removed after heating the reaction mixture with ammonium formate and Pd/C in ethanol to 120°C. Palladium catalyzed hydrogenation was ineffective in removing the methyl group under all conditions attempted. The Lewis acid boron

tribromide finally deprotected the methyl, however, a solubility problem arose when attempting to demethylate from the Bn deprotected intermediate as the solvent used for deprotection was dichloromethane, which did not solubilize the compound and therefore deprotection of the methyl was more efficient when performed prior to deprotection of the Bn (Scheme 43).

Scheme 43. Deprotection strategies for 4-(1-benzyl-1*H*-imidazol-4-yl)-6-methoxy-2-pyrimidinylamine

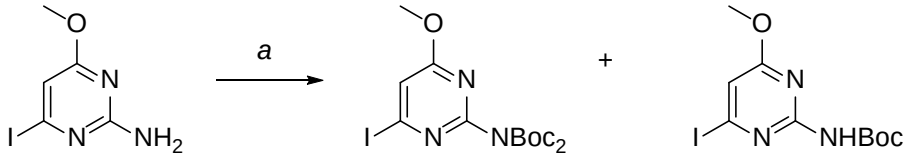


Reagents and conditions: a. ammonium formate, Pd/C, EtOH, 120°C, 48 h; b. BBr_3, CH_2Cl_2, rt, 72 h.

Furthermore, these molecules proved difficult to purify (on either silica or C_{18} solid phases), likely due to their polar nature and their ability to stack efficiently from the presence of two heteroaromatic moieties and multiple hydrogen bonding elements. In order to bypass these challenges, a trityl protected 4-iodoimidazole and

exocyclic amine of the pyrimidine was protected with a *tert*-butyloxycarbonyl (Boc) group (Scheme 44 and Scheme 45.).

Scheme 44. *tert*-Butyloxycarbonyl protection of 2-amino-6-iodo-4-methoxypyrimidine.

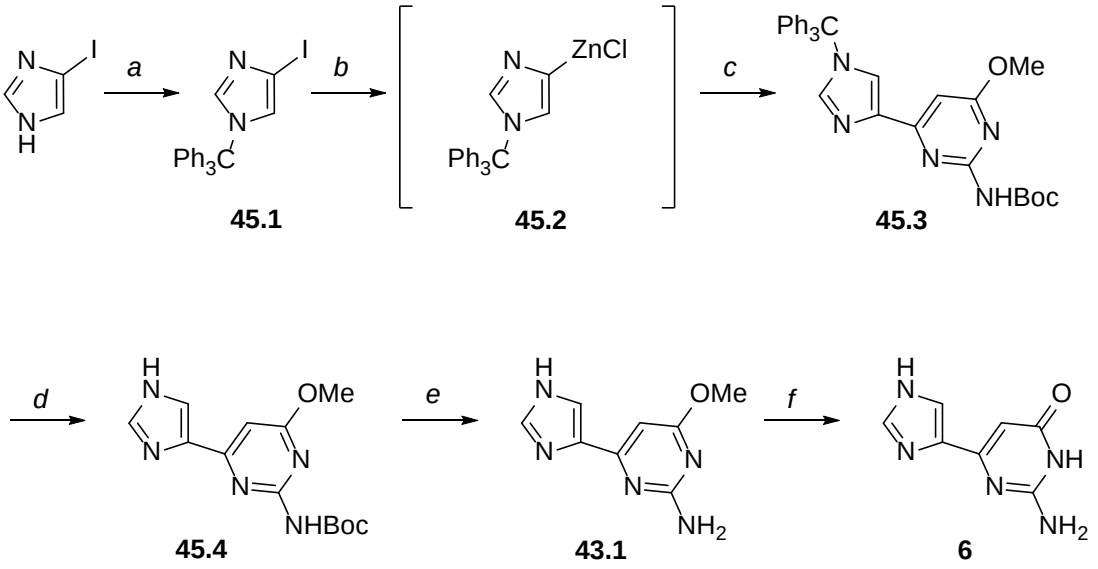


37.1 **44.1** **44.2**

Reagents and conditions: a. Di-*tert*-butyl dicarbonate, DMAP, CH_2Cl_2, rt, 18 h.

Negishi coupling using these two heterocycles proved much simpler and the cross-coupling reaction proceeded at room temperature (Scheme 45). Trityl deprotection was accomplished using acetic acid while Boc deprotection required trifluoroacetic acid. As previous mentioned, the methyl protected fleximer guanine **43.1** was insoluble in dichloromethane, however, it was found to be soluble in ethyl acetate, thus final deprotection of the methyl was done using BBr_3 once again, but this time in ethyl acetate.

Scheme 45.



Reagents and conditions: a. Trityl chloride, triethylamine, CH_2Cl_2, rt, 18 h; b. (i) EtMgBr, THF, -78°C, (ii) $ZnCl_2$, 2 h, rt; c. **44.1**, $Pd(PPh_3)_4$, CuI, THF, rt, 18 h; d. acetic acid, rt, 18 h; e. TFA, rt, 18 h; f. BBr_3, EtOAc, rt, 72 h.

With the distal fleximer **6** in hand, a NC ^{1}H NMR experiment was performed, with the help of Dr. Xiao Heng from the Summers lab (UMBC). In theory, if the fleximer did bind to the nucleic acid binding site of NC where the W34 lies, there should be a shift observed in the aromatic region of the protein (as seen in Figure 41). After having observed the ^{1}H NMR spectra of NC in D_2O without **6**, a one equivalent molar ratio of **6** was added to the sample (dissolved in DMSO-d_6). Both the aliphatic region (

Figure 45) and aromatic region (

Figure 46) were observed and no dramatic changes were witnessed.

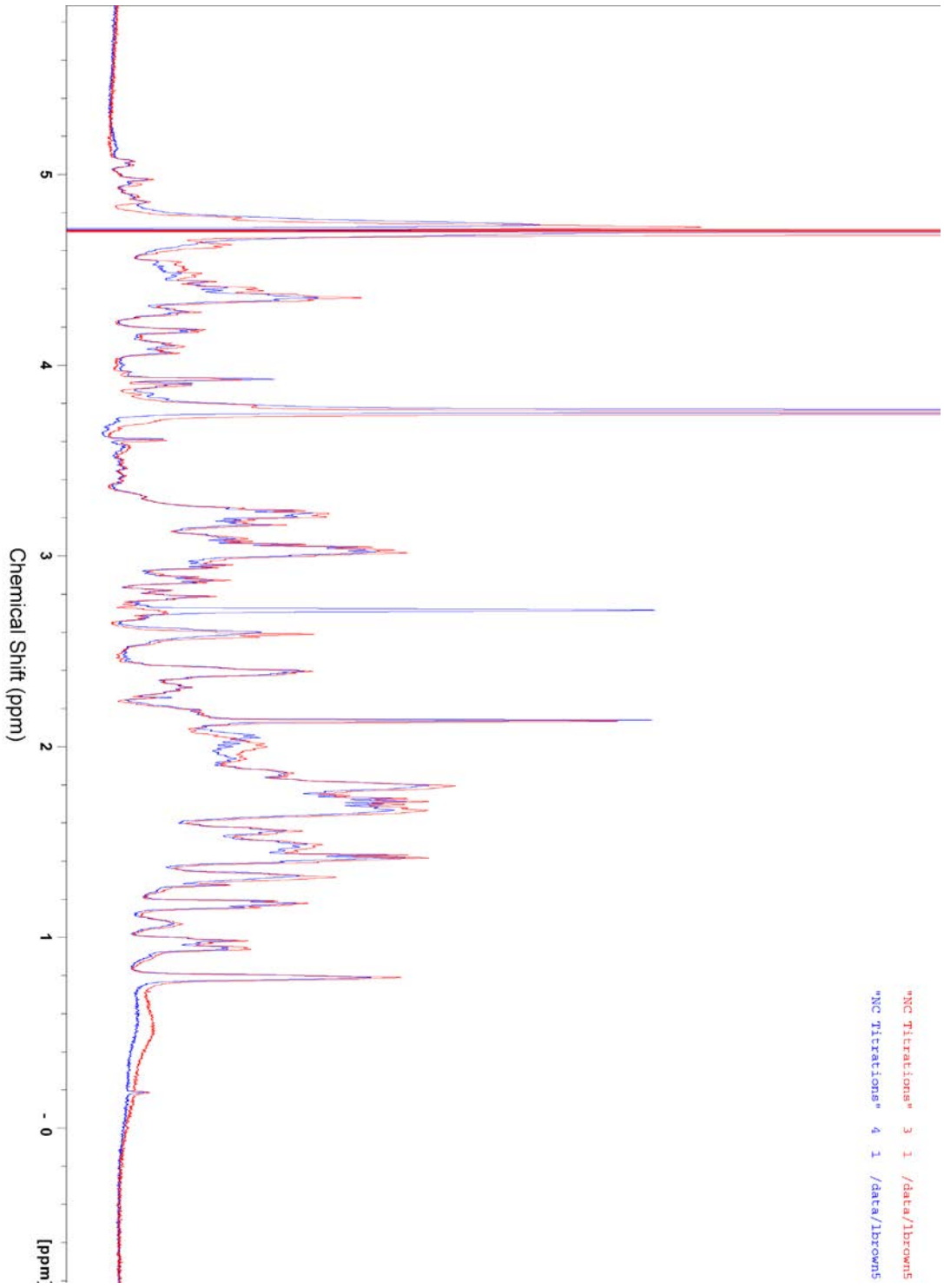


Figure 45. ^{1}H NMR of NC (red) and NC with **6** (blue), aliphatic region.

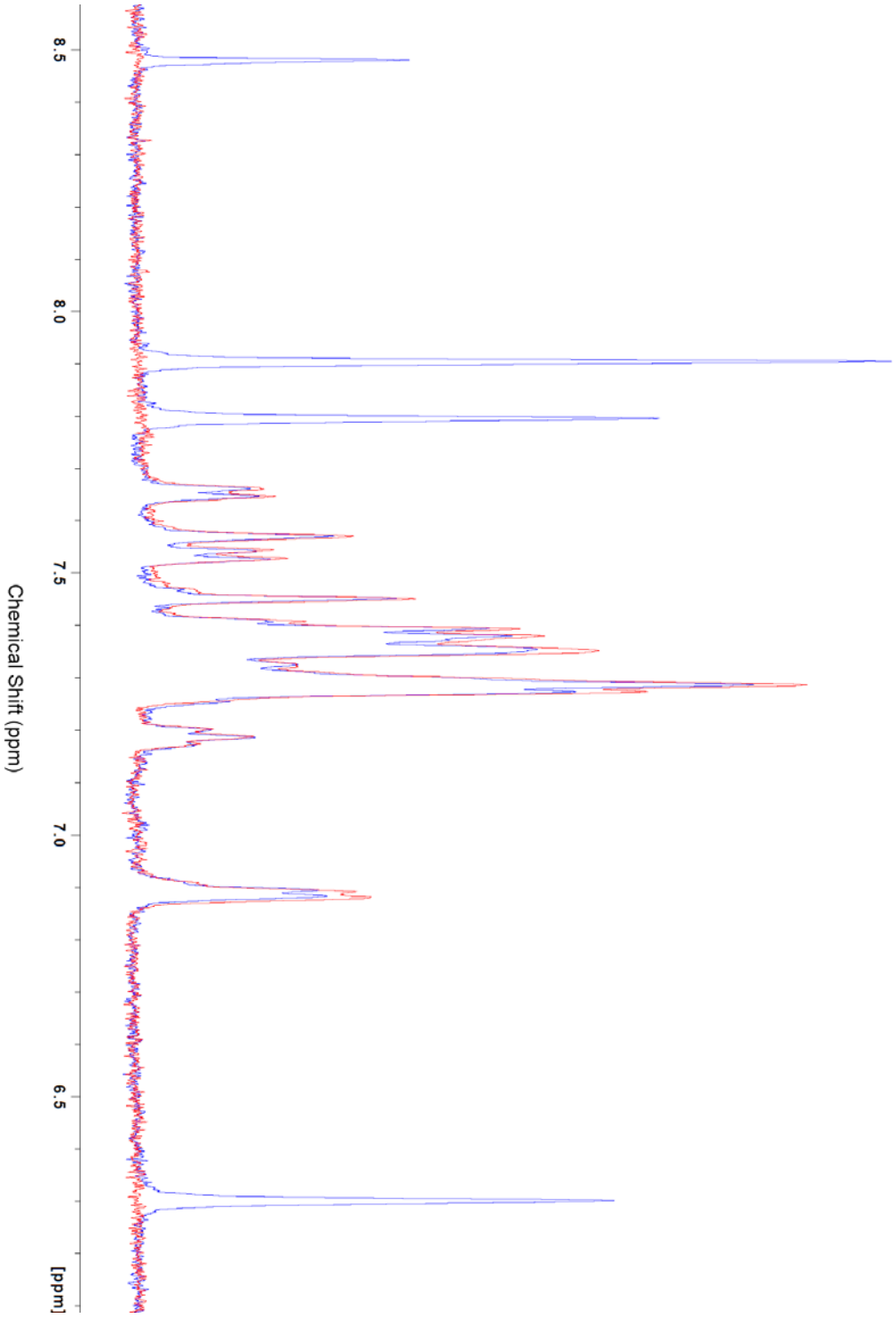
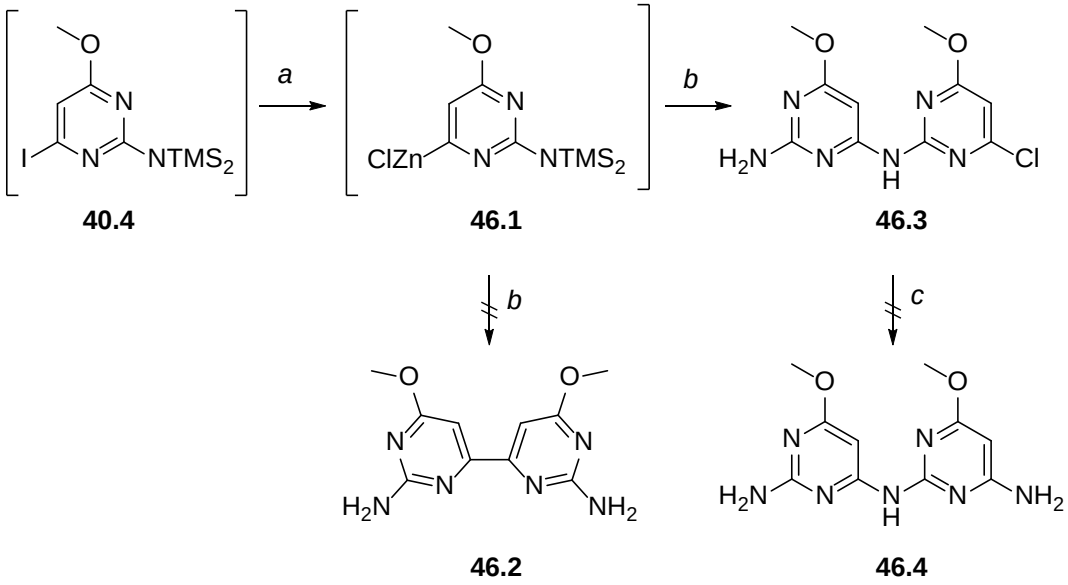


Figure 46. ^{1}H NMR of NC (red) and NC with **6** (blue), aromatic region.

While these initial results were disappointing, the bipyrimidine was computationally determined to be a better binder, thus it was the next target. Since the distal fleximer guanine was ultimately obtained through Negishi coupling, a similar strategy was employed to obtain the analogous bipyrimidine. Pyrimidine **37.1** was protected *in situ* with TMSCl (**40.4**). EtMgBr followed by $ZnCl_2$ addition should produce the organozinc pyrimidine intermediate **46.1**, and Negishi coupling using the same starting material would theoretically produce the desired bipyrimidine product **46.2**. Unexpectedly, the amine-linked compound **46.3** was produced. The compound was subjected to aminolysis to convert the chloro group to an exocyclic amine, however, no starting material or product was recovered.

Scheme 46. Synthesis of amine-linked bipyrimidine **40.4**.

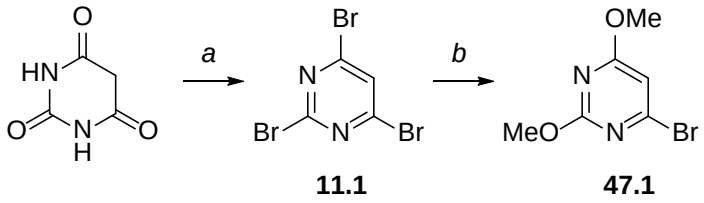


Reagents and conditions: a. (i) EtMgBr, THF, -78°C, (ii) $ZnCl_2$, 2 h, rt; b. 2-amino-4-chloro-6-methoxypyrimidine, $PdCl_2(PPh_3)_2$, CuI, THF, reflux, 18 h; c. NH_3, CH_3OH, 120°C, Parr bomb, 48 h.

In hindsight, the intended Negishi coupling did not occur; the presence of a palladium catalyst likely induced a Buchwald-Hartwig amination reaction, leading to the synthesis of **46.3** and the absence of compound **46.2**.

Because the correct cross-couplings procedures were not easily performed on the C-6 of **37.1**, a 6-bromo-2,4-dimethoxypyrimidine (**47.1** Scheme 47) was pursued instead, using Suzuki coupling, and the distal fleximer xanthosine was achieved through an analogous boronic acid **11.3** (Scheme 11). Subjecting **11.1** to two equivalents of sodium methoxide produced **47.1**.

Scheme 47. Synthesis of 6-bromo-2,4-dimethoxypyrimidine **47.1**.

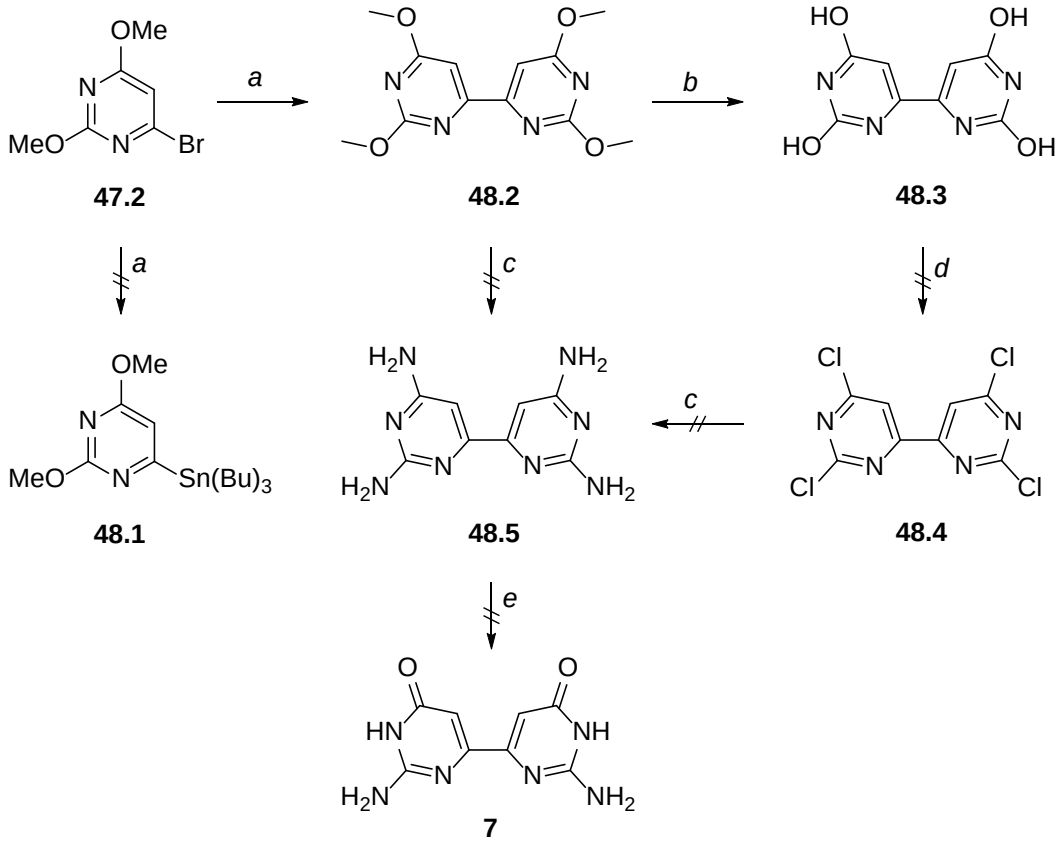


Reagents and conditions: a. $POBr_3$, *N*,*N*-dimethylaniline, toluene, 110°C, 3 h; b. sodium methoxide, methanol, 0°C to rt, 18 h.

Historically, the boronic acids our laboratory have synthesized are usually unstable and must be used crude for cross-coupling in many cases, the tributyltin intermediate was pursued instead such that it could be isolated and characterized. Interestingly, the 6-tributytin-2,4-dimethoxypyrimidine **48.1** was never synthesized, however the bipyrimidine **48.2** was recovered at good yields (80%, Scheme 48). From there, two strategies were attempted to attain the desired bipyrimidine **7**. Removal of the methyl protecting groups followed by chlorination via $POCl_3$ was

tried, and the intermediate **48.3** was used crude as it was insoluble in various purification solvents. The tetrachlorinated intermediate **48.4** could not be isolated as the crude reaction mixture was difficult to purify. The next approach was to directly convert the methoxy groups to amines (**48.5**) and enzymatically convert the "C-4" -NH_2 to -OH using adenosine deaminase. Neither the starting material nor product were recovered, likely due to the harsh conditions used.

