

Nucleoside and Oligonucleotide Modification for Targeting the DNA Damage Repair Enzyme SNM1A



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Declaration

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Ellen Fay

Abstract

The exonuclease SNM1A is a key enzyme involved in the repair of interstrand crosslinks, a highly cytotoxic form of DNA damage. As cells depleted in this enzyme display increased sensitivity to chemotherapeutic agents such as cisplatin, SNM1A is a potential target for the treatment of cancers that have developed resistance to traditional chemotherapeutics. The study of SNM1A presents formidable challenges, primarily attributed to its markedly low expression levels and the inherent difficulties associated with achieving stable overproduction from mammalian cells. There remains a need for improved chemical and biochemical tools to facilitate the study of SNM1A.

The work described within elaborates on previous results from the McGouran Group which identified that nucleosides and oligonucleotides, incorporating metal-binding groups, can successfully target the metal ions in the SNM1A active site. Initially, the expansion of established inhibitor scaffolds was investigated for generating tools to study SNM1A. This involved probing the utility of a malonate group as both a metal-binding group and a functionalisation handle. Subsequently, nucleobase modification was examined as a strategy to incorporate a functionalisation handle into established inhibitor frameworks. A thymidine derivative, based on a reported inhibitor, was designed to include a 5'-hydroxamic acid metal-binding group and a propargyl handle on the *N*³-position. Surprisingly, this compound showed limited inhibitory activity compared to its parent compound. The results indicated the importance of the *N*³-position for recognition of thymidine derivatives by SNM1A.

Novel carboxylate-rich nucleoside analogues, featuring succinic, aspartic, glutamic and citric acid-derived 5'-modifications were also evaluated for their ability to target SNM1A. The succinic acid series demonstrated particular activity. The most effective inhibitor was a 5-methyluridine derivative with a 5'-succinic acid modification. A real-time fluorescence assay was employed to determine an IC₅₀ for inhibition of SNM1A by this 5-methyluridine derivative. To further validate its mechanism of action as binding to the SNM1A active site, the ability of this inhibitor to bind to DNA was examined using a circular dichroism-based assay. The 5-methyluridine-derived inhibitor is the most potent nucleoside-based inhibitor of SNM1A reported to date.

A two-step validation method was devised to assess the suitability of metal-binding groups for targeting SNM1A. A fragment-based screening approach was first employed to identify metal-binding fragments with the ability to target the enzyme. Effective fragments were then incorporated into oligonucleotide strands *via* the copper-catalysed azide-alkyne cycloaddition reaction. These tailored oligonucleotides hindered SNM1A activity at concentrations >1000-fold lower than their fragment counterparts. This innovative strategy of integrating functional fragments into oligonucleotides holds broad applicability for crafting modified oligonucleotides with affinity for DNA damage repair enzymes.

Oligonucleotides featuring artificial backbones were explored for targeting SNM1A. A series of dinucleoside phosphoramidites, containing unnatural internucleoside linkages, were generated and incorporated into oligonucleotides *via* solid-phase oligonucleotide synthesis. Oligonucleotides containing urea, squaramide, thioacetamide and sulfinyl acetamide linkages were examined for targeting SNM1A using a gel electrophoresis-based assay. The binding ability of the oligonucleotides was further evaluated using a real-time fluorescence assay to determine IC₅₀ values. This approach proved successful in generating oligonucleotides with notable affinity for SNM1A, as all of the modified oligonucleotides demonstrated increased inhibition levels compared to an unmodified oligonucleotide.

Overall, the findings presented in this work mark substantial advancements in the tailored modification of nucleosides and oligonucleotides for targeting SNM1A. The discerned insights will not only guide the design of next-generation inhibitors but also pave the way for the construction of chemical probes, facilitating *in vivo* studies of SNM1A and, by extension, contributing significantly to the broader landscape of DNA damage repair mechanisms.

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Publications

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Fay, E. M.; Newton, A.; Berney, M.; El-Sagheer, A. H.; Brown, T.; McGouran, J. F., Two-Step Validation Approach for Tools To Study the DNA Repair Enzyme SNM1A. *ChemBioChem* **2023**, e202200756

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Berney, M.; Manoj, M. T.; Fay, E. M.; McGouran, J. F., 5'-Phosphorylation Increases the Efficacy of Nucleoside Inhibitors of the DNA Repair Enzyme SNM1A. *ChemMedChem* **2022**, 17 (5), e202100603

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Abbreviations

2'-F	2'-fluoro
2'-MOE	2'- <i>O</i> -methoxyethyl
2'-OMe	2'- <i>O</i> -Methyl
5'-FSBA	5'- <i>p</i> -fluorosulfonylbenzoyl adenosine
53BP1	p53 binding protein 1
8-oxo-dG	8-oxo-2'-deoxyguanosine
A	Adenine
ABP	activity-based probe
ABPP	activity-based protein profiling
Ac	acetyl
Ac-Gly	<i>N</i> -acetyl-glycine
AcOH	acetic acid
ACV	acyclovir
AfBP	affinity-based probe
APC	anaphase-promoting complex
APCI	atmospheric-pressure chemical ionisation
app.	apparent
aq.	aqueous
Ar	aromatic
ara-C	cytosine arabinoside
ara-CTP	arabinofuranosylcytosine triphosphate
ASO	antisense oligonucleotide
Asp	aspartic acid
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
AZT	zidovudine/azidothymidine
BAIB	(diacetoxyiodo)benzene
BER	base excision repair
BHQ	black hole quencher
BITS	break-induced telomere synthesis
Bn	benzyl

Boc	tert-butyloxycarbonyl
Boc ₂ O	di- <i>tert</i> -butyl carbonate
br	broad
BSA	bovine serum albumin
BTT	5-(benzylthio)-1 <i>H</i> -tetrazole
C	Celsius
calc.	calculated
CD	circular dichroism
conc.	concentrated
CPG	controlled-pore glass
CPSF	cleavage and polyadenylation specificity factor
CSB	Cockayne syndrome B protein
CuAAC	copper-catalysed azide-alkyne cycloaddition
Cy3	Cyanine3
Cys	cysteine
d	doublet
DCA	dichloroacetic acid
DCM	dichloromethane
dd	doublet of doublets
ddd	doublet of doublets of doublets
DE	Aspartic acid - glutamic acid dipeptide
decomp.	decomposed
DEPT	distortionless enhancement by polarisation transfer
dGTP	deoxyguanosine triphosphate
DIPA	diisopropylamine
DIPEA	<i>N,N</i> -diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DMTr	4,4'-dimethoxytrityl
DNA	deoxyribonucleic acid
DNMT	DNA cytosine 5-methyltransferase
dq	doublet of quartets

Dr	Doctor
DSB	double-strand break
dsDNA	double-stranded DNA
dt	doublet of triplets
DTT	1,4-dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
ED	Glutamic acid - aspartic acid dipeptide
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDTA	Ethylenediaminetetraacetic acid
eq.	equivalent(s)
ERCC1	excision repair cross complementation group 1
ESI	electrospray ionisation
Et	ethyl
<i>et al.</i>	<i>et alii</i> , and others
EtOAc	ethyl acetate
EtOH	ethanol
FA	Fanconi anaemia
FAM	fluorescein
FDA	U.S. Food and Drug Administration
fmol	femtomole(s)
FRET	Förster resonance energy transfer
g	gram(s)
G ₀	gap 0 phase/resting phase
G ₁	gap 1 phase/growth 1 phase
h	hour(s)
HDAC	histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HIV	human immunodeficiency virus
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	high-performance liquid chromatography
HR	homologous recombination
HRMS	high resolution mass spectrometry

HSQC	Heteronuclear Single Quantum Coherence
HSV	herpes simplex virus
Hz	hertz
IC ₅₀	half maximal inhibitory concentration
ICL	interstrand crosslink
Ile	isoleucine
IR	infrared
<i>J</i>	coupling constant
LC-MS	liquid chromatography-mass spectrometry
LG	leaving group
lit.	literature
LNA	locked nucleic acid
Lys	lysine
m ⁵ C	5-methylcytosine
m ⁵ U	5-methyluridine
m	multiplet
M	molar
<i>m</i> -CPBA	<i>meta</i> -chloroperoxybenzoic acid
<i>m/z</i>	mass to charge ratio
MALDI	Matrix Assisted Laser Desorption/Ionisation
MBG	metal-binding group
MBL	metallo-β-lactamase
Me	methyl
MeCN	acetonitrile
MeOH	methanol
mg	milligram(s)
min	minute(s)
MIT	Massachusetts Institute of Technology
mL	millilitre(s)
mM	millimolar
mmol	millimole(s)
MMP	matrix-metalloprotease
MMR	mismatch repair

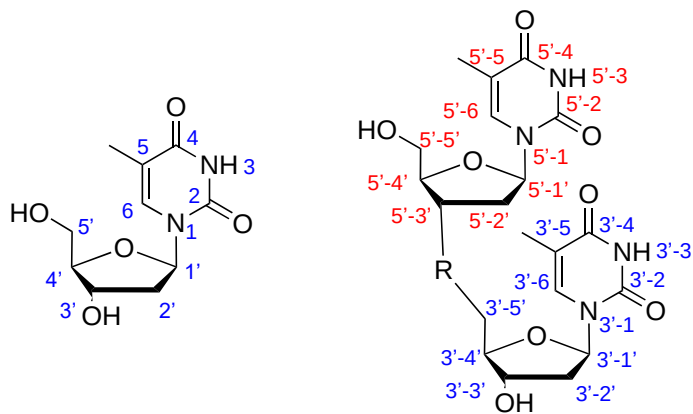
mp	melting point
MS	mass spectrometry
MsCl	methanesulfonyl chloride
NDM-1	New Delhi metallo- β -lactamase-1
NDM-5	New Delhi metallo- β -lactamase-5
NDM-7	New Delhi metallo- β -lactamase-7
NER	nucleotide excision repair
nM	nanomolar
NMM	<i>N</i> -methylmorpholine
NMR	nuclear magnetic resonance
NRTI	Nucleoside reverse transcriptase inhibitors
nt	nucleotides
PAGE	polyacrylamide gel electrophoresis
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDB	protein data bank
pet. ether	petroleum ether
PIAS1	protein inhibitor of activated STAT1
pK _a	acid dissociation constant
pmol	picomole(s)
PNA	peptide nucleic acid
ppm	parts per million
Prof.	Professor
<i>pso</i>	sensitive to psoralen
PSO2	sensitive to psoralen 2
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
q	quartet
qC	quaternary carbon
quant.	quantitative
quin	quintet
<i>R_f</i>	retention factor
RNA	ribonucleic acid
ROS	reactive oxygen species

RP-HPLC	Reversed-phased high performance liquid chromatography
RPA	replication protein A
rpm	revolutions per minute
rt	room temperature
RuAAC	Ru ^{II} -catalysed azide-alkyne cycloaddition
s	singlet
S phase	synthesis phase
SAHA	suberoylanilide hydroxamic acid
SAR	structure activity relationship
sat.	saturated
siRNA	small interfering RNA
snm1	sensitive to nitrogen mustard 1
SNM1A	sensitive to nitrogen mustard 1a
SNM1B	sensitive to nitrogen mustard 1b
SNM1C	sensitive to nitrogen mustard 1c
SNP	single nucleotide polymorphism
SPOS	solid-phase oligonucleotide synthesis
SSB	single-strand break
ssDNA	single-stranded DNA
t	triplet
T	Thymine
T4 PNK	bacteriophage T4 polynucleotide kinase
TBAF	tetrabutylammonium fluoride
TBDMS	<i>tert</i> -Butyl-dimethylsilyl
TBE	tris-borate-EDTA
<i>t</i> Bu	<i>tert</i> -butyl
TCR	transcription coupled repair
td	triplet of doublets
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxyl
TES	triethylsilane
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography

TLS	translesion synthesis
TMS-CHN ₂	trimethylsilyldiazomethane
TOCSY	total correlation spectroscopy
TOF	time-of-flight
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Uracil
UCD	University College Dublin
UNA	unlocked nucleic acid
UV	Ultraviolet
UV-Vis	Ultraviolet-visible
wt	weight
XPF	Xeroderma pigmentosum complementation group F
β-CASP	metallo-β-lactamase-associated CPSF, Artemis, SNM1, PSO2
ε	molar extinction coefficient
μL	microlitres
μM	micromolar
μmol	micromole(s)

Explanatory Note

Modified nucleosides and dinucleosides are numbered according to standard nucleoside convention as shown below.



Contents

Abstract.....	I
Acknowledgements.....	III
Publications.....	V
Abbreviations.....	VI
Explanatory Note	XIII

Chapter 1 – Introduction

1.1. DNA Damage and Repair	1
1.1.1. Interstrand Crosslink Repair	2
1.1.2. SNM1A – A Key Enzyme in Interstrand Crosslink Repair	3
1.2. SNM1A – Structure and Function	6
1.2.1. Active Site and Mechanism of Action	7
1.2.2. Need for Tools to Study SNM1A.....	9
1.3. Utilising Metal-Binding Groups to Study Enzyme Activity.....	10
1.3.1. Activity- and Affinity-Based Protein Profiling.....	11
1.3.2. Targeting Metalloenzymes with Metal-Binding Groups	12
1.4. Nucleoside and Oligonucleotide Modification to Study Enzyme Activity.....	15
1.4.1. Nucleosides/Nucleotides as Enzyme Inhibitors.....	15
1.4.2. Nucleosides/Nucleotides as Molecular Probes	19
1.4.3. Oligonucleotides as Enzyme Inhibitors	20
1.4.4. Oligonucleotides as Molecular Probes	21
1.5. Targeting SNM1A with Metal-Binding Groups and Nucleoside/Oligonucleotide Modification.....	23
1.5.1. Targeting SNM1A with Metal-Binding Groups.....	23
1.5.2. Metal-Binding Nucleosides/Oligonucleotides for Targeting SNM1A.....	26
1.6. Synthesis of Modified Nucleosides/Oligonucleotides	30
1.6.1. Synthesis of Modified Nucleosides/Nucleotides	30
1.6.2. Synthesis of Modified Oligonucleotides.....	33
1.6.2.1. Alternative Backbones for Oligonucleotides	35

1.6.2.2. Solid-Phase Oligonucleotide Synthesis	37
1.7. Project Aims and Work in this Thesis	40
Chapter 2 – Expanding Nucleoside Inhibitor Scaffolds for Targeting SNM1A	
2.1. Introduction.....	44
2.2. Malonate-Based Nucleosides as Tools to Study SNM1A.....	45
2.2.1. Biochemical Evaluation of a Series of Malonate-Based Nucleosides	45
2.2.2. Expansion of the Malonate Inhibitor Scaffold.....	48
2.3. Nucleobase Modification for Targeting SNM1A.....	51
2.3.1. Incorporation of a 5'-Hydroxamic Acid Group into 2'-Deoxycytidine.....	52
2.3.2. Synthesis and Biochemical Evaluation of an <i>N</i> ³ -Modified Thymidine Derivative.....	59
2.3.2.1. Synthesis of an <i>N</i> ³ -Propargyl Thymidine Derivative.....	60
2.3.2.2. Biochemical Evaluation of an <i>N</i> ³ -Propargyl Thymidine Derivative	62
2.3.2.3. Molecular Docking Studies.....	63
2.4. Conclusions.....	64
Chapter 3 – Carboxylate-Rich Nucleoside Analogues for Targeting SNM1A	
3.1. Introduction.....	66
3.2. Biochemical Evaluation – Gel Electrophoresis-Based Assays	67
3.3. IC ₅₀ Determination.....	74
3.4. Circular Dichroism Titrations	76
3.5. Conclusions.....	78
Chapter 4 – Two-Step Validation Approach for Tools to Study SNM1A	
4.1. Introduction.....	80
4.2. Fragment-Based Screening for Targeting SNM1A.....	82
4.2.1. Synthesis of Metal-Binding Fragments	83
4.2.2. Biochemical Evaluation of Metal-Binding Fragments	84
4.3. Triazole-Modified Oligonucleotides for Targeting SNM1A	86
4.3.1. Triazole-Modified Oligonucleotides: Flexible Linker Derivatives.....	88

4.3.1.1. Synthesis and Biochemical Evaluation of Metal-Binding Fragments Containing an Alkyne Handle	89
4.3.1.2. Synthesis and Biochemical Evaluation of Modified Oligonucleotides ..	95
4.3.2. Triazole-Modified Oligonucleotides: Thymidine-Derived Linker	99
4.3.2.1. Synthesis of Thymidine Derivatives Containing an Alkyne Handle ...	100
4.3.2.2. Synthesis and Biochemical Evaluation of Modified Oligonucleotides Containing a Triazole Internucleotide Linkage	103
4.4. Conclusions.....	105

Chapter 5 – Oligonucleotides with Alternative Backbones for Targeting SNM1A

5.1. Introduction.....	108
5.2. Synthesis of Dinucleoside Phosphoramidites	109
5.3. Incorporation of Dinucleosides into Oligonucleotides	116
5.4. Biochemical Evaluation of Modified Oligonucleotides	119
5.5. Conclusions.....	123

Chapter 6 – Conclusions and Future Directions

6. Conclusions and Future Directions	125
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Chapter 7 – Experimental

7.1. General Methods.....	130
7.2. Synthetic Methods	131
7.2.1. Synthetic Methods for Chapter 2	131
7.2.2. Synthetic Methods for Chapter 4	151
7.2.3. Synthetic Methods for Chapter 5	171
7.3. Synthesis and Characterisation of Modified Oligonucleotides.....	185
7.3.1. Sequences of Oligonucleotides	185
7.3.2. Synthesis of Fluorescent Substrate Oligonucleotide	185
7.3.3. Modified Oligonucleotides from Chapter 4.....	186
7.3.3.1. Preparation of Triazole-Linked Oligonucleotides.....	186
7.3.3.2. Mass Spectrometry of Triazole-Linked Oligonucleotides	186
7.3.4. Modified Oligonucleotides from Chapter 5	189

7.3.4.1. Preparation of Oligonucleotides <i>via</i> SPOS using the manual syringe method.....	189
7.3.4.2. Preparation of 19mer Oligonucleotide.....	190
7.3.4.3. Purification and characterisation of Oligonucleotides.....	190
7.3.5. Determination of Concentration of Oligonucleotide Solutions	192
7.4. Biochemical Methods	192
7.4.1. Gel Electrophoresis Assay of Modified Nucleosides or Fragments	192
7.4.2. Gel-Electrophoresis Assay of Modified Oligonucleotides	193
7.4.3. Real-Time Fluorescence Assay of Modified Nucleoside.....	193
7.4.4. Real-Time Fluorescence Assay of Modified Oligonucleotides	194
7.4.5. Circular Dichroism Titrations	195
 Chapter 8 – References	
8. References.....	196

Chapter 1

Introduction

1. Introduction

1.1. DNA Damage and Repair

Deoxyribonucleic acid (DNA) encodes all the genetic information necessary to the function of living organisms, and its maintenance is pivotal for sustaining life. In general, DNA damage may be defined as any modification of DNA that disrupts its coding properties or interferes with normal function in transcription or replication.¹ If left unrepaired or mis-repaired, DNA damage leads to various conditions or disease states. DNA lesions can occur in many different forms as illustrated in **Figure 1.1**. These include oxidative base lesions, abasic sites, single-strand breaks (SSBs), double-strand breaks (DSBs), DNA mismatches and DNA crosslinks.²

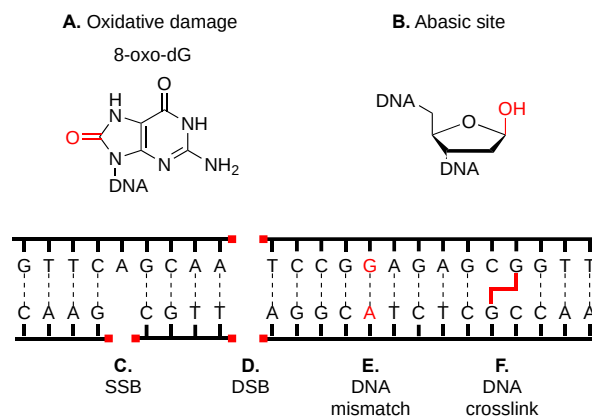


Figure 1.1: Various types of DNA damage; A. Oxidative damage, B. Abasic site, C. Single-strand break (SSB), D. Double-strand break (DSB), E. DNA mismatch and F. DNA crosslink

Oxidative base lesions, such as 8-oxo-2'-deoxyguanosine (8-oxo-dG) (**Figure 1.1A**), can occur when reactive oxygen species (ROS), typical by-products of aerobic respiration, react with DNA.³ Abasic sites (**Figure 1.1B**) are formed when the N-glycosidic bond, which links the nitrogenous base and the sugar phosphate backbone, is cleaved.² This can occur either through spontaneous hydrolysis or through cleavage by enzymes involved in DNA repair. Abasic sites are highly unstable and readily convert into SSBs. SSBs (**Figure 1.1C**) are discontinuities in one strand of the DNA double helix and are the most common form of DNA damage, occurring at a frequency of tens of thousands per cell per day.⁴ Other than being formed from abasic sites, SSBs can also occur through attack by ROS or from erroneous activity of DNA topoisomerase 1.² Although DSBs occur less frequently than SSBs,⁵ they are also highly cytotoxic and can cause genome rearrangements and cell death.⁶ DSBs occur when both strands of the DNA double helix

are severed (**Figure 1.1D**). DSBs can form through endogenous processes, including DNA replication and repair, or as a result of exposure to exogenous agents such as ionising radiation and certain chemical agents.⁷ DNA mismatches (**Figure 1.1E**) occur when two non-complementary nucleobases are aligned in the same base-pair step of a DNA duplex.⁸ Mismatches can develop during DNA replication and heteroduplex formation, through exposure to mutagenic chemicals and ionising radiation, or from spontaneous deamination. If left unrepaired, DNA mismatches can introduce mutations into the genome.⁹

DNA crosslinking (**Figure 1.1F**) is a highly cytotoxic form of DNA damage. Various lesions can result from DNA crosslinking such as interstrand crosslinks (ICLs), intrastrand crosslinks as well as DNA mono-adducts and DNA protein crosslinks.¹⁰ Crosslinking can be induced by endogenous agents, including reactive aldehydes and products derived from lipid peroxidation,^{11, 12} or exogenous agents such as environmental mutagens and anticancer chemotherapeutic agents.¹³ Although such agents may produce a variety of crosslinking lesions, cytotoxicity is primarily associated with ICLs owing to the fact that the complementary strands of the DNA duplex are covalently coupled together.¹⁴ This blocks the separation of the two DNA strands that is required for essential cellular processes, such as DNA replication and transcription. To counteract these adverse effects, sophisticated DNA repair pathways have evolved. ICL repair is thought to predominantly occur in a replication-dependent manner.¹⁴ However, there is increasing evidence for a replication-independent pathway of ICL repair that may be vital to ICL removal in non-cycling cells such as neurons.¹² Anticancer therapeutics, such as psoralen,¹⁵ mitomycin C¹⁶ and cisplatin,¹⁷ exploit the cytotoxicity of ICLs, inducing such crosslinks in tumour cells. However, overexpression of enzymes involved in ICL repair in certain cancers leads to resistance to such chemotherapeutics, rendering treatment ineffective.¹⁸⁻²¹ Therefore, the inhibition of enzymes involved in ICL repair is a potential strategy for the treatment of chemotherapy resistant cancers.

1.1.1. Interstrand Crosslink Repair

ICL repair typically involves sequential excision of the lesion from one DNA strand, then the other.²² The structure and position of an ICL play a major role in how the lesion is recognised and subsequently repaired.²³ Various factors from different DNA repair pathways cooperate to recognise and completely remove ICLs from DNA, including

proteins from the nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR), homologous recombination (HR), translesion synthesis (TLS), transcription coupled repair (TCR), and the Fanconi anaemia (FA)/breast cancer (BRCA) cancer predisposition pathways.¹⁴ The method of ICL detection and choice of repair pathway depends on the type of lesion that is detected and the phase of the cell cycle. In the synthesis phase (S phase), the stage of the cell cycle dedicated to DNA replication, ICL repair occurs in a replication-dependent manner.¹⁴ There are currently three main models for replication-dependent repair: collision of a single replication fork with an ICL, dual collision of converging replication forks and replication fork traversal past the ICL.²⁴ On the other hand, in the absence of replication in the gap 0 (G₀) or gap 1 (G₁) phases, ICLs are thought to be repaired *via* a replication-independent pathway.¹⁷ Regardless of the type of lesion and method of detection, ICL repair mechanisms are complex and require the coordination of multiple DNA repair pathways.

Although considerable progress has been made in the field of ICL repair, the precise functions of numerous repair proteins, as well as the order of events within these pathways, are not yet fully understood. Overlap and redundancy within the different repair pathways complicates the delineation of precise functions for individual repair proteins.

1.1.2. SNM1A – A Key Enzyme in Interstrand Crosslink Repair

Human sensitive to nitrogen mustard 1a (SNM1A) is a 5'-3' exonuclease that plays a pivotal role in DNA damage repair.²⁵ The importance of SNM1A in ICL repair is highlighted by the fact that cells depleted in this enzyme show increased sensitivity towards several crosslink-inducing agents, including mitomycin C and cisplatin.^{26, 27} In fact, SNM1A has been implicated in numerous repair pathways, acting as both an exonuclease²⁸ and an endonuclease.²⁹ There is also evidence of SNM1A playing a role in transcription-dependent ICL repair³⁰ as well as reports of involvement of SNM1A in cell cycle checkpoints,^{31, 32} telomere maintenance³³ and DSB repair.³⁴ The role of SNM1A in replication-dependent ICL repair is illustrated in **Figure 1.2**. The current models depict the joint action of replication protein A (RPA), Xeroderma pigmentosum complementation group F (XPF) in complex with excision repair cross complementation group 1 (ERCC1) and SNM1A to facilitate ICL unhooking.