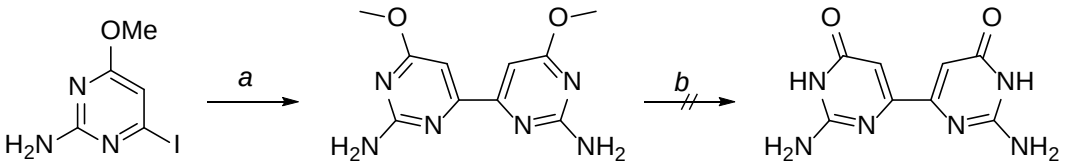
Scheme 48. 2,4-Dimethoxypyrimidine homocoupling.



Reagents and conditions: a. bis(tributyltin), $Pd(PPh_3)_2Cl_2$, 1,4-dioxane, 120°C, 18 h; b. BBr_3, CH_2Cl_2, rt, 48 h; c. NH_3, CH_3OH, 120°C, 72 h; d. $POCl_3$, reflux, 18 h; e. ADA.

Since homocoupling of the pyrimidines had occurred for the dimethoxypyrimidines, the assumption that the same conditions would produce a homocoupled product for the 2-amino-4-methoxypyrimidines as well, which fortuitously proved true (Scheme 49). Unfortunately, the product proved to be highly insoluble, and impossible to purify, and only observed *via* LRMS (APCI, predicted: 249.11 ($M+H^+$), found: 249.1).

Scheme 49. 2-Amino-6-iodo-4-methoxypyrimidine homocoupling.

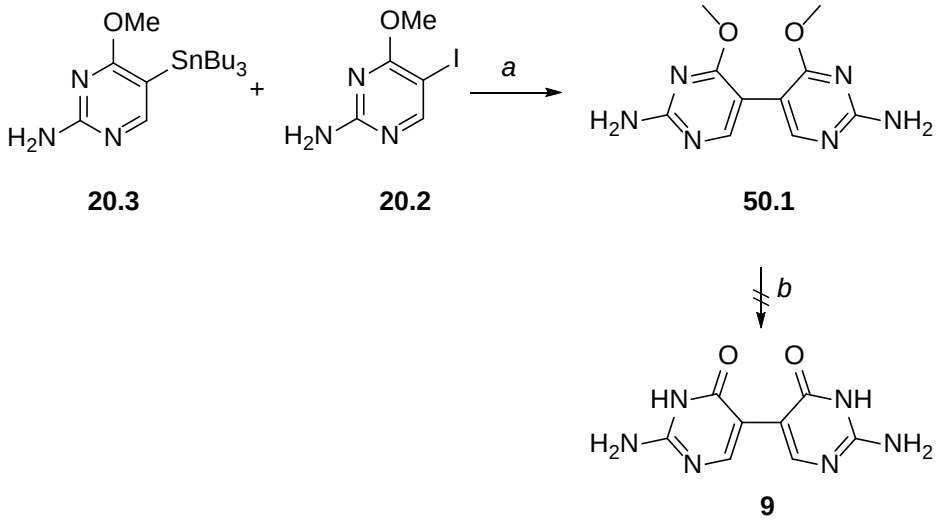


37.1 **46.2** **7**

Reagents and conditions: a. bis(tributyltin), $Pd(PPh_3)_2Cl_2$, 1,4-dioxane, 130°C, 48 h; b. BBr_3, CH_2Cl_2, rt, 48 h.

As achieving the *distal* analogues have thus far been challenging, the focus turned to synthesizing the *proximal* analogues instead. Considering the synthesis of compound **20.3** was facile, a straightforward Stille with the halogenated pyrimidine should produce the bipyrimidine **50.1** (Scheme 50). This was indeed the case, however, similar purification and solubility issues arose. Since the yield was much higher in this case (TLC visualization analysis), a series of recrystallizations using ethyl acetate, ethanol, methanol, and finally DMSO gave **50.1**. Further deprotection to obtain **9** was impossible as bipyrimidine **50.1** proved only soluble in DMSO.

Scheme 50. 2-Amino-5-iodo-4-methoxypyrimidine homocoupling.



Reagents and conditions: a. $Pd(PPh_3)_4$, DMF, 90°C, 18 h; b. BBr_3, CH_2Cl_2, 48 h.

A NC ^{1}H NMR experiment was performed using **50.1**, with the help of Dr. Jan Marchant from the Summers lab (UMBC). The same method was used for **50.1** compared to **6.** After having obtained the control ^{1}H NMR spectra of NC in D_2O, a one equivalent molar ratio of **60.1** was added to the sample (dissolved in DMSO-d_6) as well as a 2:1 compound to NC experiment. Both the aliphatic region (

Figure 47) and aromatic region (

Figure 48) were observed. Interestingly, in addition to the peak at 7.86 ppm (**50.1**), additional peaks at 5.54, 5.66 (t, J = 5.5 Hz), 5.89 (t, J = 5.2 Hz), 6.31 (d, J = 4.9), 8.16 (s), 8.23 (s) ppm appeared. Referring to the changes noted by Groudreau *et al.* (Figure 41),[301] the three signals between 5.54 and 6.31 ppm could be a shift by W37, however, the appearance of the peaks above 8 ppm would likely be related to a change in conformation of **50.1** due to the effect by the protein.

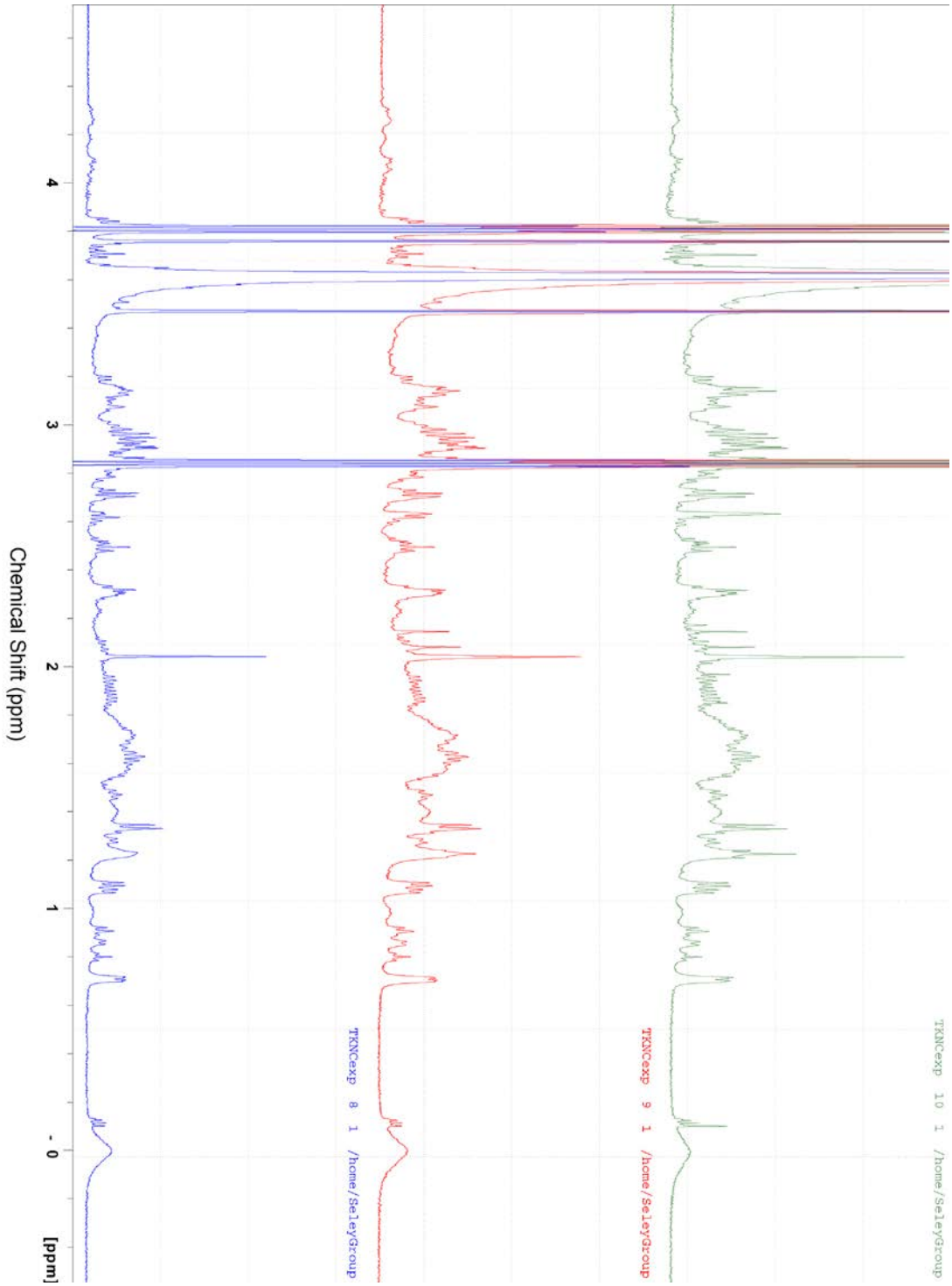


Figure 47. Aliphatic region of NC with **50.1** (blue = NC blank, red = 1:1 NC/**50.1**, green = 1:2 NC/**50.1**).

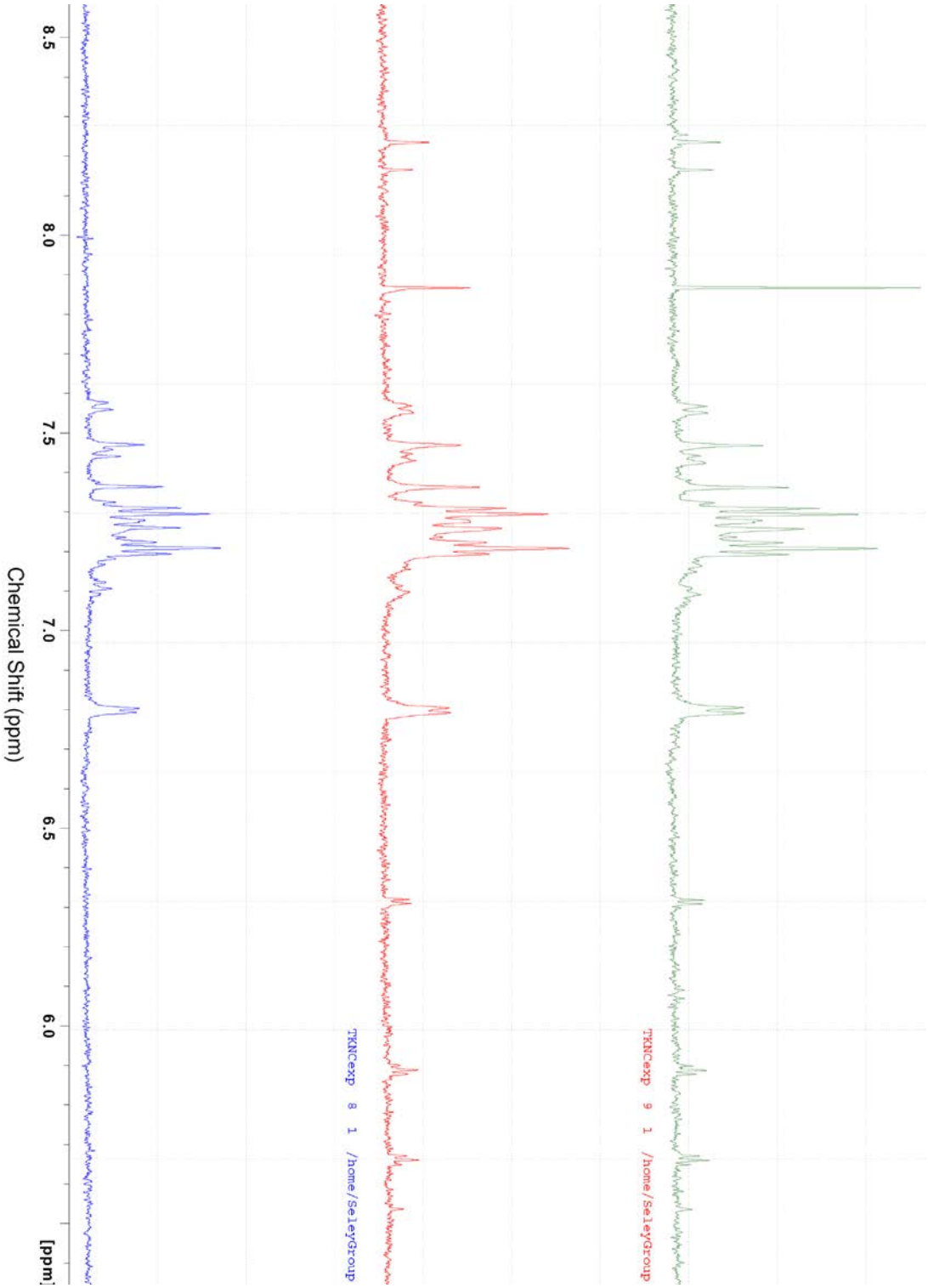
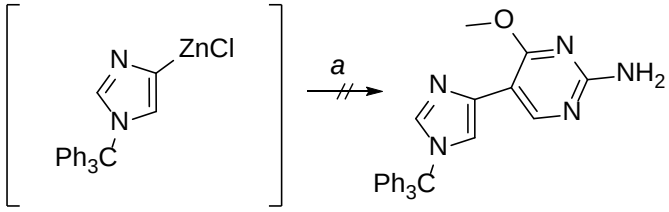


Figure 48. Aromatic region of NC with **50.1** (blue = NC blank, red = 1:1 NC/**50.1**, green = 1:2 NC/**50.1**).

To complete this series, the *proximal* fleximer guanine was also synthesized. Instead of using Stille cross-coupling techniques, the Negishi method used for achieving the *distal* fleximer guanine was employed (Scheme 51). Surprisingly, no product was observed when the reaction was allowed to stir at room temperature, and poor yields were found even after reflux.

Scheme 51. Unsuccessful attempts at Negishi coupling of **45.2** and **20.2**.



45.2

Reagents and conditions: a. **20.2**, $Pd(PPh_3)_4$, CuI, THF, rt and reflux, 18 h.

The failure of the reaction is likely due to the placement of the halogen on the electron-rich carbon of the C-5 position of the pyrimidine. As palladium prefers to react with electron-deficient carbons (Figure 49), the oxidative addition reaction between the halogenated coupling partner and palladium probably did not occur, which led to the absence of the desired coupled product. To solve this problem, the organozinc was placed on the pyrimidine in subsequent reactions.

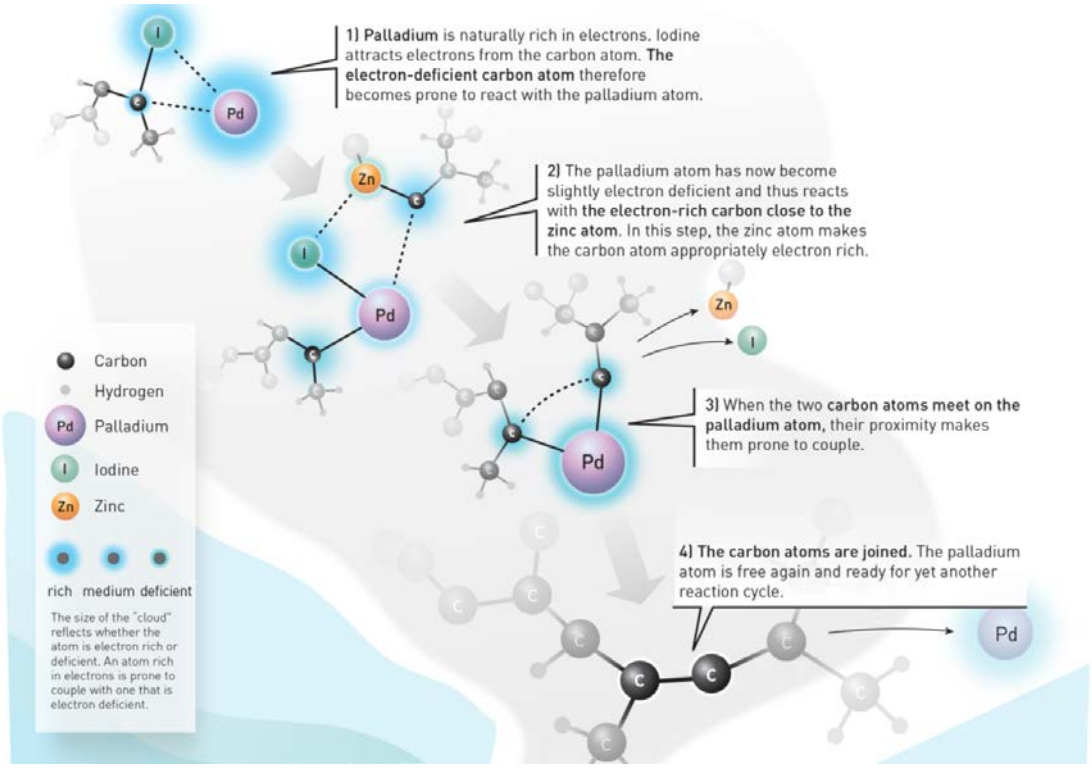
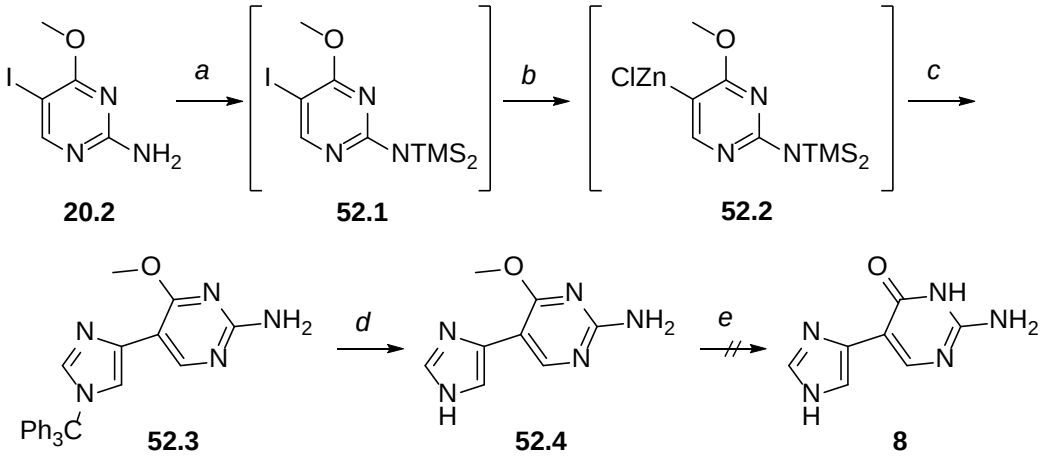


Figure 49. Palladium catalyzed cross-coupling mechanism.[304]

As predicted, the organozinc on the pyrimidine was successfully synthesized *in situ*, and Negishi cross-coupling followed by deprotection of the trityl was accomplished to yield **52.4** (Scheme 52). Regrettable, methyl deprotection to produce the *proximal* fleximer guanine was unsuccessful using BBr_3 in either CH_2Cl_2 or EtOAc as **52.4** was insoluble in both solvents, unlike **43.1**. Deprotection using catalytic sulfuric acid, TMSCl in acetic anhydride also did not produce **8** (confirmed through LRMS). The hypothesis to explain this phenomenon ties into the *in silico* results that show that the most thermodynamically stable conformation of the *proximal* fleximer bases is in a planar form.[305] This conformation could be promoting the stacking of the bases that consequently do not allow **52.4** to solubilize in usual solvents.

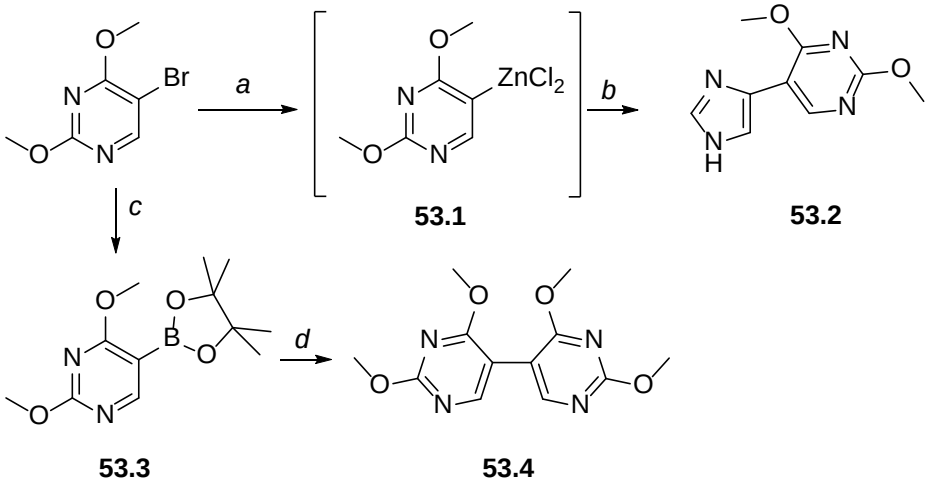
Scheme 52. Negishi cross-coupling to synthesize **8**.