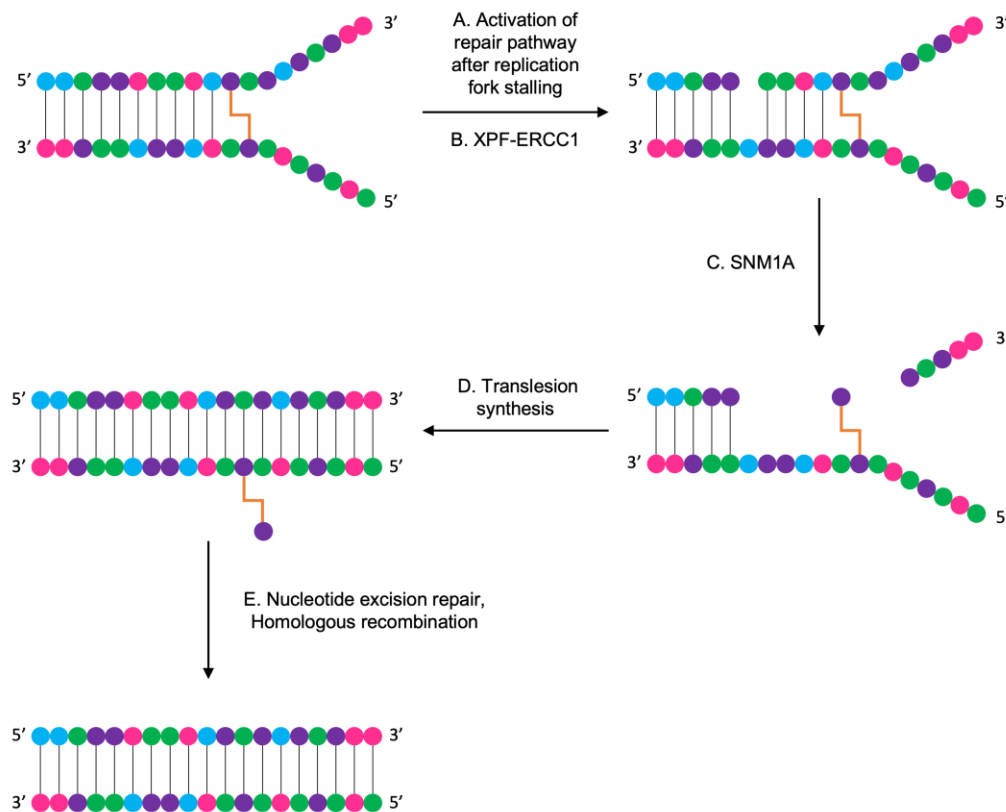


Figure 1.2: The role of SNM1A in replication-dependent ICL repair.^{24, 28} A. Stalling of a replication fork at an ICL initiates ICL repair. B. Endonuclease XPF-ERCC1 is recruited and makes an incision. C. Exonuclease SNM1A is recruited and digests the strand of DNA in a 5'-3' direction. Processes such as D. translesion synthesis, E. nucleotide excision repair and homologous recombination complete DNA repair.^{28, 30, 35, 36}

Upon encountering an ICL, a single replication fork stalls approximately 20 nucleotides away from the site of the lesion, due to steric constraints of the replication machinery helicases.³⁷ Replication fork stalling then initiates the ICL repair pathway (**Figure 1.2A**). Following helicase unloading, the replication fork can then proceed to within one nucleotide of the site of the ICL.³⁸ In the presence of RPA, endonuclease XPF-ERCC1 generates an incision in the DNA strand six nucleotides 5' to the ICL site (**Figure 1.2B**).²⁸ SNM1A is then recruited to the site of the ICL. Interactions with proliferating cell nuclear antigen (PCNA)³⁹ and protein inhibitor of activated signal transducer and activator of transcription 1 (PIAS1)²⁷ have been implicated in the recruitment of SNM1A to sites of DNA damage. SNM1A initiates digestion of the DNA backbone in a 5'-3' direction,²⁶ bypassing the ICL and unhooking the lesion, thus leaving a single mononucleotide tethered to the complementary DNA strand (**Figure 1.2C**). The digested strand then becomes a substrate for TLS polymerases,^{40, 41} replication fork restart and HR-directed repair of the damaged chromatid and the tethered mononucleotide is likely removed by NER (**Figure 1.2D and E**).²⁴ When two replication forks converge at the site of an ICL,

repair follows a similar pathway. A strand of double-stranded DNA (dsDNA) is generated, which becomes a substrate for XPF-ERCC1 incision and SNM1A lesion bypass.^{28,42} In the case of the ICL traverse model of ICL repair, a single replication fork encounters an ICL and is able to bypass the lesion and continue replication 3' to the ICL.⁴³ ⁴⁴ The structure of the post-replicative ICL-containing DNA is not known. However, it is possible that this may also be a substrate for XPF-ERCC1 and SNM1A nucleolytic processing.²⁴

Whether SNM1A acts as both an endonuclease and an exonuclease is yet to be fully understood. Endonucleases cleave phosphodiester bonds within the DNA strand, potentially at specific sites, whereas exonucleases cleave phosphodiester bonds from the end of the DNA strand, removing nucleotides one at a time. One study, utilising low concentrations of SNM1A, reported no structure-specific endonuclease activity *in vitro*.⁴⁵ However, a more recent study published by Buzon *et al.* utilised a higher concentration of SNM1A for the same quantity of DNA substrate, and structure-specific endonuclease activity of SNM1A was observed.²⁹ It is therefore possible that at lower concentrations SNM1A predominantly operates as an exonuclease, but at higher concentrations the activity has reduced specificity and some endonuclease activity can be observed.²⁴ The fact that SNM1A displays both exonuclease and endonuclease activity suggests that the enzyme may have a more extensive role in DNA damage repair than previously thought.

SNM1A also plays a role in transcription-dependent repair. Upon stalling at the site of an ICL, RNA polymerase II is bound by Cockayne syndrome B protein (CSB), which facilitates transcription-dependent repair. It has been demonstrated that CSB recruits SNM1A to ICL sites in human cells and stimulates SNM1A's exonuclease activity.³⁰ Although a comprehensive mechanism linking SNM1A and CSB has yet to be proposed, this finding shows that SNM1A also functions outside of replication-dependent repair pathways.

The role of SNM1A in DNA repair extends beyond ICL repair pathways. A recent study used proteomics of isolated chromatin segments to capture the telomeric DNA damage response proteome during break-induced telomere synthesis (BITS).³³ Interestingly, as well as the break-induced replisome, SNM1A was substantially enriched at damaged telomeres. Furthermore, it was shown that SNM1A is recruited to telomeres by ubiquitinated PCNA and SNM1A is required for end resection during BITS. Another

recent study found that SNM1A is crucial for the repair of DSBs in human cells.³⁴ Cells deficient in SNM1A displayed profound sensitivity to DSBs induced by radiation and were defective in the repair of these lesions. This prompted comprehensive characterisation of SNM1A's role in DSB repair. Taken together, these studies depict the extensive role of SNM1A in DNA damage repair, not only in ICL repair but also in telomere maintenance and DSB repair pathways.

Aside from DNA damage repair, SNM1A has also been implicated in the regulation of cell cycle checkpoints. SNM1A has been found to interact with p53 binding protein 1 (53BP1)⁴⁶ and the anaphase-promoting complex (APC),³¹ both of which have been implicated in mitotic stress checkpoints. Furthermore, it was reported that cells deficient in SNM1A failed to arrest at a mitotic checkpoint.³¹ Although its exact role has not yet been fully elucidated, these results suggest that SNM1A plays an important function in the mitotic checkpoint. SNM1A is also required for an efficient G₁ checkpoint arrest in response to ionising radiation. It has been observed to localise to the site of DSBs induced by ionising radiation, in a manner dependent on interaction with the ataxia telangiectasia mutated (ATM) kinase.³² SNM1A has also been shown to be a phosphorylation substrate of ATM *in vitro* and to interact with ATM *in vivo*, particularly after exposure of cells to ionising radiation.³² In addition, in the absence of SNM1A, the activation of the downstream ATM target p53 is reduced.³² These findings suggest that SNM1A acts with ATM to promote the G₁ cell cycle checkpoint.

There is increasing evidence of SNM1A playing a pivotal role in many clinically relevant pathways including ICL repair, telomere maintenance, DSB repair and cell cycle checkpoints. While the precise function of the enzyme in ICL repair remains unclear, the fact that cells depleted in SNM1A show increased sensitivity to ICLs induced by anticancer agents^{26,27} makes SNM1A an ideal target for inhibition to specifically sensitise cells to ICL-inducing agents.

1.2. SNM1A – Structure and Function

In order to gain deeper insights into the function of SNM1A, it is important to examine its structure as well as the structures of closely related enzymes. The founding member of the sensitive to nitrogen mustard 1 (SNM1)/sensitive to psoralen 2 (PSO2) nuclease family was identified in the early 1980s.⁴⁷⁻⁵⁰ Genetic screens utilising *Saccharomyces*

cerevisiae unveiled mutant strains that displayed sensitivity to bifunctional alkylating agents, while remaining unaffected by monofunctional alkylating agents, ionising radiation, or ultraviolet (UV) light. The mutated loci, *pso2* and *snm1* were later found to be allelic.⁵¹ To date, ten *pso* genes have been identified, all of which play a role in DNA damage repair.⁵² There are three vertebrate analogues of SNM1/PSO2: SNM1A, SNM1B/Apollo and SNM1C/Artemis (**Figure 1.3**).⁵³⁻⁵⁵ All are members of the metallo- β -lactamase (MBL) structural superfamily of enzymes.⁵⁶ The characteristic structural motif of MBLs is a fold domain enabling transition metal-binding which is essential for enzyme activity.⁵⁷ A subset of the nucleic acid processing MBLs is delineated into the self-defining MBL-associated cleavage and polyadenylation specificity factor (CPSF), Artemis, SNM1, PSO2 (β -CASP) subfamily. They have an appended MBL domain and an inserted β -CASP domain.⁵⁶ Although MBLs are often associated with bacterial antibiotic resistance, in humans MBLs perform a wide variety of functions including amide, ester, thioester and phosphodiester hydrolysis.⁵⁷



Figure 1.3: Conservation of the catalytic domains of yeast PSO2 and human orthologues SNM1A, SNM1B/Apollo and SNM1C/Artemis. The MBL domain is shown in dark blue with the canonical MBL motifs (1-4) indicated in light blue. The β -CASP domain is displayed in orange and motifs A-C of this region are in yellow. Adapted from Baddock *et al.* 2020.²⁴

1.2.1. Active Site and Mechanism of Action

There are three main structural features that are important for the activity of SNM1A: a metal-containing active site, a phosphate binding pocket and a distal DNA binding groove. Whether SNM1A contains one or two metal ions in its active site, as well as the identity of these metal ions, has not yet been elucidated. A crystal structure of truncated SNM1A obtained by Allerston *et al.* (Protein Data Bank (PDB): 5AHR),⁵⁸ shows a single zinc(II) ion in the active site, coordinated by three histidine (His) residues and one aspartic acid (Asp) residue (**Figure 1.4A**). This is supported by another crystal structure of truncated SNM1A (PDB: 4B87) that also shows one zinc ion in the active site.

However, crystal structures of SNM1A in complex with ceftriaxone, an inhibitor of the enzyme (PDB: 5NZW and 5NZX), show one nickel(II) ion in the SNM1A active site. It is postulated that the active form of SNM1A accommodates a second metal cation, possibly zinc, as in the crystal structure of SNM1B (PDB: 5AHO) (**Figure 1.4B**).^{45, 58} All residues involved in the coordination of the zinc ions in SNM1B are conserved in SNM1A. These residues are all in the same conformation except for one residue, Asp736, which corresponds to Asp35 in SNM1B (**Figure 1.4A and B**). This difference in conformation may be due to the fact that Asp35 in SNM1B is hydrogen bonded to an asparagine residue that is absent in SNM1A. Activity of SNM1A is dependent on Asp736 as substitution of this residue leads to loss of enzymatic activity.^{26, 45, 59} It has been proposed that in SNM1A the second zinc ion is more loosely bound and hence not observed in the crystal structure of the protein. The two metal ions likely activate a water molecule or hydroxide ion for nucleophilic attack on the phosphodiester backbone, and potentially serve to stabilise the build-up of negative charge on the phosphodiester as it undergoes hydrolysis (**Figure 1.4C**).⁶⁰ SNM1A also contains a phosphate binding pocket adjacent to the active site. This is a region of positive charge that interacts with the terminal phosphate at the 5'-end of substrate DNA. This terminal phosphate moiety in the substrate is required for SNM1A activity.⁵⁹ Furthermore, SNM1A contains a distal DNA binding region (**Figure 1.4D**), a wide groove that can accommodate large DNA strands containing bulky ICL lesions.⁵⁸ Mutational analysis identified lysine (Lys) residues in this distal DNA binding region that are required for efficient digestion of DNA.⁵⁸

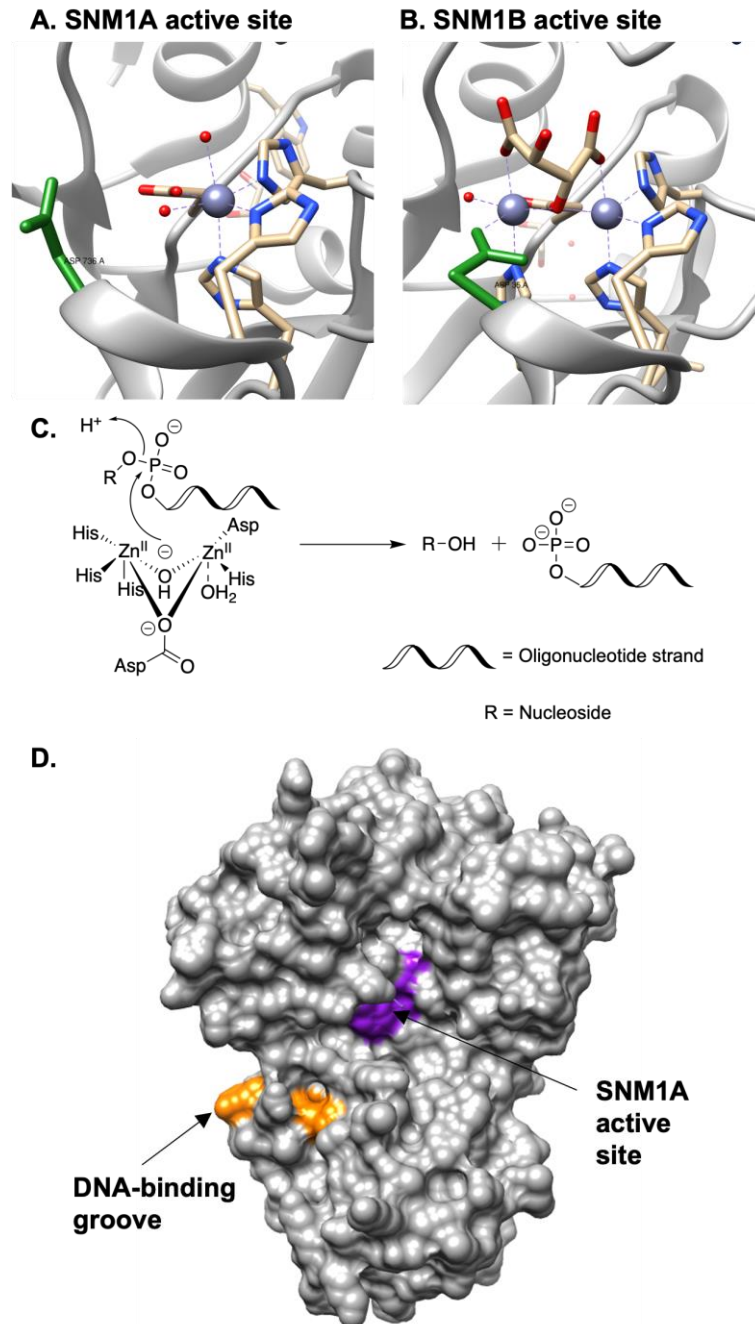


Figure 1.4: Structure of SNM1A. A. Crystal structure of SNM1A (PDB: 5AHR) showing the active site. A single zinc ion is coordinated by three His residues and one Asp residue. Asp736 (green) is not in the correct conformation for coordination to a second metal ion. B. Crystal structure of SNM1B (PDB: 5AHO) showing the active site containing two zinc ions. Asp35 (green) corresponds to Asp736 in SNM1A but is in a different conformation, allowing it to coordinate the second zinc ion. C. Putative mechanism for the hydrolysis of DNA by SNM1A based on the mechanisms of other metallo- β -lactamase enzymes.⁶⁰ D. Crystal structure of SNM1A (PDB: 5AHR) showing the active site (purple) and distal DNA-binding groove (orange). Graphics created using UCSF Chimera.⁶¹

1.2.2. Need for Tools to Study SNM1A

Given its variety of roles in vital cellular processes, such as DNA damage repair and cell cycle checkpoints, SNM1A is a potential candidate for the development of targeted

therapies. SNM1A has been associated with both cancer risk and survival outcomes. Several genetic studies have identified single nucleotide polymorphisms (SNPs) in the human *SNM1* gene as being associated with cancer risk. For example, one SNP was tenuously associated with increased small cell lung carcinoma risk.⁶² Another SNP was identified as a candidate for predisposition to breast cancer.⁶³ Increased expression levels of SNM1A have also been associated with decreased survival rates in ovarian cancer.⁶⁴ Although the exact role of SNM1A in cancer has not yet been elucidated, no doubt it is a potential target in the treatment of cancers that have become resistant to traditional crosslinking chemotherapeutics. Cells depleted in SNM1A show increased sensitivity to crosslinking agents including cisplatin,²⁵ chlorambucil,²⁵ mitomycin C²⁶ and SJG-136.²⁶ However, much remains unknown about SNM1A including its exact functions, posttranslational modifications and interaction partners. Research in this area would be accelerated by the availability of improved tools for its investigation.

The study of SNM1A presents significant challenges due to its notably low expression levels and the inherent difficulty in achieving stable overproduction from mammalian cells.^{46, 59, 65} To date, *in vitro* biochemical and crystallographic studies of SNM1A have relied on recombinant human SNM1A produced from insect cells,^{58, 59} yeast cells²⁶ or truncated human SNM1A expressed in *Escherichia coli*.²⁶ SNM1A expressed in different organisms may lack essential post-translational modifications specific to human cells, which may modulate its functionality. Truncation of SNM1A may impact its enzymatic activity and its interactions with other proteins, a phenomenon well-documented in the context of other truncated proteins and often manipulated in protein engineering.⁶⁶⁻⁷⁰ Furthermore, conventional *in vitro* studies may not fully capture the intricate interplay between SNM1A and various interaction partners which may be present in cellular environments. There is, therefore, an unmet need for methods and tools to enable the study of SNM1A.

1.3. Utilising Metal-Binding Groups to Study Enzyme Activity

As SNM1A employs metal ions for its enzymatic activity, targeting these metal ions with metal-binding groups (MBGs) is a promising approach for studying this enzyme. In fact, the incorporation of MBGs into inhibitors and probes has proven an effective method of targeting a vast array of metalloenzymes to date.⁷¹⁻⁸² The following section provides an

overview of inhibitors and probes that target metalloenzymes through binding to their active site metal ions.

1.3.1. Activity- and Affinity-Based Protein Profiling

Molecular probes featuring MBGs have proven effective in the targeting of a variety of enzymes that utilise metal ions for catalysis.⁷¹⁻⁷⁶ Activity-based probes (ABPs) and affinity-based probes (AfBPs) are particularly powerful tools for the identification of enzymes in biological samples and have enabled a range of discoveries including the characterisation of new enzymes and drug targets.⁸³⁻⁸⁵ Such probes typically consist of three key elements: a chemical warhead or reactive group, which binds to the enzyme; a linker region that confers specificity, usually by mimicking the natural substrate of the target enzyme; and a reporter tag for enrichment or visualisation (**Figure 1.5**).

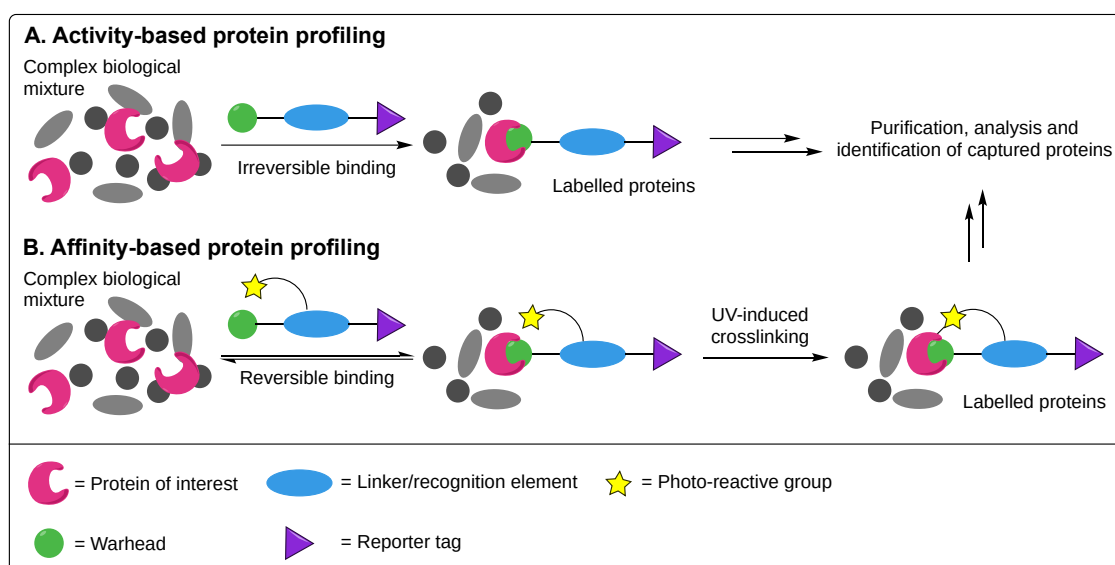


Figure 1.5: Activity- and affinity-based protein profiling are powerful techniques that enable the identification of new enzymes and drug targets. A. Activity-based protein profiling. Irreversible binding of the warhead enables direct labelling of the protein of interest. B. Affinity-based protein profiling. Reversible binding of the warhead to the protein of interest is followed by UV irradiation which enables the crosslinking of the photo-reactive group to the protein, bringing about covalent protein labelling. Once proteins are covalently labelled, uncaptured proteins may be removed and labelled proteins may be analysed.

Enzymes with conserved nucleophilic residues in the active site may be targeted with electrophilic warhead groups.⁸⁶ Irreversible protein binding occurs when the electrophilic warhead reacts with the nucleophilic residue to form a covalent probe-protein complex (**Figure 1.5A**). Enzymes lacking in a catalytically active nucleophilic residue, such as metalloenzymes, may be targeted using AfBPs (**Figure 1.5B**).⁸⁵ Metalloenzymes contain a metal centre which is vital for catalysis and may be targeted with warheads

incorporating MBGs. This results in a reversible, non-covalent probe-protein interaction. Inclusion of a photo-reactive group, such as a diazirine or benzophenone moiety,^{71, 73, 87-93} enables covalent capture of the probe-protein complex upon irradiation with UV light.^{94, 95} A linker region connects the warhead, reporter tag and photo-reactive group (if included). The linker region can increase selectivity of the probe *via* the incorporation of a recognition element, often inspired by natural substrates or inhibitors of the enzyme of interest. The reporter tag is usually a fluorescent group or an affinity tag,⁹⁶ and may be present during labelling or can be added in a subsequent step using a bioorthogonal reaction.⁹⁷⁻¹⁰⁰ Once target proteins are covalently labelled, uncaptured proteins may be removed. The reporter tag allows for target proteins to be identified and analysed *via* a variety of techniques such as gel electrophoresis-based analysis, mass spectrometry (MS) based analysis, fluorescence microscopy or non-invasive imaging *in vivo*.¹⁰¹

Although probes for various metalloenzymes have been reported,⁷¹⁻⁷⁶ to date, there are no reported probes targeting SNM1A or any of the SNM1 family of enzymes. Valuable insights into methods to build suitable tools to study SNM1A can be drawn from established inhibitors and probes for metalloenzymes.

1.3.2. Targeting Metalloenzymes with Metal-Binding Groups

Due to the essential roles played by metalloenzymes in critical cellular processes, the generation of metal-binding inhibitors and AfBPs targeting these enzymes has been thoroughly investigated. Matrix-metalloproteases (MMPs) are a class of hydrolytic enzymes dependent on zinc ions for their activity.⁷⁷ Many substrate-mimic inhibitors, featuring MBGs, have been reported for MMPs including the broad spectrum inhibitors marimastat and GM6001 (**1** and **2**, **Figure 1.6**).⁷⁷ They both consist of a peptide scaffold containing a hydroxamic acid MBG to target the zinc-containing active site. These inhibitors inspired the design of HxBP-Rh (**3**, **Figure 1.6**), a broad spectrum probe that targets MMPs.⁷¹ The HxBP-Rh probe incorporates a hydroxamic acid MBG, a photo-reactive benzophenone group and a rhodamine reporter tag. HxBP-Rh was shown to inhibit several MMPs with potencies that were only slightly lower than that of marimastat and GM6001 (**Figure 1.6**), demonstrating that the incorporation of the benzophenone and rhodamine groups have minimal effect on metal binding. Moreover, it has been shown that replacing the rhodamine reporter tag with an alkyne handle increases the labelling efficiency of these probes.⁹³

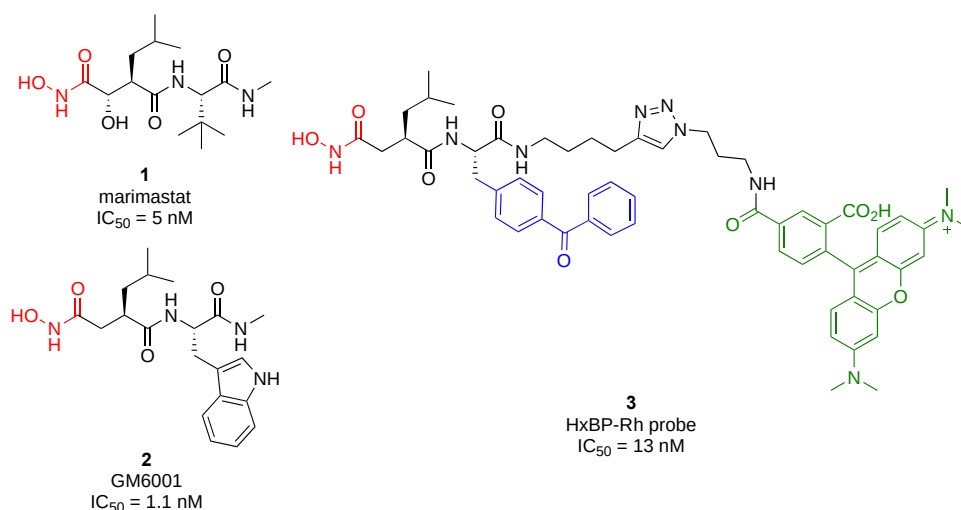


Figure 1.6: Inhibitors and an inhibitor-based probe, featuring MBGs, for targeting MMPs. IC_{50} values are for inhibition of MMP-2 by **1**, **2** and **3**.^{71, 77} MBGs depicted in red, photo-reactive group in blue and reporter tag in green.

AfBPs based on inhibitor frameworks integrating MBGs have also been explored for histone deacetylase (HDAC) enzymes (**Figure 1.7**). This class of enzymes utilises a catalytic zinc ion to deacetylate histone Lys residues. Suberoylanilide hydroxamic acid (SAHA) (**4**) is a reversible HDAC inhibitor with a hydroxamate moiety that acts through chelation of the active site zinc ion.^{72, 73} SAHA-BPpyne (**5**) is a HDAC AfBP based on the SAHA molecular template, incorporating a benzophenone group for photoaffinity labelling and an alkyne moiety as a handle for latent affinity tag incorporation.^{72, 73} SAHA-BPpyne showed effective labelling of HDAC in multiple complex biological systems including cell lysate and live cells.