Reagents and conditions: a. EtMgBr, TMSCl, THF, -78°C; b. (i) EtMgBr, (ii) $ZnCl_2$, 2 h, rt; c. **45.1**, $Pd(PPh_3)_4$, CuI, THF, 40°C, 18 h; d. AcOH, rt, 48 h; e. BBr3, CH_2Cl_2/EtOAc, or TMSCl, cat. H_2SO_4, Ac_2O, rt, 48 h.

The dimethoxypyrimidine series was also pursued and proved more facile to obtain as no extra protection steps were required. The *proximal* compounds were synthesized using the pyrimidine as the organometallic moiety (Scheme 53).

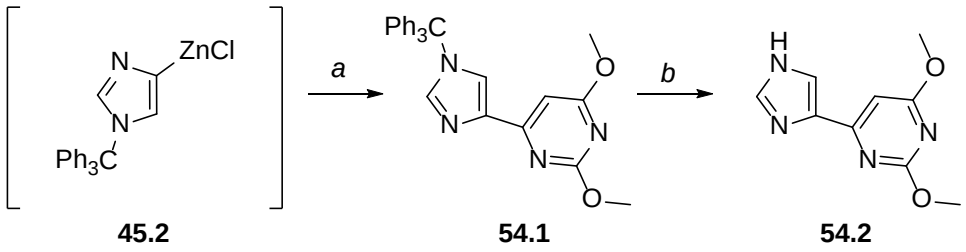
Scheme 53. Negishi cross-coupling reactions with 2,4-dimethoxypyrimidine.



Reagents and conditions: a. (i) EtMgBr, THF, -78°C, (ii) $ZnCl_2$, 2 h, rt; b. 4(5)-iodoimidazole, $Pd(PPh_3)_4$, CuI, THF, 60°C, 18 h; c. bis(pinacolato)diboron, KOAc, $PdCl_2(dppf)_2 \cdot CH_2Cl_2$, 100°C, 1 h; d. 5-bromo-2,4-dimethoxypyrimidine, $PdCl_2(dppf)_2 \cdot CH_2Cl_2$, Cs_2CO_3, 105°C, 1 h.

Similar conditions were used to synthesize the dimethyl protected *distal* fleximer xanthosine as Scheme 45 (Scheme 54).

Scheme 54. Negishi coupling of imidazole **45.2** with pyrimidine **47.1**.



45.2 **54.1** **54.2**

Reagents and conditions: a. **47.1**, $Pd(PPh_3)_4$, CuI, THF, 6 h; b. AcOH, rt, 48 h.

All final compounds were send to NCI (NIH) to be tested against HIV-1 by Dr. Eric Freed. Disappointingly, none showed activity. In lieu of the lack of activity towards HIV-1, the bases synthesized have been repurposed for transglycosylation (to be discussed in Chapter 5).

Conclusions

The original aim of this project entailed the synthesis of fleximer bases along with their geminal pyrimidine counterparts *via* palladium-catalyzed cross-coupling for the purpose of finding small molecules that could potentially inhibit the

interaction between HIV-1 nucleocapsid NCp7 and viral nucleic acids, and as a result, inhibit HIV-1 replication. Several compounds were realized through the desired cross-coupling methodologies, although through routes that were not initially planned. Unfortunately, the hypothesis that these molecules may interact with NCp7 was not proven through preliminary ^{1}H NMR studies. In addition, none of the compounds showed antiviral activity towards HIV-1. It is possible that the concentration of protein utilized in the NMR studies were not high enough to observe the desired ineractions and future studies could potentially provide more information.

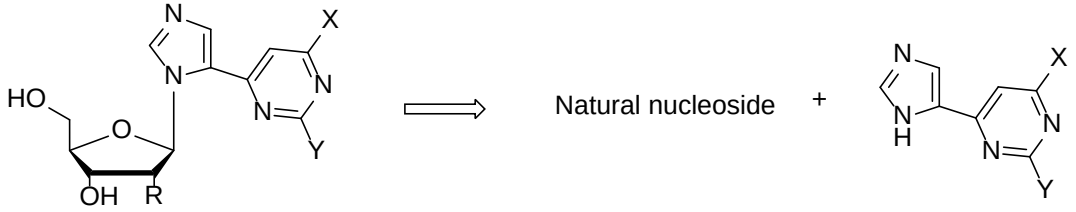
While the biological results were disappointing, this project has expanded our group's understanding in palladium-catalyzed cross-coupling strategies in terms of choosing the optimum cross-coupling partners, which has proven advantageous for other ongoing projects.

Furthermore, although the fleximer bases were ineffective for their original purpose, they are now being tested for their ability to act as a substrate for enzymatic transglycosylation by our collaborator Dr. Sylvie Pochet. The significance of this new project will be discussed in the next chapter.

Chapter 5: Transglycosylation

Summary

Synthesis of *distal* fleximers has thus far been tedious and low yielding. The protection of the furanose sugar functional groups and subsequent construction of the tricyclic base to achieve the final *distal* fleximer nucleosides is inefficient when compared to the palladium catalyzed cross-coupling methodologies employed to produce the *proximal* fleximer nucleosides. Previous efforts at coupling the tricyclic base to the sugar have yielded only the undesired N7 connected isomer, and because the *distal* fleximer base has not been previously synthesized, there have been no attempts at coupling a *distal* fleximer base to a ribofuranose sugar. As mentioned in Chapter 1, the laboratory of Dr. Sylvie Pochet has been able to couple a series of *proximal* fleximer bases to both ribose and 2'-deoxyribose sugars using the *N*-deoxyribosyltransferase II of *L. leichmanii (Ll*-NDT*)* and *E. coli* purine nucleoside phosphorylase (PNP). In a collaborative effort, Dr. Pochet is performing the same transglycosylation experiments with fleximer and tricyclic nucleobases synthesized in Chapter 4 to determine whether the *distal* fleximer nucleosides can be obtained in this fashion thus potentially shortening the route to these products.



R = OH for ribonucleoside
R = H for 2'-deoxyribonucleoside

Specific Aims

***Specific Aim 1*:** A series of *distal* and *proximal* fleximer bases were synthesized *via* palladium-catalyzed cross-coupling.

***Specific Aim 2*:** Dr. Sylvie Pochet (Pasteur Institute) will test the compounds from Aim 1 for their ability to act as substrates for enzymatic transglycosylation using *Ll*-NDT and PNP as catalysts.

***Specific Aim 3*:** Dr. Sylvie Pochet will determine the optimum conditions to achieve the nucleoside isomers in the desired conformation.

Background and Significance

As indicated through previous chapters, synthesis of nucleosides analogues has been classically achieved through chemical methodologies.[306, 307] However, as can be seen in Chapter 2 and 3, chemical synthesis typically involves difficult and time-consuming multistep processes. Suitable protection of various functional groups is typically required on the nucleos(t)ide sugar and/or on the heterocyclic base moieties, and subsequent deprotection steps often results in overall lower yields. Another significant problem, faced in Chapter 3, is the stereospecific control of configuration at the anomeric center (α versus β nucleos(t)ide). Enzymatic syntheses of nucleoside analogues, on the other hand, do not usually require protecting groups and are highly stereospecific. Nucleoside phosphorylases (NP's) and *N*-deoxyribosyltransferases (NDT's) have been the predominant classes of enzymes used in the synthesis of nucleosides by mediating the transglycosylation reaction of a nucleoside sugar to a free heterocyclic base.[308-335]

I. Nucleoside Phosphorylase

NP's catalyze the reversible 1'-monophosphorylation of ribo- or deoxyribonucleosides, leading to the corresponding α-D-ribose- or α-D-deoxyribose-1-phosphate (R-1-P) and the release of the nucleobase (Figure 50).[309] There exists both pyrimidine and purine NP's. The presence of another nucleobase results in the formation of a new nucleoside product.

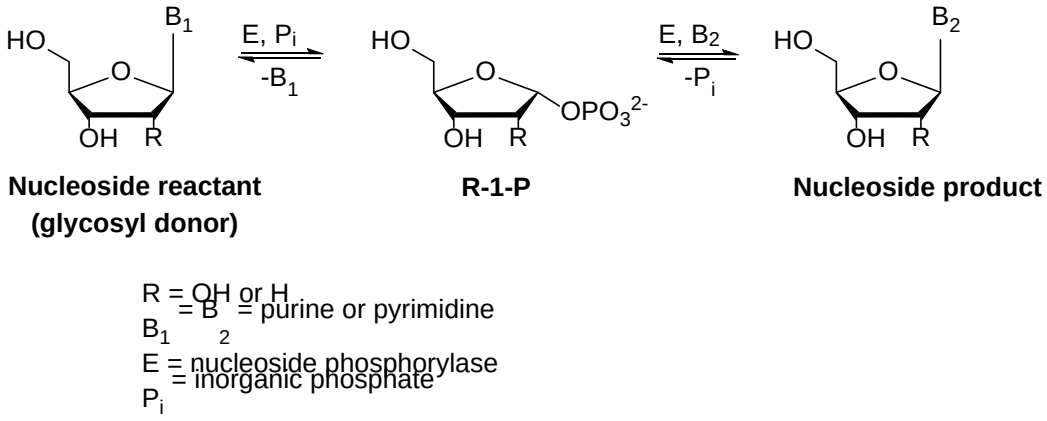


Figure 50. Synthesis of a nucleoside analogue using nucleoside phosphorylase in the transglycosylation reaction.[309]

Transglycosylation can be accomplished using isolated enzymes[310] or whole cells that can be modified to overexpress the necessary enzymes.[311-317] Conventionally, when performing a one pot synthesis with all reactants present (enzyme, inorganic phosphate and heterobase B_2), only a catalytic amount of phosphate is required to drive the reaction towards product, however, a mixture of starting and product nucleosides can be generated as the reaction is equilibrium driven (Figure 50).[318]

To prevent resynthesizing the nucleoside reactant, R-1-P could first be isolated such that only the nucleoside product would be generated, instead of a mixture of both.[319, 320] In addition, using *N*-7 alkylated purine nucleosides as the glycosyl donor is highly advantageous whether to produce a purine or pyrimidine nucleoside product as the liberated *N*-7-alkyl purines are insoluble in the buffered solution, thus driving the equilibrium to the right.[321, 322]

Another strategy is to use a coupled enzymatic system with both pyrimidine and purine NP's (Figure 51). With this system, if the aim is to synthesize a purine nucleoside, using a pyrimidine nucleoside as the reactant would prevent competitive inhibition of the purine NP as the pyrimidine NP would catalyze the phosphorylation step and the purine NP would subsequently couple the purine base to R-1-P.[318, 323-325]

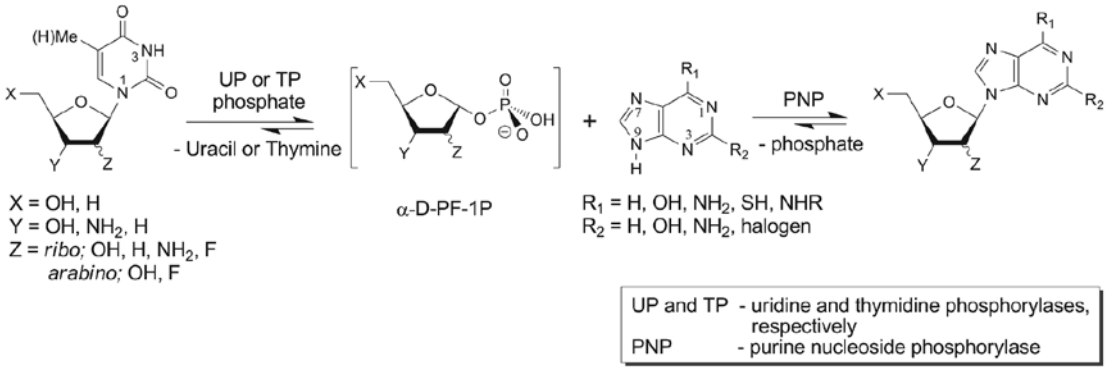


Figure 51. Transglycosylation with a coupled enzyme system.[326, 327]

II. <u>*N*-deoxyribosyltransferase</u>

NDT's catalyze the transfer of 2-deoxyribose between two nucleobases (Figure 52). There are two types of NDT's: type I catalyzes the switch between two purine bases and type II catalyze the switch between pyrimidine or purine bases.[328,

[329] Although NDT's can perform essentially the same reaction as NP's, they are more regioselective (preference towards *N*-1 glycosylation in pyrimidines and *N*-9 in purines), and more stereoselective (only β-anomers products).[330-335] Interestingly, compared to NP's, NDT's have a lower tolerance towards nucleosides with modified sugars, but they can couple a wider range of modified nucleobases, including expanded size purines.[330-335]

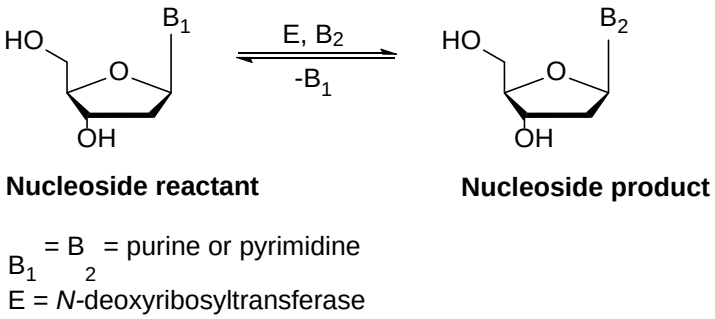


Figure 52. Transglycosylation reaction using *N*-deoxyribosyltransferase.[309]

NP's and NDT's can be used to transglycosylate a wide array of natural and unnatural furanose sugars and nucleobases. Modified bases can be coupled to ribose and/or deoxyribose sugars by using the corresponding natural nucleosides as glycosyl donors. To obtain nucleoside analogues with modified sugars, the glycosyl donor would have a natural nucleobase coupled to the modified sugar and, through transglycosylation, the nucleobase (whether modified or naturally occuring) would be subsequently coupled to the unnatural sugar once the R-1-P analogue is established. We intend to take advantage of these options, and determine the ability of the fleximer bases to be transglycosylated with 1) natural nucleosides and 2) sugar modified nucleosides.

Fleximer Transglycosylation

As mentioned in Chapter 1, the Pochet group has successfully synthesized a series of imidazole nucleosides *via* transglycosylation methodologies. Of these, four products resemble the Seley-Radtke *proximal* fleximers (Scheme 24 and Figure 53).

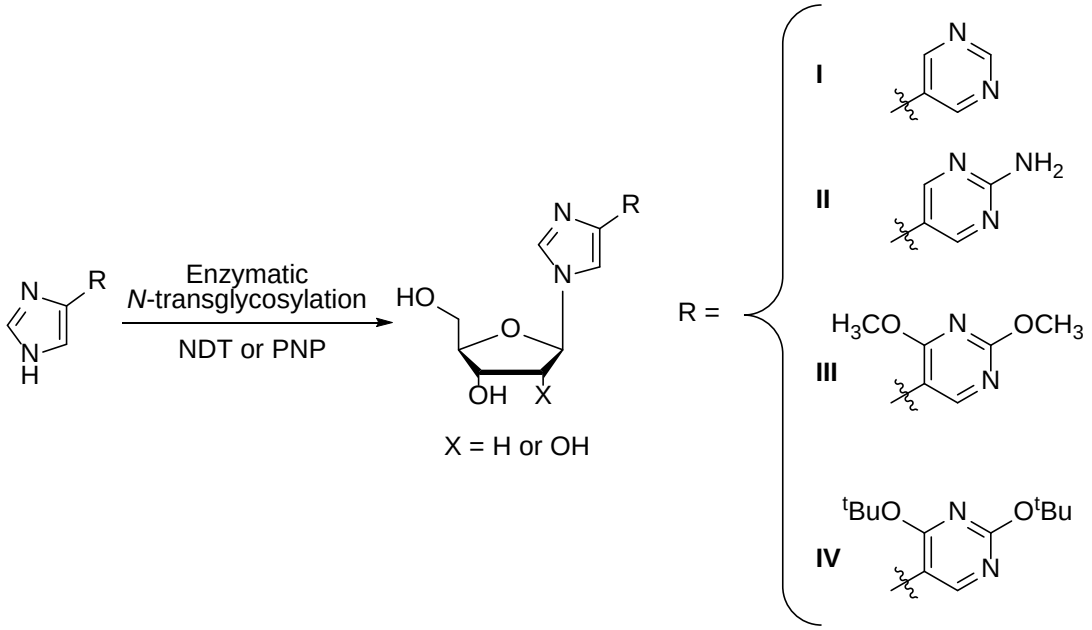


Figure 53. Enzymatic transglycosylation of *proximal* fleximers.[182-184]

The fleximer bases were synthesized through microwave-assisted Suzuki-Miyaura cross-coupling of 4(5)-iodoimidazole and the boronic acid pyrimidine partners (Figure 53).[184] Subsequent transglycosylation using *E. coli* PNP for ribonucleosides and *Ll*-NDT for 2'-deoxyribonucleosides produced the corresponding *proximal* fleximer nucleosides as major products. Interestingly, NDT also provided unexpected byproducts (Figure 54 and Figure 55).[182, 183]

Figure 54 shows the products from transglycosylation of Figure 53 **III** and thymidine using NDT at t = 0, 2 and 16 h.[182] While the major product was the *proximal* fleximer nucleoside of **III** at 16 h (Figure 54, panel C, **b**), its isomer (Figure

54, **c**) is present almost in equimolar amount at t = 2 h. Vichier-Guerre *et al.* hypothesized that these findings indicate that the *proximal* fleximer is the thermodynamic product whereas the isomer is the kinetic product, however, this hypoethesis has yet to be proven by experiments. Furthermore, glycosylation at multiple sites of a modified purine is not a unique phenomenon,[217, 336] and does occur for **IV**.[182]

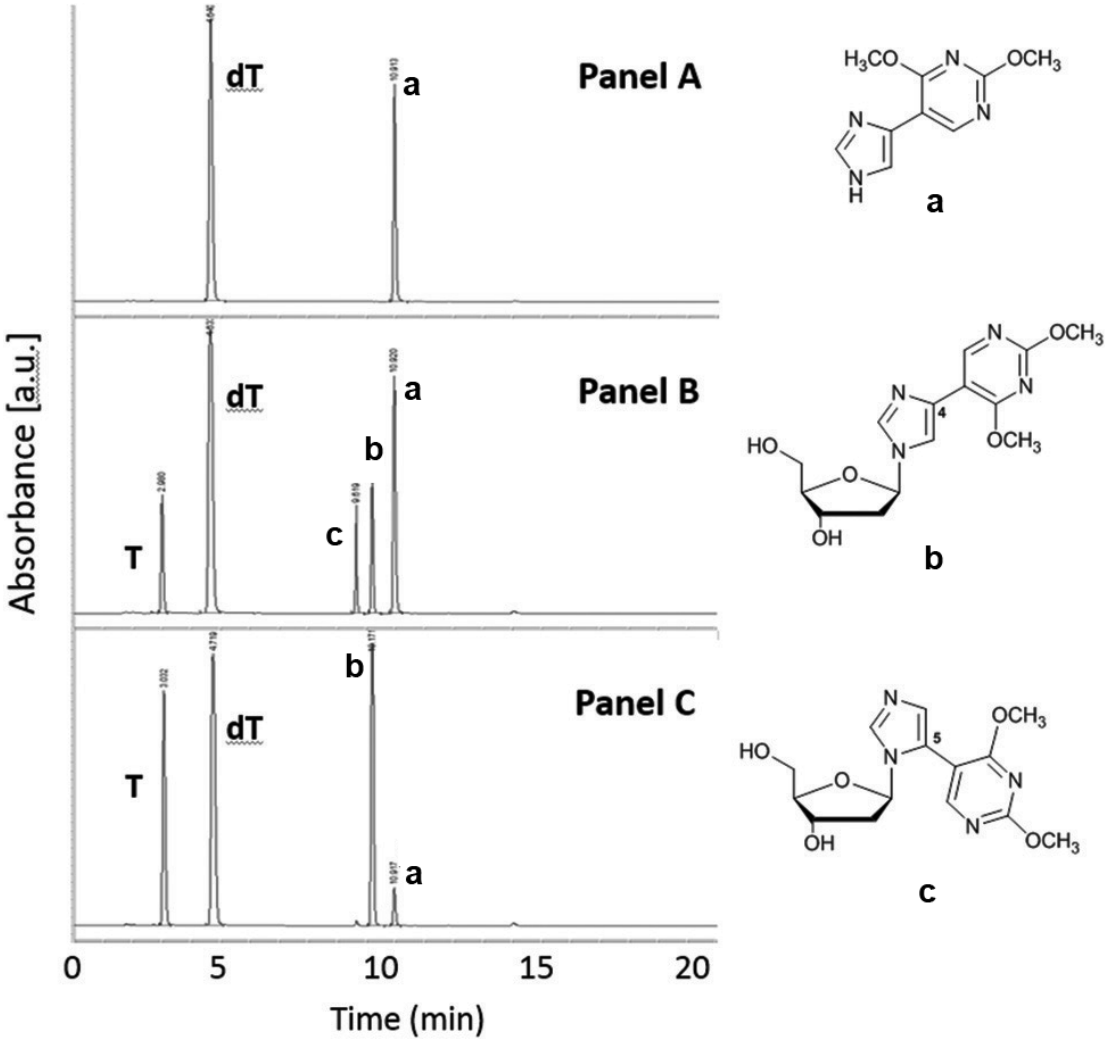


Figure 54. RP-HPLC analysis of the crude reaction mixture of **a** and 2'-deoxythymidine (dT) in the presence of NDT at t = 0 (panel A), after 2 h (panel B), and after 16 h incubation (panel

C). Conditions: 5-60% linear gradient of CH_3CN in 10 mM TEAA buffer pH 6.0 over 20 min at a flow rate of 1 mL/min. Detections at 254 nm.[182]

The other side reaction witnessed in this study was the emergence of a double-glycosylated product (Figure 55, **d**).[182] Since the removal of the *t*-Bu group on the pyrimidine allows for tautomerization, subsequent glycosylation of the pyrimidine at the "*N-1*" was possible (confirmed through HMBC experiments).