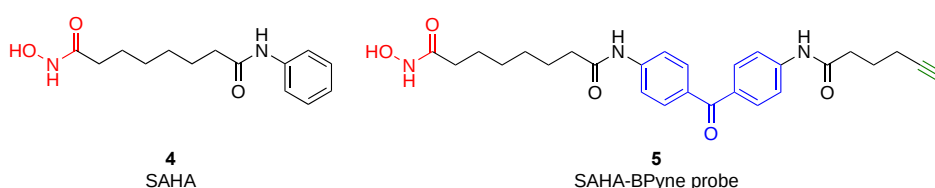


Figure 1.7: An inhibitor and an inhibitor-based probe, featuring MBGs, for targeting HDACs.^{72, 73} MBGs depicted in red, photo-reactive group in blue and affinity tag in green.

MBLs, the subclass of metalloenzyme to which SNM1A belongs, have also been explored using molecular probes. As MBLs have primarily been studied in the context of their role in bacterial antibiotic resistance, reported substrate mimic probes are based on β -lactams, modified with an alkyne handle.⁷⁴ However, examples of MBLs being targeted with inhibitors and probes incorporating MBGs have been described (**Figure 1.8**). A multistep docking methodology followed by experimental validation successfully

identified a series of NDM-1 inhibitors based on a sulfonamide scaffold.⁷⁸ These inhibitors, particularly **6**, inspired the design of a library of zinc-binding AfBPs, including **7**, for NDM-5 and NDM-7.⁷⁶ These photoaffinity probes incorporate various zinc-binding groups onto a previously described azidonaphthalimide template with a built-in fluorophore and photo-crosslinker.^{76, 102} The examination of inhibitors and inhibitor-based probes for MBLs, as well as metalloenzymes in general, provides valuable insights into how MBGs can be used to target these enzymes and how such an approach may be applied to target SNM1A.

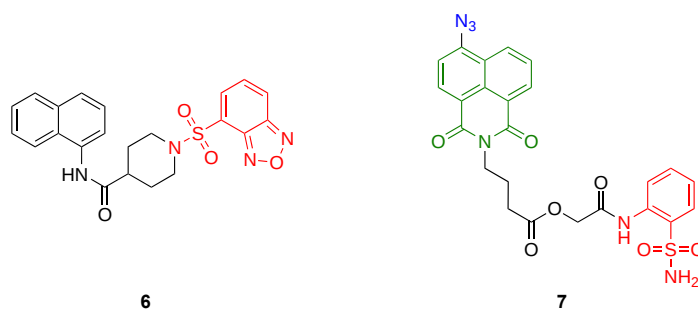


Figure 1.8: Inhibitor and inhibitor-based probe, featuring MBGs, for targeting MBLs.^{76, 78} MBGs depicted in red, photo-reactive group in blue and reporter tag in green.

Like SNM1A, human immunodeficiency virus 1 (HIV-1) integrase is both a metalloenzyme and a nucleic acid-processing enzyme. Therefore, examination of inhibitors and probes targeting HIV-1 integrase may provide useful insights into appropriate methods of targeting SNM1A. HIV-1 integrase utilises a di-metal active site for catalysis. The diketoacid motif, as seen in **8** and **9** (**Figure 1.9**), has proven successful in metal binding and HIV-1 integrase inhibition.^{79, 80} A number of photoaffinity probes based on the diketoacid inhibitor scaffold have been developed for this enzyme, incorporating various photo-reactive groups.^{81, 82} A biotinylated probe has also been synthesised to enable identification of binding sites of these diketoacid inhibitors (**10**).¹⁰³ The use of MBGs is, therefore, a suitable approach for targeting nucleic acid-processing enzymes that rely on metal ions for catalysis and has the potential to be applied in the study and characterisation of SNM1A.

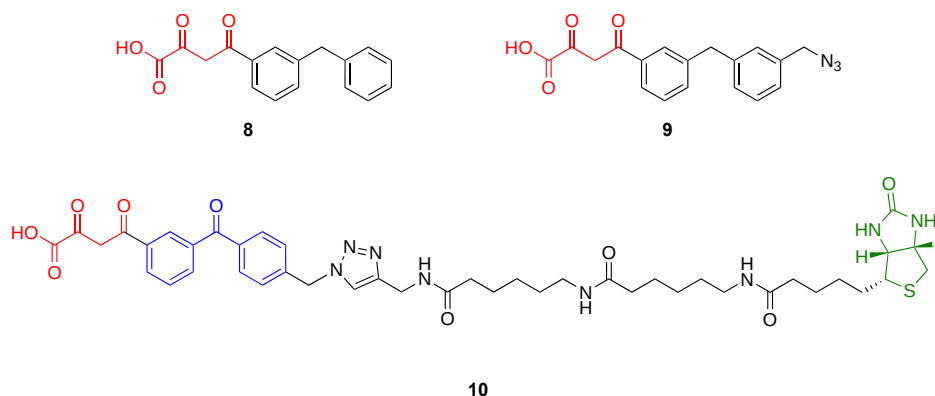


Figure 1.9: Inhibitors and an inhibitor-based probe, featuring MBGs, for targeting nucleic acid-processing enzyme HIV-1 integrase. ⁷⁹⁻⁸² MBGs depicted in red, photo-reactive group in blue and reporter tag in green.

1.4. Nucleoside and Oligonucleotide Modification to Study Enzyme Activity

Nucleic acid-processing enzymes hold significant relevance in disease prevention and cancer treatment, making them an important class of enzymes for investigation. Despite this, tools to study these enzymes are limited and, in particular, activity-based protein profiling (ABPP) of these enzymes has yet to be extensively explored. A rational strategy in the design of inhibitors and probes targeting nucleic-acid processing enzymes is the utilisation of substrate-inspired nucleoside and oligonucleotide scaffolds. Several instances demonstrate the application of modified nucleosides/oligonucleotides as inhibitors and probes for nucleic acid-processing enzymes. A comprehensive analysis of these structures offers valuable insights into the potential avenues for developing substrate-inspired inhibitors and molecular probes targeting SNM1A.

1.4.1. Nucleosides/Nucleotides as Enzyme Inhibitors

Nucleoside-based enzyme inhibitors play a crucial role in modern medicine. Many important antiviral and anticancer therapies involve the use of nucleoside-based enzyme inhibitors.¹⁰⁴⁻¹⁰⁸ Typically, nucleosides act as pro-drugs, undergoing conversion to their active phosphate derivatives in cells. Although many nucleoside-based inhibitors operate through targeting nucleic acid-processing enzymes, diverse mechanisms exist through which these compounds can function such as inhibition of viral genome methylation, nucleotide pool depletion and immunomodulation.¹⁰⁹

Certain viruses, such as HIV, contain genetic information which is encoded by RNA rather than DNA.¹¹⁰ Such viruses, termed retroviruses, exhibit a unique method of

replication. Once the viral genome is inside the host cell, the viral RNA is transcribed into double stranded DNA by viral reverse transcriptase. This DNA is then incorporated into the host's genome by DNA integrase, allowing viral proteins and genomic material to be produced by the host cell machinery. Reverse transcriptase plays a major role in the replication of retroviruses and is, therefore, a major target in the design of antiretroviral therapeutics.¹⁰⁸ Nucleoside reverse transcriptase inhibitors (NRTIs) are an important class of antiretroviral drug. NRTIs are classed as prodrugs as they undergo intracellular modification, converting them to the active form of the drug. Examples of well-established nucleoside-based antivirals are illustrated in **Figure 1.10**.

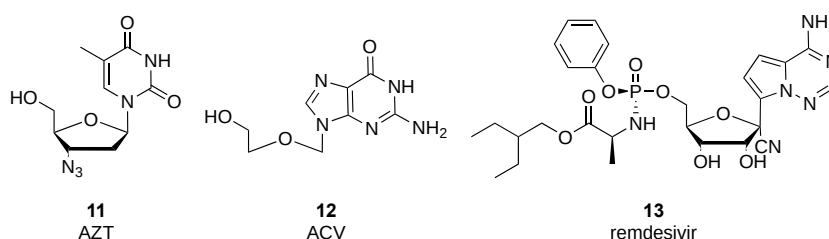


Figure 1.10: Selected antivirals based on modified nucleosides.¹¹¹⁻¹¹³

Zidovudine (AZT) (**11, Figure 1.10**) is a nucleoside prodrug active against HIV. It was approved by the U.S. Food and Drug Administration (FDA) in 1987 as an anti-HIV therapeutic and was both the first drug approved for the treatment of AIDS and the first NRTI drug.¹¹¹ AZT is a thymidine analogue in which the 3'-hydroxyl group has been replaced with an azido group. AZT is active against HIV-1,¹¹⁴ HIV-2,¹¹⁴ hepatitis B virus¹¹⁵ and Epstein-Barr virus.¹¹⁶ This NRTI completely inhibits viral DNA synthesis and is highly selective for HIV, compared to normal host cells.¹¹⁷ AZT permeates cell membranes by non-facilitated diffusion¹¹⁸ and is converted into its active form, AZT triphosphate, *via* phosphorylation by kinase enzymes.¹¹⁹ AZT triphosphate competes with deoxythymidine triphosphate for incorporation into DNA by HIV reverse transcriptase.¹¹¹ Once AZT triphosphate has been incorporated into the DNA strand, the absence of a 3'-hydroxyl group leads to chain termination.

Acyclovir (ACV) (**12, Figure 1.10**) is a nucleoside-based drug which is active against herpes simplex virus (HSV). ACV is an acyclic guanosine derivative. In HSV infected cells, ACV is phosphorylated into its active triphosphate form.¹¹² ACV triphosphate competitively inhibits viral DNA polymerase by acting as an analogue to deoxyguanosine triphosphate (dGTP). Incorporation of ACV triphosphate into the HSV DNA results in

chain termination.¹¹² ACV has also shown to be effective in the deceleration of HIV progression.¹²⁰ In HIV infected cells, ACV is phosphorylated and the active ACV triphosphate acts as an inhibitor of HIV reverse transcriptase.¹²¹

Recently, interest in nucleoside-based inhibitors has surged due to the approval of remdesivir for the treatment of COVID-19. Remdesivir (**13**, **Figure 1.10**), a broad-spectrum antiviral drug, is an adenosine derivative with some distinct chemical modifications. The ribose ring is modified with a 1'-cyano group which increases inhibitory activity and selectivity.¹¹³ Remdesivir is a C-nucleoside; the glycosidic chain is connected to the heterocyclic base by a C-C bond instead of the C-N bond of adenosine, making it resistant to chemical and enzymatic hydrolytic cleavage of the glycosidic bond.¹²² Remdesivir is a phosphoramidite nucleotide prodrug; the 5'-phosphate group is masked, enabling the molecule to cross the plasma membrane.¹²³⁻¹²⁶ Once inside cells, remdesivir is metabolised to an alanine metabolite. This is then hydrolysed to liberate the monophosphate derivative and subsequently phosphorylated to the active nucleoside triphosphate derivative.¹²⁷ This active nucleoside triphosphate is integrated into viral RNA by the viral RNA-dependent RNA polymerase, inducing delayed chain termination and inhibiting viral replication.¹²⁷ Remdesivir was originally investigated as a treatment for hepatitis C¹¹³ and respiratory syncytial virus.¹²⁸ However, its most prominent development occurred in the context of the Ebola virus outbreak in West Africa, in 2014.^{128, 129} Given the urgent global need for COVID-19 treatments, remdesivir underwent accelerated testing and, in 2020, the FDA granted emergency use for remdesivir to treat COVID-19.¹²⁷ While it did not prove to be a definitive cure, it has been used in some cases to mitigate the severity of symptoms in COVID-19 patients, particularly in hospitalised individuals.¹²⁷

Nucleoside-based drugs also play an important role in anticancer chemotherapy (**Figure 1.11**).¹⁰⁴⁻¹⁰⁷ One of the first anticancer nucleoside analogues to be developed was cytarabine (or cytosine arabinoside, ara-C) (**14**, **Figure 1.11**), an analogue of 2'-deoxycytidine.¹⁰⁷ Ara-C contains an arabinose ring rather than a deoxyribose ring, as in 2'-deoxycytidine. The 2'-hydroxyl group of the arabinose moiety is in a *trans* position with respect to the 3'-hydroxyl group (instead of a *cis* position, as in the ribose), causing steric hindrance to rotation of the base around the glycosidic bond.¹³⁰ Similar to NRTIs, ara-C is a nucleoside prodrug. Once ara-C has entered cancer cells, it is phosphorylated

to its active form, arabinofuranosylcytosine triphosphate (ara-CTP).^{131, 132} Cytotoxicity of the drug arises from the inhibition of DNA polymerase α or incorporation of ara-CTP into DNA.^{133, 134} Moreover, incorporation of arabinofuranosylcytosine monophosphate into DNA prevents elongation of the DNA chain.^{135, 136} ara-C is used in the treatment of acute myeloid lymphoma and non-Hodgkin's lymphoma.¹⁰⁷

Another example of a nucleoside-based inhibitor used in cancer treatment is gemcitabine (**15**, **Figure 1.11**). Gemcitabine is a 2'-deoxycytidine derivative which has been modified at the 2'-position by the substitution of two fluorine atoms. Gemcitabine must be activated *via* phosphorylation to produce the active form of the drug, gemcitabine triphosphate.^{137, 138} Gemcitabine triphosphate competes with 2'-deoxycytidine triphosphate for incorporation into the DNA of the cell. Gemcitabine triphosphate brings about masked chain termination; once a molecule of gemcitabine has been incorporated, an additional deoxyribonucleotide is added to the DNA strand and thereafter, DNA synthesis can no longer proceed.¹³⁹ DNA proofreading enzymes are unable to remove gemcitabine from this position and the drug is therefore locked into the DNA strand. Furthermore, gemcitabine diphosphate acts as an inhibitor of ribonucleotide reductase, a key enzyme in DNA synthesis.¹⁴⁰⁻¹⁴² Gemcitabine is used in the treatment of cancers such as breast cancer, ovarian cancer and pancreatic cancer.¹⁰⁷

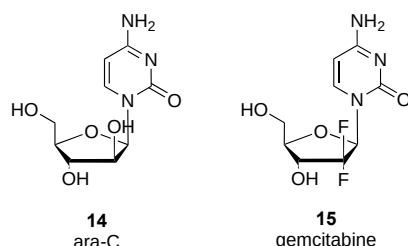


Figure 1.11: Selected anticancer drugs based on modified nucleosides.^{107, 137, 138}

Modified nucleoside inhibitors of nucleic-acid processing enzymes play a pivotal role in contemporary medicine and are significant in numerous antiviral and anticancer therapies. These specialised compounds have continued to contribute significantly to the development of modern therapeutic strategies, as exemplified by the recent approval of remdesivir as a treatment for COVID-19. Although first established in the 1950's, the use of modified nucleosides as inhibitor scaffolds remains crucial in contemporary chemical research. This approach may be potentially fruitful in the targeting of SNM1A and related enzymes.

1.4.2. Nucleosides/Nucleotides as Molecular Probes

As well as potential therapeutics, modified nucleoside/nucleotide scaffolds hold promise in the development of molecular probes to study enzyme activity (**Figure 1.12**). Extensive work has been carried out by Colman and co-workers in the development of nucleotide scaffolds modified with reactive groups to enable the study of nucleotide-binding proteins.¹⁴³⁻¹⁴⁵ The reactive groups incorporated into these probes are usually alkylating, acylating (sulfonylating), or photolytic groups. Kinases, enzymes that catalyse the transfer of adenosine triphosphate (ATP) to substrate proteins, have been well characterised using this approach. Molecular probes for kinases typically target conserved Lys residues in the active site of these enzymes.⁸⁵ Adenosine derivative, 5'-*p*-fluorosulfonylbenzoyl adenosine (5'-FSBA) (**16**, **Figure 1.12**) is a prime example of a modified nucleoside suitable for use as a proteomic tool for kinase profiling.¹⁴⁶⁻¹⁴⁹ The electrophilic fluorosulfonyl warhead reacts with nucleophilic Lys residues to form covalent adducts. 5'-FSBA scaffolds modified with a biotin tag in the 2'- or 3'- position have also been reported as ABPs for protein kinases.¹⁵⁰ Furthermore, multiple ATP-derived probes have been reported for targeting the conserved Lys residues of protein kinases (**17**, **Figure 1.12**).¹⁵¹⁻¹⁵⁷ These probes consist of an ATP scaffold with a reporter tag appended to the 5'-position *via* an electrophilic acyl phosphate warhead on the terminal phosphate of the nucleotide. Nucleophilic attack by a Lys residue and ATP release results in the covalent attachment of a reporter tag. These ABPs, based on a mononucleotide scaffolds, are typically broad spectrum probes due to the large number of nucleotide-binding proteins. Nevertheless, the utility of modified nucleosides in the generation of probes for nucleic acid-processing enzymes indicates the potential viability of this approach in the development of probes targeting SNM1A and related enzymes.

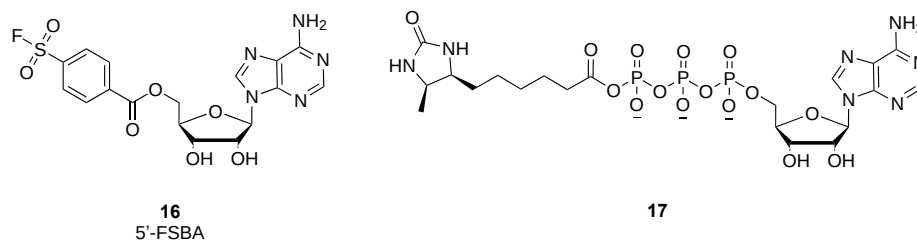


Figure 1.12: Selected molecular probes for nucleic acid-processing enzymes based on modified nucleosides/nucleotides.^{146-149, 151-157}

1.4.3. Oligonucleotides as Enzyme Inhibitors

The use of modified oligonucleotides as enzyme inhibitors or molecular probes represents a potential approach for the targeting of SNM1A. In recent years, the field of oligonucleotide therapeutics has experienced significant growth, exemplified by the development of the mRNA vaccine for COVID-19. Several oligonucleotide-based therapeutic agents have surfaced on the market,¹⁵⁸ marking substantial progress in this field. The majority of oligonucleotide-based therapeutics currently in development are antisense oligonucleotides (ASOs) or small interfering RNA (siRNA) oligonucleotides.¹⁵⁸ Both of these therapeutics target complementary mRNA strands, therefore inhibiting expression of particular proteins.¹⁵⁹ Immunomodulatory oligonucleotide therapeutics harness an often undesirable side effect of certain ASOs, interacting with proteins of the immune system *via* discrete motifs.¹⁶⁰ Aptamers are another type of modified oligonucleotide widely used in modern medicine, from diagnostics to therapeutics. Oligonucleotide aptamers fold into three dimensional structures and bind non-covalently to a variety of biological targets with high affinity. Aptamers are also known as “chemical antibodies” and, although most therapeutically relevant aptamers primarily act through inhibition of protein-protein interactions,¹⁶¹ they have also found utility as inhibitors for nucleic-acid processing enzymes.¹⁶² Numerous RNA/DNA aptamer inhibitors of HIV-1 reverse transcriptase have been reported, and their ability to disrupt viral replication has been evaluated.¹⁶³⁻¹⁷⁰

A series of oligonucleotide-based inhibitors of DNA cytosine 5-methyltransferase (DNMT) has been reported.¹⁷¹ DNMT catalyses the methylation of the C-5 position of cytosine residues in specific sequences of DNA. The oligonucleotides incorporated electrophilic C-nucleosides as warheads (**Figure 1.13**).¹⁷¹ This facilitated the attack by the catalytic Cys residue of DNMT leading to a covalent DNA-protein complex and subsequent DNMT inhibition. Although these oligonucleotides were only evaluated as enzyme inhibitors, they have the potential to undergo further modification, such as the addition of a reporter tag, to generate ABPs. The use of modified oligonucleotides as ABPs represents a promising yet underutilised strategy for the study of nucleic-acid processing enzymes.

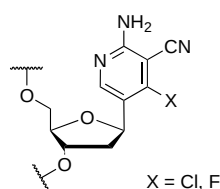


Figure 1.13: Structure of electrophilic C-nucleosides incorporated into oligonucleotide inhibitors of DNMT.¹⁷¹

1.4.4. Oligonucleotides as Molecular Probes

The use of modified oligonucleotides as molecular probes has enabled extensive investigation into the functions of nucleic acid-processing enzymes and proteins involved in DNA damage repair (**Figure 1.14**). A commonly employed strategy utilises oligonucleotides modified with a photo-reactive group. One of the first instances of this involved the modification of the *lac* operator DNA sequence with 5-bromodeoxyuridine, a photo-reactive nucleotide. Exposure to UV light resulted in covalent binding of the modified DNA sequence to the *lac* repressor protein and the specificity of this interaction was confirmed.¹⁷² This photoaffinity strategy has since been extended to facilitate the study of various DNA-protein interactions. Oligonucleotide sequences incorporating photo-reactive modifications have been designed to mimic intermediates in repair pathways, enabling capture of the proteins that bind to such structures. This strategy has been instrumental in unravelling the functions of several proteins within DNA repair pathways, including the BER pathway¹⁷³⁻¹⁷⁷ and NER pathway.¹⁷⁸⁻¹⁸¹ Furthermore, modified oligonucleotides incorporating a DNA lesion such as an ICL,¹⁸² thymidine dimer,¹⁸³ or 8-oxoguanine,^{183, 184} a photo-reactive group, and a reporter tag have also been used to study proteins involved in DNA repair (**Figure 1.14A**). The DNA lesion acts as molecular “bait” for DNA repair proteins. The photo-reactive group enables covalent capture of the protein and the reporter tag allows for analysis of the binding event and isolation of the protein responsible for lesion binding. Although these probes can facilitate the identification of proteins that are recruited to the site of a lesion, they are not specific for active enzymes. Photoaffinity probes can be employed in conjugation with complementary techniques to validate active enzymes involved in DNA damage repair.¹⁸⁴⁻¹⁸⁶

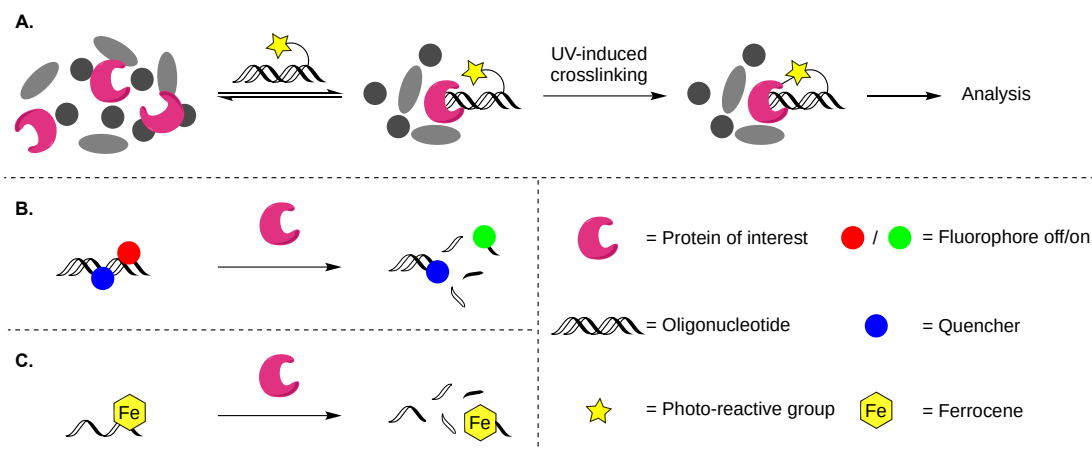


Figure 1.14: Modified oligonucleotides as probes for enzymes and proteins that interact with nucleic acids. **A.** Photoaffinity labelling of proteins using an oligonucleotide containing a photo-reactive group.¹⁷²⁻¹⁸⁴ **B.** FRET assay using an oligonucleotide probe containing a fluorophore and a quencher.¹⁸⁷⁻¹⁹³ **C.** Electrochemistry-based assay using a ferrocene-labelled oligonucleotide.¹⁹⁴⁻¹⁹⁶

Several luminescence-based strategies have been used to study proteins involved in DNA repair.¹⁹⁷⁻¹⁹⁹ Förster resonance energy transfer (FRET) based assays, utilising oligonucleotides modified with a fluorophore and a quencher, have been used in the study of nucleases and other DNA repair enzymes (**Figure 1.14B**).¹⁸⁷⁻¹⁹³ In general, enzymatic processing of the oligonucleotide causes the fluorophore and quencher to be separated, leading to an increase in fluorescence. Oligonucleotides modified with a ferrocene label have facilitated the study of nucleases using electrochemical methods (**Figure 1.14C**).¹⁹⁴⁻¹⁹⁶ Nuclease digestion of the ferrocene-labelled oligonucleotide yields lower molecular weight ferrocene-containing fragments.¹⁹⁶ These fragments diffuse faster in solution and, therefore, a quantifiable increase in current is observed.

Oligonucleotide modification is a promising strategy for the generation of molecular tools suitable for targeting nucleic acid-processing enzymes. While oligonucleotide-based photoaffinity probes can covalently capture proteins recruited to DNA lesions, oligonucleotide assays based on luminescence and electrochemical methods enable kinetic studies of enzyme activity. Although oligonucleotide-based inhibitor scaffolds present an opportunity for further refinement to produce highly effective molecular probes, there are no examples of modified oligonucleotides as ABPs reported in the literature to date. The incorporation of electrophilic warheads into oligonucleotides, as discussed in **Section 1.4.3**, successfully yielded inhibitors targeting DNMT. Such oligonucleotide-derived inhibitors could be further modified with a reporter tag to generate effective ABPs. Furthermore, there are various DNA-protein conjugation

strategies, based on modified oligonucleotides, that have yet to be explored in terms of the generation of oligonucleotide-based probes. Several DNA-protein conjugates based on reductive amination^{200, 201} or conjugate addition²⁰² of a Lys residue or disulfide formation of a Cys residue^{203, 204} have been reported. Modified oligonucleotides containing a vinylsulfonamide²⁰⁵ or chloroacetamide²⁰⁶ in their nucleobases have also been used for bioconjugation. Each of these strategies holds promise for the development of oligonucleotide-based probes. Given the success of oligonucleotide modification in probing a number of nucleic-acid processing enzymes, the application of this approach may be useful for the characterisation of SNM1A.

1.5. Targeting SNM1A with Metal-Binding Groups and Nucleoside/Oligonucleotide Modification

Due to its crucial involvement in numerous clinically significant pathways, the ability to study the activity of SNM1A is becoming increasingly important. Furthermore, as cells depleted in this enzyme show increased sensitivity to certain chemotherapeutic agents,^{26, 27} inhibition of SNM1A is a potential approach for the treatment of cancers that have developed resistance to traditional chemotherapeutics. There remains a limited number of inhibitors, and no reported probes, suited to the study of SNM1A to date. Strategies for the study of enzyme activity discussed thus far, such as the use of MBGs (**Section 1.3**) and/or the application of nucleoside and oligonucleotide modification (**Section 1.4**) have proven successful in the study of SNM1A. To date, a number of non-nucleoside as well as nucleoside/oligonucleotide inhibitors have been reported. Examination of these inhibitors may provide important insights for the future development of molecular tools targeting SNM1A.