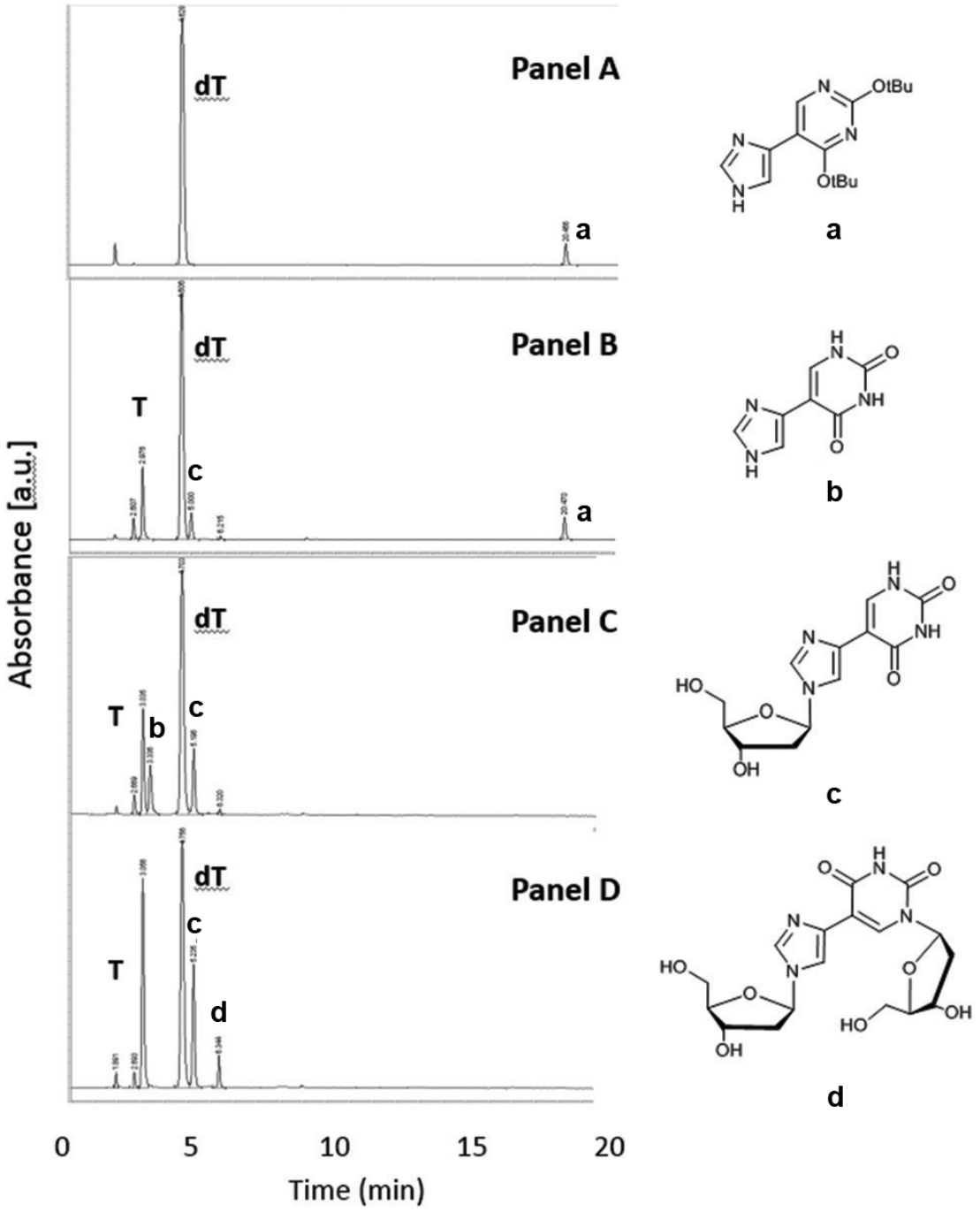


Figure 55. RP-HPLC analysis of the crude reaction mixture of **a** and thymidine in the presence of NDT at t = 0 (panel A), after 2 h (panel B), after 16 h incubation (panel C) and after 2 days (panel D). Conditions: 5-60% linear gradient of CH_3CN in 10 mM TEAA buffer pH 6.0 over 20 min at a flow rate of 1 mL/min. Detections at 254 nm.[182]

These results, while unexpected, are highly advantageous as they demonstrate that transglycosylation using NDT can provide a more diverse set of products that may be explored for their biological, and potentially, therapeutic properties.

In addition to Dr. Pochet's group's efforts, Dr. Elena Matyugina from the Engelhardt Institute of Molecular Biology (Moscow, Russia), who is also a former Seley-Radtke graduate student, achieved the fleximer nucleosides shown in Figure 56 through transglycosylation (personal communication). The conversion to nucleoside **A** was about 50%, however, the product was not isolated. The conversion to nucleoside **B** was more successful, with a yield exceeding 80%.

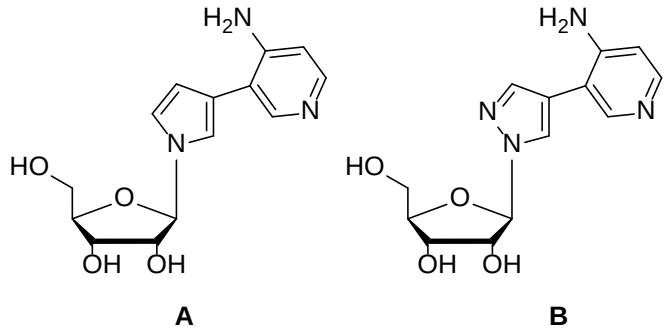


Figure 56. Dr. Matyugina's fleximer nucleosides synthesized through enzymatic transglycosylation.(personal communication)

Studies Currently Underway

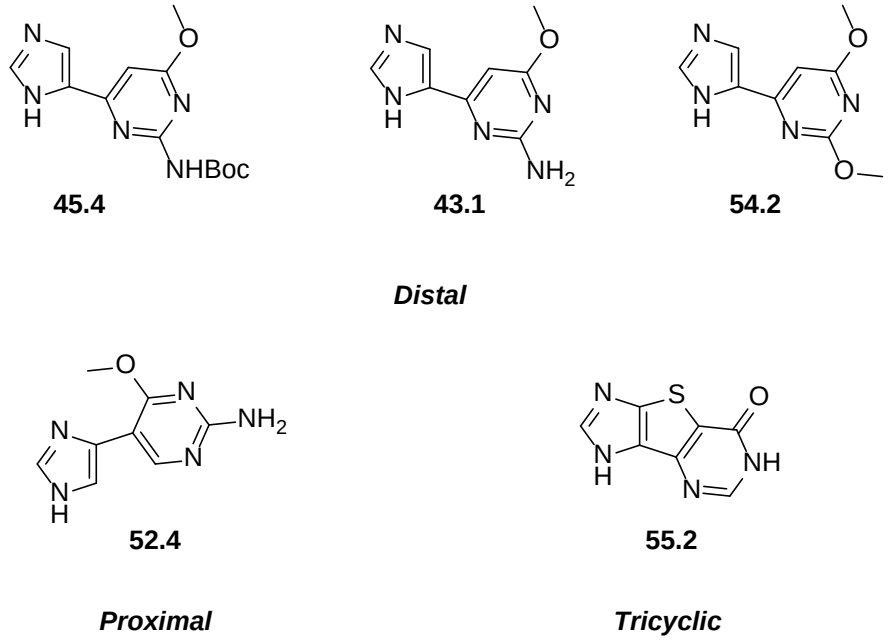


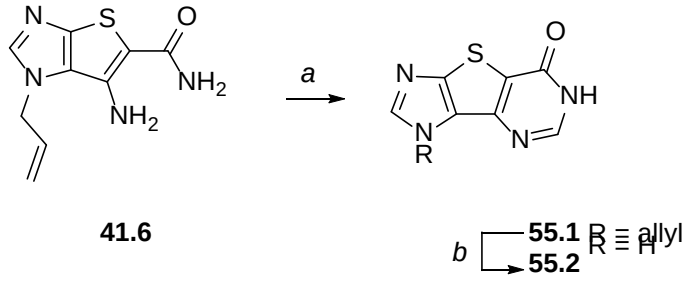
Figure 57. Modified purine bases for transglycosylation

The fleximer and tricyclic bases from Figure 57 have been sent to Dr. Pochet for transglycosylation experiments with both *Ll*-NDT and *E. coli* PNP. It is hoped that the results will identify the thermodynamic product, and potentially any kinetic products, for the *distal* fleximers as **54.2** is an isomer of **a** in Figure 54. As unexpected glycosylation of the exocyclic amines did not occur previously,[182] it is unlikely to occur with these bases, however, the Boc protected intermediate **45.4** was provided as a precaution.

The tricyclic inosine was also provided as previous attempts at coupling this base to β-D-ribofuranose-1,2,3,5-tetraacetate (mentioned in Chapter 2) through Vorbrüggen coupling methodologies yielded only the undesired N-7 coupled

product.[123, 139] A comparison between chemical and enzymatic glycosylation reactions and rates could thus be established through this study.

Scheme 55. Synthesis of tricyclic inosine base.



Reagents and conditions: a. triethyl orthoformate, Ac_2O, 135°C, 3 h; b. phenylsilane, $Pd(PPh_3)_4$, AcOH/CH_2Cl_2 (1:1), 40°C, 48 h.[139]

Expected Outcomes and Future Direction

The desired outcome for this collaboration is to establish a facile method to synthesize the *distal* fleximer nucleosides and bypass the traditional and tedious tricyclic nucleoside route. A realistic expectation is that an isomeric mixture will be observed where substitution at the *N-1* and *N-3* of the imidazole (Figure 58, **A** and **B**) could occur, with product **B** being the major product. Only one product is predicted to be produced for the *proximal* fleximer base **52.4** (Figure 58, **C**) as this configuration has been determined to be thermodynamically favored.

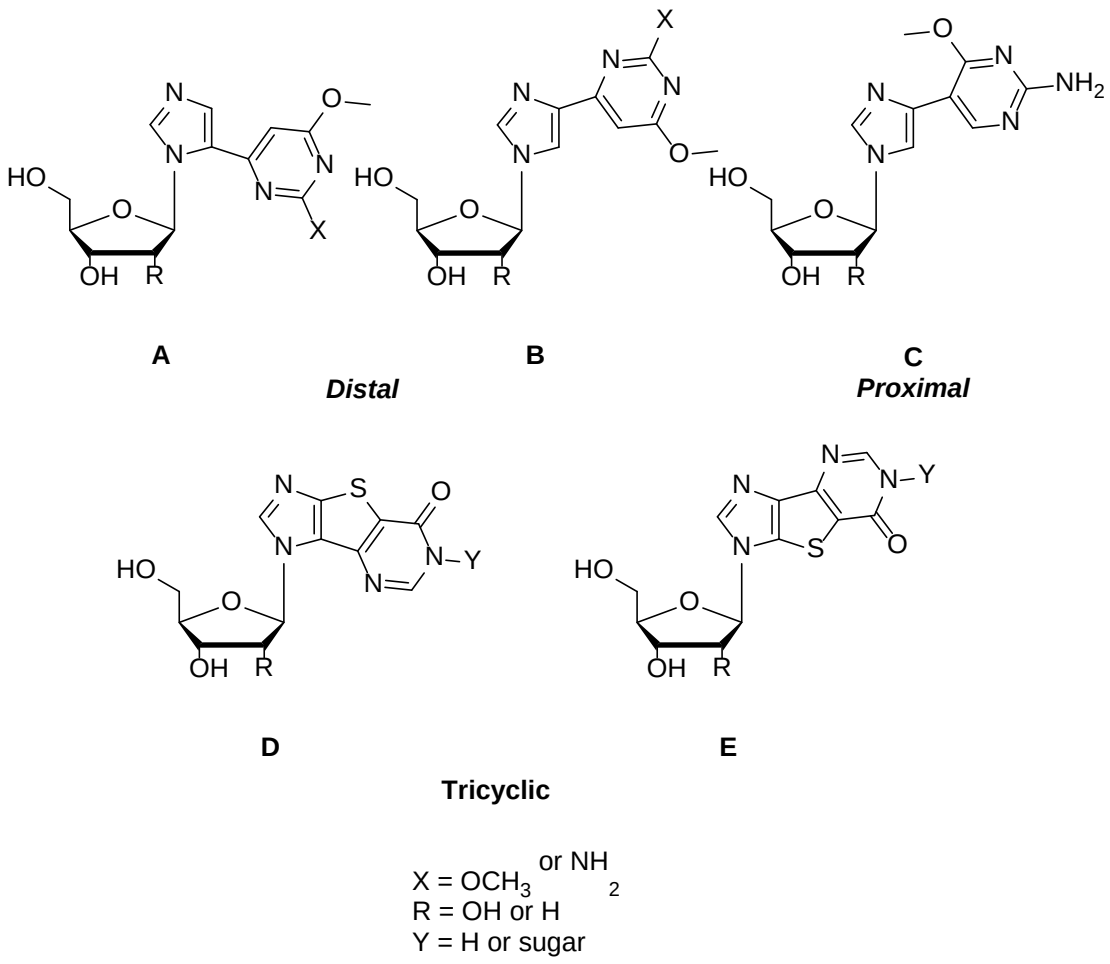


Figure 58. Potential transglycosylation products.

To prevent double-glycosylation on the nucleobase, the methyl protecting group on the exocyclic oxygen was not removed for the fleximer base targets, however, the tricyclic inosine (**55.2**) does not have this protection, thus there is a possibility that double-*N*-glycosylation could occur. Furthermore, as no information on tricyclic base transglycosylation has been gathered, both isomers (Figure 58, **D** and **E**) could theoretically be synthesized.[139]

The results from these studies will be published and future studies could be planned based on the outcome.

Chapter 6: Experimental Procedures

All chemicals were obtained from commercial sources and used without further purification unless otherwise noted. Anhydrous DMF, CH_3OH, DMSO and EtOH were purchased from Fisher Scientific. Anhydrous THF, acetone, CH_2Cl_2, CH_3CN, and ether were obtained using a solvent purification system (mBraun Labmaster 130). NMR solvents were purchased from Cambridge Isotope Laboratories (Andover, MA). All ^{1}H, ^{13}C, ^{19}F and ^{31}P NMR spectra were obtained either on a JEOL ECX 400 MHz NMR, operated at 400 and 100 MHz, respectively, or a Bruker AVANCE III HD 500 MHz NMR, operated at 500 and 125 MHz, respectively, and referenced to internal tetramethylsilane (TMS) at 0.0 ppm. The spin multiplicities are indicated by the symbols s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), m (multiplet), and br (broad). Reactions were monitored by thin-layer chromatography (TLC) using 0.25 mm Whatman Diamond silica gel 60-F_{254} pre-coated plates. Purification was performed on a Teledyne Isco CombiFlash Rf 200, and eluted with the indicated solvent system. Yields refer to chromatographically and spectroscopically (^{1}H, ^{13}C, ^{19}F and ^{31}P NMR) homogeneous materials. Mass Spectra were recorded at the UMBC MCAC for nominal using Bruker APOLLO™ II ESI/APCI - MALDI Dual Source for apex(R)-Qe FTMS or Johns Hopkins Mass Spectrometry Facility for high resolution using VG Analytical VG-70SE Magnetic Sector Mass Spectrometer.

Synthesis of 3,5-dibenzoyloxy-2-deoxy-2-fluoro-2-C-methyl-D-ribono-γ-lactone (32.1a).[161]

To a stirred solution of diol **31.7a** (31.5 g, 0.192 mol) in anhydrous anhydrous Py (400 mL) at 0°C under nitrogen, was added BzCl (108 g, 0.768 mol) slowly. The resulting mixture was allowed to warm to room temperature and was stirred for 30 min. Then cold water (120 mL) was added and the mixture was stirred for 5 min to form a suspension. The precipitated product was collected by filtration. The filter cake was suspended in cold water (400 mL), and the solid was collected. This was repeated three times followed by Combi-Flash chromatography (PE/EtOAc = 100:1-1:2) to give a white solid (57.2 g, 80%). R_f = 0.50, (PE/EtOAc = 4:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.66 (d, 3H, J = 24.3 Hz), 4.60-4.72 (m, 2H), 5.10-5.14 (m, 1H), 5.74 (dd, 1H, J = 6.8, 18.3 Hz), 7.43-7.56 (m, 4H), 7.60-7.71 (m, 2H), 7.91-7.93 (m, 2H), 7.99-8.01 (m, 2H). Agrees with literature.[164]

Synthesis of 3,5-dibenzoyloxy-2-deoxy-2-fluoro-2-C-methyl-D-ribono-γ-lactol (32.2a).[161]

To a stirred solution of protected lactone **32.1a** (54.0 g, 0.145 mol) in anhydrous THF (600 mL) was added lithium tri-*tert*-butoxyaluminum hydride (42.5 g, 0.167 mol) in several batches at -20°C under nitrogen, then slowly warmed to room temperature. After 6 h, the reaction was complete based on TLC analysis. Cold saturated NH_4Cl was added slowly to quench the reaction. The solvent was removed, and the residue was purified by silica gel column chromatography to give compound **32.2a** (46.5 g,

85%) as a white solid. R_f = 0.45, (PE/EtOAc = 4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.52-1.60 (m, 3H), 3.56-3.58 (d, 1H), 4.43-4.69 (m, 3H), 5.27-5.35 (m, 1H), 5.37-5.68 (m, 1H), 7.33-7.62 (m, 6H), 7.97-8.01 (m, 4H).