1.5.1. Targeting SNM1A with Metal-Binding Groups

One of the first reported inhibitors of SNM1A was the metal-binder *o*-phenanthroline.⁴⁵ *O*-Phenanthroline is relatively specific towards zinc chelation,^{207, 208} and hence, the fact that it effectively inhibits SNM1A supports the hypothesis that at least one zinc ion is key for SNM1A activity. In 2016, a panel of β -lactam antibiotics, prototypical MBL inhibitors, were screened for inhibition of SNM1A and SNM1B.²⁰⁹ These compounds are believed to bind to the metal ions in MBLs through the carbonyl on the β -lactam ring and the adjacent carbonyl on the 5- or 6-membered fused ring.²¹⁰⁻²¹⁴ Interestingly, only

compounds with a cephalosporin core (7-aminocephalosporinic acid, cephalosporin C, ceftriaxone and cefotaxime, **18-21**, **Figure 1.15**) were found to be effective inhibitors of SNM1A.²⁰⁹ Compounds with a penicillin, carbapenem or monobactam core did not show inhibition of SNM1A. 7-Aminocephalosporinic acid, ceftriaxone and cefotaxime inhibited SNM1A with IC₅₀ values of 4-7 μ M and were more active against SNM1A than SNM1B. Since V_{max} was shown to be independent of inhibitor concentration, it is likely that these compounds act as competitive reversible inhibitors. Moreover, crystal structures of SNM1A in complex with ceftriaxone and cefotaxime have been reported. A crystal structure of SNM1A in complex with ceftriaxone (PDB: 5NZW) shows the inhibitor coordinating to an active site nickel(II) ion through the α,β -dicarbonyl moiety. A second crystal structure (PDB: 5NZX) shows ceftriaxone bound to SNM1A at an alternative allosteric site. A crystal structure of SNM1A in complex with cefotaxime (PDB: 5NZY) shows two molecules of the inhibitor bound to the enzyme, but neither are coordinating to the active site metal ion. Cephalosporins were designed to inhibit bacterial cell wall synthesis through an extracellular mechanism and, therefore, display low membrane permeability. This means that although they show potent inhibition of SNM1A *in vitro*, they are not suitable for *in vivo* inhibition of the enzyme. Despite this, the cephalosporin inhibitor scaffold has the potential for further modification to enhance permeability and may be expanded to generate probes enabling the study of SNM1A.

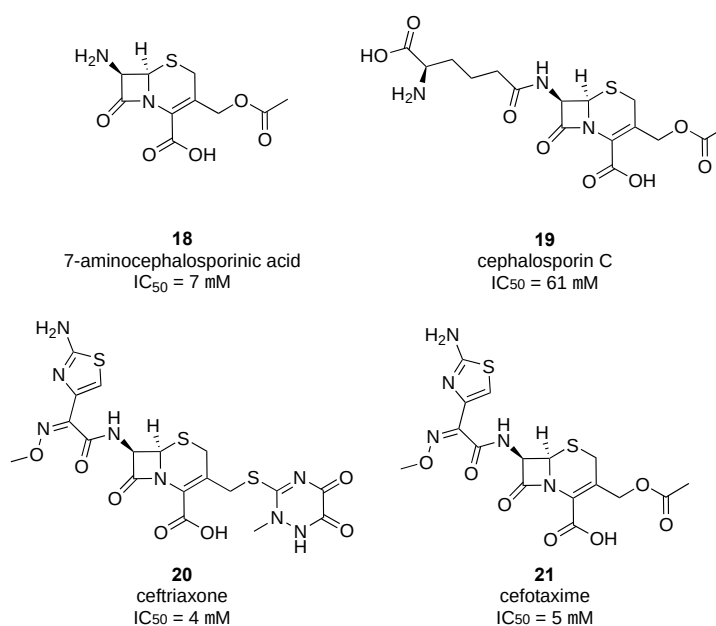


Figure 1.15: Cephalosporin derivatives that have demonstrated inhibition of SNM1A.²⁰⁹

In an attempt to identify membrane-permeable inhibitors of SNM1A, Buzon *et al.* carried out a screening of a library of 3941 bioactive compounds.²¹⁵ A number of inhibitors of SNM1A were successfully identified (**22-30**, **Figure 1.16**). However, these compounds did not show specificity for SNM1A over SNM1B. Furthermore, as they were not specifically designed to target the active site of SNM1A, their mechanism of action remains unclear. Follow-up studies evaluating these compounds in an SNM1A knockout or knockdown in cells would be useful in the investigation of their specificity for SNM1A. Structure-activity relationship studies could improve the specificity of these compounds for SNM1A over SNM1B and other nucleases. Determining inhibitor-bound structures of SNM1A would also be beneficial for elucidating the mechanism of inhibition.

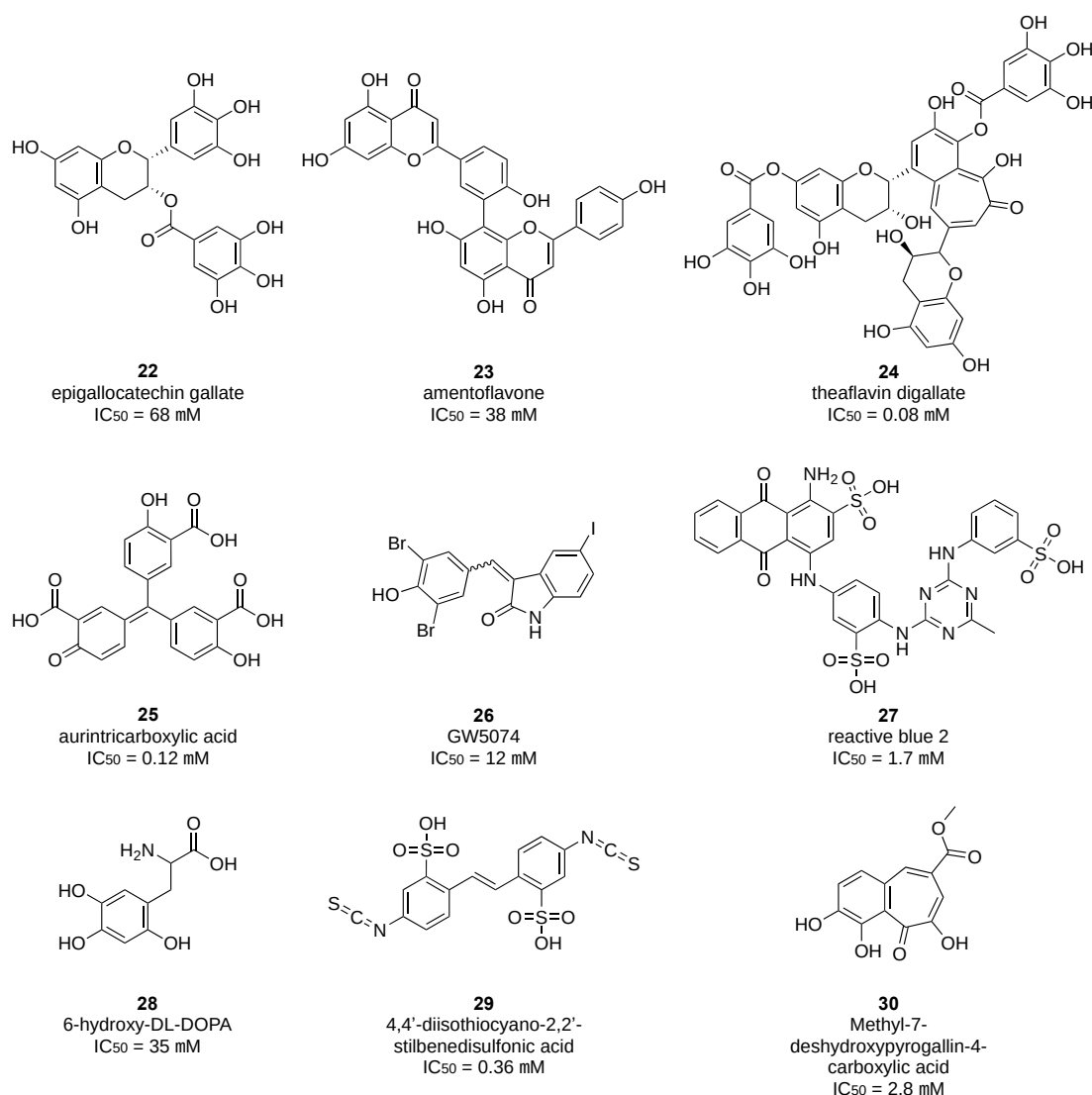


Figure 1.16: Inhibitors of SNM1A identified during a screen of bioactive molecules. IC₅₀ values calculated for inhibition of exonuclease activity of SNM1A by **22-30**.²¹⁵

1.5.2. Metal-Binding Nucleosides/Oligonucleotides for Targeting SNM1A

Various substrate-inspired nucleoside and oligonucleotide inhibitors of SNM1A have been reported by the McGouran Group.²¹⁶⁻²²⁰ These inhibitors are based on a nucleoside scaffold for recognition by the enzyme and incorporate MBGs to target the active site metal ions. These MBGs bind to the metal centre of the enzyme, inhibiting its ability to bind and digest the phosphodiester backbone of DNA. Initial efforts focused on the incorporation of a 5'-hydroxamic acid, a well-established MBG, into a nucleoside scaffold (**31**, **Figure 1.17A**).²²⁰ Thymidine derivative **31** successfully inhibited SNM1A but it was not a particularly potent inhibitor and had an IC₅₀ of only 139 μ M. Furthermore, **31** was not membrane permeable as determined by a parallel artificial membrane permeability assay (PAMPA).²²⁰ Investigations into the incorporation of the hydroxamic acid MBG through a malonate scaffold were carried out with the aim of enhancing inhibitory activity (**Figure 1.17B**). It was postulated that malonate derivatives may have the ability to coordinate to both metal ions in the active site of SNM1A. A series of modified nucleosides featuring malonate-derived MBGs were designed to inhibit SNM1A (**32-43**, **Figure 1.17B**).²¹⁸ Malonate MBGs were linked to a thymidine framework either through a carbon-carbon bond (C-linked malonates, **32-34**) or through an amide bond (N-linked malonates, **35-40**). In the case of the N-linked malonates, modification of the 3'-position (**38-40**) led to increased inhibitory activity compared to installation at the 5'-position (**35-38**). 5'-C-linked malonates (**32-34**) were the most active inhibitors of this series, with **34** displaying inhibition as low as 33 μ M. However, 3'-C-linked malonates were not generated for comparison due to synthetic inaccessibility. A number of dinucleosides containing a malonate linkage were also reported in this study (**41-43**). The dinucleosides showed a slight increase in inhibitory activity compared to their 3'-modified analogues (**38-40**). This points towards the importance of a second nucleoside residue for enzyme recognition. However, the dinucleosides were less effective inhibitors than the 5'-C-linked mononucleosides. This could be attributed to the fact that attachment of a second nucleoside residue through an amide bond prohibited the inclusion of a second carboxylate or hydroxamic acid moiety as part of the MBG. The formation of the dinucleoside may also have constrained the flexibility of the malonate, therefore preventing it from assuming the ideal conformation required for binding to the active site metal ion(s).

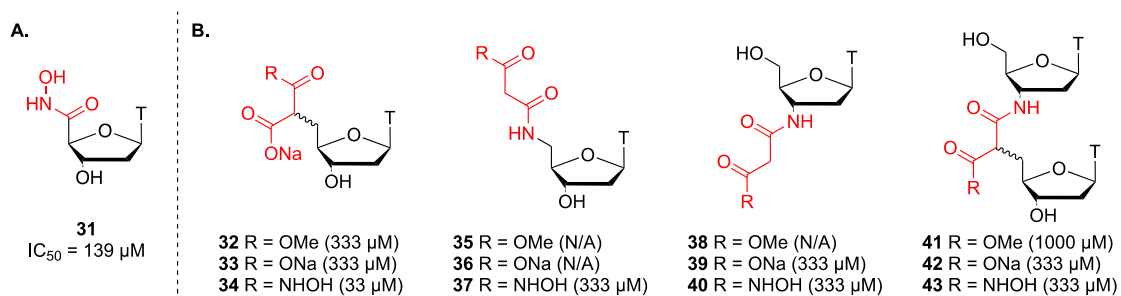


Figure 1.17: Targeting the active site of SNM1A through nucleoside modification. A. 5'-Hydroxamic acid **31** was the first lead compound identified as an effective nucleoside-based inhibitor of SNM1A.²²⁰ B. Malonate-based nucleoside inhibitors of SNM1A.²¹⁸ Numbers in brackets indicate the lowest inhibitory concentration for inhibition of SNM1A by the malonate derivatives. N/A indicates that the compound did not inhibit SNM1A at 1 mM. MBG modifications are indicated in red. T = thymine

The synthesis of nucleosides with squaramide and thiosquaramide modifications also proved an effective method of generating inhibitors of SNM1A (**44-48**, **Figure 1.18**).²¹⁶ Interestingly, nucleosides bearing a squaramide group at the 5'-position were not effective inhibitors but nucleoside derivatives **44-46**, bearing a 3'-squaramide moiety, successfully inhibited SNM1A activity. Thymidine derivative **44**, featuring an *N*-hydroxysquaramide at the 3'-position, inhibited SNM1A as low as 333 μM whereas its uridine analogue **46** only displayed inhibition at 1 mM. This finding aligns with the enzyme's well-established preference for DNA over RNA.^{45, 59} Compound **45**, featuring a squaric acid moiety at the 3'-position was a more effective inhibitor of SNM1A ($IC_{50} = 456 \mu M$) than either of the *N*-hydroxysquaramides **44** and **46**. Contrary to the trend observed for the oxo-squaramides, 5'-thiosquaramide **48** showed increased inhibitory activity ($IC_{50} = 238 \mu M$) relative to 3'-thiosquaramide **47**, which did not inhibit SNM1A at concentrations lower than 1 mM. It is possible that the incorporation of the more sterically bulky thiosquaramide moiety at the 3'-position caused the nucleobase and the deoxyribose ring to be displaced from their optimal binding position in the SNM1A active site. Of this series, 5'-thiosquaramide **48** was the most potent inhibitor. However, it did not show increased inhibitory activity compared to lead inhibitor **31** and had an IC_{50} of only 238 μM . Despite this, the series exhibited improved membrane permeability compared to lead inhibitor **31**. Squaramides **44** and **45** showed a high degree of membrane permeability, while the most potent inhibitor 5'-thiosquaramide **48** was permeable to a lesser extent.

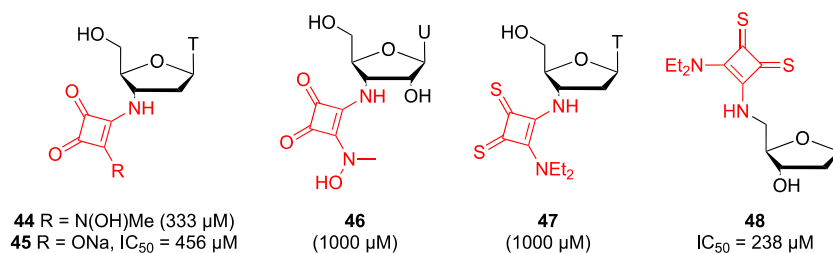


Figure 1.18: Squaramide- and thio-squaramide-based inhibitors of SNM1A.²¹⁶ Numbers in brackets indicate the lowest inhibitory concentration for inhibition of SNM1A. MBG modifications are indicated in red. T = Thymine, U = Uracil

Targeting both the active site and the phosphate-binding pocket of SNM1A has been explored by the McGouran Group through the generation of nucleosides containing both a 3'-metal binding modification and a 5'-phosphate group (**Figure 1.19**).²¹⁷ This study demonstrated that 5'-phosphorylation is an effective approach for generating nucleoside-based inhibitors with increased efficacy. Nucleotide derivatives containing a 5'-phosphate group and a 3'-2-thiophene carboxamide (**49**) or 3'-sulfonamide (**50**) displayed effective inhibition, with IC₅₀ values of 25.7 μ M and 19.7 μ M respectively, while the corresponding nucleoside derivatives with a 5'-alcohol did not exhibit effective inhibition.²¹⁷ Similarly, thymidine derivatives **52** and **54** bearing a 5'-phosphate and a 3'-squaramide were shown to be effective inhibitors of SNM1A, with IC₅₀ values of 46.7 μ M and 26.2 μ M respectively, while their previously reported unphosphorylated analogues did not display such activity.^{216, 217} This study also further explored the preference of SNM1A for DNA substrates over RNA,^{45, 59} with the results indicating that deoxyribonucleotide derivatives inhibited SNM1A more effectively than ribonucleotide derivatives. For instance, squaramide-bearing uridine derivative **51**, had an IC₅₀ of 46.3 μ M, while its thymidine analogue **53** was a more potent inhibitor, with an IC₅₀ value of 31.7 μ M. However, ribonucleotide derivatives also displayed some inhibitory activity towards SNM1A. The choice of MBG is likely an important factor as sulfonamide-containing uridine derivative **50** was the most potent of any of the nucleoside-derived inhibitors reported to date, with an IC₅₀ of 19.7 μ M. Utilising a ribonucleotide scaffold also allows for additional modification at the 2'-position, which could be valuable for future optimisation of lead compounds. The generation of inhibitors incorporating a 5'-phosphate for *in vivo* applications may be facilitated through the exploration of prodrug strategies.²²¹⁻²²⁴ While phosphate moieties typically exhibit limited membrane permeability, approaches such as the use of bio-cleavable phosphate

protecting groups,^{225, 226} or *in vivo* phosphorylation by nucleoside kinases,²²⁷ may be employed to address such challenges.

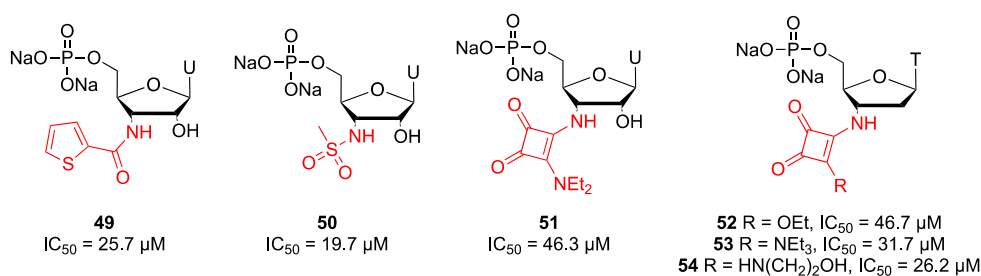


Figure 1.19: Targeting the active site and phosphate-binding pocket of SNM1A. Modified nucleotides containing a 5'-phosphate group and 3'-MBG are effective inhibitors of SNM1A.²¹⁷ MBG modifications are indicated in red. T = thymine, U = Uracil

Incorporation of MBGs into the 5'-position of oligonucleotide strands has enabled the concurrent targeting of the metal ion(s) in the active site and the DNA-binding groove.²¹⁹ Modified DNA 21mer oligonucleotides containing a squaramide at the 5'-end (**55**, **56**, **Figure 1.20**) were shown to inhibit SNM1A at much lower concentrations compared to their mononucleoside analogues, with the modified oligonucleotides showing inhibition of SNM1A as low as 80 nM.^{216, 219} It has been postulated that the oligonucleotide strand had the effect of anchoring the MBG within the active site of the enzyme. Inclusion of the oligonucleotide strand brings its own difficulties as oligonucleotide-based therapeutics face various challenges regarding membrane permeability.¹⁵⁸ However, there have been advances in enhancing oligonucleotide drug delivery including chemical modification of the DNA backbone, nucleobase, 2'-ribose substitutions, ribose modifications, bridged nucleic acids and alternative chemistries such as peptide nucleic acids (PNA).¹⁵⁸ Bioconjugation of oligonucleotides to biomolecules such as lipids, cell-targeting or cell-penetrating peptides, antibodies or sugars is another strategy to enable oligonucleotide drug delivery.¹⁵⁸ As well as the generation of inhibitors, modification of oligonucleotides with MBGs may also be used to build tools for the study of SNM1A and related enzymes.

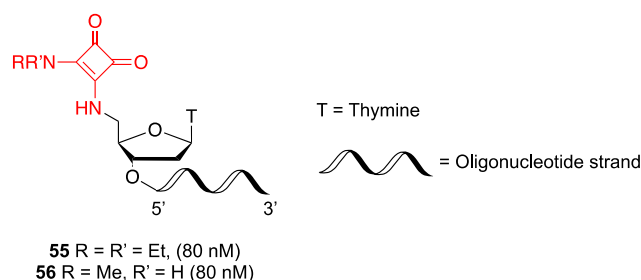


Figure 1.20: Targeting the active site and DNA-binding groove of SNM1A. Incorporation of a 5'-squaramide modification into an oligonucleotide strand generated an effective inhibitor of SNM1A.²¹⁹ Numbers in brackets indicate the lowest inhibitory concentration for inhibition of SNM1A. MBG modifications are indicated in red.

Taken together, this body of work demonstrates a novel approach for potential therapeutic development through the use of rationally designed nucleoside and oligonucleotide derived inhibitors. Although this recent progress is promising, there remains a need for the development of inhibitors of SNM1A that strike a balance between excellent membrane permeability and low IC₅₀ values. Further exploration into the use of modified nucleosides/oligonucleotides to target SNM1A is necessary. Understanding the synthesis of such modified scaffolds is crucial for the effective implementation of this investigative method.

1.6. Synthesis of Modified Nucleosides/Oligonucleotides

To date, the use of modified nucleosides/oligonucleotides has proven successful in the generation of effective inhibitors of SNM1A (**Section 1.5.2**).²¹⁶⁻²²⁰ However, further elaboration of these inhibitor scaffolds may solve membrane permeability and potency issues and facilitate the development of probes targeting the enzyme. In **Section 1.4**, diverse instances of the application of nucleoside/oligonucleotide modification for targeting nucleic-acid processing enzymes were described. These examples offer only a glimpse into the extensive spectrum of nucleoside/oligonucleotide modifications reported to date. Examination of diverse modifications, and their synthesis, may offer inspiration for the design of inhibitors and probes targeting SNM1A.

1.6.1. Synthesis of Modified Nucleosides/Nucleotides

The synthesis of modified nucleosides/nucleotides is an area of considerable interest that has been widely reviewed in the literature.^{105, 228-230} The potential sites for modification within the nucleotide scaffold are depicted in **Figure 1.21**.

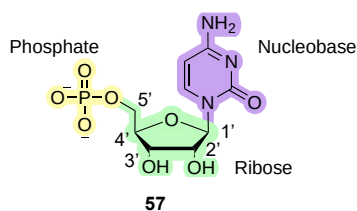


Figure 1.21: Potential sites for modification within the nucleotide scaffold.

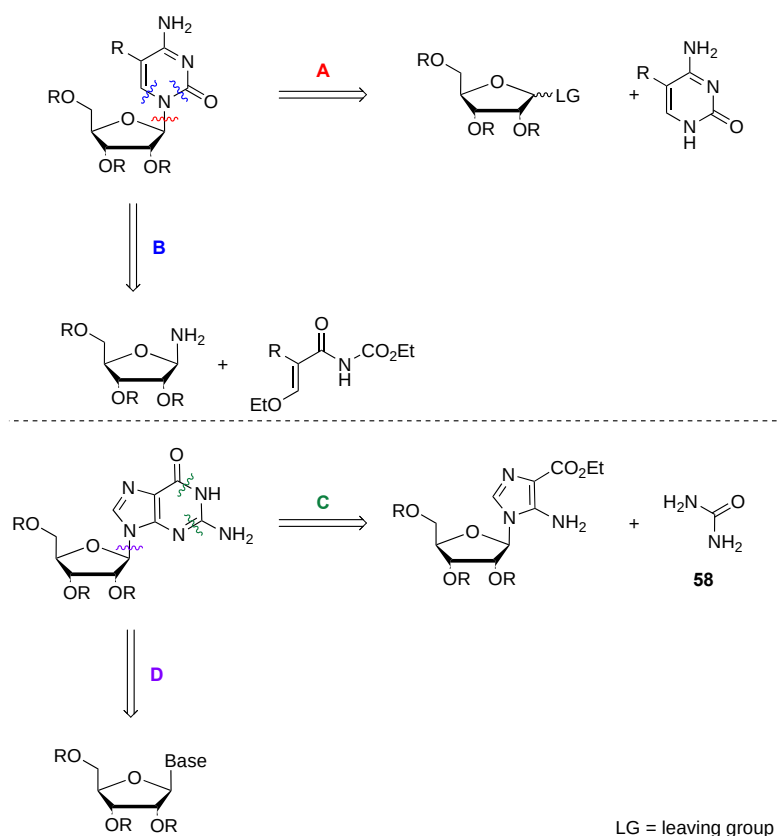
The ribose ring exhibits versatile modification avenues, including, but not limited to, substitution of the oxygen atom with a carbon or a heteroatom,²²⁸ alteration of the 5-membered ring to 3-, 4- or 6-membered configurations,²³¹⁻²³⁶ introduction of an internal or external double bond on the ring²³⁷⁻²⁴¹ and exchange of the cyclic ribose scaffold for an acyclic derivative.^{112, 120, 121} The 1'-, 2'-, 3'-, 4'- and 5'- positions also offer opportunities for modification through substitution. For example, the incorporation of fluoride,¹³⁷ methyl,²⁴² azido,¹¹¹ and cyano¹¹³ groups at various positions has been explored.

The nucleobase also offers extensive prospects for modification. C-Nucleosides, or C-glycosides, are compounds in which the C-N glycosidic bond of natural nucleosides is replaced with a C-C glycosidic bond.²³⁰ This modification was designed to tackle instability associated with the C-N glycosidic bond due to cleavage by glycoside hydrolases. Additional explored modifications include nucleobase functionalisation,²⁴¹ nitrogen substitutions and rearrangements within the nucleobase,¹¹³ utilisation of expanded purine nucleobases²⁴³⁻²⁴⁶ and the replacement of nucleobases with diverse heterocyclic moieties.²⁴⁷

Phosphate modifications explored in nucleotide modification include single-atom substitutions such as phosphorothioate, phosphoroselenoate, boranophosphate, fluorophosphate, phosphoramidate, methylenebisphosphonate and imidodiphosphate moieties, as well as their combinations and substitutions with bulkier groups.²⁴⁸ Phosphate prodrug modifications have also been explored and have proven successful in the generation of antivirals, as in the case of remdesivir (**13**, **Figure 1.10**, **Section 1.4.1**).¹²⁷

The generation of these modified nucleosides involves a diverse array of synthetic pathways. These synthetic routes, which have been widely reviewed,^{105, 228-230, 249-255} enable the creation of tailored nucleosides/nucleotides, with pivotal roles spanning from

medicinal chemistry to molecular biology. Some common disconnections towards nucleosides are depicted in **Scheme 1.1**. Nucleosides are often generated through glycosidic bond formation (**Scheme 1.1A**). Pyrimidine nucleobases can be constructed around a 1'-substituted ribose to give the nucleoside (**Scheme 1.1B**). Purines and their analogues can be constructed around a ribose ring containing an imidazole moiety at the 1'-position (**Scheme 1.1C**). Transglycosylation reactions typically transfer ribose rings from pyrimidines to more basic purines (**Scheme 1.1D**). This can be carried out either enzymatically or chemically. Although many of these methods were initially investigated for the synthesis of naturally occurring nucleic acids, they remain relevant routes in the synthesis of modified nucleosides/nucleotides.