Synthesis of 2'-deoxy-2'-fluoro-2'-C-methyl-1'-(4,5-diiodoimidazol-3-yl]-α/β-D-ribofuranose (32.4a).[161]

To a stirred solution of PPh_3 (40.0 g, 0.153 mol), 4,5-diiodoimidazole (45.1 g, 0.141 mol) and compound **32.2a** (44.0 g, 0.118 mol) in anhydrous THF (4.0 L) at 0°C, was added DIAD (30.9 g, 0.153 mol) dropwise. The resulting mixture was stirred at this temperature for 30 min, and then the reaction mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 3:1) to give mixture product **32.3a** (53.0 g) as off-white crude foam. The above mixture **32.3a** (53.0 g, 78.4 mmol) was dissolved in reagent methanol (1.2 L) and $NaOCH_3$ (8.70 g, 0.161 mol) was added in 5 batches at 0°C for 15 min. The resulting solution was stirred at this temperature for 45 min and then acetic acid was added to neutralize the solution. The mixture was concentrated under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 50:1-10:1) twice to give a mixture of two isomers **32.4a** (α:β = 2.2:1); α isomer (20.1 g, 37%) as an off-white foam. R_f = 0.24, (CH_2Cl_2/CH_3OH = 20:1). ^{1}H NMR (400 MHz, CD_3OD) δ 1.47 (d, 3H, J = 22.0 Hz), 3.71 (dd, 1H, J = 3.6, 12.4 Hz), 3.89 (dd, 1H, J = 1.8, 12.4 Hz), 4.14-4.28 (m, 2H), 5.93 (d, 1H, J = 19.2 Hz), 7.94 (d, 1H, J = 3.7 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 20.5 (d, J = 24.8 Hz) 64.2, 76.8 (d, J = 18.1 Hz), 87.0, 95.8, 98.5,

101.7 (d, J = 189.7 Hz), 102.6, 146.2; β isomer (9.1 g, 17%) as an off-white solid. R_f = 0.25, (CH_2Cl_2/CH_3OH = 20:1). ^{1}H NMR (400 MHz, CD_3OD) δ 1.15 (d, 3H, J = 22.0 Hz), 3.79 (dd, 1H, J = 1.8, 12.4 Hz), 3.96-4.03 (m, 2H), 4.13 (dd, 1H, J = 9.2, 23.8 Hz), 5.90 (d, 1H, J = 17.0 Hz), 8.38 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 16.2 (d, J = 24.8 Hz), 58.6, 70.4 (d, J = 17.2 Hz), 82.0, 93.7, 94.1, 95.3, 100.7 (d, J = 181.2 Hz), 140.7.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-(4,5-diiodoimidazol -3-yl]-β-D-ribofuranose (32.5a).[161]

To a solution of **32.4a** (2.50 g, 5.34 mmol) in anhydrous DMF (60 mL) at 0°C was added a suspension of NaH (95%, 322 mg, 13.4 mmol) in anhydrous DMF (20 mL) dropwise over a period of 10 min. The resulting mixture was stirred at 0°C for 2 h, and then BnBr (2.5 mL, 21.3 mmol) and TBAI (197 mg, 0.533 mmol) were added slowly. The mixture was stirred at room temperature for an additional 4 h. The reaction mixture was quenched by cold water and neutralized by AcOH at 0°C. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 5:1) to give the product (2.82 g, 81%) as an off-white syrup. R_f = 0.50, (PE/EtOAc = 5:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.12 (d, 3H, J = 22.2 Hz), 3.63 (dd, 1H, J = 2.0, 13.4 Hz), 3.93 (dd, 1H, J = 1.8, 11.4 Hz), 4.13-4.26 (m, 2H), 4.52-4.66 (m, 4H), 5.86 (d, 1H, J = 16.4 Hz), 7.29-7.42 (m, 10H), 8.25 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.9 (d, J = 24.9 Hz), 66.8, 73.6, 74.3, 76.9, 79.7, 80.7, 93.7 (d, J = 38.1 Hz), 97.3, 101.7 (d, J = 184.0 Hz), 127.8, 128.3

(m), 137.2, 140.5; HRMS (FAB) calculated for $C_{23}H_{23}FI_2N_2O_3$ [M + H^+] 648.9860; Found, 648.9863.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2',2'-difluoro-1'-(4,5-diiodoimidazol-3-yl]-α/β-D-ribofuranose (32.5b).[162]

To a solution of **32.4b** (2.2 g, 4.7 mmol) in anhydrous DMF (40 mL) at 0°C was added a suspension of NaH (95%, 268 mg, 11.2 mmol) in anhydrous DMF (20 mL) dropwise over a period of 10 min. The resulting mixture was stirred at 0°C for 2 h, and then BnBr (1.9 mL, 16.3 mmol) was added slowly. The mixture was stirred at room temperature for an additional 4 h. The reaction mixture was quenched by cold water and neutralized by AcOH at 0°C. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 20:1 - 2:1) to give **32.5b** (2.5 g, 82%) as off-white syrup, β:α = 1:2.6; β isomer: R_f = 0.25, (PE/EtOAc = 4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.57-3.60 (m, 1H), 3.76-3.79 (m, 1H), 4.13-4.15 (m, 1H), 4.28-4.35 (m, 1H), 4.47-4.56 (m, 3H), 4.81 (d, 1H, *J* = 11.5 Hz), 5.83 (dd, 1H, *J* = 3.6, 9.6 Hz), 7.24-7.37 (m, 10H), 8.04 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 66.7, 73.3, 73.6, 79.3, 88.9, 97.3, 122.2, 128.6 (m), 136.3, 137.1, 140.8; ^{19}F NMR (376 MHz, $CDCl_3$) δ -106.6, -121.0; HRMS (FAB) calculated for $C_{22}H_{20}F_2I_2N_2O_3$ [M + H^+] 652.96096; Found, 652.96065. α isomer: R_f = 0.24, (PE/EtOAc = 4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.57-3.68 (m, 2H), 4.36-4.41 (m, 2H), 4.49-4.59 (m, 3H), 4.84 (d, 1H, *J* = 11.4Hz), 6.00 (t, 1H, *J* = 6.4 Hz), 7.26-7.39 (m, 10H), 7.92 (d, 1H, *J* = 2.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ

68.4, 73.5, 73.7, 82.3, 89.2, 97.2, 122.5, 128.6 (m), 136.2, 137.4, 141.1; ^{19}F NMR (376 MHz, $CDCl_3$) δ -106.6, -121.0; MS (ESI, pos, CH_3OH), m/z: 653.0 ($M + H^+$).

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-iodo-4-carbaldehyde)-imidazole-3-yl]-β-D-ribofuranose (32.6a).[161]

Compound **32.5a** (1.60 g, 2.47 mmol) was dissolved in anhydrous THF (50 mL) at 0°C, and then EtMgBr (1M, 2.7 mL, 2.72 mmol) was added dropwise under nitrogen. The reaction mixture was stirred at 0°C for 30 min. Anhydrous DMF (2 mL) was added and the reaction mixture was stirred overnight, quenched with water. The solvent was removed, and the residue was purified by silica gel column chromatography (PE/EtOAc = 2:1) to give (1.10 g, 81%) a colorless syrup. R_f = 0.45, (PE/EtOAc = 5:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.06 (d, 3H, *J* = 22.0 Hz), 3.59 (dd, 1H, *J* = 1.8, 13.3 Hz), 3.93 (dd, 1H, *J* = 1.8, 13.3 Hz), 4.06-4.41 (m, 2H), 4.47-4.61 (m, 4H), 6.66 (d, 1H, *J* = 15.6 Hz), 7.29-7.43 (m, 10H), 8.62 (s, 1H), 9.61 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 16.6 (d, *J* = 24.9 Hz), 66.8, 73.6, 74.3, 79.7, 89.7 (d, *J* = 39.3 Hz), 99.4, 101.7 (d, *J* = 93.9 Hz), 126.7, 127.8, 128.3 (m), 137.1 (d, *J* = 17.2 Hz), 143.1, 181.3; MS (ESI, pos, CH_3OH), m/z: 551.1 [$M + H^+$].

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2',2'-difluoro-1'-[(5-iodo-4-carbaldehyde) imidazole-3-yl]-β-D-ribofuranose (32.6b).[162]

Compound **32.5b** (715 mg, 1.1 mmol) was dissolved in anhydrous THF (20 mL), and then EtMgBr (1M, 1.2 mL, 1.2 mmol) was added dropwise under nitrogen. The reaction mixture was stirred at 0°C for 30 min. Anhydrous DMF (1.5 mL) was added

and the reaction mixture was stirred overnight, quenched with water. The solvent was removed, and the residue was purified by silica gel column chromatography (PE/EtOAc = 10:1-2:1) to give **32.6b** (432 mg, 72%) as colorless syrup. R_f = 0.45, (PE/EtOAc = 5:1).

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-iodo-4-carbonitrile)-imidazole-3-yl]-β-D-ribofuranose (32.8a).[161]

To a stirred solution of **32.6a** (1.10 g, 2.00 mmol) in anhydrous EtOH (30 mL) was added hydroxylamine hydrochloride (556 mg, 8.00 mmol) and $NaHCO_3$ (672 mg, 8.00 mmol). The resulting mixture was refluxed for 5 h. The solvent was removed under reduced pressure, and the crude product was extracted in EtOAc (2 x 25 mL) and excess water. The combined organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure to give the crude oxime intermediate **32.7a** as syrup (1.20 g), which was then used for the next step without further purification. Oxime intermediate **32.7a** (1.20 g) was dissolved in anhydrous THF (25 mL) and CDI (973 mg, 6.0 mmol) was added to the mixture. The resulting solution was stirred at reflux for 8 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 8:1) to provide the cyano nucleoside as a light yellow syrup (820 mg, 75% in 2 steps). R_f = 0.75, (PE/EtOAc = 3:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15 (d, 3H, *J*= 22.0 Hz), 3.60 (dd, 1H *J*= 2.3, 11.5 Hz), 3.91 (dd, 1H, *J* = 1.8, 11.4 Hz), 4.07-4.15 (m, 1H), 4.26-4.28 (m, 1H), 4.47-4.63 (m, 4H), 5.83 (d, 1H, *J* = 14.7 Hz), 7.21-7.39 (m, 10H), 8.25 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.0 (d, *J* = 24.8 Hz), 66.8, 73.7, 74.3, 80.5, 91.2 (d, *J*

= 38.1 Hz), 96.5, 100.0 (d, *J* = 189.9 Hz), 110.6, 128.3, 128.4, 128.5, 128.6, 128.8, 128.9, 136.9, 140.7; HRMS [FAB] calculated for $C_{24}H_{23}FIN_3O_3$ [M + H^+] 548.0846; Found, 548.0850.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2',2'-difluoro-1'-[(5-iodo-4-carbonitrile) imidazole-3-yl]-β-D-ribofuranose (32.8b).[162]

To a stirred solution of **32.6b** (800 mg, 1.4 mmol) in anhydrous EtOH (30 mL) was added hydroxylamine hydrochloride (400 mg, 5.7 mmol) and $NaHCO_3$ (480 mg, 5.7 mmol). The resulting mixture was refluxed for 4 h. The solvent was removed under reduced pressure, and the crude product was extracted in EtOAc (2 x 25 mL) and excess water. The combined organic layer was dried over sodium sulfate, filtered, and concentrated under reduced pressure to give the crude oxime intermediate **32.7b** as syrup (920 mg), which was then used for the next step without further purification. Oxime intermediate **32.7b** (920 mg) was dissolved in anhydrous THF (30 mL) and CDI (1.3 g, 8.0 mmol) was added to the mixture. The resulting solution was stirred at reflux for 6 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 8:1) to provide the cyano nucleoside **32.8b** as a light yellow syrup (362 mg, 45% in 2 steps). R_f = 0.40, (PE/EtOAc = 3:1).

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-carboxamide)[2, 3-d]imidazole-3-yl]-β-D-ribofuranose (32.10a).[161]

To a solution of **32.8a** (3.00 g, 5.48 mmol) in anhydrous DMF (600 mL), was added mercaptoacetamide (3.00 g, 32.9 mmol) and K_2CO_3 (3.80 g, 27.5 mmol). The resulting mixture was heated to 65°C for 24 h under nitrogen. The crude mixture was filtered over a pad of Celite and the solid was washed with DMF. The solvent was removed under reduced pressure to give crude mixture, which was then refluxed in a NaOEt (21%, 12 mL and anhydrous EtOH (200 mL) mixture for 2 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 50:1-10:1) to afford the product (1.46 g, 52%) as a yellow foam. R_f = 0.50, (CH_2Cl_2/CH_3OH = 15:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.15 (d, 3H, *J* = 22.9 Hz), 3.63 (d, 1H, *J* = 11 Hz), 3.90 (d, 1H, *J* = 11.0 Hz), 4.09-4.12 (m, 2H), 4.43-4.58 (m, 3H), 4.76 (d, 1H, *J* = 11.9 Hz), 5.26 (br, 2H), 5.89 (d, 1H, *J* = 18.3 Hz), 6.45 (br, 2H), 7.25-7.41 (m, 10H), 7.84 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 19.6 (d, *J* = 25.7 Hz), 66.1, 73.7, 77.9(d, *J* = 15.3 Hz), 79.8, 93.0 (d, *J* = 40.0 Hz), 97.9 (d, *J* = 189.7 Hz), 99.0, 128.2, 128.3, 128.5 (m), 128.7, 128.9, 136.6, 137.1, 139.4, 142.8, 146.0, 168.2; HRMS (FAB) calculated for $C_{26}H_{27}FN_4O_4S$ [M + H^+] 511.1815; Found, 511.1796.

Synthesis of 3',5'-dibenzoyloxy-2'-deoxy-2',2'-difluoro-1'-[(5-carboxamide)[2, 3-d]imidazole-3-yl]-β-D-ribofuranose (32.10b).[162]

To a solution of **32.8b** (350 mg, 0.6 mmol) in anhydrous DMF (80 mL), was added mercaptoacetamide (347 mg, 3.8 mmol) and K_2CO_3 (439 mg, 3.2 mmol). The resulting mixture was heated to 65°C for 24 h under nitrogen. The crude mixture was filtered over a pad of Celite and the solid was washed with DMF. The solvent was

removed under reduced pressure to give crude mixture **32.9b**, which was then refluxed in a NaOEt (21%, 0.5 mL) and anhydrous EtOH (40 mL) mixture for 4 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 50:1 - 10:1) to afford compound **32.10b** as a yellow foam (185 mg, 57%). R_f = 0.50, (CH_2Cl_2/CH_3OH = 15:1). ^{1}H NMR (400 MHz, CD_3OD) δ 3.60-3.64 (m, 1H), 3.91-3.93(m, 1H), 4.12-4.14 (m, 1H), 4.36-4.62 (m, 4H), 4.79-4.87 (m, 1H), 6.18 (dd, 1H, *J* = 7.8, 11.4 Hz), 7.22-7.38 (m, 10H), 8.16 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 65.7, 73.0, 73.3, 74.8, 79.3, 86.8, 98.3, 125.4, 127.6, 127.7, 127.9, 128.0, 128.1, 128.2, 128.3, 128.4, 136.8, 139.3, 143.7, 145.4, 169.4; ^{19}F NMR (376 MHz, CD_3OD) δ -111.9, -120.4.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(2-amino-imidazo-[4',5':4,5]-thieno-[3,2-d]-pyrimidin-3-yl-7-one)]-β-D-ribofuranose (32.11a).[161]

To a stirred solution of **32.10a** (1.50 g, 2.94 mmol) in anhydrous CH_3OH (45 mL) was added NaOH (588 mg, 14.7 mmol). The resulting mixture was stirred at room temperature until the mixture was homogeneous. CS_2 (1.34 g, 17.6 mmol) was added, and the resulting mixture was heated in a steel bomb for 18 h at 150°C. The mixture was cooled to 0°C. H_2O_2 (8 mL, 30%) was then added dropwise and allowed to stir at 0 °C for 2 h. The resulting suspension was added to a steel bomb and anhydrous ammonia was bubbled in at -40 °C for 20 min. The bomb was then heated at 130°C for 12 h. The solvent was removed under reduced pressure, and the crude yellow residue was purified by silica gel column chromatography to afford **32.11a** (787 mg,

50%) as a yellow foam. R_f = 0.35, (CH_2Cl_2/CH_3OH = 15:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.20 (d, 3H, *J* = 22.7 Hz, Me), 3.75 (dd, 1 H, *J* = 2.7, 9.2 Hz, H-5), 3.95 (dd, 1 H, *J* = 1.6, 9.3 Hz, H-5'), 4.21 (d, 1H, *J* = 7.4Hz, H-4), 4.47 (dd, 1 H, *J* = 11.9, 19.4 Hz, H-3), 4.58 (dd, 2 H, *J* = 9.5, 25.4 Hz, CH_2), 4.72 (dd, 2 H, *J* = 12.8, 22.1 Hz, CH_2), 6.45 (d, 1H, *J* = 13.4 Hz, H-1), 6.59 (s, 2H, NH_2), 8.43 (s, 1H, Ar), 7.32-7.39 (m, 10H, Ar), 11.07 (s, 1H, NH); HRMS (FAB) calculated for $C_{27}H_{26}FN_5O_4S$ [M + H^+] 536.1768; Found, 536.1767.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2',2'-difluoro-1'-[(2-amino-imidazo-[4',5':4,5]-thieno-[3,2-d]pyrimidin-3-yl-7-one)]-β-D-ribofuranose (32.11b).[162]

To a stirred solution of **32.10b** (135 mg, 0.26 mmol) in anhydrous CH_3OH (12 mL) was added NaOH (52 mg, 1.3 mmol). The resulting mixture was stirred at room temperature until the mixture was homogeneous. CS_2 (120 mg, 1.6 mmol) was added, and the resulting mixture was heated in a steel bomb for 18 h at 150°C. The mixture was cooled to 0°C. H_2O_2 (0.9 mL, 30%) was then added dropwise and allowed to stir at 0°C for 2 h. The resulting suspension was added to a steel bomb and anhydrous ammonia was bubbled in at -40°C for 20 minutes. The bomb was then heated at 130°C for 12 h. The solvent was removed under reduced pressure, and the crude yellow residue was purified by silica gel column chromatography to afford **32.11b** (77mg, 54%) as a yellow foam. HRMS (FAB) calculated for $C_{26}H_{23}F_2N_5O_4S$ [M + H^+] 540.15171; Found, 540.15032.

Synthesis of 2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(2-amino-imidazo-[4',5':4,5]-thieno-[3,2-d]-pyrimidin-3-yl-7-one)]-β-D-ribofuranose (1).[161]

To a solution of **32.11a** (150 mg, 0.280 mmol) in anhydrous CH_2Cl_2 (30 mL) was added EtSH (622 mL, 8.40 mmol) and $BF_3.Et_2O$ (48%, 2.2 mL, 8.40 mmol) at 0°C. The reaction was allowed to proceed for 72 h at room temperature before TLC analysis confirmed complete product formation. The solvent was removed under reduced pressure, and the residue was purified by Combi-flash silica column chromatography ($CHCl_3/CH_3OH$ =100:1-1:1) to afford target nucleoside **1** (80.0 mg, 80%) as white powder. R_f = 0.15, (CH_2Cl_2/CH_3OH = 5:1). ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.11 (d, 3H, J = 22.6 Hz, Me), 3.72 (d, 1H, J = 11.9 Hz, H-5), 3.90-3.96 (m, 2H, H-5' and H-4), 4.20-4.25 (m, 1H, H-3), 5.43 (br, 2H, OH), 6.45 (d, 1H, J = 16.3Hz, H-1), 6.59 (s, 2H, NH_2), 8.62 (s, 1H, Ar), 11.08 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.7 (d, J = 24.9 Hz), 59.3, 70.3 (d, J = 17.2 Hz), 82.6, 90.0 (d, J = 39.3 Hz), 101.7 (d, J = 180.1 Hz), 110.3, 128.0, 143.0, 145.9, 148.4, 155.4, 158.9; HRMS (FAB) calculated for $C_{13}H_{14}FN_5O_4S$ [M + H^+] 356.0829; Found, 356.0826.

Synthesis of 6-(3-{(2*R*,5*R*)-4-(benzyloxy)-5-[(benzyloxy)methyl]-3-fluoro-3-methyltetrahydrofur-2-yl}-3*H*-imidazol-4-yl)-2-amino-3*H*-pyrimidin-4-one (32.12).[161]

To a solution of **32.11a** (360 mg, 0.67 mmol) in CH_3OH (15 mL) and H_2O (15 mL) was added Raney nickel. The resulting mixture reaction was heated to 90°C for 48 h. TLC analysis confirmed complete product formation. The mixture was filtered, the solvent was removed under reduced pressure, and the residue was purified by Combi-

flash silica column chromatography ($CHCl_3/CH_3OH$ =10:1-5:1) to afford **32.12** (204 mg, 60%) as a yellow oil; ^1H NMR (400 MHz, $CDCl_3$) δ 1.10 (d, 3H, *J* = 22.4 Hz), 3.63 (d, 1H, *J* = 10.5 Hz), 3.92 (d, 1H, *J* = 11.0 Hz), 4.11-4.19 (m, 2H), 4.49-4.58 (m, 1H), 4.59-4.68 (m, 3H), 5.85 (br, 2H), 6.07 (s, 1H), 7.13 (d, 1H, *J* = 17.8 Hz), 7.26-7.40 (m, 10H), 7.53 (s, 1H), 8.31 (s, 1H), 11.71 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 17.8 (d, *J* = 24.8 Hz), 67.1, 73.6, 74.3, 76.8, 77.1, 77.5, 79.1, 90.3 (d, *J* = 12.09 Hz), 98.0, 100.2, 102.0, 128.2, 128.2, 128.3, 128.4, 128.6, 128.7, 132.9, 137.4 (d, *J* = 4.76), 139.4, 155.1, 157.5, 165.2; MS (ESI, pos, CH_3OH), m/z: 506.2 ($M + H^+$).

6-{3-[(2*R*,5*R*)-3-Fluoro-4-hydroxy-5-(hydroxymethyl)-3-methyltetrahydrofur-2-yl]-3*H*-imidazol-4-yl}-2-amino-3*H*-pyrimidin-4-one (2).[161]

To a solution of **32.12** (150 mg, 0.297 mmol) in anhydrous CH_2Cl_2 (50 mL) was added EtSH (439 mL, 5.93 mmol) and BF_3•Et_2O (48%, 1.6 mL, 5.93 mmol) at 0°C. The reaction was allowed to proceed for 72 h at room temperature before TLC analysis confirmed complete product formation. The solvent was removed under reduced pressure, and the residue was purified by Combi-flash silica column chromatography ($CHCl_3/CH_3OH$ =100:1-1:1) to afford target nucleoside **2** (49.0 mg, 51%) as white powder; ^1H NMR (400 MHz, DMSO-d_6) δ 1.03 (d, 3H, *J* = 22.4 Hz), 3.72 (d, 1H, *J* = 11.0), 3.94 (d, 2H, *J* = 7.4), 4.03 (dd, 1H, *J* = 9.16, 26.7, 1H) 6.06 (s, 1H), 6.83 (br, 1H), 6.96 (s, 1H), 7.09 (s, 1H), 7.21 (s, 1H), 7.46 (d, *J* = 15.6, 1H), 8.15 (br, 1H), 9.31 (br, 1H), 11.08 (br, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 16.8 (d, *J* = 24.8 Hz), 49.1, 55.4, 58.8, 68.6 (d, *J* = 17.2), 82.6, 90.5 (d, *J* = 39.1 Hz),

100.2, 100.7, 102.5, 129.9, 156.6, 163.0; ^{19}F NMR (376 MHz, CD_3OD) δ -148.0; MS (ESI, pos, CH_3OH), m/z: 326.1 ($M + H^+$).