Scheme 1.1: Common disconnections towards modified nucleoside/nucleotides.

Nucleoside/nucleotide modification can also be carried out from commercially available parent nucleoside/nucleotide scaffolds through the manipulation of protecting groups, as will be introduced in Chapter 2 (**Section 2.3.1**). Protecting groups can be strategically placed on 2'-, 3'- and 5'-OH groups as well as the exocyclic amines of the nucleobases to enable chemical modification at other positions. This approach enables the creation of a diverse range of modified nucleosides and nucleotides tailored for specific applications.

The strategic modification of nucleosides/nucleotides has emerged as a powerful strategy for the creation of inhibitors and probes targeting nucleic acid-processing enzymes. There are numerous well-established synthetic methodologies for the production of modified nucleosides and nucleotides. By utilising these established synthetic routes and integrating specific modifications, a focused and strategic approach can be devised to design inhibitors and probes targeting SNM1A.

1.6.2. Synthesis of Modified Oligonucleotides

The application of a modified oligonucleotide scaffold is also a suitable approach for the generation of inhibitors and probes targeting SNM1A (**Sections 1.4.3 and 1.4.4**).²¹⁹ The widespread use of oligonucleotide therapeutics has significantly intensified research in the field of oligonucleotide modification. Key characteristics to be considered in the design of modified oligonucleotides for therapeutic applications include binding affinity for complementary DNA/RNA strands, resistance to degradation by nucleases, membrane permeability and pharmacokinetic properties.²⁵⁶ The potential sites for modification within the oligonucleotide framework are similar to those discussed in relation to nucleosides/nucleotides (**Figure 1.21, Section 1.6.1**). Modification can occur at the ribose scaffold, nucleobase or phosphodiester backbone.

Oligonucleotides are frequently modified at the 2'-position of the ribose scaffold (**Figure 1.22**). 2'-*O*-Methyl (2'-OMe) (**Figure 1.22A**) and 2'-*O*-methoxyethyl (2'-MOE) (**Figure 1.22B**) modifications are commonly used in oligonucleotide therapeutics.¹⁵⁸ Such modifications enhance binding affinity for complementary RNA.²⁵⁷⁻²⁶⁰ Furthermore, these modifications increase nuclease resistance leading to improved stability in plasma, increased tissue half-lives and prolonged drug effects.²⁵⁷⁻²⁶⁰ 2'-Fluoro (2'-F) (**Figure 1.22C**) modification is the best mimic of the 2'-OH group in terms of size and charge.²⁶¹ 2'-F modification enhances binding affinity for complementary oligonucleotide strands but does not increase nuclease resistance.²⁶² Incorporation of other types of chemical modifications into 2'-F oligonucleotides enable enhancement of nuclease stability.

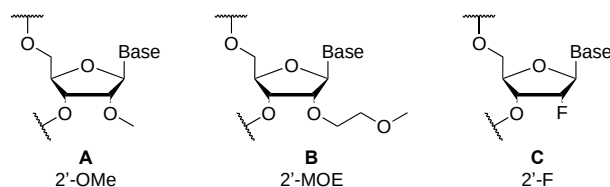


Figure 1.22: Common modifications incorporated at the 2'-position of oligonucleotides.

The ribose scaffold can also be modified in a variety of other ways (**Figure 1.23**). Locked nucleic acid (LNA) is a conformationally restricted oligonucleotide analogue bearing monomer units with a 2'-*O*-4'-*C*-methylene bridge (**Figure 1.23A**). This locks the molecule in a C-3'-*endo*-sugar configuration, ensuring the oligonucleotide adopts the A-form helix, enhancing duplex stability.²⁶³⁻²⁶⁸ LNAs enhance both nuclease stability²⁶⁹ and the affinity of the oligonucleotide for complementary RNA.²⁶³⁻²⁶⁸ Whereas LNA is conformationally restricted, unlocked nucleic acid (UNA) (**Figure 1.23B**) is an oligonucleotide modification that is highly flexible.²⁷⁰ UNA is an analogue of RNA in which the 2'-3' bond has been cleaved.²⁷⁰ Although incorporation of UNA modifications into an oligonucleotide decreases its binding affinity for target DNA/RNA, UNA can be used in combination with LNA to modulate binding affinity of modified oligonucleotides.²⁷⁰ UNA modifications have found utility in siRNA oligonucleotides used in RNA interference.^{271, 272} UNA-modified siRNA molecules were found to be stable in serum, have lower toxicity and reduce off-target effects.^{271, 272}

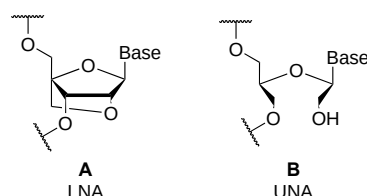


Figure 1.23: Common modifications to the ribose scaffold of oligonucleotides.

Strategies to modify nucleobase chemistry are under investigation (**Figure 1.24**). Pyrimidine methylation, the use of 5-methylcytidine (m^5C , **Figure 1.24A**) and 5-methyluridine/ribothymidine (m^5U , **Figure 1.25B**), increases the oligonucleotide's binding affinity for complementary DNA/RNA.²⁷³ Incorporation of 5-methylcytidine into oligonucleotide strands enhances nuclease stability and reduces the immunostimulatory profiles of certain DNA oligonucleotides.²⁷³ Abasic sites (**Figure 1.24C**), nucleotides lacking a nucleobase, prevent base pairing and can alleviate steric clashes. Oligonucleotides containing abasic sites have been successfully used in

siRNAs to eliminate miRNA-like off-target repression while preserving on-target activity.²⁷⁴

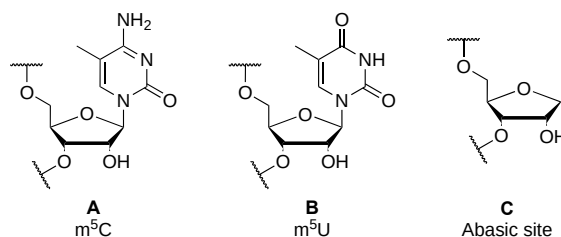


Figure 1.24: Common nucleobase modifications used in oligonucleotides.

1.6.2.1. Alternative Backbones for Oligonucleotides

In recent years, intensive research efforts have been directed toward the modification of the DNA backbone, replacing the phosphodiester bonds (**Figure 1.25A**) with alternative groups (**Figure 1.25B-J**). Alternative backbones are introduced for various purposes including to augment binding affinity to complementary strands, to increase resistance to degradation by nucleases and to improve membrane permeability, all of which are important in the development of effective oligonucleotide therapeutics.²⁵⁶

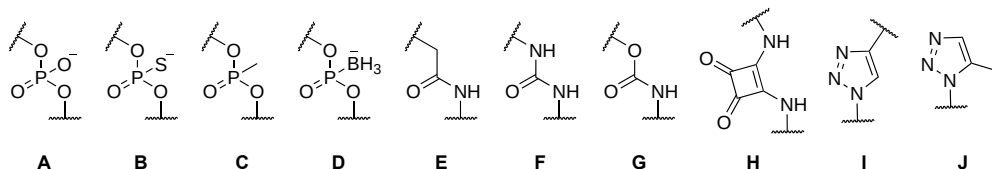


Figure 1.25: Selected alternative backbones for oligonucleotides.

Phosphorothioates (**Figure 1.25B**), in which one of the non-bridging phosphate oxygen atoms is replaced with a sulphur atom, were the first backbone modification to be extensively used in oligonucleotide therapeutics.²⁵⁶ Phosphorothioates are of particular interest as they exhibit enhanced nuclease stability compared to their phosphodiester counterparts,²⁵⁶ as well as improved pharmacokinetic profiles.²⁷⁵ Methylphosphonates (**Figure 1.25C**) also display enhanced nuclease resistance.²⁵⁶ However, methylphosphonates have low water solubility due to the replacement of one negatively charged oxygen atom with a methyl group. Boranophosphate oligonucleotides (**Figure 1.25D**), in which a borane (BH₃) moiety replaces one of the phosphate non-bridging oxygens, exhibit compelling characteristics for therapeutic applications.²⁷⁶ Boranophosphate modifications demonstrate enhanced nuclease stability^{277, 278} and

lipophilicity compared to their phosphate counterparts, while retaining high water solubility.²⁷⁹⁻²⁸¹ Despite these advantages, there are still no bioactive molecules employing boranophosphate as a substitute of the natural phosphodiester linkage.²⁵⁶ All aforementioned phosphorous-based phosphate mimics are chiral at the phosphorous atom, as one hydroxyl group is substituted by a different functional group. This increased complexity leads to heterogeneous oligonucleotide mixtures if multiple phosphodiester groups are replaced.²⁸²

Amides (**Figure 1.25E**) have also emerged as viable options for phosphate backbone replacements.²⁵⁶ In PNA, the amide backbone replaces both the phosphodiester linkage and deoxyribose sugar moieties.²⁸³ Alternatively, the amide linkage can be used to replace only the phosphodiester without replacing the deoxyribose sugar.²⁵⁶ Amide gapmers, combining amide modifications and natural phosphate linkages, have shown enhanced nuclease resistance, and their neutral backbone is lipophilic enough to allow cellular uptake, while maintaining water solubility to enable biological activity.²⁸⁴ Urea²⁸⁵ (**Figure 1.25F**) and carbamate²⁸⁶ (**Figure 1.25G**) modifications have also been investigated as suitable neutral phosphate backbone replacements stable to enzymatic hydrolysis. The use of squaramide moieties (**Figure 1.25H**) as backbone replacements is of interest due to their strong polarisation and interaction with metal ions.²⁸⁷⁻²⁸⁹ They have been employed both as standard 3'-5' linkages as well as unnatural 2'-5' linkages.^{288, 289}

Over the last few years, there has been extensive research into the generation of biocompatible oligonucleotides with a triazole linkage (**Figure 1.25I-J**) replacing the phosphodiester bond.²⁹⁰⁻³⁰³ The utilisation of various combinations involving 3'-azides and 5'-alkynes or 3'-alkynes and 5'-azides, coupled with the use of the CuAAC reaction to generate 1,4-triazoles (**Figure 1.25I**) or the Ru^{II}-catalysed azide-alkyne cycloaddition (RuAAC) reaction to generate 1,5-triazoles (**Figure 1.25J**), has enabled the generation of a vast toolbox of biocompatible triazole-containing oligonucleotides.²⁹⁰⁻³⁰³ One of the most promising is an oligonucleotide containing a 1,4-disubstituted 1,2,3-triazole modification (**Figure 1.25I**) synthesised through reaction of a 3'-alkyne and a 5'-azide. DNA with triazole modifications of this type can be replicated and transcribed by polymerases in cells while retaining high fidelity readthrough.^{296, 301-303} However, this modification displays a reduction in binding affinity to a complementary DNA/RNA

target^{295, 304-306} and induces a slowdown in PCR replications compared to other triazole modifications.³⁰³ Most recently, a modified oligonucleotide containing a 1,5-disubstituted 1,2,3-triazole (**Figure 1.25J**) generated through RuAAC reaction of a 3'-azide and 5'-alkyne was reported.³⁰⁰ This modified oligonucleotide displays good binding affinity with DNA/RNA targets and the readthrough kinetics and amplification of this linkage by DNA polymerases exceeded that of all other triazole-modified oligonucleotides.³⁰⁰

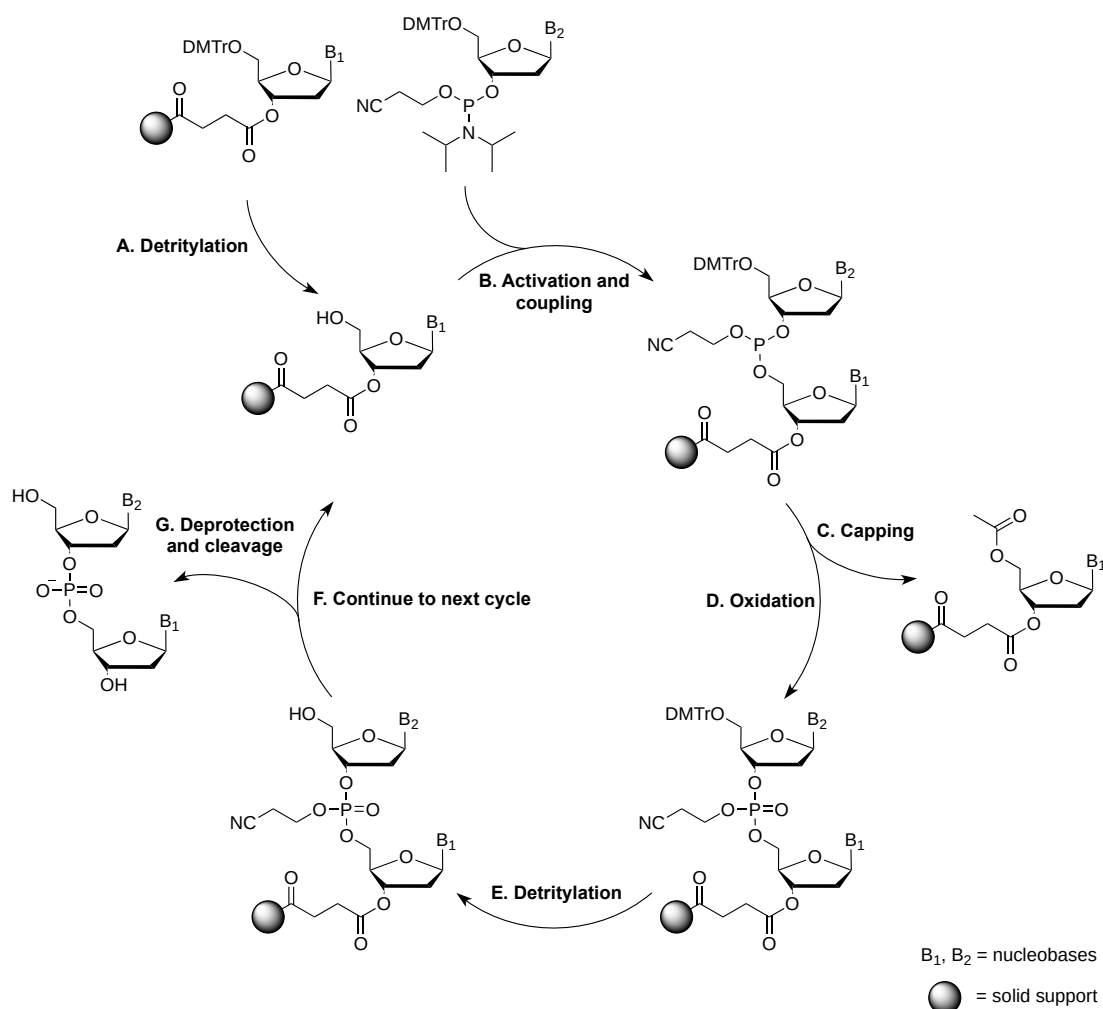
The exploration of modifications within the ribose scaffold, nucleobase or the phosphate backbone of oligonucleotides stands as a focal point in the pursuit of oligonucleotide-based therapeutics. Enhanced affinity for targets, elevated stability, improved membrane permeability and optimised pharmacokinetic properties are critical factors in the design and synthesis of oligonucleotide therapeutics. The feasibility of incorporating modifications into oligonucleotide scaffolds *via* standard methodologies is also pivotal. Modifications that seamlessly integrate into oligonucleotides using established procedures are not only scientifically compelling but also industrially relevant, making them highly favourable for widespread adoption in oligonucleotide therapeutics.

1.6.2.2. Solid-Phase Oligonucleotide Synthesis

In 1963, Bruce Merrifield pioneered the solid-phase chemical synthesis of peptides, for which he was awarded the Nobel Prize in Chemistry in 1984.³⁰⁷ Since then, solid-phase synthesis has been extensively applied in various disciplines, especially in the synthesis of peptides,³⁰⁸ oligosaccharides³⁰⁹ and oligonucleotides.³¹⁰ Solid-phase oligonucleotide synthesis (SPOS) has become an indispensable tool in both research laboratories and industrial settings due to its scalability and automation capabilities, ensuring efficiency and consistency in oligonucleotide manufacturing. Automated SPOS can be used to make small quantities (often ≤ 40 nmol), as is required for most biochemical and biological applications, but can also facilitate multi-kilogram scale synthesis of oligonucleotides intended for use as drugs.³¹¹ Synthesis of oligonucleotides incorporating non-natural functionalities can also be facilitated with SPOS, with modifications being installed either during SPOS, or post-synthetically.³¹²⁻³¹⁴

SPOS is carried out on a solid support or resin, commonly controlled-pore glass (CPG) or polystyrene,³¹¹ contained within columns equipped with filters, allowing for the unhindered passage of reagents and solvents.³¹⁵ Large excesses of solution-phase reagents can be used to drive reactions to completion quickly. Purification is performed simply by washing excess reagents off the resin surface between reactions, therefore eliminating the need for chromatographic purification between steps and greatly expediting the synthetic process. Various purification methods are used to isolate oligonucleotides after SPOS including high-performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE).³¹⁰

The phosphoramidite method, pioneered by Marvin Caruthers in the 1980s,^{316, 317} and enhanced by the application of solid-phase technology and automation, is the preferred choice for oligonucleotide synthesis. Phosphoramidite oligonucleotide synthesis proceeds in the 3'-5' direction, with one nucleotide being added per synthetic cycle. The steps involved in the phosphoramidite DNA synthetic cycle are outlined in **Scheme 1.2**.³¹⁰



Scheme 1.2: The key steps involved in synthesis of DNA oligonucleotides *via* the phosphoramidite method and solid-phase oligonucleotide synthesis.

SPOS enables the construction of oligonucleotides featuring a diverse array of modifications, thereby enhancing their functionality and adaptability for various applications. Modifications such as 2'-substitution, LNA, UNA, methylated pyrimidine and abasic modifications discussed in **Section 1.6.2**, may be incorporated into oligonucleotides *via* SPOS using the corresponding commercially available phosphoramidites. Furthermore, phosphoramidites incorporating functionalities that enable post-synthetic modifications such as azide and alkyne groups are also commercially available as well as phosphoramidites containing useful modifications such as fluorophores and affinity tags.³¹⁸ Phosphoramidites with various modifications may also be prepared *via* phosphitylation of a suitable precursor.³¹⁹ The preparation of oligonucleotides with alternative backbones primarily relies on SPOS. Artificial backbones are typically incorporated through modified on-resin coupling of a monomer to form the unnatural backbone, or by coupling of a dinucleoside phosphoramidite

featuring the artificial linkage.³²⁰ On-resin formation of artificial backbones has been reported for phosphorothioate,³¹⁰ methylphosphonate,^{321, 322} boranophosphate,²⁷⁶ amide²⁸⁴ and, to a lesser degree, triazole³²³ modifications. The dinucleoside approach is more popular for urea,²⁸⁵ carbamate,³²⁴ squaramide^{288, 289} and triazole²⁹⁰⁻³⁰³ modifications, as these linkages can be more difficult to form on a solid support.

Undoubtedly, SPOS is a remarkably valuable technique for the production of customised oligonucleotides and should be carefully considered when developing oligonucleotides suitable for use as enzyme inhibitors or molecular probes.

1.7. Project Aims and Work in this Thesis

Although a number of inhibitors of SNM1A have been reported to date,^{209, 215-220} there remains a need for inhibitors that combine high potency with excellent membrane permeability. Moreover, there is a lack of molecular tools to facilitate the characterisation of this elusive enzyme. This thesis outlines efforts to develop novel inhibitors and probes for targeting SNM1A through nucleoside/oligonucleotide modification. The aim of this work is to build on initial results in the McGouran Group which have shown that modified nucleosides/oligonucleotides incorporating MBGs can effectively target SNM1A (Section 1.5.2).²¹⁶⁻²²⁰

The aim of Chapter 2 is to expand previously reported nucleoside-based inhibitors of SNM1A through the incorporation of functionalisation handles. In principle, such handles may enable further modification of the nucleoside scaffold for the exploration of peripheral binding pockets of SNM1A and potential probe development. The objective is to generate modified nucleosides containing both an MBG, to target the active site of SNM1A, and a functionalisation handle, to allow further modification (**Figure 1.26**).

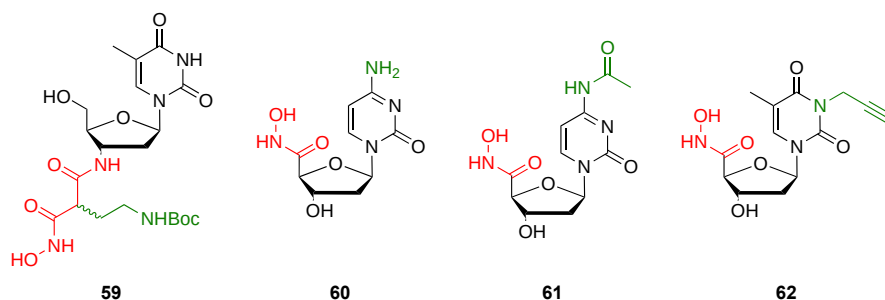


Figure 1.26: Target compounds that will be explored in Chapter 2. MBGs are indicated in red, functionalisation handles are highlighted in green.

The objective of Chapter 3 is to evaluate a series of carboxylate-rich nucleoside analogues as inhibitors of SNM1A (**Figure 1.27**). These modified nucleosides, featuring MBGs that have not yet been explored for SNM1A inhibition, were synthesised by Dr Christine Arbour, Imperiali Group, Massachusetts Institute of Technology (MIT), using a novel solid-phase synthetic method. The library includes 5-methyluridine, thymidine and 3'-azido thymidine scaffolds and incorporates succinate, *N*-acetyl-glycine, dipeptide and citrate modifications at the 5'-position for targeting the active site of SNM1A. Biochemical evaluation using a gel electrophoresis-based assay will enable identification of the most promising SNM1A inhibitors. Compounds found to be effective inhibitors may be further investigated using a real-time fluorescence assay, to determine IC₅₀ values, and a circular dichroism (CD) based assay, to validate the mechanism of action as binding to the active site of SNM1A.

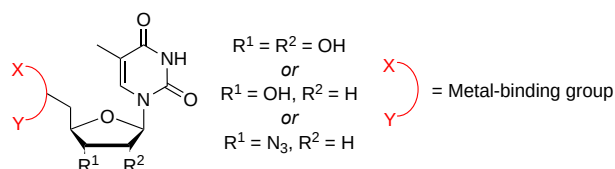


Figure 1.27: Carboxylate-rich nucleoside analogues that will be discussed in Chapter 3.

Although previous work in the McGouran Group has identified a number of nucleoside and oligonucleotide inhibitors of SNM1A (**Section 1.5.2**),²¹⁶⁻²²⁰ there remains a need for the generation of chemical tools to facilitate the study of this poorly characterised enzyme. The aim of Chapter 4 is to develop a two-step validation approach to evaluate the suitability of MBGs for targeting SNM1A (**Figure 1.28**). Initially, a fragment-based screening approach will be used to identify MBGs suitable for targeting the active site of the enzyme. Effective fragments may then be incorporated into oligonucleotides using the CuAAC reaction to generate a series of triazole-modified oligonucleotides. The modified oligonucleotides may be capable of targeting both the SNM1A active site and the DNA-binding groove. The modified oligonucleotides will be evaluated for their ability to inhibit SNM1A using a gel electrophoresis-based assay.

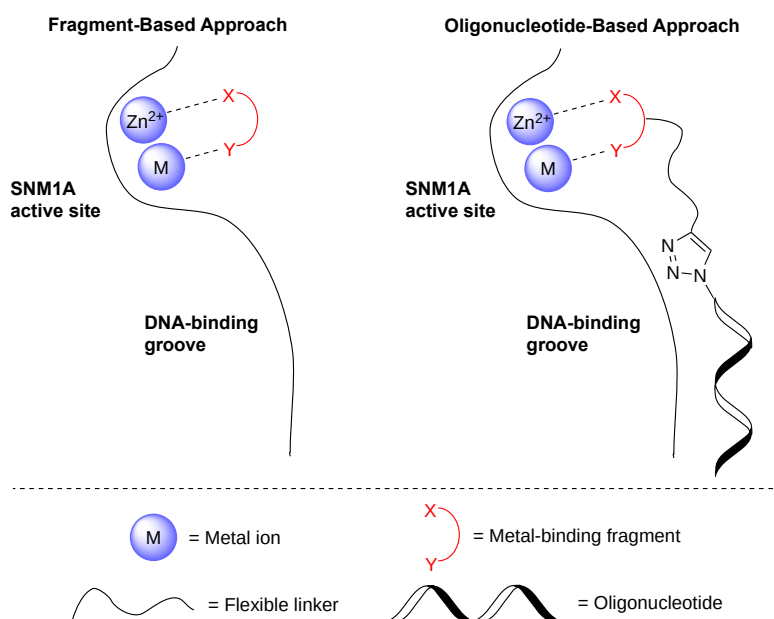


Figure 1.28: Two-step validation method for the identification of MBGs suitable for targeting SNM1A that will be discussed in Chapter 4.

The aim of Chapter 5 is to investigate a series of modified oligonucleotides, with alternative backbones, for their ability to target SNM1A (**Figure 1.29**). This work was carried out in collaboration with Dr Mark Berney, Ferguson Group, University College Dublin (UCD). The design and synthesis of oligonucleotides in which one phosphodiester linkage is replaced with a non-natural internucleotide linkage will be described. The bridging groups that will be explored include: (thio)urea, oxyurea, *N*-oxyamide, (thio)squaramide, and thio/sulfinyl/sulfonyl acetamide groups. The objective is to synthesise a number of dinucleoside phosphoramidites featuring unnatural linkages and to incorporate these phosphoramidites into oligonucleotide strands *via* manual SPOS. The ability of the modified oligonucleotides to target SNM1A will be evaluated using a gel electrophoresis-based inhibition assay and a real-time fluorescence assay.

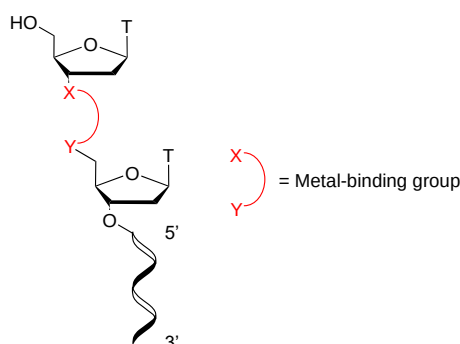


Figure 1.29: Modified oligonucleotides with non-natural linkages that will be described in Chapter 5.

Chapter 6 will conclude the work in this thesis and potential future directions will be briefly discussed.

Chapter 7 will describe experimental details and characterisation data of compounds synthesised during the course of this work.

Chapter 2

Expanding Nucleoside Inhibitor Scaffolds for Targeting SNM1A

2. Expanding Nucleoside Inhibitor Scaffolds for Targeting SNM1A

2.1. Introduction

Previous studies on cellular sensitivity to ICLs induced by anticancer agents have identified SNM1A as a potential target for cancer therapy.²⁶ However, little is understood about SNM1A and its related enzymes as there is a lack of tools to facilitate their study. To date, various substrate-inspired nucleoside and oligonucleotide inhibitors of SNM1A, containing MBGs at the 3'- and 5'-positions, have been reported (**Section 1.5.2**).²¹⁶⁻²²⁰ However, there remains an unmet need for the generation of inhibitors of SNM1A with enhanced potency and membrane permeability. The modification of reported nucleoside-derived inhibitors offers a promising avenue for addressing such challenges. The expansion of nucleoside inhibitor scaffolds also has the potential to enable the exploration of peripheral binding pockets of SNM1A. Functionalisation of core inhibitor scaffolds with secondary binding motifs has proven an effective method of generating small molecule inhibitors of enzyme classes including protein tyrosine kinases³²⁵ and MMPs.³²⁶ Furthermore, modification of established inhibitor scaffolds may allow incorporation of useful functionalities, such as a photo-reactive group or reporter tag,^{83, 84} which would enable the generation of targeted probes for SNM1A. This chapter describes the modification of established SNM1A inhibitor scaffolds in order to facilitate the exploration of peripheral binding pockets and to potentially enable the generation of probes targeting the enzyme. Target compounds featured both an MBG, to target the metal ion in the active site of SNM1A, and a functionalisation handle, to enable the expansion of the inhibitor scaffold (**Figure 2.1**). Initially, integration of a malonate-derived functionalisation handle at the 3'-position of the ribose scaffold was explored (**59**, **Figure 2.1**). Functionalisation through nucleobase modification was also investigated (**60-62**, **Figure 2.1**).

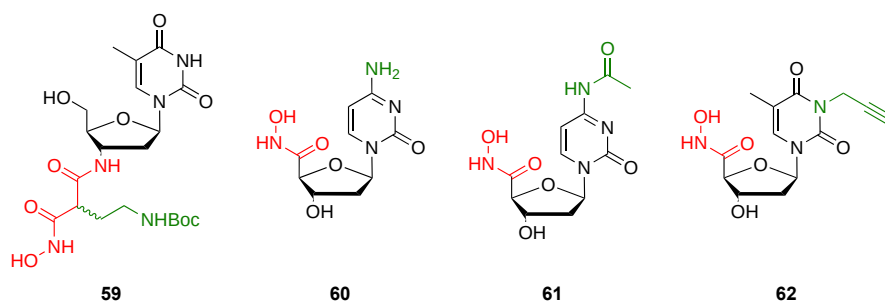


Figure 2.1: Target compounds explored in this chapter. MBGs are depicted in red while functionalisation handles are in green.

2.2. Malonate-Based Nucleosides as Tools to Study SNM1A

As nucleoside analogues featuring metal-binding modifications have proven effective inhibitors of SNM1A,^{216-218, 220} further exploration of diverse MBGs may enable the generation of inhibitors with enhanced potency. Malonate-derived MBGs are of particular interest in terms of targeting SNM1A as this functional group possesses the potential to chelate both metal ions in the SNM1A active site. The malonate group has also been previously reported as a scaffold for the hydroxamic acid group,³²⁷⁻³³⁴ a well-established MBG,³³⁵ and as a phosphate mimic.³³⁶⁻³⁴¹ Furthermore, a reported crystal structure of SNM1A (PDB: 5Q1Q), shows malonate bound to the metal ion in the active site.³⁴² The malonate scaffold also has the capacity to act as both an MBG and a functionalisation handle. Therefore, the incorporation of a malonate group into nucleoside scaffolds may generate effective SNM1A inhibitors with the potential for diversification. A series of malonate derivatives of thymidine were designed and synthesised by Dr. Eva-Maria Dürr (**32-43**, **Figure 2.2**).²¹⁸

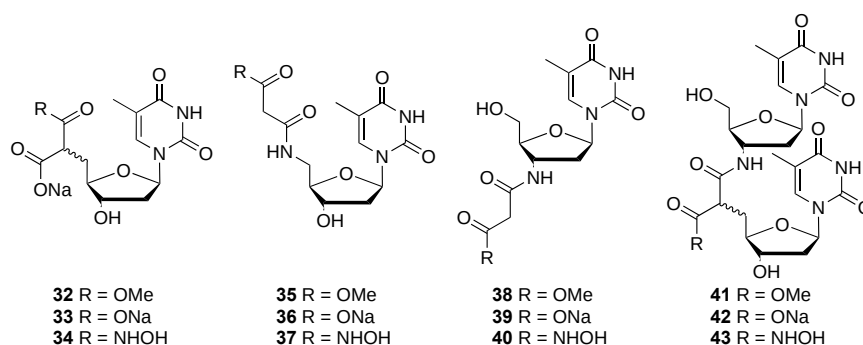
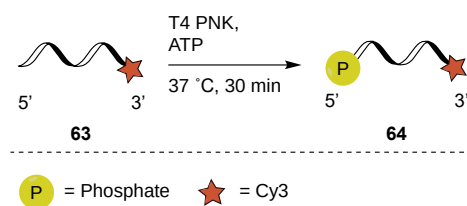


Figure 2.2: Malonate derivatives **32-43** were designed and synthesised by Dr Eva-Maria Dürr.²¹⁸

2.2.1. Biochemical Evaluation of a Series of Malonate-Based Nucleosides

The malonate derivatives **32-43** were evaluated as inhibitors of SNM1A using a previously described gel electrophoresis-based assay.^{209, 220} A 21mer oligonucleotide **63** (sequence: 5'-TTAG CAG TCA GTC AGT CAT CGY-3', Y = Cy3) labelled with a Cyanine3 (Cy3) fluorophore at the 3'-end was provided by the research group of Prof. Tom Brown, University of Oxford. Oligonucleotide **63** was phosphorylated at the 5'-end using bacteriophage T4 polynucleotide kinase (T4 PNK) to afford fluorescent substrate oligonucleotide **64** (sequence: 5'-XTAG CAG TCA GTC AGT CAT CGY-3', X = thymidine 5'-phosphate, Y = Cy3, **Scheme 2.1**).



Scheme 2.1: Enzymatic phosphorylation of oligonucleotide **63** to afford fluorescent substrate oligonucleotide **64**. Sequence of fluorescent substrate oligonucleotide **64**: 5'-XTAG CAG TCA GTC AGT CAT CGY-3', X = thymidine 5'-phosphate, Y = Cy3

Oligonucleotide **64**, containing a 5'-phosphate group and a 3'-Cy3, was then used as a substrate for SNM1A in a gel electrophoresis-based assay to determine the ability of **32-43** to inhibit SNM1A.^{209, 220} The presence of the Cy3 fluorophore enables visualisation of reaction products. A simple schematic of the assay is depicted in **Figure 2.3**.

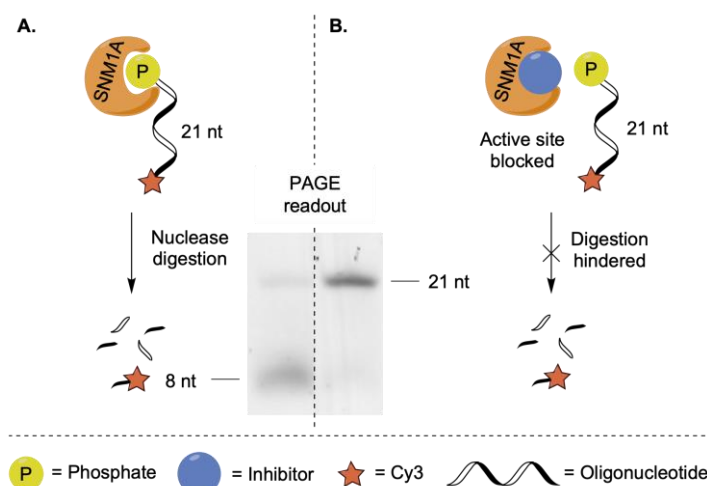


Figure 2.3: Simple schematic depicting the gel electrophoresis-based assay used to determine the efficacy of SNM1A inhibitors. A. In the absence of an inhibitor, the 21mer fluorescent substrate oligonucleotide **64** is digested and shorter fluorescent products are observed in the PAGE readout. Full activity of the enzyme results in a product 8 nucleotides in length. B. In the presence of an effective inhibitor, **64** is not digested and is visible in the PAGE readout. nt = nucleotides

In the absence of an inhibitor, SNM1A digests **64** with 5'-3' directionality to produce shorter fluorescent products (**Figure 2.3A**). SNM1A does not show significant nuclease activity on DNA strands of 8 nucleotides or less and, therefore, digestion of oligonucleotide **64** to this length indicates full activity of the enzyme in this assay.⁵⁹ In the presence of an effective inhibitor, the active site is blocked, **64** is not digested and the full length 21mer **64** is visible in the PAGE readout (**Figure 2.3B**).

Replicates of the gel electrophoresis-based assays were carried out and results were in agreement with those obtained by Dr Eva-Maria Dürr.²¹⁸ Nucleosides **32-43** (1 mM) were pre-incubated with SNM1A at 37 °C for 5 min followed by addition of the fluorescent

substrate oligonucleotide **64** and incubation was continued for 1 h. Digestion of **64** was analysed by urea PAGE (**Figure 2.4**).

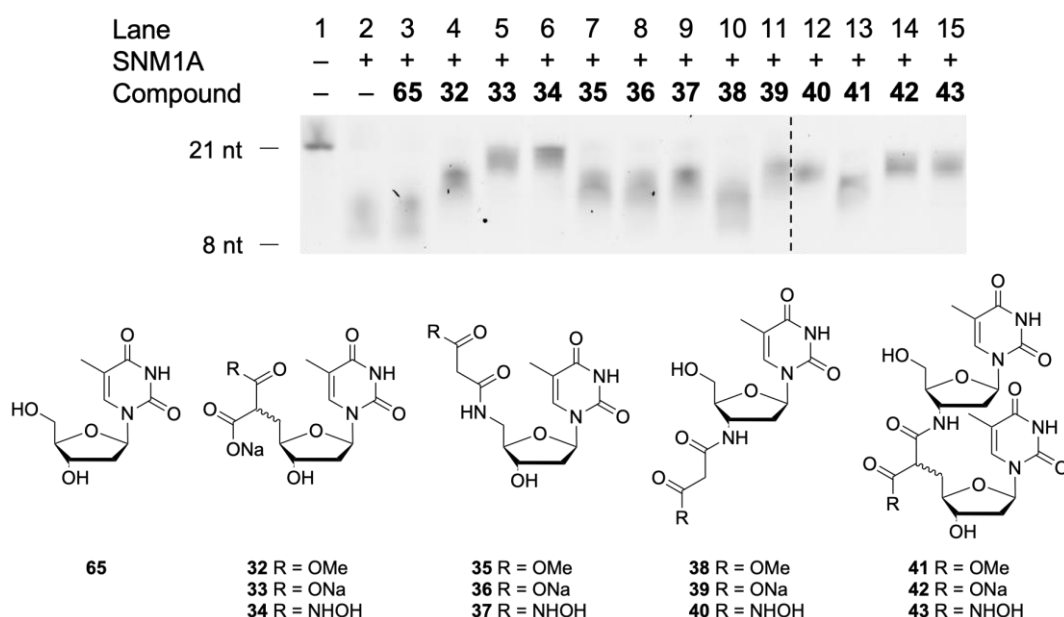
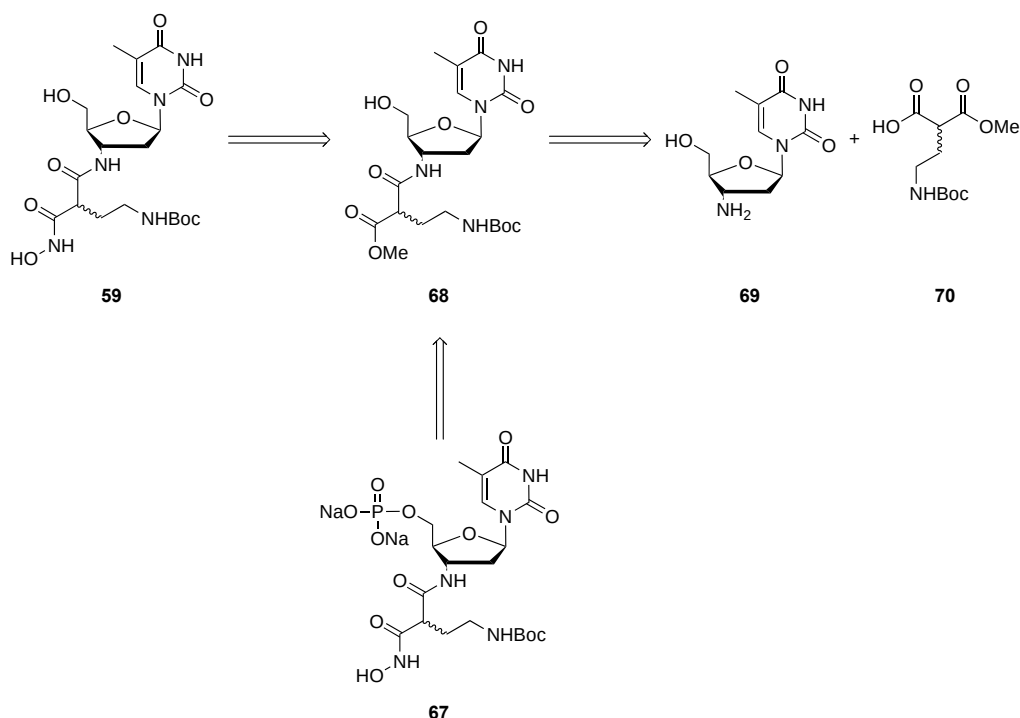


Figure 2.4: Evaluation of malonate derivatives **32-43** synthesised by Dr Eva-Maria Dürr as inhibitors of SNM1A. Thymidine, **65**, was incubated with SNM1A at 1 mM to act as a control. Nucleosides **32-43** (1 mM) were pre-incubated with SNM1A (25 fmol, 2.5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. Results are in agreement with those published by Dürr and McGouran in 2021.²¹⁸ nt = nucleotides

The negative control for digestion contained only oligonucleotide **64** and therefore the full-length 21mer can be observed in the PAGE readout (**Figure 2.4**, Lane 1). The positive control for digestion contained SNM1A and the fluorescent substrate oligonucleotide **64** and therefore oligonucleotides of approximately 8-10 nucleotides in length are observed in the PAGE readout (**Figure 2.4**, Lane 2).⁵⁹ Thymidine (**65**) was evaluated for activity against SNM1A (**Figure 2.4**, Lane 3). Digestion of **64** was seen, demonstrating that **65** did not inhibit the enzyme. It is clear from the PAGE readout that malonate-containing nucleosides can act as effective inhibitors of SNM1A, with only **38** (**Figure 2.4**, Lane 10) showing limited inhibition of enzymatic activity. In particular, the hydroxamic acid derivative of each series of compounds seems to be the most effective (**34**, **37**, **40** and **43**, **Figure 2.4**, Lanes 6, 9, 12 and 15) with **34**, containing a 5'-C-linked malonate hydroxamic acid, emerging as the most effective inhibitor (**Figure 2.4**, Lane 6). These results demonstrate that malonate derived nucleosides and dinucleosides are effective inhibitors of SNM1A and, therefore, may be considered as scaffolds for potential probes targeting the enzyme.

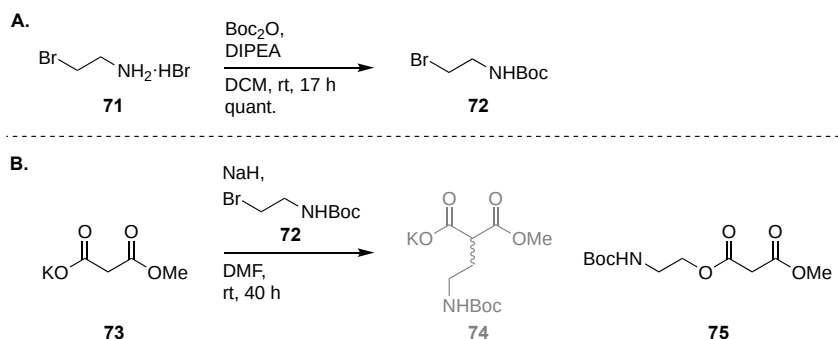
2.2.2. Expansion of the Malonate Inhibitor Scaffold

Based on the initial screening discussed in **Section 2.2.1**, **40** was selected as a suitable scaffold for modification. Expansion of this established inhibitor scaffold could enable the exploration of peripheral binding pockets of SNM1A and the generation of probes targeting the enzyme. This 3'-N-linked nucleoside has a malonate group that has the potential to act as both an MBG and a functionalisation handle. Attachment of functionalities to the malonate moiety *via* the α -carbon may be advantageous in increasing inhibitor potency or enhancing membrane permeability. Probes targeting SNM1A could be generated through the attachment of a photo-reactive group and/or a reporter tag at this position. Furthermore, incorporation of a phosphate group at the 5'-position of 3'-N-linked nucleosides may enhance their ability to inhibit SNM1A. A 5'-phosphate group in substrate oligonucleotides is essential for activity of SNM1A,^{26, 59} and it has been demonstrated that incorporation of a 5'-phosphate group improves the inhibitory activity of other nucleoside-derived SNM1A inhibitors.²¹⁷ Compound **59** and its phosphorylated derivative **67** (**Scheme 2.2**), both containing a 3'-N-linked malonate group with an extended linker and a *tert*-butoxycarbonyl (Boc) protected amino handle were designed. Evaluation of these key intermediates as inhibitors of SNM1A may determine whether modification at this position is tolerated by the enzyme. Both **59** and **67** can be generated from a common intermediate (**Scheme 2.2**). This common intermediate, **68**, can be formed from 3'-amino thymidine, **69**, and malonate derivative **70**.



Scheme 2.2: Retrosynthetic analysis of target compounds **59** and **67**.

Initial efforts focused on the synthesis of **74**, the potassium salt of **70** – a suitable candidate for amide coupling with 3'-amino thymidine (**Scheme 2.3**).



Scheme 2.3: Initial efforts in the synthesis of **74**.

Boc protection of 2-bromoethylamine hydrobromide **71** via a literature procedure using di-*tert*-butyl carbonate (Boc_2O) and DIPEA afforded compound **72** in quantitative yield (**Scheme 2.3A**).³⁴³ Attempts to synthesise **74** via reaction of **72** with monomethyl potassium malonate **73** in the presence of NaH yielded **75** instead of the desired product **74**, as confirmed by ^1H nuclear magnetic resonance (NMR) spectroscopy (**Scheme 2.3B**). Therefore, focus turned to the generation of **77** via reaction of **72** with protected malonate **76** (Table 2.1).

Removal of the benzyl (Bn) group *via* hydrogenation furnished **70** in 93% yield. 3'-Amino thymidine, **69**, was synthesised by Dr Mark Berney by reduction of AZT. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) mediated amide coupling was attempted but no reaction progress was seen by thin layer chromatography (TLC) analysis after 25 h. No additional coupling conditions were attempted.

Further testing of the malonate series **32-43** for inhibition of SNM1A at various concentrations by Dr Eva-Maria Dürr demonstrated that the lowest concentration at which **40** displayed inhibition was 333 μM .²¹⁸ However, inhibitor scaffolds must be highly active prior to further functionalisation in order to minimise any resulting loss in efficacy. It was determined that **40** lacked sufficient activity and the generation of **59** and **67** was no longer pursued. An alternative strategy, nucleobase modification, was devised and explored for targeting SNM1A.

2.3. Nucleobase Modification for Targeting SNM1A

Attention turned to lead inhibitor **31** (Figure 2.5A) for the exploration of peripheral binding pockets of SNM1A. **31** is a thymidine analogue featuring a 5'-hydroxamic acid MBG which coordinates to the metal ion(s) in the active site of SNM1A, inhibiting its ability to digest the phosphodiester backbone of DNA. **31** inhibits SNM1A with an IC_{50} of 139 μM ,²²⁰ making it a more effective SNM1A inhibitor compared to malonate derivative **40**.²¹⁸ This section describes the expansion of the **31** inhibitor scaffold *via* nucleobase modification (**60-62**, Figure 2.5B), a strategy that has not yet been explored for targeting SNM1A. Evidence that nucleobase modifications could be tolerated by SNM1A has been provided by ICL inducing agents, such as SJG-136.²⁶ SJG-136 is responsible for the covalent linkage of two strands of DNA, *via* the modification of guanine nucleobases, generating bulky lesions (Figure 2.5C).³⁴⁶ SNM1A has been shown to digest past these DNA lesions,²⁶ indicating that there is a potential binding pocket that can accommodate bulkier moieties and could favour further interactions upon nucleobase modification. The extended functionalisation of nucleoside inhibitor scaffolds would allow the exploration of this binding pocket, which may enable an increase in inhibitor potency and selectivity. With this in mind, compounds **60-62** (Figure 2.5B) were designed based on inhibitor **31**.²²⁰ Nucleosides **60** and **61** are 2'-deoxycytidine derivatives bearing 5'-hydroxamic acid groups. The free exocyclic amine of **60** can act as a functionalisation handle. Testing **61**, a precursor of **60** containing a 5'-hydroxamic

acid and an N^4 -acetyl group, as an inhibitor of SNM1A, may determine whether modification at the N^4 -position of the nucleobase is tolerated by the enzyme. Nucleoside **62** is a thymidine derivative bearing an alkyne functionalisation handle at the N^3 -position of the nucleobase. These amine and alkyne handles, as well as the 3'-OH on the deoxyribose ring, may enable further modification to enhance inhibitor potency, explore peripheral binding pockets or incorporate probe components. Testing **60-62** for inhibition of SNM1A may determine the suitability of these compounds as probe scaffolds.

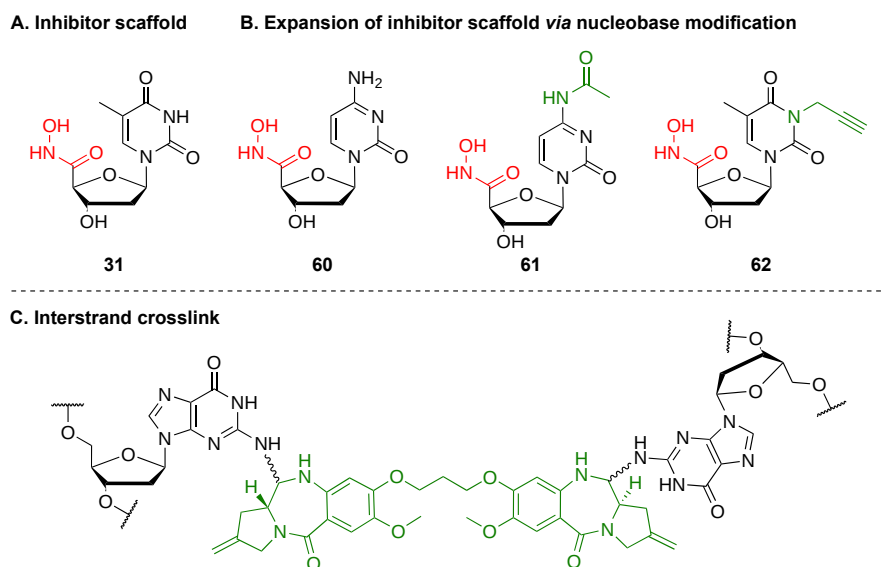
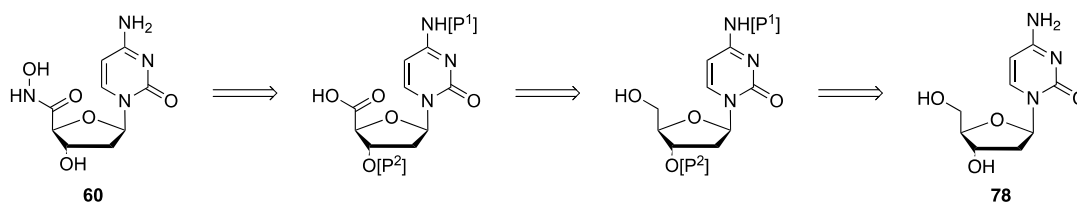


Figure 2.5: Nucleobase modification for targeting SNM1A. A. **31**, a reported inhibitor of SNM1A, contains a 5'-hydroxamic acid group.²²⁰ B. Target compounds **60-62**, based on reported inhibitor **31**. C. Structure of ICL induced by crosslinking agent SJG-136.³⁴⁶ MBGs are depicted in red while nucleobase modifications are in green.

2.3.1. Incorporation of a 5'-Hydroxamic Acid Group into 2'-Deoxycytidine

Nucleoside analogue **60** (Figure 2.5), a 2'-deoxycytidine derivative containing a 5'-hydroxamic acid moiety was designed. Modified cytidine derivatives incorporating MBGs have not yet been tested for inhibition of SNM1A and, therefore, testing of **60** prior to further modification is an important step. Moreover, direct comparison of the inhibitory activity of **60** and lead inhibitor **31** may provide insights into the nucleobase specificity of SNM1A. This strategy may also allow the evaluation of **61** (Figure 2.5) for inhibition of SNM1A. 2'-Deoxycytidine derivative **61**, a precursor of **60**, contains a 5'-hydroxamic acid and an N^4 -acetyl group therefore enabling the investigation of whether modification at the N^4 -position is suitable for the exploration of peripheral binding pockets and/or the generation of molecular probes targeting SNM1A.

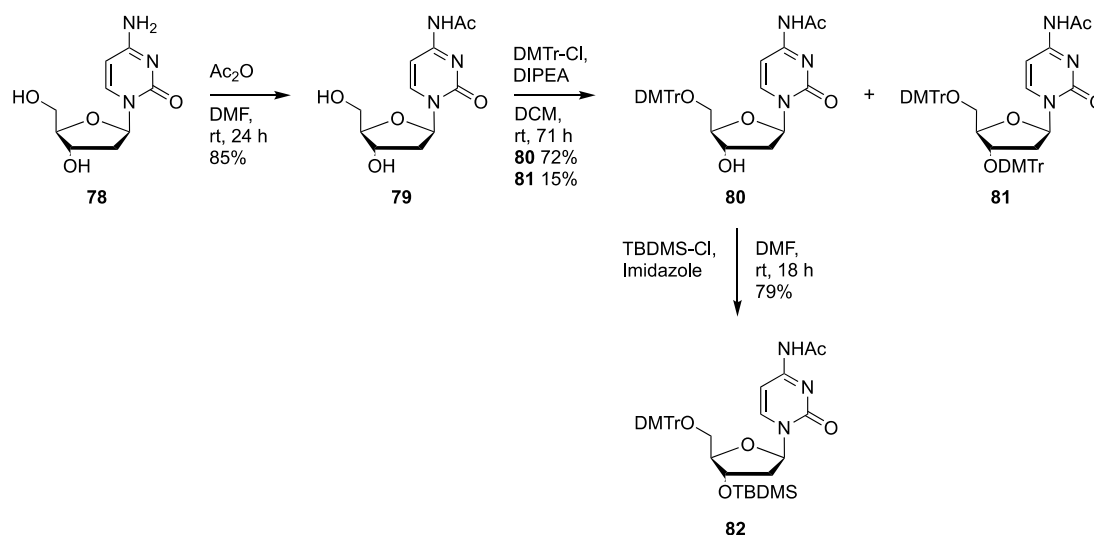
The initial synthetic strategy chosen for the generation of **60** is illustrated in **Scheme 2.5**. This synthetic strategy was based on the previously reported synthesis of lead inhibitor **31** and involved the coupling of a 5'-carboxylic acid-containing nucleoside derivative with a suitably protected hydroxylamine derivative. Full protection of 2'-deoxycytidine (**78**) was required followed by selective deprotection of the 5'-position to allow for 5'-modification. 5'-Modification included oxidation and amide coupling. Deprotection may generate the desired product **60**, containing a free hydroxamic acid.