Synthesis of 2'-deoxy-2',2'-difluoro-1'-[(2-amino-imidazo-[4',5':4,5]-thieno-[3, 2-d]pyrimidin-3-yl-7-one)]-β-D-ribofuranose (4).[162]

To a solution of **32.11b** (95 mg, 0.18 mmol) in anhydrous CH_2Cl_2 (8 mL) was added EtSH (0.3 mL, 3.5 mmol) and BF_3•Et_2O (48%, 0.9 mL, 3.5 mmol) at 0°C. The reaction was allowed to proceed for 72 h at room temperature before TLC analysis confirmed complete product formation. The solvent was removed under reduced pressure, and the residue was purified by Combi-flash silica column chromatography ($CHCl_3$/CH_3OH = 100:1 - 1:1) to afford target nucleoside **4** (51 mg, 81%) as off-white powder. R_f = 0.32, (CH_2Cl_2/CH_3OH = 4:1). ^1H NMR (400 MHz, CD_3OD) δ 3.83-3.86 (m, 1H, H-5), 3.99-4.01 (m, 2H, H-5' and H-4), 4.58-4.66 (m, 1H, H-3), 6.53 (dd, 1H, J = 6.0 Hz, J = 8.7 Hz, H-1), 8.47 (s, 1H, Ar); ^{19}F NMR (376 MHz, CD_3OD) δ -119.2, -122.0; MS (ESI, pos, CH_3OH), m/z: 359.9 ($M + H^+$).

Synthesis of 3,5-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-2-fluoro-2-C-methyl-D-ribono-γ-lactone (33.1).[161]

To a stirred solution of diol **31.7a** (620 mg, 3.78 mmol) in anhydrous pyridine (15 mL) was added $TIPDSCl_2$ (1.31 g, 4.15 mmol) slowly at 0°C under nitrogen. The mixture was then stirred at room temperature for 16 h. The solvent was removed under reduced pressure, and residue was purified by silica gel column chromatography (hexanes/EtOAc = 20:1-5:1) to give a colorless syrup (1.29 g, 84%).

R_f = 0.55, (hexanes/EtOAc = 15:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.03-1.10 (m, 28H), 1.60 (d, 3H, *J* = 22.9 Hz), 4.06-4.21 (m, 3H), 4.37-4.40 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 12.7, 13.0, 13.8, 17.0-17.4 (m), 59.1, 72.5 (d, *J* = 17.2 Hz), 81.2, 92.5 (d, *J* = 184.0 Hz), 169.8 (d, *J* = 20.0 Hz); MS (ESI, pos, CH_3OH), m/z: 429.1 ($M + Na^+$).

Synthesis of 3,5-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2-deoxy-2-fluoro-2-C-methyl-D-ribono-γ-lactol (33.2).[161]

To a stirred solution of protected lactone **33.1** (1.30 g, 3.20 mmol) in anhydrous THF (30 mL) was added lithium tri-*tert*-butoxyaluminum hydride (980 mg, 3.85 mmol) at -20°C under nitrogen, then warm to 10°C. After 3h, the mixture was quenched with saturated NH_4Cl solution. THF was removed under reduced pressure and the aqueous layer was extracted with EtOAc. The combined organic extracts were washed with water, brine and dried over Na_2SO_4. Organic layer was removed under reduced pressure and the residue was purified by silica gel column chromatography (hexanes/EtOAc = 10:1-5:1) to give a white solid (1.15 g, 90%). R_f = 0.70, (hexanes/EtOAc = 8:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.04-1.10 (m, 28H), 1.44-1.51 (m, 3H), 3.56-3.61 (m, 1H), 3.94-4.05 (m, 3H), 4.16-4.25 (m, 1H), 5.07-5.20 (m, 1H); MS (ESI, pos, CH_3OH), m/z: 431.1 ($M + Na^+$).

Synthesis of 3',5'-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-deoxy-2'-fluoro-2'-C-methyl-1'-(4,5-diiodoimidazol-3-yl]-α/β-D-ribofuranose (33.3).[161]

To a mixture of 4,5-diiodoimidazole (646 mg, 2.02 mmol), Ph_3P (530 mg, 2.02 mmol) and lactol **33.2** (550 mg, 1.35 mmol) in THF (150 mL) was dropwise added DIAD (408 mg, 2.02 mmol) at 0°C. The resulting mixture was stirred for 24 h at room temperature. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (hexanes/EtOAc = 20:1-1:1) to give a mixture of the two isomers of **33.3**. α isomer (516 mg, 54%) as colorless syrup: R_f = 0.60, (PE/EtOAc = 10:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.05-1.09 (m, 28H), 1.51 (d, 3H, *J* = 21.4 Hz), 4.00-4.10 (m, 2H), 4.24-4.30 (m, 2H), 5.76 (d, 1H, *J* = 18.8 Hz), 7.79 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.7, 12.8, 13.1, 13.8, 17.0, 17.1, 17.2, 17.3, 17.3, 17.4, 18.2 (d, *J* = 25.7 Hz), 60.4, 73.7 (d, *J* = 17.2 Hz), 80.9, 82.4, 91.1 (d, *J* = 14.3 Hz), 96.4, 97.3 (d, *J* = 195.5 Hz), 141.8 (d, *J* = 7.6 Hz); HRMS (FAB) calculated for $C_{21}H_{37}FI_2N_2O_4Si_2$ [M + H^+] 711.0444; Found, 711.0437; β isomer (172 mg, 18%) as colorless syrup: R_f = 0.80, (PE/EtOAc = 10:1). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.03-1.14 (m, 28H), 1.24 (d, 3H, *J* = 22.0 Hz), 4.02-4.10 (m, 2H), 4.15-4.28 (m, 2H), 5.84 (d, 1H, *J* = 16.8 Hz), 8.01 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 13.0, 13.1, 13.8, 17.0 (m), 17.4, 17.5, 17.6, 17.7, 59.5, 71.0 (d, *J* = 17.2 Hz), 80.3, 80.9, 93.3 (d, *J* = 38.1 Hz), 97.4, 100.3 (d, *J* = 185.9 Hz), 139.9; HRMS (FAB) calculated for $C_{21}H_{37}FI_2N_2O_4Si_2$ [M + H^+] 711.0444; Found, 711.0439.

Synthesis of 3',5'-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-iodo-4-carbaldehyde)-imidazole-3-yl]-β-D-ribofuranose (33.4).[161]

To a stirred solution of **33.3** (1.60 g, 2.25 mmol) in anhydrous THF (40 mL) at 0°C, was dropwise added EtMgBr (3M, 900 µL, 2.70 mmol) under nitrogen. The reaction mixture was stirred for 0.5 h at 0°C. Anhydrous DMF (2 mL) was added and the reaction mixture was stirred overnight, quenched with water, and extracted with EtOAc (50 mL × 2). The combined organic layers were washed with brine (20 mL) and dried over Na_2SO_4. The solvent was removed, and the residue was purified by silica gel column chromatography (PE/EtOAc = 10:1) to give **33.4** (993 mg, 72%) as colorless syrup. R_f = 0.60, (PE/EtOAc = 9:1). ^{1}H NMR (400 MHz, acetone-d_6) δ 1.01-1.24 (m, 31H), 4.12-4.34 (m, 4H), 6.59 (d, 1H, J = 15.6 Hz), 8.30 (s, 1H), 9.65 (d, 1H, J = 0.9 Hz); ^{13}C NMR (100 MHz, acetone-d_6) δ 12.6, 12.8, 12.9, 13.5, 15.6, 15.9, 16.5, 16.6, 16.7, 16.8, 16.9, 17.0, 17.1, 60.0, 71.0 (d, J = 17.2 Hz), 80.9, 89.4 (d, J = 38.1 Hz), 100.4 (d, J = 185.9 Hz), 102.1, 129.5, 141.9, 181.2; HRMS (FAB) calculated for $C_{22}H_{38}FIN_2O_5Si_2$ [M + H^+] 613.1426; Found, 613.1442.

Synthesis of 3',5'-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-iodo-4-carbonitrile)-imidazole-3-yl]-β-D-ribofuranose (33.6).[161]

To a stirred mixture of nucleoside **33.4** (900 mg, 1.47 mmol) in anhydrous EtOH (40 mL) were added hydroxylamine hydrochloride (511 mg, 7.35 mmol) and $NaHCO_3$ (617 mg, 7.35 mmol). The resulting mixture was refluxed for 5 h. The solvent was removed under reduced pressure and the crude product was extracted in EtOAc (2 x 30 mL) and excess water. The combined organic layer was dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give the crude oxime

intermediate **33.5** as a syrup (1.10 g). This was dissolved in anhydrous THF (30 mL) and 1,1'-carbonyldiimidazole (574 mg, 3.54 mmol) was added to the mixture. The resulting solution was stirred at reflux for 16 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (PE/EtOAc = 8:1) to provide the cyano nucleoside **33.6** as a yellow syrup (625 mg, 70% in 2 steps). R_f = 0.60, (PE/EtOAc = 10:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.01-1.10 (m, 28H), 1.29 (d, 3H, *J* = 22.0 Hz), 4.01-4.05 (m, 1H), 4.10-4.19 (m, 2H), 4.25-4.28 (m, 1H), 5.84 (d, 1H, *J* = 14.6 Hz), 8.04 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.6, 12.9, 13.0, 13.8, 16.5, 16.7, 16.9 (m), 17.1, 17.3, 17.4, 17.5, 59.6, 70.6 (d, *J* = 17.2 Hz), 81.6, 90.8 (d, *J* = 37.2 Hz), 96.7, 99.9 (d, *J* = 186.8 Hz), 110.6 (d, *J* = 16.2 Hz), 140.0; HRMS (FAB) calculated for $C_{22}H_{37}FIN_3O_4Si_2$ [M + H^+] 610.1430; Found, 610.1435.

Synthesis of 3',5'-(1,1,3, 3-tetraisopropyldisiloxane-1,3-diyl)-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-carboxamide)-[2,3-d]-imidazole-3-yl]-β-D-ribofuranose (33.8).[161]

To a stirred mixture of **33.6** (500 mg, 0.820 mmol) and mercaptoacetamide (299 mg, 3.28 mmol) in anhydrous DMF (100 mL) was added anhydrous K_2CO_3 (453 mg, 3.28 mmol). The reaction was heated to 65°C for 24 h under nitrogen. The crude mixture was filtered over a pad of Celite and the solid was washed with a little DMF. The solvent was removed under reduced pressure to give crude mixture **33.7**, which was refluxed in EtONa (21%, 500 µL) and EtOH (20 mL) mixture for 2 h before solvent was removed under reduced pressure. The residue was purified by silica gel column

chromatography (PE/EtOAc = 1:2) to give the intermediate **33.8** as a yellow syrup (215 mg, 46% in two steps); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.01-1.17 (m, 31H), 3.98-4.10 (m, 2H), 4.26-4.35 (m, 2H), 5.43 (br, 2H), 5.94 (d, 1H, *J* = 21.0 Hz), 6.35 (br, 2H), 7.93 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.5, 13.0, 13.6, 13.7, 17.0, 17.1, 17.2, 17.3, 17.5, 17.7, 58.9, 71.3 (d, *J* = 16.2 Hz), 80.6, 93.2 (d, *J* = 40.0 Hz), 98.9 (d, *J* = 187.8 Hz), 100.1, 126.4, 139.1, 142.5, 146.0, 168.3; MS (ESI, pos, CH_3OH), m/z: 573.3 ($M + H^+$) , 595.3 ($M + Na^+$).

Synthesis of 2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(5-carboxamide)-[2,3-d]-imidazole-3-yl]-β-D-ribofuranose (5).[161]

To a solution of compound **33.8** (240 mg, 0.419 mmol) in anhydrous THF (5 mL) was added TBAF (1 M in THF, 4.2 ml, 4.20 mmol). The reaction mixture was stirred at room temperature for 2 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 30:1-10:1) to give **5** (99 mg, 71%) as a colorless powder. R_f = 0.15, (CH_2Cl_2/CH_3OH = 25:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 0.96 (d, 3H, *J* = 22.4 Hz), 3.69-3.71 (m, 1H,), 3.86-3.88 (m, 2H), 4.06-4.08 (m, 1H), 5.44-5.43 (m, 1H), 5.68 (d, 1H, *J* = 6.9Hz), 6.42 (d, 1H, *J* = 17.4 Hz), 6.72 (br, 2H), 6.92 (br, 2H), 8.44 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 17.1 (d, *J* = 24.8 Hz), 58.6, 70.2 (d, *J* = 17.2 Hz), 82.0, 90.0 (d, *J* = 39.3 Hz), 100.8, 100.9 (d, *J* = 182.1 Hz), 126.9, 139.0, 143.4, 144.5, 168.2; HRMS (FAB) calculated for $C_{12}H_{15}FN_4O_4S$ [$M + H^+$] 331.0876; Found, 331.0863.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-(imidazo[4',5':4,5]- thieno-[3,2-d]-pyrimidin-3-yl-7-one]-β-D-ribofuranose (35.1).[161]

A mixture of **32.10a** (200 mg, 0.39 mmol), triethylorthoformate (25 mL), and 4Å molecular sieves (oven dried before use) was refluxed for 6 h. The reaction mixture was filtered and the excess solvent was evaporated and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, 50:1-20:1) to afford compound **35.1** (164 mg, 80.4%) as a hygroscopic white foam. R_f = 0.40, (CH_2Cl_2/CH_3OH = 20:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.13 (d, 3H, *J* = 22.0 Hz), 3.66-3.69 (m, 1H), 3.98-4.01 (m, 1H), 4.27-4.34(m, 2H), 4.51-4.68 (m, 4H), 6.55 (d, 1H, *J* = 15.6 Hz), 7.26-7.43 (m, 11H), 8.16 (s, 1H), 8.59 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.0 (d, *J* = 24.8 Hz), 67.4, 73.7, 74.3, 76.8, 80.0, 90.5 (d, *J* = 38.1 Hz), 100.6 (d, *J* = 183.1 Hz), 122.1, 128.7 (m), 137.2, 143.6, 145.3, 150.9, 160.4; HRMS (FAB) calculated for $C_{27}H_{25}FN_4O_4S$ [M + H^+] 520.1580; Found, 520.1662.

Synthesis of 3',5'-dibenzyloxy-2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(7-aminoimidazo [4',5':4,5]-thieno-[3,2-d]-pyrimidin-3-yl]-1-β-D-ribofuranose (35.5).[161]

To a mixture of **35.1** (150 mg, 0.29 mmol), DMAP (141 mg, 1.15 mmol), NEt_3 (2 ml) in CH_3CN (6 ml) was added TPSCl (349 mg, 1.15 mmol) portion wise over 5 min. The mixture was stirred at room temperature for 3 h and the solvent was removed under reduced pressure. THF (5 ml) was added and the resulting mixture was transferred to a bomb and cooled to -78°C and ammonia was bubbled in for 10 min.

The bomb was sealed and then left to stir overnight at room temperature. After which, the solvent was removed and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 25:1) to afford compound **35.5** (82 mg, 55%) as a yellow foam. R_f = 0.35, (CH_2Cl_2/CH_3OH = 20:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14 (d, 3H, *J* = 22.0 Hz), 3.67-3.70 (m, 1H), 3.97-4.00 (m, 1H), 4.33-4.40 (m, 2H), 4.51-4.68 (m, 4H), 5.32 (s, 2H), 6.63 (d, 1H, *J* = 15.6 Hz), 7.26-7.42 (m, 10H), 8.53 (s, 1H), 8.57 (s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 17.0 (d, *J* = 24.8 Hz), 67.4, 73.7, 74.3, 79.9, 90.7 (d, *J* = 38.1 Hz), 100.6 (d, *J* = 183.1 Hz), 114.0, 114.4, 127.9, 128.2, 128.2, 128.4, 128.5, 128.7, 128.8, 137.3, 142.9, 146.4, 148.5, 154.7, 158.0; MS (ESI, pos, CH_3OH), m/z: 520.1 ($M + H^+$).

Synthesis of 2'-deoxy-2'-fluoro-2'-C-methyl-1'-[(7-aminoimidazo-[4',5':4,5]-thieno-[3,2-d]-pyrimidin-3-yl]-1-β-D-ribofuranose (3).[161]

To a solution of **35.5** (150 mg, 0.29 mmol) in anhydrous CH_2Cl_2 (8 mL) was added EtSH (0.6 mL, 8.1 mmol) and $BF_3 \cdot OEt_2$ (2.1 mL, 8.0 mmol) at 0°C. The reaction mixture was allowed to warm up to room temperature and stirred for 72 h. The solvent was removed, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 25:1) to afford compound **3** (50 mg, 51%) as a white powder. R_f = 0.15, (CH_2Cl_2/CH_3OH = 5:1). ^{1}H NMR (400 MHz, CD_3OD) δ 1.14 (d, 3H, *J* = 22.5 Hz), 3.90 (dd, 1H, *J* = 2.8, 13.3 Hz), 4.08-4.11 (m, 2H), 4.35 (dd, 2H, *J* = 9.2, 23.4 Hz), 6.63 (d, 1H, *J* = 16.0 Hz), 8.43 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 59.0, 15.4 (d, *J* = 24.8 Hz), 82.3, 70.4 (d, *J* = 17.2 Hz), 91.0 (d, *J* = 39.1 Hz), 100.7 (d, *J* = 180.2 Hz), 113.9, 119.2, 134.5, 145.4, 148.1, 154.3, 159.0; ^{19}F

NMR (376 MHz, CD_3OD) δ -155.0; HRMS (FAB) calculated for $C_{13}H_{14}FN_5O_3S$ [M + H^+] 340.0880; Found, 340.0877.

Synthesis of 2'-deoxy-2'-fluoro-2'-C-methyl-1'-(imidazo-[4',5':4,5]-thieno-[3,2-d]- pyrimidin-3-yl-7-one]-β-D-ribofuranose (35.2).[161]

To a solution of **35.1** (120 mg, 0.23 mmol) in anhydrous CH_2Cl_2 (8 mL) was added EtSH (0.3 mL, 4.6 mmol) and $BF_3 \cdot OEt_2$ (1.2 mL, 4.6 mmol) at 0°C. The reaction mixture was allowed to warm up to room temperature and stirred for 72 h. The solvent was removed, and the residue was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH = 5:1) to afford compound **35.2** (44 mg, 56%) as off-white powder. R_f = 0.20, (CH_2Cl_2/CH_3OH = 5:1). ^1H NMR (400 MHz, CD_3OD) δ 1.14 (d, 3H, *J* = 22.5 Hz), 3.87-3.92 (m, 1H), 4.11-4.19 (m, 2H), 4.31-4.39 (m, 1H), 6.62 (d, 1H, *J* = 16.0 Hz), 6.76 (s, 1H), 8.34 (s, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 14.9 (d, *J* = 24.8 Hz), 58.5, 69.5 (d, *J* = 18.1 Hz), 83.2, 91.7 (d, *J* = 42.0 Hz), 100.8 (d, *J* = 187.8 Hz), 114.1, 124.6, 132.8, 143.8, 148.1, 152.2, 158.2; ^{19}F NMR (376 MHz, CD_3OD) δ -156.2; HRMS (FAB) calculated for $C_{13}H_{13}FN_4O_4S$ [M + H^+] 341.0720; Found, 341.0717.

General Procedure for the Preparation of Compounds 36.2-36.6.[161, 162]

Method A: To a stirred solution of free nucleoside **1**, **2**, **4** and **5** (10-20 mg, 1 eq.) in anhydrous DMF (2-4 mL), was added tert-butyl magnesium chloride (1.20 eq., 1 M solution in THF) slowly at -78°C. After completion of the addition, the mixture was

stirred at room temperature for 30 min. To the above mixture was added freshly prepared phosphorous reagent *N*-(chlorophenoxyphosphinyl)-L-alanine-1-methylethyl ester (**36.1**, 1.20 eq., 1M solution in THF) dropwise at -78°C, and the resulting mixture was then slowly allowed to warm to room temperature for 4 h. The mixture was quenched with cold water, and the aqueous phase was extracted with EtOAc (20 mL × 3). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The residue was purified by Combi-flash silica gel column chromatography (CH_2Cl_2/CH_3OH = 100:1-10:1) twice to give target nucleoside prodrugs **36.2-36.6** respectively in a diastereomeric mixture.

Method B: To a stirred solution of free nucleoside **1**, **2**, **4** and **5** (10-20 mg, 1.00 eq.) in anhydrous DMF (3-6 mL), was added tert-butyl magnesium chloride (1.50 eq., 1 M solution in THF) slowly at 0°C. After completion of the addition, the mixture was stirred at 0 °C for 1 h. To the above mixture was added freshly prepared phosphorous reagent (S)-2-[(2,3,4,5,6-pentafluoro-phenoxy)-phenoxy-phosphorylamino] propionic acid isopropyl ester (1.15 eq., 1 M solution in THF) dropwise, and the resulting mixture was stirred at 0°C for 1 h and then slowly allowed to warm to room temperature overnight. The mixture was quenched with cold water, and the aqueous phase was extracted with EtOAc (20 mL × 3). The combined organic layers were dried over Na_2SO_4 and filtered, and the solvent was removed under reduced pressure. The residue was purified by Combi-Flash silica gel column chromatography (CH_2Cl_2/CH_3OH = 100:1-10:1) twice to give target nucleoside prodrugs **36.2-36.6**, respectively in a diastereomeric mixture.