Scheme 2.5: Retrosynthesis for the generation of target compound **60**.

The initial approach utilised the Bn protecting group for the 5'-hydroxamic acid. Nucleoside protecting groups must, therefore, be stable to catalytic hydrogenation, as required for the removal of the Bn group, as well as oxidation and amide coupling conditions. The protecting group strategy must also enable selective modification of the various sites on the nucleoside, enabling selective incorporation of probe elements. Nucleoside protecting groups were carefully selected based on their stability to conditions used during the synthesis and their extensive utilisation in nucleoside and oligonucleotide chemistry. 4,4'-Dimethoxytrityl (DMTr) was selected as a suitable protecting group for the 5'-alcohol. This group is selective for primary alcohols and is easily removed in mildly acidic conditions.³⁴⁷ *tert*-Butyl-dimethylsilyl (TBDMS) was selected for the 3'-alcohol because of its stability to the mildly acidic conditions required for DMTr deprotection, oxidation mediated by (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO) and (diacetoxyiodo)benzene (BAIB) and general amide coupling conditions. TBDMS can be easily removed using tetrabutylammonium fluoride (TBAF).³⁴⁸ The most common protecting groups used for the exocyclic amine of cytidine are acetyl (Ac) and benzoyl groups.³⁴⁹ The acetyl group was used in this case due to the fact that it is orthogonal to the DMTr and TBDMS groups and therefore allows for selective modification of the *N*⁴-, 3'- and 5'-positions. Furthermore, facile acetyl protection is carried out using acetic anhydride (Ac₂O),³⁵⁰ and deprotection is carried out under mild conditions, either using a methanolic solution of potassium carbonate or a mixture of

33% aqueous ammonia and 40% aqueous methylamine.³⁴⁹ This strategy would also facilitate the testing of **61** (Figure 2.5), a precursor of **60** containing a 5'-hydroxamic acid and an *N*⁴-acetyl group, for recognition by SNM1A. This would interrogate whether modification at the *N*⁴-position is suitable for the exploration of peripheral binding pockets and/or generation of molecular probes. The synthetic route to fully protected 2'-deoxycytidine **82** is illustrated in Scheme 2.6.



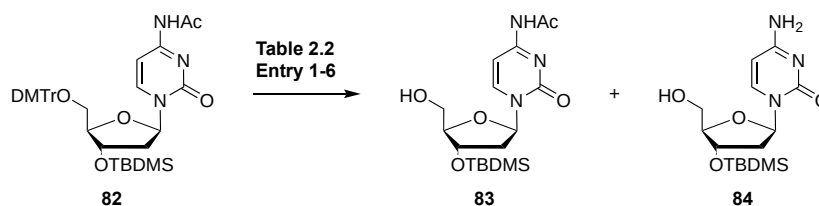
Scheme 2.6: Generation of fully protected 2'-deoxycytidine **82**.

Acetyl protection of the exocyclic amine of 2'-deoxycytidine was carried out according to a literature procedure using Ac₂O in dimethyl formamide (DMF) to generate the desired product **79** in 85% yield.³⁵⁰ DMTr protection was initially carried out using DMTr-Cl in pyridine but this afforded only 33% of **80**. An alternative method, using DMTr-Cl and *N,N*-diisopropylethylamine (DIPEA) in dichloromethane (DCM), furnished the 5'-protected nucleoside **80** in 72% yield as well as 15% of the bis-DMTr protected nucleoside **81**. The formation of **81** was unexpected as the steric bulk of DMTr tends to increase the selectivity for 5'-OH groups.^{351, 352} The generation of **81** is likely due to the high concentration of **79** used in the reaction (0.3 M) as well as the extended reaction time of 71 h. TBDMS protection yielded fully protected 2'-deoxycytidine **82** with a 79% yield.

DMTr deprotection was then carried out in order to obtain the free 5'-OH and enable 5'-modification. DMTr is highly acid labile and is routinely used in the synthesis of nucleosides and oligonucleotides.^{351, 352} Despite also being regularly used in nucleoside and oligonucleotide synthesis,³⁴⁹ the *N*⁴-acetyl group displayed unexpected acid lability

during DMTr deprotection. The N^3 -position of cytosine has a $pK_a \sim 4.2$,³⁵³ and consequently, protonation of this position in the acidic conditions required for DMTr removal may promote hydrolysis of the N^4 -acetyl group. Conditions were optimised for the removal of the 5'-DMTr group while keeping the N^4 -acetyl group intact. The optimisation of DMTr deprotection is illustrated in **Table 2.2**.

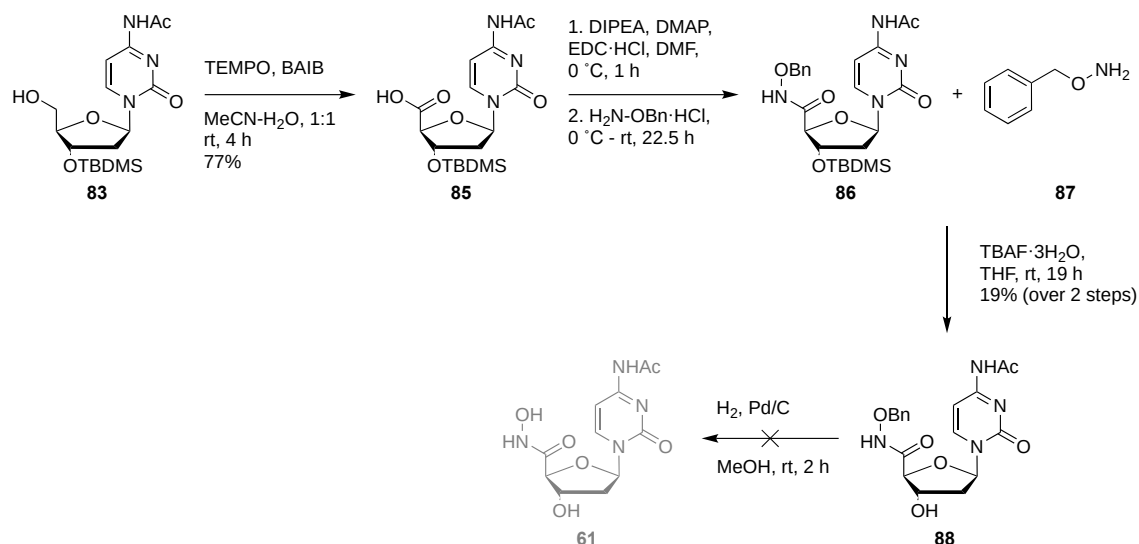
Table 2.2: Optimisation of DMTr deprotection conditions. rt = room temperature



Entry	Deprotection reagents	Temp.	Time	Result
1	80% AcOH in H ₂ O	rt	45 min	83 : 24%
2	80% AcOH in H ₂ O	rt	1.5 h	83 : 44% 82 : 28% 84 : observed by TLC
3	80% AcOH in H ₂ O	0 °C	2 h	83 : 40% 82 : 35% 84 : 25%
4	Chloroform	40 °C	24 h	Trace 83 observed by TLC
5	1% TFA in DCM, TES (5 eq.)	rt	40 min	83 : 30% 82 , 84 observed by TLC
6	3% TFA in DCM, TES (5 eq.)	rt	10 min	83 : 88%

DMTr deprotection was first attempted using 80% acetic acid (AcOH) in H₂O (**Table 2.2**, Entry 1). This was initially carried out for 45 min, after which only 24% of the desired product **83** was isolated. A longer reaction time of 1.5 h increased the yield to 44% but the undesired N^4 -deprotection product **84** was also generated (**Table 2.2**, Entry 2). Carrying out the reaction at 0 °C for 2 h did not improve the yield of desired product **83** and also generated 25% of N^4 -deprotection product **84** (**Table 2.2**, Entry 3). DMTr deprotection was also attempted by stirring in chloroform at 40 °C for 24 h (**Table 2.2**, Entry 4). This was based on a reported “warming up” strategy for DMTr deprotection,³⁵⁴ as well as observations in the McGouran Group that DMTr can be cleaved in chloroform. However, little DMTr-deprotection was observed by TLC analysis and hence, this

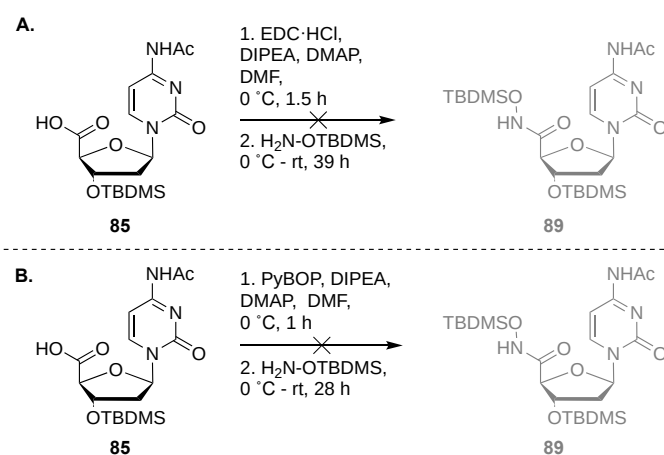
method was deemed insufficient. Finally, DMTr-deprotection was attempted using trifluoroacetic acid (TFA) in DCM with triethylsilane (TES) as a scavenger. Although 1% TFA in DCM did not improve the yield of desired product **83** (Table 2.2, Entry 5), stirring in 3% TFA in DCM for 10 min afforded 88% of **83** (Table 2.2, Entry 6). Following the generation of 5'-alcohol **83**, 5'-modification was carried out as illustrated in Scheme 2.7.



Scheme 2.7: Attempted synthesis of hydroxamic acid **61** via amide coupling with *O*-benzylhydroxylamine followed by catalytic hydrogenation.

The first step in 5'-modification was transformation of the 5'-alcohol into a carboxylic acid *via* TEMPO/BAIB mediated oxidation to furnish the desired product **85** in 77% yield. EDC mediated amide coupling with *O*-benzylhydroxylamine generated the desired product **86**. Purification of **86** was challenging as it co-eluted with *O*-benzylhydroxylamine **87** during flash column chromatography. The crude product was washed with 5% aqueous (aq.) citric acid in an attempt to remove excess amine. Caution was taken not to use harsh acid to prevent unintentional removal of the *N*⁴-acetyl group. Despite these efforts, *O*-benzylhydroxylamine could not be completely removed and the product was carried through as 5:1 **86**:**87**. Deprotection of the 3'-TBDMS group using TBAF afforded the desired product **88** in 19% yield over two steps. Deprotection of the hydroxamic acid was attempted prior to *N*⁴-deprotection to furnish hydroxamic acid **61**. The aim was for **61** to be tested alongside compound **60** in order to establish whether the exocyclic amine is necessary for recognition by SNM1A. Catalytic hydrogenation was carried out in order to generate the free hydroxamic acid **61**. During the reaction, TLC

analysis showed consumption of starting material and the precipitation of a white solid was observed. However, after filtering through celite and removing the solvent *in vacuo*, a red solid was obtained and multiple products were observed by TLC and NMR analysis. It is possible that the metal-binding ability of the hydroxamic acid moiety caused the product to be contaminated with Pd. Due to the poor solubility of the crude product purification was not feasible and hydroxamic acid **61** was not isolated. Therefore, an alternate strategy to hydroxamic acid synthesis was devised. TBDMS was selected as an alternate protecting group for the hydroxamic acid. *O*-TBDMS-hydroxylamine is commercially available and synthesis of **89** *via* amide coupling was attempted as illustrated in **Scheme 2.8**.

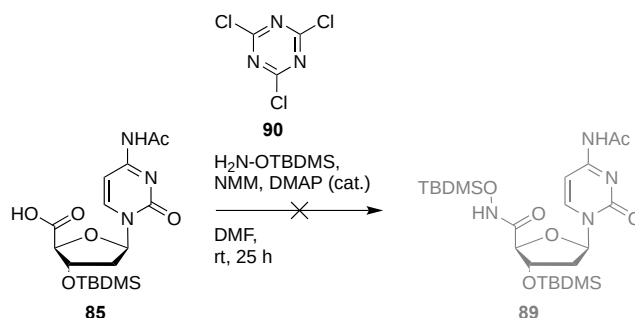


Scheme 2.8: Attempted amide coupling between **85** and *O*-TBDMS-hydroxylamine *via* A. EDC-mediated amide coupling,²²⁰ and B. PyBOP-mediated amide coupling.²²⁰

Incorporation of the TBDMS protected hydroxylamine was first attempted *via* EDC-mediated amide coupling (**Scheme 2.8A**). However, after 39 h, TLC analysis did not show any consumption of starting material and no formation of product was observed. Amide coupling was next attempted using benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as the coupling reagent (**Scheme 2.8B**). Unfortunately, after 28 h no reaction progress was observed by TLC analysis.

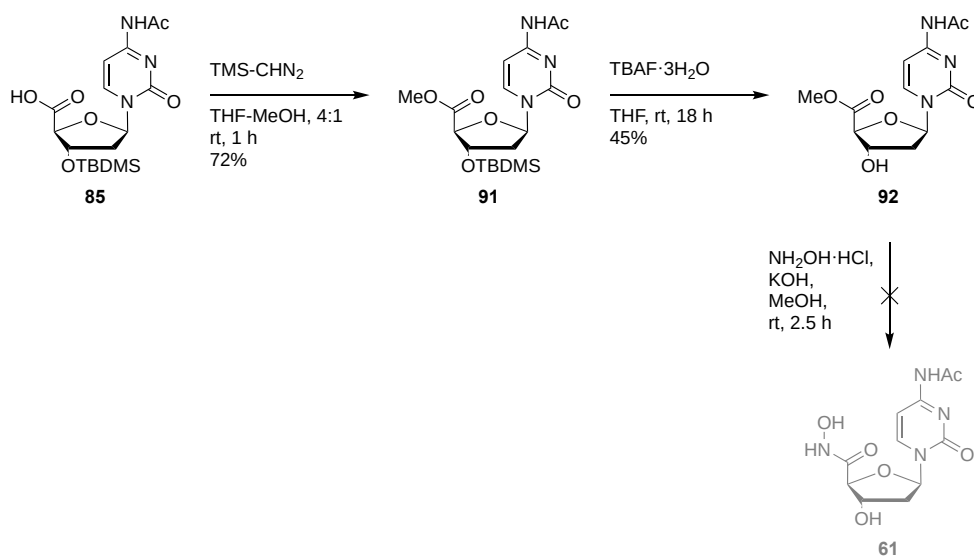
Failure of these amide coupling reactions may be due to the low nucleophilicity and large steric bulk of *O*-TBDMS-hydroxylamine. Therefore, synthesis of the TBDMS protected hydroxamic acid **89** was attempted *via* reaction of *O*-TBDMS-hydroxylamine with the acid chloride derivative of carboxylic acid **85**. Due to the previously observed acid

lability of the *N*⁴-acetyl group, caution was taken not to use the harsh conditions of conventional chlorinating agents such as SOCl₂ or PCl₅. The chosen alternative was cyanuric chloride, **90**, a mild chlorinating agent that has been reported for the one-pot installation of hydroxamic acids from carboxylic acids *via* acid chlorides in the presence of acid labile Boc protecting groups (**Scheme 2.9**).³⁵⁵



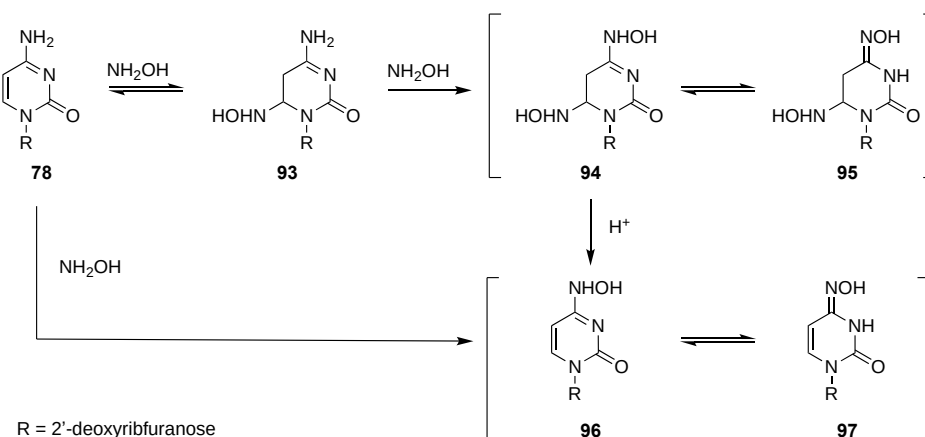
Scheme 2.9: Attempted amide coupling between **85** and *O*-TBDMS-hydroxylamine *via* acid chloride synthesis with cyanuric chloride.³⁵⁵

Cyanuric chloride was used to generate the acid chloride followed by amide coupling with *O*-TBDMS-hydroxylamine. However, after 25 h, no reaction progress was observed by TLC analysis and only carboxylic acid **85** was isolated from the reaction. After these failed attempts at amide coupling with *O*-TBDMS-hydroxylamine, an alternative route to **61** was explored. Synthesis of the 5'-hydroxamic acid *via* aminolysis with hydroxylamine was investigated (**Scheme 2.10**).



Scheme 2.10: Attempted synthesis of hydroxamic acid **61** *via* aminolysis.

Methylation of carboxylic acid **85** with trimethylsilyldiazomethane (TMS-CHN₂), utilising conditions adopted from the literature,³⁵⁶ furnished methyl ester **91** in 72% yield. Deprotection of the 3'-position afforded 45% of the desired product **92**. Aminolysis with NH₂OH·HCl and KOH in MeOH was attempted using a modified literature procedure.³⁵⁷ The *N*⁴-acetyl group is also base labile³⁴⁹ which enabled simultaneous *N*⁴-deprotection and introduction of the hydroxamic acid group. After 2.5 h, TLC analysis showed complete consumption of methyl ester **92**. However, after column chromatography, the desired product **61** was not isolated. It is possible that the reaction of hydroxylamine with the cytosine nucleobase generated multiple products, as previously reported for other cytosine derivatives (**Scheme 2.11**).³⁵⁸ Aminolysis with hydroxylamine was then considered unsuitable for the synthesis of **61**. Due to the multiple issues encountered during the attempted synthesis of **61**, an alternative target compound was designed.



Scheme 2.11: Addition of hydroxylamine to cytosine to yield multiple products as reported by Phillips (adapted from Phillips, 1967).³⁵⁸

2.3.2. Synthesis and Biochemical Evaluation of an *N*³-Modified Thymidine Derivative

Returning to the original inhibitor scaffold **31** (**Figure 2.6**), it was considered whether this thymidine derivative itself could be expanded to explore peripheral binding pockets and/or generate molecular tools to study SNM1A. Although **31** was not a highly potent inhibitor, (IC₅₀ 139 μM) it is possible that the activity of this inhibitor could be increased through functionalisation with a secondary binding motif. Inhibitor **31** was also found not to be membrane permeable,²²⁰ and modification of **31** may enhance membrane permeability. Terminal alkynes are small functionalisation handles that are

non-perturbing to most biological systems.³⁵⁹ They have been extensively used as handles for functionalisation not only within synthetic chemistry,³⁶⁰ but also for modification of biologically active molecules,^{361, 362} proteins,³⁶³ and DNA.³¹² Alkynes have versatile reactivity and are used for functionalisation *via* a wide range of reactions including CuAAC reactions,^{364, 365} coupling reactions such as Sonogashira and Glaser coupling reactions,^{366, 367} alkyne metathesis reactions,³⁶⁸ thiol-yne reactions³⁶⁹ and nucleophilic addition/substitution reactions of acetylide anions.³⁶⁵ Compound **62** (Figure 2.6), an analogue of lead inhibitor **31**, containing a terminal alkyne in the *N*³-position was designed in order to determine whether modification at this position is a suitable approach for the generation of inhibitors and probes targeting SNM1A. As SNM1A is capable of digesting past bulky DNA lesions *in vitro*,^{26, 36} it was proposed that this *N*³-propargyl group would not have a significant detrimental effect on enzyme recognition. The *N*³-propargyl group would allow for functionalisation at this position, facilitating exploration of possible peripheral binding pockets. Thymidine derivative **62** was synthesised and its biochemical activity was evaluated and compared to that of lead inhibitor **31**.

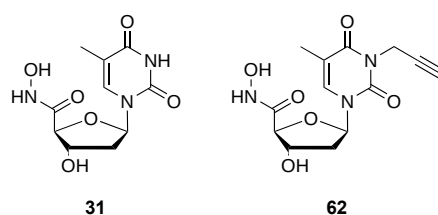
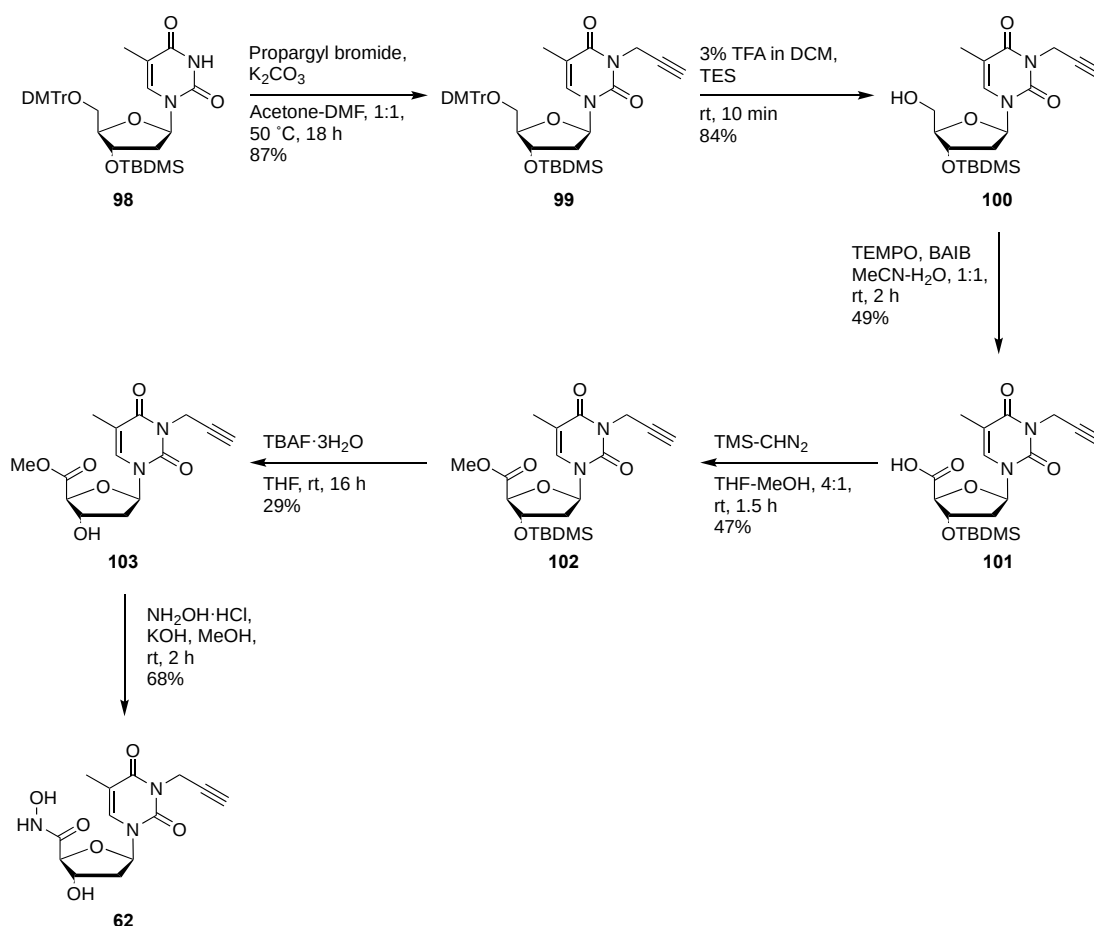


Figure 2.6: *N*³-modification of thymidine for targeting SNM1A.

2.3.2.1. Synthesis of an *N*³-Propargyl Thymidine Derivative

Lead inhibitor **31** was synthesised in the McGouran Group by Dr William Doherty as previously reported.²²⁰ Target compound **62** was synthesised in six steps from protected thymidine **98**, with the propargyl group being installed early in the synthetic route (Scheme 2.12).



Scheme 2.12: Synthesis of compound **62** from protected thymidine **98**.

Protected thymidine **98** was synthesised from thymidine by Dr Eva-Maria Dürr.²²⁰ The *N*³-position was propargylated, utilising modified literature conditions,³⁷⁰ to generate **99** in 87% yield. DMTr deprotection was carried out using TFA to afford alcohol **100** in 84% yield. TEMPO/BAIB mediated oxidation of the 5'-alcohol furnished carboxylic acid **101** in 49% yield. Methylation of carboxylic acid **101** using TMS-CHN₂ yielded 47% of methyl ester **102**. TBDMS deprotection generated **103** in 29% yield. Low recovery of **103** is presumed to be caused by the extended reaction time of 16 h, leading to product degradation. This phenomenon with TBAF treatment of similar nucleoside analogues was previously observed in the McGouran Group and higher isolated yields were reported when a shorter reaction time was used.²¹⁷ Finally, aminolysis of **103** with NH₂OH furnished target compound **62** in 68% yield. The ¹H NMR spectrum of the purified product no longer displayed a singlet at 3.81 ppm, corresponding to the CH₃ group of the methyl ester. Further characterisation using ¹³C, heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) NMR experiments as well

as infrared (IR) spectroscopy and high resolution mass spectrometry (HRMS) confirmed the structure of **62** prior to biochemical evaluation.

2.3.2.2. Biochemical Evaluation of an *N*³-Propargyl Thymidine Derivative

Thymidine analogue **62** was evaluated for inhibition of SNM1A using the gel electrophoresis-based assay described in **Section 2.2.1**. To determine the effect of the *N*³-propargyl modification, the assay was carried out with both lead inhibitor **31** and *N*³-propargyl 5'-hydroxamic acid **62** and their inhibitory activities were compared (**Figure 2.7**). Thymidine (**65**) was incubated with SNM1A as a control, to show that any inhibition displayed by **31** or **62** was due to nucleoside modification. Nucleosides **31** and **62** were pre-incubated with SNM1A at various concentrations from 1 mM – 1 μ M at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** was then added and the reaction mixture was further incubated for 1 h. Digestion of the fluorescent substrate oligonucleotide **64** was examined by PAGE (**Figure 2.7**).