Synthesis of tricyclic nucleoside prodrug (36.2).[161]

Off-white powder; R_f = 0.75, (CH_2Cl_2/CH_3OH = 10:1). ^1H NMR (500 MHz, $CDCl_3$) δ 0.78-1.54 (m, 12H), 3.98-4.06 (m, 2H), 4.37-4.45 (m, 1H), 4.71 (s, 1H), 5.12-5.25 (m, 3H), 5.89-6.14 (m, 2H), 6.71-6.84 (m, 2H), 7.14 (s, 1H), 7.22-7.30 (m, 4H), 7.66 (s, 1H), 11.10-11.31 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ -157.5; ^{31}P NMR (400 MHz, $CDCl_3$) δ 5.76, 5.97; HRMS (FAB) calculated for $C_{25}H_{30}FN_6O_8PS$ [M + H^+] 625.1646; Found, 625.1645.

Synthesis of fleximer nucleoside prodrug (36.3).[161]

White powder; ^1H NMR (500 MHz, $CDCl_3$) δ 1.19-1.36 (m, 16H), 3.85-4.21 (m, 3H), 4.29-4.57 (m, 3H), 4.99 (s, 1H), 5.87 (br, 1H), 6.07 (s, 1H), 7.14 (s, 2H), 7.49-7.57 (m, 1H), 7.72-7.97 (m, 1H), 11.35-11.68 (m, 1H); ^{19}F NMR (376 MHz, $CDCl_3$) δ -163.8; ^{31}P NMR (400 MHz, $CDCl_3$) δ 4.39, 4.98; MS (ESI, pos, CH_3OH), m/z: 595.2 (M + H^+).

Synthesis of bicyclic nucleoside prodrug (36.4).[161]

Colorless syrup; R_f = 0.45, (CH_2Cl_2/CH_3OH = 25:1). ^1H NMR (400 MHz, CD_3OD) δ 1.07-1.20 (m, 9H), 1.27-1.34 (m, 3H), 3.85-3.98 (m, 2H), 4.04-4.22 (m, 3H), 4.92-4.96 (m, 1H), 6.29 (d, 1H, J = 17.8 Hz), 7.15-7.18 (m, 1H), 7.25-7.35 (m, 4H), 8.47 (s, 1H); ^{19}F NMR (400 MHz, $CDCl_3$) δ -161.3; ^{31}P NMR (162 MHz, CD_3OD) δ 3.4, 4.3; HRMS (FAB(calculated for $C_{24}H_{30}FN_5O_8PS$ [M + H^+] 600.1693; Found, 600.1687.

Synthesis of tricyclic nucleoside prodrug (36.5).[162]

Off-white powder; ^{1}H NMR (400 MHz, CD_3OD) δ 1.10-1.29 (m, 9H), 3.83-3.91 (m, 1H), 4.14-4.20 (m, 1H), 4.45-4.60 (m, 2H), 4.69-4.82 (m, 1H), 4.92-4.96 (m, 1H), 6.56-6.60 (m, 1H), 7.14-7.23 (m, 3H), 7.28-7.32 (m, 2H), 8.25 (s, 1H); ^{19}F NMR (376 MHz, CD_3OD) δ -118.4, -120.1; ^{31}P NMR (400 MHz, CD_3OD) δ 4.53, 4.58; HRMS (FAB) calculated for $C_{24}H_{28}F_2N_6O_8PS$ [M + H^+] 629.13950; Found, 629.13985.

Synthesis of tricyclic nucleoside prodrug (36.6).[162]

Colorless syrup; ^{1}H NMR (400 MHz, CD_3OD) δ 1.03-1.14 (m, 10H), 1.18-1.38 (m, 8H), 3.61-3.72 (m, 1H), 3.93-4.00 (m, 1H), 4.23-4.42 (m, 2H), 4.55-4.83 (m, 2H), 4.96-5.01 (m, 2H), 6.39-6.44 (m, 1H), 7.05-7.36 (m, 10H), 8.29 (s, 1H); ^{19}F NMR (376 MHz, CD_3OD) δ -114.0, -117.2; ^{31}P NMR (400 MHz, CD_3OD) δ 4.02, 4.21, 5.49, 5.63; HRMS (FAB) calculated for $C_{36}H_{44}F_2N_7O_{12}P_2S$ [M + H^+] 898.22120; Found, 898.22221.

Cell-Based Inhibition and Cytotoxicity Assays.[161, 249]

The Huh 5.2 HCV subgenomic replicon-containing cells were provided by Prof. R. Bartenschlager (University of Heidelberg, Heidelberg, Germany).

The inhibitory potency (EC_{50} values) and cytotoxicity (CC_{50} values) of the compounds were evaluated in Huh 5.2 cells as described previously.[337]

In brief, Huh 5.2 cells, containing the hepatitis C virus genotype 1b I389luc-ubi-neo/NS3-3′/5.1 replicon,[338] were subcultured in DMEM supplemented with 10%

FCS, 1% nonessential amino acids, 1% penicillin/streptomycin, and 2% Geneticin at a ratio of 1:3 to 1:4 and grown for 3 to 4 days in 75 cm^2 tissue culture flasks. One day before addition of the compound, cells were harvested and seeded in an assay medium (DMEM, 10% FCS, 1% nonessential amino acids, 1% penicillin/streptomycin) at a density of 6500 cells/well (100 μL/well) in 96-well tissue culture microtiter plates for the evaluation of the antimetabolic effect and in a CulturPlate (PerkinElmer) for the evaluation of the antiviral effect. The microtiter plates were incubated overnight (37°C, 5% CO_2, 95−99% relative humidity), yielding a nonconfluent cell monolayer. Compounds were subsequently added to the plates at the indicated concentrations. Following assay setup, the microtiter plates were incubated for 72 h (37°C, 5% CO2, 95−99% relative humidity). For the evaluation of antimetabolic effects, the assay medium was aspirated, replaced with 75 μL of a 5% MTS solution in phenol red-free medium, and incubated for 1.5 h (37°C, 5% CO_2, 95−99% relative humidity). Absorbance was measured at a wavelength of 498 nm (Safire2, Tecan), and optical densities (OD values) were converted to the percentage of untreated controls. For the evaluation of antiviral effects, the assay medium was aspirated and the cell monolayers were washed with PBS. The wash buffer was aspirated, and 25 μL of Glo lysis buffer (Promega) was added, allowing cell lysis to proceed for 5 min at room temperature. Subsequently, 50 μL of the luciferase assay system (Promega) was added, and the luciferase luminescence signal was quantified immediately (1000 ms integration time/well, Safire2, Tecan). Relative luminescence units were converted to the percentage of untreated controls.

EC_{50} and EC_{90} (values calculated from the dose−response curve) represent the concentrations at which 50 and 90% inhibition, respectively, of viral replication are achieved. CC_{50} (value calculated from the dose−response curve) represents the concentration at which the metabolic activity of the cells is reduced by 50% as compared to that of untreated cells.

The concentration of the compound is considered to elicit a genuine antiviral effect in the HCV replicon system when the antireplicon effect is significant at concentrations where no antimetabolic activity is observed.

Chapter 4 Experimentals

Synthesis of 2-amino-4-iodo-6-methoxypyrimidine (37.1).

Commercially available 2-amino-4-chloro-6-methoxypyrimidine (5.0 g, 31.3 mmol) was suspended in 20 mL of 57 wt. % HI in H_2O at 0°C. The mixture was stirred at room temperature for 72 h. The resulting sludge was diluted in 20 mL H_2O and neutralized to pH 7-8 using sat. Na_2CO_3. The precipitate was filtered and recrystallized in EtOH to yield a white solid (4.1g, 16.3 mmol, 52%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H), 6.07 (s, 1H), 7.15 (br, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 40.7, 94.7, 160.3, 163.4, 171.5. MS (ESI, pos, CH_3OH) calculated for $C_5H_6IN_3O$ $[M + H]^+$ 251.96, found 251.96.

Synthesis of 2-amino-4-methoxy-6-tributylstannylpyrimidine (38.1).

37.1 (139 mg, 0.55 mmol) was dissolved under N_2 in 20 mL of anhydrous THF and cooled to -78°C. EtMgBr (3.0 M, 0.20 mL, 0.61 mmol) was added dropwise and allowed to stir for 2 min. TMSCl (0.08 mL, 0.61 mmol) was added and allowed to stir

for 5 min. Again, EtMgBr (3. 0M, 0.20 mL, 0.61 mmol) was added dropwise and allowed to stir for 2 min, then TMSCl (0.08 mL, 0.61 mmol) was added and allowed to stir for 5 min. *n*-Butyllithium (1.6 M, 0.4 mL, 0.61 mmol) was added dropwise and allowed warm to room temperature and stirred for 3.5 h. Tributyltin chloride (0.3 mL, 1.11 mmol) was added and the mixture was stirred for 18 min. The reaction was quenched using 10 mL NH_4Cl and the solvent was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (20 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 9:1 – 3:1) to yield a yellow oil (126 mg, 0.30 mmol, 55 % yield). R_f = 0.80, (hexanes/EtOAc = 4:1). Compound decomposed rapidly. ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.85-0.93 (m, 9H), 1.04-1.08 (m, 6H), 1.25-1.39 (m, 6H), 1.50-1.67 (m, 6H), 3.84 (s, 3H), 4.93 (br, 2H), 6.26 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 9.8, 13.7, 27.4, 29.0, 52.9, 107.6, 161.9, 168.4, 183.8. MS (APCI, pos, CH_3CN) calculated for $C_{17}H_{34}N_3OSn$ $[M + H]^+$ 416.17, found 416.17.

Synthesis of 4-(1-benzyl-1*H*-imidazol-4-yl)-6-methoxy-2-pyrimidinylamine (42.3).

1-Benzyl-4-iodo-1*H*-imidazole (118 mg, 0.42 mmol) was dissolved under N_2 in 25 mL of anhydrous THF and cooled to -78°C. EtMgBr (3.0 M, 0.15 mL, 0.44 mmol) was added dropwise and allowed to stir for 10 min. $ZnCl_2$ (0.7 M in THF, 1.2 mL, 0.84 mmol) was subsequently added dropwise, stirred at -78°C for 10 min, warmed to room temperature and stirred for 2 h. The organozinc was added dropwise to a

mixture of **37.1** (105 mg, 0.42 mmol), $Pd(PPh_3)_4$ (48 mg, 0.04 mmol), CuI (19 mg, 0.1 mmol) in 40 mL of anhydrous THF and allowed to stir at room temperature for 24 h. The reaction was quenched using 10 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 1:1 – 0:1) to yield a white solid (25 mg, 0.10 mmol, 24% yield). R_f = 0.35, (EtOAc). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.78 (s, 3H), 5.23 (s, 2H), 6.41 (s, 1H), 6.46 (br, 2H), 7.29-7.37 (m, 5H), 7.66 (s, 1H), 7.89 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 51.4, 53.8, 92.2, 120.3, 127.6, 128.6, 129.2, 135.5, 138.0, 140.6, 160.6, 162.6, 171.9. HRMS (FAB) calculated for $C_{15}H_{15}N_5O$ $[M + H]^+$ 282.1355, found 282.1351.

Synthesis of 4-(3*H*-imidazol-4-yl)-6-methoxy-2-pyrimidinylamine (43.1).

39.4 (100 mg, 0.34 mmol) was dissolved in 20 mL trifluoroacetic acid and stirred for 48 h. The solvent was removed and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 19:1 – 4:1) to yield a white solid (59 mg, 0.31 mmol, 91% yield). R_f = 0.50, (CH_2Cl_2/CH_3OH = 4:1). ^{1}H NMR (400 MHz, CF_3COOD) δ 3.81 (s, 3H), 6.59 (s, 1H), 8.18 (s, 1H), 8.72 (s, 1H). ^{13}C NMR (100 MHz, CF_3COOD) δ 55.7, 97.4, 121.6, 123.8, 136.8, 141.5, 156.4, 172.8. ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.77 (s, 3H), 6.44 (s, 1H), 7.56 (s, 1H), 7.70 (s, 1H), 10.62 (br, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 53.3, 90.3, 119.6 (m), 136.9, 137.7 (m),

160.9, 163.7, 171.2043. MS (APCI, pos, CH_3OH) calculated for $C_8H_9N_5O$ $[M + H]^+$ 192.09, found 192.1.

2-Amino-6-(1-benzyl-1*H*-imidazol-4-yl)-3*H*-pyrimidin-4-one (43.2).

42.3 (25 mg, 0.10 mmol) was dissolved in 15 mL anhydrous CH_2Cl_2 under N_2 and cooled to -78°C. BBr_3 (3M, 0.4 mL, 1.2 mmol) was added dropwise and the reaction was allowed to warm to room temperature and stirred for 72 h. The mixture was dripped slowly into 20 mL iced water and stirred for 30 min. The solvent was removed *in vacuo* and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 9:1 – 4:1) to yield a white solid (16 mg, 0.06 mmol, 60% yield). R_f = 0.70, (CH_2Cl_2/CH_3OH = 9:1). ^1H NMR (400 MHz, CD_3OD) δ 5.29 (s, 2H), 6.23 (s, 1H), 7.32-7.38 (m, 5H), 7.74 (s, 1H), 7.96 (s, 1H). MS (APCI, pos, CH_3OH) calculated for $C_{14}H_{14}N_5O$ $[M + H]^+$ 268.12, found 268.11.

Synthesis of 2-(di-*tert*-butoxycarbonylamino)-4-iodo-6-methoxypyrimidine (44.1) & 4-iodo-6-methoxy-2-(*tert*-butoxycarbonylamino)pyrimidine (44.2).

2-amino-4-iodo-6-methoxypyrimidine (4.1 g, 16.3 mmol) was suspended in 50 mL CH_2Cl_2 under N_2. Di-*tert*-butyl decarbonate (8.9g, 40.8 mmol) and 4-dimethylaminopyridine (5.0g, 40.9 mmol) were added. The reaction was allowed to stir at room temperature for 18 h. TLC showed absence of starting material and two products **38.1** and **38.2**. The solvent was removed under pressure and crude material was purified using silica gel column chromatography (hexanes/EtOAc = 4:1 – 3:2) to yield **44.1** as a colorless oil (2.9 g, 6.5 mmol, 40% yield). R_f = 0.85, (hexanes/EtOAc

= 4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.39 (s, 18H), 3.85 (s, 3H), 7.01 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) 27.9, 54.5, 83.6, 116.4, 126.8, 150.2, 156.4, 169.8. MS (APCI, pos) calculated for $C_{15}H_{23}IN_3O_5$ $[M + H]^+$ 452.07, found 452.05, and **44.2** as a white solid (2.8g, 8.0 mmol, 49%). R_f = 0.70, (hexanes/EtOAc = 4:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.47 (s, 9H), 3.93 (s, 3H), 6.35 (s, 1H), 7.68 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 28.2, 54.6, 81.8, 101.5, 150.0, 157.0, 161.0, 171.4. MS (APCI, pos, CH_3CN) calculated for $C_{10}H_{15}IN_3O_3$ $[M + H]^+$ 352.02, found 351.98.

Synthesis of 6-methoxy-2-(*tert*-butoxycarbonylamino)-4-(1-trityl-1*H*-imidazol-4-yl)pyrimidine (45.3).

Commercially available 4-iodo-1-trityl-1*H*-imidazole (500 mg, 1.15 mmol) was dissolved under N_2 in 25 mL of anhydrous THF and cooled to -78°C. EtMgBr (3.0 M, 0.4 mL, 1.20 mmol) was added dropwise and allowed to stir for 10 min. $ZnCl_2$ (0.7 M in THF, 3.3 mL, 2.3 mmol) was subsequently added dropwise, stirred at -78°C for 10 min, warmed to room temperature, and stirred for 2 h. The organozinc was added dropwise to a mixture of **44.1** (451 mg, 1.0 mmol), $Pd(PPh_3)_4$ (115 mg, 0.1 mmol) and CuI (10 mg, 0.05 mmol) in 40 mL of anhydrous THF and allowed to stir at room temperature for 24 h. The reaction was quenched using 10 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 1:1 – 0:1) to yield a yellow oil (267 mg, 0.5 mmol, 43% yield). R_f = 0.60, (hexanes/EtOAc = 1:2). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.45 (s, 9H), 3.98 (s,

3H), 6.99 (s, 1H), 7.14-7.16 (m, 6H), 7.32-7.33 (m, 9H), 7.47 (br, 1H), 7.60 (s, 1H), 7.61 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 28.2, 53.9, 75.8, 80.8, 95.9, 122.2, 128.2, 128.2, 129.8, 139.0, 139.9, 142.0, 150.7, 157.3, 161.4, 171.5. MS (APCI, pos, CH_3CN) calculated for $C_{32}H_{32}N_5O_3$ $[M + H]^+$ 534.25, found 534.24.

Synthesis of 4-(3*H*-imidazol-4-yl)-6-methoxy-2-(*tert*-butoxycarbonylamino)-pyrimidine (45.4).

45.3 (267 mg, 0.50 mmol) was dissolved in 20 mL acetic acid and stirred for 48 h. The solvent was removed and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 19:1 – 4:1) to yield an off-white solid (140 mg, 0.48 mmol, 96% yield). R_f = 0.85, (CH_2Cl_2/CH_3OH = 9:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.43 (s, 9H), 3.87 (s, 3H), 6.80 (s, 1H), 7.27 (br, 1H), 7.64 (s, 1H), 7.76 (s, 1H), 9.67 (br, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 28.52, 54.02, 79.82, 95.05, 119.68, 128.94, 133.60, 137.51, 151.56, 158.09, 171.01. MS (APCI, pos, CH_3OH) calculated for $C_{13}H_{18}N_5O_3$ $[M + H]^+$ 292.14, found 292.12.

Synthesis of 2-amino-6-(3*H*-imidazol-4-yl)-3*H*-pyrimidin-4-one (6).

37.1 (41 mg, 0.21 mmol) was dissolved in 20 mL anhydrous EtOAc under N_2 and cooled to -78°C. Boron tribromide (1.0 M, 0.6 mL, 0.6 mmol) was added dropwise. The reaction was allowed to warm to room temperature and stirred for 36 h. The mixture was dripped slowly into 20 mL iced water and stirred for 30 min. The solvent was removed *in vacuo* and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 9:1 – 2:1) to yield a white solid (32 mg, 0.18

mmol, 86% yield). R_f = 0.25, (CH_2Cl_2/CH_3OH = 2:1). ^1H NMR (400 MHz, CD_3COOD) δ 6.42 (s, 1H), 8.12 (s, 1H), 8.61 (s, 1H). ^{13}C NMR (100 MHz, CD_3COOD) δ 97.6, 120.1, 131.0, 137.0, 150.7, 155.1, 164.4. HRMS (FAB) calculated for $C_7H_7N_5O$ $[M + H]^+$ 178.0729, found 178.0727.

Synthesis of 6,6'-dimethoxy-4,4'-bipyrimidine-2,2'-diamine (46.2).

37.1 (101.3 mg, 0.40 mmol) was dissolved in 30 mL degassed 1,4-dioxane in a glass tube. Bis(tributyltin) (0.2 mL, 0.4 mmol) was added, followed by $Pd(PPh_3)_2Cl_2$ (28.3 mg, 0.04 mmol). The glass tube was sealed and heated to 130°C for 48 h. The tube was cooled to 0°C, opened and warmed to room temperature. The crude content was filtered over a pad of Celite and the solvent was removed. The crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 9:1 – 2:1) to yield an impure mixture. Another attempt at purification with silica gel column chromatography using the Pharmasset conditions (EtOAc/CH_3OH/acetone/H_2O = 6:1:1:0.5) still yielded impure mixture. R_f = 0.15, (EtOAc/CH_3OH/acetone/H_2O = 6:1:1:0.5). MS (APCI, pos, DMSO/CH_3OH = 1:1) calculated for $C_{12}H_{14}N_4O_4$ 249.11 ($M+H^+$), found: 249.1.

Synthesis of 4-(4-chloro-6-methoxy-2-pyrimidinylamino)-6-methoxy-2-pyrimidinamine (46.3).

37.1 (212 mg, 0.84 mmol) was dissolved under N_2 in 20 mL of anhydrous THF and cooled to -78°C. EtMgBr (3.0 M, 0.3 mL, 0.93 mmol) was added dropwise and allowed to stir for 2 min. TMSCl (0.1 mL, 0.93 mmol) was added and allowed to stir

for 5 min. Again, EtMgBr (3.0 M, 0.3 mL, 0.93 mmol) was added dropwise and allowed to stir for 2 min, then TMSCl (0.1 mL, 0.93 mmol) was added and allowed to stir for 5 min. EtMgBr (3.0 M, 0.3 mL, 0.93 mmol) was added dropwise and allowed to stir for 10 min followed by addition of $ZnCl_2$ (1 M in THF, 1.7 mL, 1.7 mmol) dropwise, stirred at -78°C for 10 min, warmed to room temperature and stirred for 2 h. The organozinc was added dropwise to a mixture of 2-amino-6-chloro-4-methoxypyrimidine (80 mg, 0.50 mmol), $PdCl_2(PPh_3)_2$ (59 mg, 0.08 mmol) and CuI (16 mg, 0.08 mmol) in 30 mL of anhydrous THF and allowed to stir at reflux for 24 h. The reaction was quenched using 20 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (30 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 1:3) to yield a white solid (107 mg, 0.38 mmol, 76% yield). R_f = 0.50, (hexanes/EtOAc = 1:3). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.76 (s, 3H), 3.91 (s, 3H), 6.30 (br, 2H), 6.56 (s, 1H), 6.85 (s, 1H), 9.80 (br, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 53.7, 54.8, 84.7, 99.7, 158.2, 159.5, 160.6, 162.4, 171.2, 172.2. HRMS (FAB) calculated for $C_{10}H_{11}ClN_6O_2$ $[M + H]^+$ 283.0710, found 283.0788.

Synthesis of 2,4,6-tribromopyrimidine (11.1).

Commercially available barbituric acid (2.0 g, 15.6 mmol) was suspended in 30 mL of anhydrous toluene under N_2 and cooled to 0°C. Phosphorus (V) oxybromide (17.9 g, 62.4 mmol) was added and *N*,*N*-dimethylaniline (3.6 mL, 28.4 mmol) was added dropwise. The mixture was heated to 110°C and stirred vigorously for 3 h. The

reaction was cooled to room temperature and quenched with 30 mL iced water. The mixture was transferred to a separatory funnel and the remaining insoluble gum was washed with EtOAc (10 mL x 3). All organic layers were combined and washed with sat. $NaHCO_3$ (10 mL x 3), then brine (10 mL x 2), and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 99:1 – 9:1) to yield **11.1** as a while solid (3.6 g, 11.4 mmol, 73% yield). R_f = 0.80, (hexanes/Et_2O = 4:1). ^1H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 128.2, 150.8, 153.2. Agrees with literature values.[129]

Synthesis of 4-bromo-2,6-dimethoxypyrimidine (47.1).

2,4,6-tribromopyrimidine **11.1** (1.04g, 3.28 mmol) was dissolved in 50 mL methanol and cooled to 0°C. A sodium methoxide solution (0.5 M, 13.2 mL, 6.60 mmol) was added dropwise and the mixture was warmed to room temperature and stirred for 18 h. The reaction was quenched using 20 mL NH_4Cl and the solvent was removed. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 19:1 – 4:1) to yield a white solid (575 mg, 2.62 mmol, 80% yield). R_f = 0.75, (hexanes/Et_2O = 4:1). ^1H NMR (400 MHz, $CDCl_3$) δ 3.89 (s, 3H), 3.92 (s, 3H), 6.51 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.4, 55.4, 105.0, 152.0, 164.5, 171.7. MS (APCI, pos, CH_3CN) calculated for $C_6H_7BrN_2O_2$ $[M + H]^+$ 218.98 and 220.97, found 218.94 and 220.93.

Synthesis of 2,2',6,6'-tetramethoxy-4,4'-bipyrimidine (48.2).

47.1 (157 mg, 0.72 mmol) was dissolved in 20 mL degassed 1,4-dioxane in a glass tube. Bis(tributyltin) (0.4 mL, 0.72 mmol) was added, followed by $Pd(PPh_3)_4$ (83 mg, 0.07 mmol). The glass tube was sealed and heated to 120°C for 18 h. The tube was cooled to 0°C, opened and warmed to room temperature. The crude content was filtered over a pad of Celite and the solvent was removed. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 1:0 – 9:1) to yield a white fluffy solid (81 mg, 0.29 mmol, 80% yield). R_f = 0.80, (hexanes/EtOAc = 9:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.01 (s, 6H), 4.06 (s, 6H), 7.41 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.3, 55.0, 99.4, 162.8, 165.5, 173.1. MS (ESI, pos, CH_3CN) calculated for $C_{12}H_{14}N_4O_4$ $[M + H]^+$ 279.11, found 279.1.

Synthesis of 4,4'-dimethoxy-5,5'-bipyrimidine-2,2'-diamine (50.1).

20.2 (124 mg, 0.5 mmol) and **20.3** (206 mg, 0.5 mmol) were dissolved in 50 mL degassed DMF. $Pd(PPh_3)_4$ (57 mg, 0.05 mmol), CuI (19 mg, 0.1 mmol) and CsF (150 mg, 0.99 mmol) were added. The reaction was allowed to stir at 90°C for 18 h. The contents were cooled and filtered over Celite. The solvent was removed and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 4:1 – 2:1) to yield contaminated **50.1**. The contaminated sample was recrystallized in EtOAc, followed by ethanol, followed by methanol and finally DMSO to obtain a white solid (15 mg, 0.06 mmol, 12 % yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.73 (s, 6H), 6.54 (br, 4H), 7.78 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 53.5, 103.9,

158.9, 163.3, 167.4. MS (APCI, pos, DMSO/CH_3OH = 1:1) calculated for $C_{10}H_{12}N_6O_2$ $[M + H]^+$ 249.11, found 249.0.

Synthesis of 4-methoxy-5-(1-trityl-1*H*-imidazol-4-yl)-2-pyrimidinylamine (52.3). **20.2** (500 mg, 1.99 mmol) was dissolved under N_2 in 50 mL of THF and cooled to -78°C. EtMgBr (3.0 M, 0.7 mL, 2.10 mmol) was added dropwise and allowed to stir for 2 min. TMSCl (0.3 mL, 2.19 mmol) was added and allowed to stir for 5 min. Again, EtMgBr (3. 0M, 0.7 mL, 2.10 mmol) was added dropwise and allowed to stir for 2 min, then TMSCl (0.3 mL, 2.19 mmol) was added and allowed to stir for 5 min. EtMgBr (3.0 M, 0.7 mL, 2.10 mmol) was added dropwise and allowed to stir for 10 min followed by addition of $ZnCl_2$ (0.7 M in THF, 5.7 mL, 3.98 mmol) dropwise, stirred at -78°C for 10 min, warmed to room temperature and stirred for 2 h. The organozinc was added dropwise to a mixture of **45.1** (850 mg, 1.95 mmol), $Pd(PPh_3)_4$ (230 mg, 0.2 mmol), CuI (20 mg, 0.1 mmol) in 80 mL of THF and allowed to stir at room temperature for 24 h. The reaction was quenched using 10 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 1:1 – 0:1) to yield a yellow solid (252 mg, 0.58 mmol, 29% yield). R_f = 0.45, (EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.86 (s, 3H), 4.92 (br, 2H), 7.28 (s, 1H), 7.33-7.37 (m, 15H), 7.46 (s, 1H), 8.86 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 53.4, 75.4, 106.5, 119.9, 128.0, 129.9, 135.0, 138.5, 142.5, 155.3, 161.0, 166.1.

Synthesis of 5-(1*H*-imidazol-4-yl)-4-methoxy-2-pyrimidinylamine (52.4).

52.3 (252 mg, 0.58 mmol) was dissolved in 20 mL acetic acid and stirred for 48 h. The solvent was removed and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 19:1 – 4:1) to yield an off-white solid (108 mg, 0.56 mmol, 97% yield). R_f = 0.60, (CH_2Cl_2/CH_3OH = 4:1). ^1H NMR (500 MHz, CD_3OD) δ 4.04 (s, 3H), 7.36 (d, 1H, J = 1.10 Hz), 7.70 (d, 1H, J = 1.15 Hz), 8.50 (s, 1H). ^{13}C NMR (125 MHz, CD_3OD) δ 52.7, 104.4, 116.8, 134.9, 147.1, 153.6, 161.7, 166.3. MS (APCI, pos, DMSO/CH_3OH = 1:5) calculated for $C_8H_9N_5O$ $[M + H]^+$ 192.09, found 192.04.

Synthesis of 5-(1*H*-imidazol-4-yl)-2,4-dimethoxypyrimidine (53.2).

Commercially available 5-bromo-2,4-dimethoxypyrimidine (200 mg, 0.91 mmol) was dissolved under N_2 in 20 mL of anhydrous THF and cooled to -78°C. EtMgBr (3.0 M, 0.3 mL, 0.96 mmol) was added dropwise and allowed to stir for 10 min. $ZnCl_2$ (0.7 M in THF, 2.6 mL, 1.83 mmol) was subsequently added dropwise, stirred at -78°C for 10 min, warmed to room temperature and stirred for 2 h. The organozinc was added dropwise to a mixture of 4(5)-iodo-1*H*-imidazole (155 mg, 0.8 mmol), $Pd(PPh_3)_4$ (92 mg, 0.08 mmol) and CuI (8 mg, 0.04 mmol) in 30 mL of THF and allowed to stir at reflux for 18 h. The reaction was quenched using 10 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH

= 1:0 – 9:1) to yield a white solid (72 mg, 0.35 mmol, 44% yield). R_f = 0.75, (CH_2Cl_2/CH_3OH = 9:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.87 (s, 3H), 4.00 (s, 3H), 7.41 (s, 1H), 7.72 (s, 1H), 8.81 (s, 1H), 12.25 (br, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 54.5, 54.9, 110.1, 117.0, 131.3, 136.2, 154.9, 163.3, 166.7. MS (APCI, pos, CH_3OH) calculated for $C_9H_{10}N_4O_2$ $[M + H]^+$ 207.09, found 207.03.

Synthesis of 2-(2,4-dimethoxy-5-pyrimidinyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (53.3).

Commercially available 5-bromo-2,4-dimethoxypyrimidine (578 mg, 2.64 mmol) was suspended in 30 mL degassed DMF under N_2, followed by addition of bis(pinacolato)diboron (804 mg, 3.17 mmol), potassium acetate (777 mg, 7.92 mmol) and $PdCl_2(dppf)_2$•$CHCl_3$ (108 mg, 0.13 mmol). The mixture was heated to 100°C and stirred for 1 h. The reaction was cooled and transferred to 50 mL dH_2O. The mixture was extracted in EtOAc/toluene (1:1, 20 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The solvent was removed *in vacuo* and the crude product was used without further purification.

Synthesis of 2,2',4,4'-tetramethoxy-5,5'-bipyrimidine (53.4).

Commercially available 5-bromo-2,4-dimethoxypyrimidine (250 mg, 1.14 mmol) and crude **53.3** (2.64 mmol) were suspended in 25 mL degassed 1,4-dioxane/dH_2O (4:1) under N_2 in a sealed glass flask. Cs_2CO_3 (1.12 g, 3.44 mmol) and $PdCl_2(dppf)_2$•$CHCl_3$ (46 mg, 0.06 mmol). The glass flask was sealed, heated to 105°C and stirred for 1 h. The flask was chilled to 0°C, opened, and warmed to room temperature. The crude

content was filtered over a pad of Celite and the solvent was evaporated *in vacuo*. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 2:1 – 3:7) to yield a white solid (212 mg, 0.76 mmol, 67% yield). R_f = 0.40, (hexanes/EtOAc = 2:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.96 (s, 6H), 4.02 (s, 6H), 8.18 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 54.2, 55.0, 108.2, 158.8, 165.1, 168.6. MS (ESI, pos, CH_3CN) calculated for $C_{12}H_{14}N_4O_4$ $[M + H]^+$ 279.11, found 279.19.

Synthesis of 2,4-dimethoxy-6-(1-trityl-1*H*-imidazol-4-yl)pyrimidine (54.1).

4-iodo-1-trityl-1*H*-imidazole (500 mg, 1.15 mmol) was dissolved under N_2 in 25 mL of THF and cooled to -78°C. EtMgBr (3.0 M, 0.4 mL, 1.20 mmol) was added dropwise and allowed to stir for 10 min. $ZnCl_2$ (0.7 M in THF, 3.3 mL, 2.3 mmol) was subsequently added dropwise, stirred at -78°C for 10 min, warmed to room temperature and stirred for 2 h. The organozinc was added dropwise to a mixture of **47.1** (219 mg, 1.0 mmol), $Pd(PPh_3)_4$ (115 mg, 0.1 mmol), CuI (10 mg, 0.05 mmol) in 40 mL of THF and allowed to stir at room temperature for 6 h. The reaction was quenched using 10 mL sat. EDTA solution and THF was removed *in vacuo*. The crude material was extracted into CH_2Cl_2 (50 mL x 3), washed with brine (10 mL x 2) and the organic layer was dried over $MgSO_4$. The crude material was purified using silica gel column chromatography (hexanes/EtOAc = 2:1 – 1:4) to yield a white solid (307 mg, 0.68 mmol, 68% yield). R_f = 0.50, (hexanes/EtOAc = 1:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.89 (s, 3H), 3.91 (s, 3H), 6.98 (s, 1H), 7.12-7.13 (m, 6H), 7.27-7.28 (m, 9H), 7.46 (d, 1H, J = 1.36 Hz), 7.6518 (d, 1H, J = 1.84 Hz). ^{13}C NMR (100 MHz,

$CDCl_3$) δ 53.9, 54.6, 75.9, 95.0, 122.6, 128.3, 129.8, 139.3, 139.8, 142.1, 162.4, 165.4, 171.1, 172.5.

Synthesis of 4-(1*H*-imidazol-4-yl)-2,6-dimethoxypyrimidine (54.2).

54.1 (307 mg, 0.68 mmol) was dissolved in 30 mL acetic acid and stirred for 48 h. The solvent was removed and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 1:0 – 9:1) to yield a white solid (133 mg, 0.64 mmol, 95% yield). R_f = 0.80, (CH_2Cl_2/CH_3OH = 9:1). ^{1}H NMR (400 MHz, CD_3COOD) δ 4.00 (s, 3H), 4.03 (s, 3H), 6.95 (s, 1H), 8.22 (s, 1H), 9.03 (s, 1H). ^{13}C NMR (100 MHz, CD_3COOD) δ 54.0, 54.7, 97.0, 119.6 (m), 130.7, 136.0, 154.2, 165.8, 172.9. MS (APCI, pos, CH_3OH) calculated for $C_9H_{10}N_4O_2$ $[M + H]^+$ 207.09, found 207.04.

Nucleocapsid NMR Studies

Expression and purification of recombinant HIV-1 NC protein (Performed by Summers lab).[339]

The HIV-1 NC coding region in pNL4-3[340] was PCR amplified using the 5'-primer CCAGCTACCATACATATGCAGAAAGGC (*Nde*I site underlined) and the 3'-primer GGCCGGATCCTCCCTAACTAATTAGCCTGTC-TCTC (*Bam*HI and stop codon underlined). The expression vector pET-3a (Novagen, Madison, WI) was doubly digested with *Nde*I and *Bam*HI and treated with calf intestinal alkaline phosphate. The PCR product was purified by phenol-extraction and ethanol-precipitation and doubly digested with NdeI and BamHI. The insert and vector were

ligated using phage T4 DNA ligase at 16°C for five hours and transformed into competent HMS174. DNA from transformants were sequenced and found to be identical with the HIV-1 NC coding sequence in pNL4-3. A clone, designated as pRD2, overexpressed the 55-residue NC protein with the sequence M Q K G N F R N Q R K T V K C F N C G K E G H I A K N C R A P R K K G C W K C G K E G H Q M K D C T E R Q A N (the two zinc knuckles are underlined). Ion-spray mass spectrometry confirmed the mass of the apoprotein to be 6369(±2) Da (calculated 6369 Da) and 6501(±2) Da (calculated 6500 Da) for the Zn-bound protein.

For protein expression of HIV-1 NC in *Escherichia coli*, pRD2 was transformed into BL21(DE3) pLysE. The purification scheme for the recombinant HIV-1 NC was adapted from Ji *et al.*[341] and You & McHenry.[342] Culture media were supplemented with 100 μg/l ampicillin and 34 μg/L chloramphenicol. A starter culture of 20 ml of ZB[343] inoculated from a single colony was grown at 37°C overnight. The starter culture was added to 2l of M9ZB[343] supplemented with 0.1 mM $ZnCl_2$ and grown at 37°C to an absorbance at 600 nm of 0.5 to 0.6 before induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). After three hours, the cells were harvested by centrifugation, resuspended in 30 ml of lysis buffer (50 mM Tris-HCl (pH 8.0), 10% (v/v) glycerol, 0.1 M NaCl, 0.1 mM $ZnCl_2$, 5 mM dithiothreitol, 2 mM EDTA), and stored at 70°C. To lyse the cells, the cells were thawed in ice-water, and 172 ml of 10 mM PMSF (phenylmethylsulfonyl fluoride), 30 ml of 1 mg/ml pepstatin A, and 2.1 ml of 1% (w/v) sodium deoxycholate were added. The cells were sonicated by five bursts of 20 seconds to reduce the viscosity. The nucleic acids were precipitated by

adding 4% (w/v) polyethyleneimine (pH 7.9) dropwise to a final concentration of 0.4% and stirred for 15 minutes before centrifugation at 23,000 *g* for 30 minutes at 4°C. The supernatant was collected (42 ml), filtered (0.45 μm pore size), and loaded at 1 ml/minute onto a 20 mL Q-Sepharose and a 20 ml SP-Sepharose column (Pharmacia) connected in series and previously equilibrated with 200 ml of buffer A (50 mM Tris-HCl (pH 8.0), 10% glycerol, 0.1 M NaCl, 0.1 mM $ZnCl_2$, 10 mM BME (β-mercaptoethanol)). After washing with 60 ml of buffer A, the Q-Sepharose column was detached, and the SP-Sepharose column was washed with 1.5 column volumes of buffer A. A ten column volume linear gradient from 40% to 50% buffer B (50 mM Tris-HCl (pH 8.0), 10% glycerol, 1.0 M NaCl, 0.1 mM $ZnCl_2$, 10 mM BME) was applied to elute the HIV-1 NC protein. The protein fractions were pooled (15 ml) and loaded at 0.5 ml/minute onto a 320 ml Sephadex G-50 column (Pharmacia) pre-equilibrated with two volumes of buffer C (50 mM Tris-HCl (pH 7.0), 10% glycerol, 0.1 M NaCl, 0.1 mM ZnCl2, 10 mM BME). The NC protein eluted at 175 ml and fractions were pooled (35 ml) for concentration and dialysis into NMR buffer (see below).

Sample preparation (Performed by Summers Lab).[339]

NMR buffer (10mM Tris-HCl, pH 7.0, 140mM KCl, 10mM NaCl, 1mM $MgCl_2$) was deoxygenated by sparging with argon for 15 minutes and filter-sterilized (0.2 μm pore size). The protein sample was dialyzed using Centricon-3 by adding NMR buffer five or six times (total volume 40 to 50 ml). The buffered protein sample was lyophilized for ease of storage.

NMR data collection and analysis (Assisted by Summers Lab).

25 μM protein samples were made in 500 μl of D_2O and loaded into a 5 mm NMR tube. After taking the blank ^1H spectrum, the test compound was titrated into the protein sample such that 1:1 and 2:1 ratios of compound/protein could be established. Data for ^1H NMR signal assignments were collected at a sample temperature of 10°C with a Bruker DMX 600 MHz (^1H) NMR and Bruker AVANCE III HD 500 MHz NMR spectrometers.

Chapter 5 Experimentals

Synthesis of 3-Allyl-7-thia-3.5.10.12-tetraazatricyclo[6.4.0.0^{2,6}]dodeca-1(8),2(6),4,11-tetraen-9-one (55.1)

Triethyl orthoformate (3mL) was added to a suspension of **35.6** (60 mg, 0.27 mmol) in Ac_2O (3 mL) under N_2. The mixture was heated to 135°C and stirred for 3 h. The solvent was evaporated *in vacuo* and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 1:0 – 6:1) to yield **49.1** as an off-white solid (46 mg, 0.20 mmol, 74% yield). R_f = 0.30, (CH_2Cl_2/CH_3OH = 19:1). ^1H NMR (400 MHz, DMSO-d_6) δ 4.99 (d, J = 5.52, 2H), 5.05-5.09 (dd, J = 1.36 Hz, 16.69 Hz, 1H), 5.16-5.19 (dd, J = 1.40, 10.08, 1H), 6.11-6.20 (m, 1H), 8.21 (s, 1H), 8.22 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 48.6, 118.4, 121.1, 129.1, 134.1, 143.6, 146.3, 147.8, 148.4, 158.2. MS (ESI, pos, CH_3CN) calculated for $C_{10}H_8N_4OS$ $[M + H]^+$ 233.05, found 233.02.

Synthesis of Tricyclic inosine (55.2)

Allyl protected tricyclic inosine (46 mg, 0.20 mmol) was suspended in degassed methylene chloride (10 mL) and acetic acid (8 mL) under N_2. $Pd(PPh_3)_4$ was added followed by dropwise addition of phenylsilane. The mixture was heated to 40°C and stirred for 18 h. The solvent was removed *in vacuo* and the crude material was purified using silica gel column chromatography (CH_2Cl_2/CH_3OH = 9:1 – 2:1) to yield an off-white solid (17 mg, 0.09 mmol, 45% yield). R_f = 0.20, (CH_2Cl_2/CH_3OH = 3:1). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.21 (s, 1H), 8.22 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 120.8, 129.5, 144.0, 144.6, 147.6, 150. 6, 158.4. Agrees with literature.[139]

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