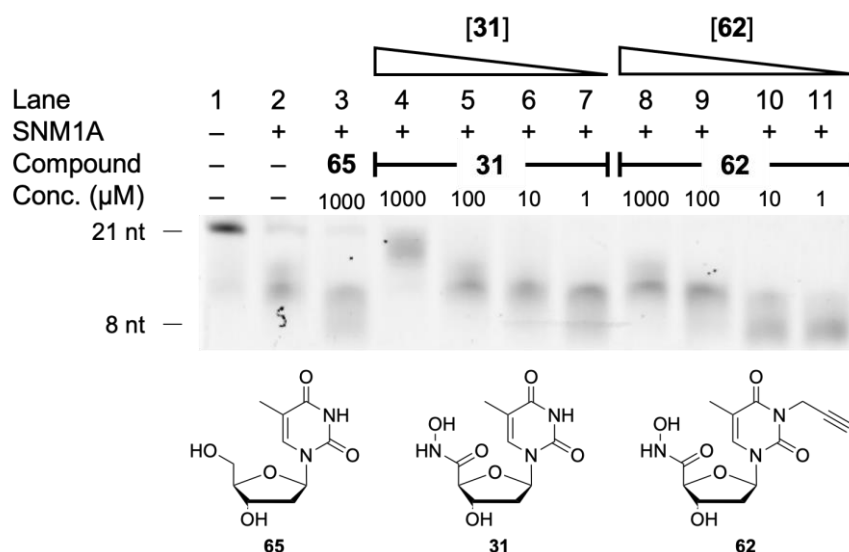


Figure 2.7: Evaluation of **31** and **62** as inhibitors of SNM1A at various concentrations. Thymidine **65** was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **31** and **62** were pre-incubated with SNM1A (25 fmol, 2.5 nM in reaction) at 37 °C for 5 min at various concentrations; 1 mM, 100 μ M, 10 μ M and 1 μ M. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

As before, **Figure 2.7** Lanes 1 and 2 display the negative and positive controls for digestion of the fluorescent substrate oligonucleotide **64**, respectively. Thymidine (**65**) was included in the assay (**Figure 2.7**, Lane 3) and did not display any activity against SNM1A. Recognition of lead compound **31** by SNM1A was evaluated at various concentrations (1 mM – 1 μ M, **Figure 2.7**, Lanes 4-7) and showed notable inhibition of

enzyme activity at high concentration (1 mM, **Figure 2.7**, Lane 4), as previously reported.²²⁰ Compound **62** was also evaluated for recognition by SNM1A at a range of concentrations (1 mM – 1 μ M, **Figure 2.7**, Lanes 8-11). Digestion of **64**, to a similar extent as with thymidine (**65**) (**Figure 2.7**, Lane 3), was observed at all concentrations of **62** (**Figure 2.7**, Lanes 8-11), indicating that modified nucleoside **62** displayed no inhibition of SNM1A. SNM1A does not, therefore, tolerate an alkyne modification at the N^3 -position of thymidine. As even the relatively modest steric requirements of the alkyne disrupt the binding of this inhibitor, functionalisation at this position is unlikely to be suitable for the generation of inhibitors targeting SNM1A.

2.3.2.3. Molecular Docking Studies

To investigate whether the N^3 -position of thymine is important for the binding of **31** to the active site of SNM1A, molecular docking studies were carried out using UCSF Chimera and Autodock Vina.^{61, 371, 372} The results from these studies, as seen in **Figure 2.8**, indicated that when **31** is in the correct position for coordination to the zinc ion in the active site, the N^3 -position of thymine forms a hydrogen bond to the backbone of the protein *via* isoleucine (Ile) 881. It is therefore possible that the N^3 -position is important for the binding of **31** to the active site. When docking studies were carried out with the propargyl bearing nucleoside analogue **62**, no coordination of the hydroxamic acid to the metal active site was observed, indicating that incorporation of the propargyl group in **62** may disrupt this interaction and impede the binding of **62** to SNM1A. This result corroborates the lack of inhibition displayed by compound **62** in the gel electrophoresis-based assay. Despite its similarity to lead inhibitor **31**, alkyne modified **62** was not recognised by SNM1A to any significant extent. Modification of the nucleobase with this minimal functionalisation handle perturbed binding to the enzyme. Therefore, modification at this position is not suitable for expanding inhibitor scaffolds for targeting SNM1A.

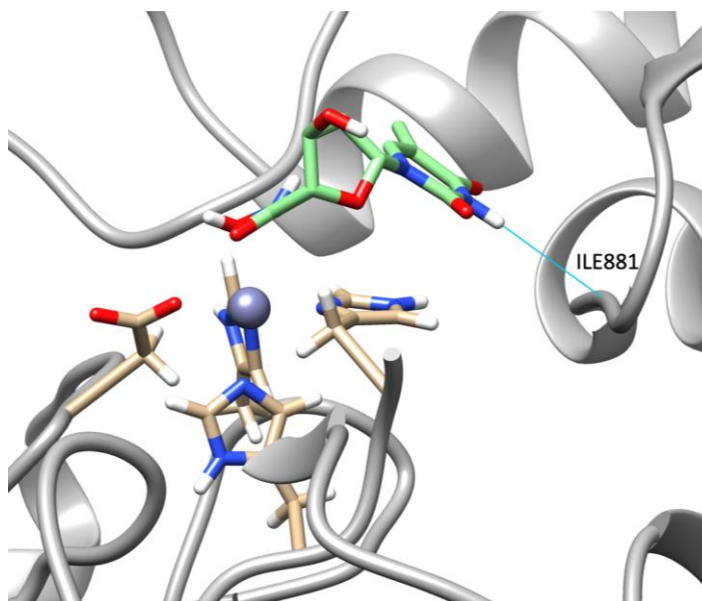


Figure 2.8: Molecular docking studies were used to model interaction of lead inhibitor **31** with the active site of SNM1A (PDB: 5AHR). The hydroxamic acid of **31** binds to the metal active site. The N³-position of **31** forms a hydrogen bond to the backbone of the protein via Ile881. Modelling carried out using USCF Chimera and AutoDock Vina.^{61, 371, 372}

2.4. Conclusions

This chapter describes the expansion of established inhibitor scaffolds as a strategy for targeting SNM1A. Functionalisation of inhibitor scaffolds has the potential to enhance inhibitory activity and membrane permeability, explore peripheral binding pockets and incorporate probe elements for AfBPs targeting the enzyme. The expansion of inhibitor frameworks for targeting SNM1A was initially explored using a malonate group as both an MBG and a functionalisation handle. A series of nucleosides featuring malonate-derived MBGs were tested as inhibitors of SNM1A. Of this series, **40** seemed to be a promising inhibitor. Nucleoside derivatives **59** and **67** were target compounds designed based on this inhibitor scaffold. Nucleosides **59** and **67** incorporated both a malonate MBG and a protected amine moiety which may be deprotected to enable further functionalisation and probe synthesis. Efforts towards the synthesis of **59** and **67** were carried out. However, it was determined that **40** lacked sufficient inhibitory activity and therefore, synthesis of **59** and **67** was abandoned.

Nucleobase modification was then explored for targeting SNM1A. Synthesis of **60** and **61**, 2'-deoxycytidine derivatives bearing 5'-hydroxamic acid groups, was unsuccessful. Thymidine derivative **62** was successfully synthesised and its biochemical activity was

evaluated and compared to lead inhibitor **31**. Thymidine derivative **62** contained a 5'-hydroxamic acid group to target the active site of SNM1A and a terminal alkyne attached *via* the N^3 -position of the nucleobase to facilitate further functionalisation. Despite its similarity to lead inhibitor **31**, **62** was not recognised by SNM1A to any significant extent. Modification of the nucleobase with this minimal functionalisation handle perturbed binding to the enzyme and prevented enzyme inhibition, providing important structure activity relationship (SAR) conclusions regarding this position. Molecular docking studies modelling the interaction of **31** with SNM1A supported the hypothesis that the N^3 -position may be important for the binding of **31** to the active site of SNM1A. The N^3 -position potentially stabilises the binding of **31** to the active site *via* a hydrogen bond interaction with Ile881. Functionalisation at the N^3 -position therefore holds less promise for the generation of potent and selective inhibitors of SNM1A. Modifications at other positions are more suitable and hence, nucleosides with modifications at the 3'- and 5'-position of the ribose ring as well as dinucleosides with unnatural linkages hold more potential for the generation of effective inhibitors and probes targeting SNM1A.^{216-218, 220} The generation of highly potent inhibitors is crucial to the development of molecular tools for targeting SNM1A. Inhibitor scaffolds must be highly potent prior to functionalisation with probe elements, in order to minimise any resulting loss in efficacy.

Chapter 3

Carboxylate-Rich Nucleoside Analogues for Targeting SNM1A

3. Carboxylate-Rich Nucleoside Analogues for Targeting SNM1A

3.1. Introduction

The development of highly potent inhibitors is essential for the design of molecular tools for targeting SNM1A. As discussed in Chapter 2, **Section 2.2**, inhibitor scaffolds must exhibit significant potency prior to integration of probe elements in order to mitigate any potential loss in efficacy. A number of MBGs have previously been investigated for targeting the metal ion(s) in the active site of SNM1A, through incorporation into nucleoside scaffolds.²¹⁶⁻²²⁰ Therefore, further exploration of these types of nucleoside modifications may prove fruitful in the discovery of highly potent inhibitors of the enzyme. As reported by the McGouran Group, the integration of malonate MBGs into nucleoside scaffolds successfully generated effective inhibitors of SNM1A, with one derivative displaying inhibition as low as 33 μ M.²¹⁸ However, the synthesis of nucleosides containing these modifications required considerable route optimisation and involved the manipulation of malonate-derived compounds, which are prone to decarboxylation.^{218, 373} Thus, there remains a need to develop facile and versatile synthetic methods to rapidly generate nucleoside derivatives featuring MBGs for targeting SNM1A. Ideally, such methods would facilitate SAR analysis of inhibitor compounds and enable rapid diversification for subsequent probe development.

Various carbonyl-rich compounds have proven effective for targeting enzymes with metal ions in their active site (**Section 1.3.2**).^{79-82, 103, 335} Derivatives of succinic, aspartic, glutamic and citric acids have also shown to be successful phosphate mimics.³⁷⁴ This chapter describes the investigation of nucleoside analogues, incorporating such carboxylate functionalities in the 5'-position, as inhibitors of SNM1A. Integration of the MBG at the 5'-position renders the 2'- and/or 3'-positions available for functionalisation. To demonstrate the diversity of this approach, derivatives of 5-methyluridine, thymidine, and 3'-azidothymidine were generated. The compounds were synthesised by Dr Christine Arbour, Imperiali Group, MIT and tested for inhibition of SNM1A using a gel electrophoresis-based assay. A real-time fluorescence assay was carried out to calculate an IC₅₀ value for the most promising inhibitor. A CD-based assay was developed to evaluate the ability of this inhibitor to bind ssDNA, and to validate its mechanism of action as binding to the active site of SNM1A.

3.2. Biochemical Evaluation – Gel Electrophoresis-Based Assays

A series of carboxylate-rich nucleoside analogues were synthesised by Dr Christine Arbour, Imperiali Group, MIT. The compounds were synthesised using a novel solid-phase synthetic methodology that involved the attachment of 5-methyluridine and thymidine derivatives to the resin *via* the 5'-position, enabling incorporation of MBGs at the 5'-position as well as downstream diversification at the 2'- and/or 3'-positions. Furthermore, 3'-azido derivatives provide future opportunities for elaboration of the inhibitor scaffold *via* the CuAAC reaction. This method enabled SAR analysis of the nucleoside inhibitor scaffold.

The first nucleoside analogues investigated featured succinic acid derived modifications (**104-112**, **Figure 3.1A**). As a 1,3-dicarbonyl scaffold, the malonate group facilitated the targeting of the metal ion(s) in the SNM1A active site.²¹⁸ It was hypothesised that a 1,4-dicarbonyl moiety, such as the succinic acid scaffold, may also bind to the active site of SNM1A in a similar manner. Being more flexible, the latter could possess enhanced binding properties towards SNM1A. This series included derivatives of 5-methyluridine, thymidine, and 3'-azidothymidine (**104**, **105** and **106**, respectively). To further highlight the diversity of compounds accessible *via* this succinate scaffold, compounds **107-112** were synthesised. The synthesis was enabled by the commercial availability of diverse succinic anhydride building blocks. The *gem*-dimethyl-functionalised succinic acid derivative **107** was generated in order to sterically bias the conformation at the MBG and investigate whether this affects recognition by SNM1A. The inhibitor scaffold was further explored through the generation of a number of fluorine-containing 5-methyluridine analogues (**108-110**). As well as providing important SAR information, such analogues also present future opportunities for fluorine-19 NMR applications.³⁷⁵ L- /D-Aspartic acid (Asp) residues were also installed at the 5'-position to interrogate whether SNM1A binding is sensitive to the stereocentre in the 5'-modification (**111**, **112**). The ability of nucleosides **104-112** to inhibit SNM1A was evaluated using the inhibition assay described in **Section 2.2.1** (**Figure 3.1B**) and the results were observed *via* PAGE (**Figure 3.1C**).^{209, 220}

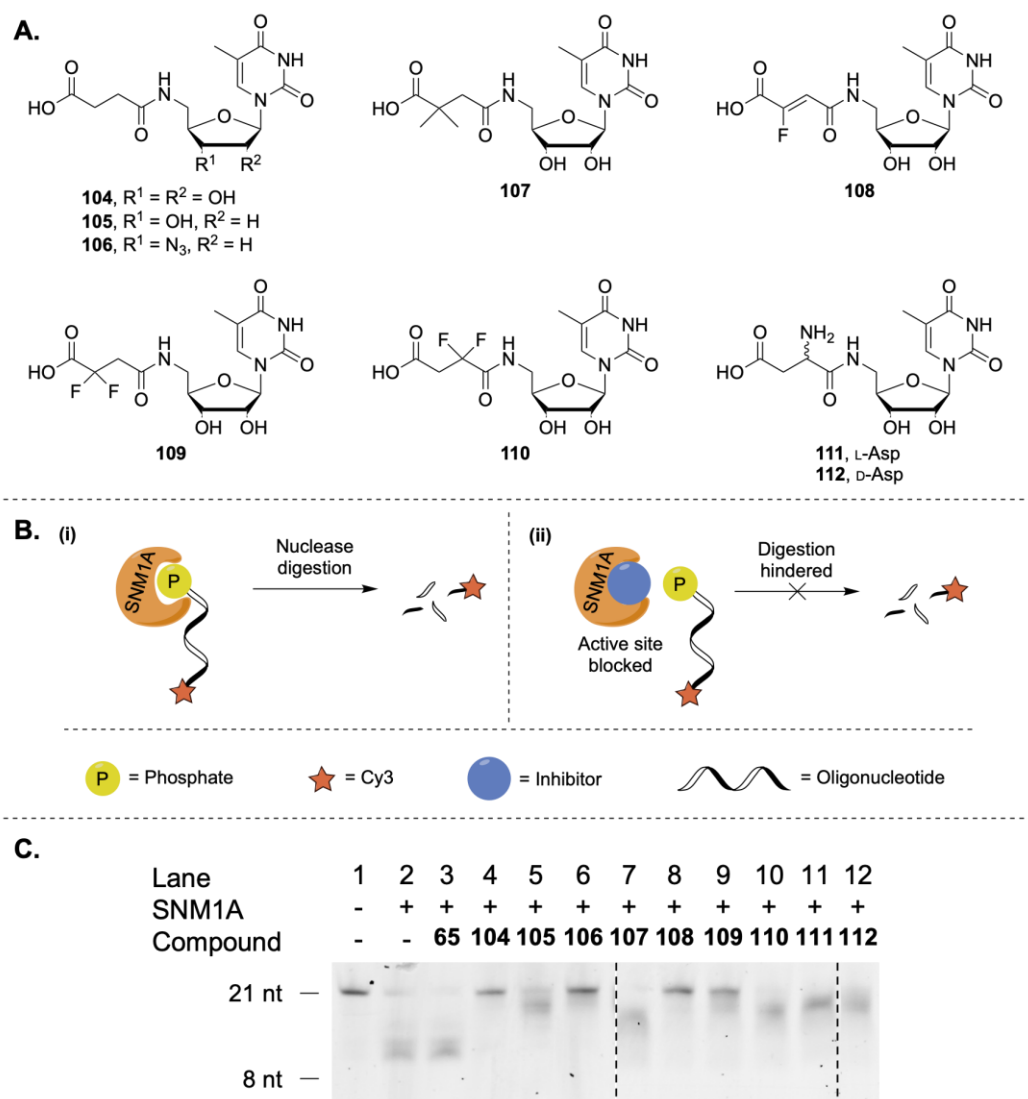


Figure 3.1: A. Nucleoside analogues containing succinic acid-derived modifications in the 5'-position synthesised by Dr. Christine Arbour. B. Gel electrophoresis-based assay (i) SNM1A digests the fluorescent substrate oligonucleotide **64** in a 5'-3' direction. (ii) In the presence of an inhibitor, the active site of SNM1A is blocked and digestion of the fluorescent substrate oligonucleotide **64** is hindered. C. Evaluation of **104-112** as inhibitors of SNM1A. Thymidine (**65**) was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **104-112** (1 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

In the absence of an inhibitor, SNM1A digests the fluorescent substrate oligonucleotide **64** in a 5'-3' direction, resulting in shorter oligonucleotide products (**Figure 3.1B(i)**). In the presence of an inhibitor, the active site is blocked, thus, fluorescent substrate oligonucleotide **64** cannot access the active site and digestion is subsequently hindered (**Figure 3.1B(ii)**). The compounds were initially screened at a concentration of 1 mM. Nucleoside analogues **104-112** were pre-incubated with SNM1A at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** was then added and the reaction mixture was further incubated for 1 h. Digestion of the fluorescent substrate oligonucleotide **64** was

examined by PAGE (**Figure 3.1C**). As previously described, Lanes 1 and 2 (**Figure 3.1C**) represent controls without and with SNM1A, respectively, whereas Lane 3 features a control with thymidine, **65**. The latter was in place of a modified nucleoside, to ensure that any inhibitory effect of **104-112** was due to modifications. Encouragingly, all of the nucleoside analogues exhibited some degree of inhibition of SNM1A at 1 mM (**Figure 3.1C**, Lanes 4-12). Compounds **104**, **106**, **108** and **109** (**Figure 3.1C**, Lanes 4, 6, 8 and 9) exhibited particularly good levels of inhibition at this concentration, as minimal digestion of substrate oligonucleotide **64** was observed.

For comparison, analogues with *N*-acetyl-glycine (Ac-Gly) in the 5'-position were generated (**113-115**, **Figure 3.2**). This modification is similar in size to the succinic acid modification but excludes the carboxylic acid functionality to investigate the importance of the negatively charged groups for metal-binding. Nucleoside analogues **113-115** were evaluated for inhibition of SNM1A at 1 mM using the gel electrophoresis-based assay (**Figure 3.2**).

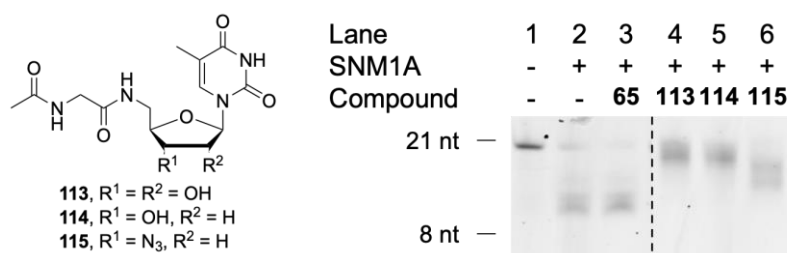


Figure 3.2: Nucleoside analogues containing *N*-acetyl-glycine modifications in the 5'-position were synthesised by Dr. Christine Arbour. Nucleoside analogues **113-115** were evaluated as inhibitors of SNM1A. Thymidine (**65**) was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **113-115** (1 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

Once again, the negative and positive controls for digestion as well as a thymidine control are displayed in **Figure 3.2**, Lanes 1-3. All of the nucleoside analogues featuring a 5'-*N*-acetyl-glycine modification exhibited some degree of inhibition of SNM1A at 1 mM (**Figure 3.2**, Lanes 4-6). To further investigate these types of modifications, the aspartic acid inhibitor scaffold (**116-121**) was expanded using glutamic acid – aspartic acid (ED) dipeptides (**116-118**) and aspartic acid – glutamic acid (DE) dipeptides (**119-121**) (**Figure 3.3**). The dipeptide modifications contained two carboxylic acids in order to introduce a charge mimicking the nucleotide phosphate group.

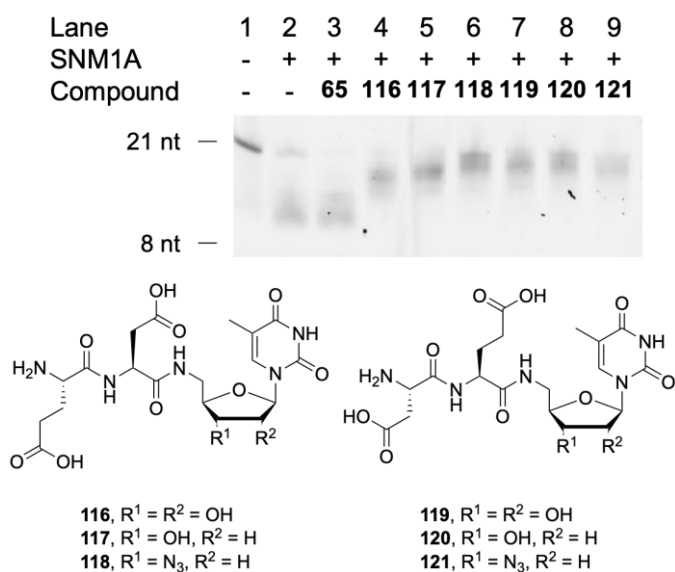


Figure 3.3 Nucleoside analogues containing dipeptide modifications in the 5'-position were synthesised by Dr Christine Arbour and examined for inhibition of SNM1A. Thymidine (**65**) was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **116-121** (1 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

The negative and positive controls for digestion as well as a thymidine control are displayed in **Figure 3.3**, Lanes 1-3. Gratifyingly, all of the nucleoside analogues featuring a dipeptide modification at the 5'-position exhibited some degree of inhibition of SNM1A at 1 mM (**Figure 3.3**, Lanes 4-9). These results suggest that the dipeptide moieties, featuring two carboxylic acid groups, successfully targeted the metal ion(s) in the SNM1A active site. Furthermore, the increased steric bulk in the 5'-position, introduced by the dipeptide modification, did not negatively impact recognition of the nucleoside scaffold. It is therefore possible that other branched and/or sterically bulky, metal-binding motifs may prove successful in the targeting of SNM1A. Citric acid is a branched, densely functionalised small molecule that readily complexes with divalent cations.^{376, 377} Citric acid is, therefore, an MBG worth exploring for targeting the metal ion(s) in the active site of SNM1A. A series of nucleoside analogues featuring a citric acid-derived modification in the 5'-position were generated and screened for inhibition of SNM1A (**122-124**, **Figure 3.4**).

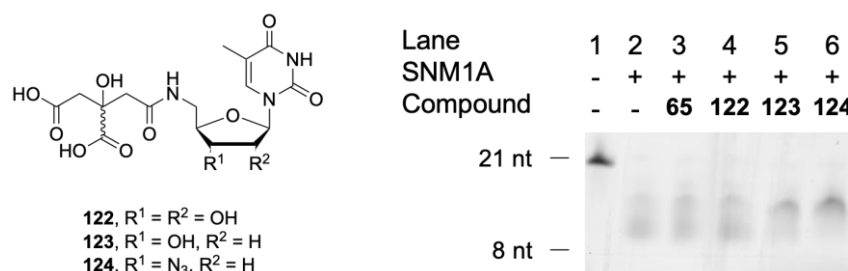


Figure 3.4: Nucleoside analogues containing citric acid modifications in the 5'-position synthesised by Dr. Christine Arbour. Thymidine (**65**) was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **122-124** (1 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

Nucleoside analogues **122-124**, containing a 5'-citric acid derived modification were examined for inhibition of SNM1A using the gel electrophoresis-based assay. Digestion of substrate oligonucleotide **64** was seen in all cases (**Figure 3.4**, Lanes 4-6), indicating that nucleoside analogues **122-124** did not inhibit SNM1A. Although the citric acid series did not display effective inhibition of SNM1A, the other 18 nucleoside analogues tested exhibited some degree of inhibition at 1 mM (**104-121**, **Figures 3.2-3.4**). Given the inhibitory activity displayed by compounds **104-121**, they were further screened at a lower concentration of 100 μ M in order to differentiate compounds with less inhibitory activity from those warranting further evaluation (**Figure 3.5**).

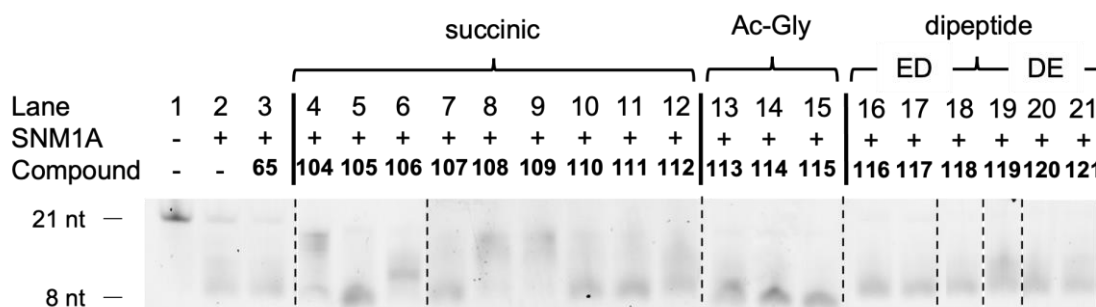


Figure 3.5: Evaluation of **104-121** as inhibitors of SNM1A at 100 μ M. Thymidine (**65**) was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **104-121** (100 μ M) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

The screening at 100 μ M (**Figure 3.5**) identified a number of compounds with moderate to good inhibition of SNM1A, all of which featured a succinic acid-derived modification at the 5'-position. Nucleosides **104**, **106**, **108** and **109** (**Figure 3.5**, Lanes 4, 6, 8 and 9) were the most promising compounds, showing moderate to good inhibition of SNM1A at this concentration. These nucleoside analogues were selected for further evaluation in concentration dependence assays. Despite displaying low inhibitory activity at 100 μ M

(**Figure 3.6**, Lane 5), **105** was also included in the concentration dependence assays in order to enable comparison of the activity of the 5-methyluridine, thymidine and 3'-azido thymidine derivatives (**104**, **105** and **106**) and investigate the effect of the 2'-OH, 3'-OH and 3'-azido groups. This is of particular interest as typically SNM1A displays preference for DNA over RNA substrates.^{45, 59} The concentration dependence of the inhibitory activity of **104-106**, **108** and **109** was explored. The gel electrophoresis-based assay was carried out with varying concentrations of the nucleoside analogues, ranging from 1 mM to 0.3 μ M (**Figure 3.6A-E**). To determine whether the 5-methyluridine scaffold had any effect on inhibitory activity, 5-methyluridine, **125**, was evaluated for inhibition of SNM1A using a similar concentration dependence assay (**Figure 3.6F**).

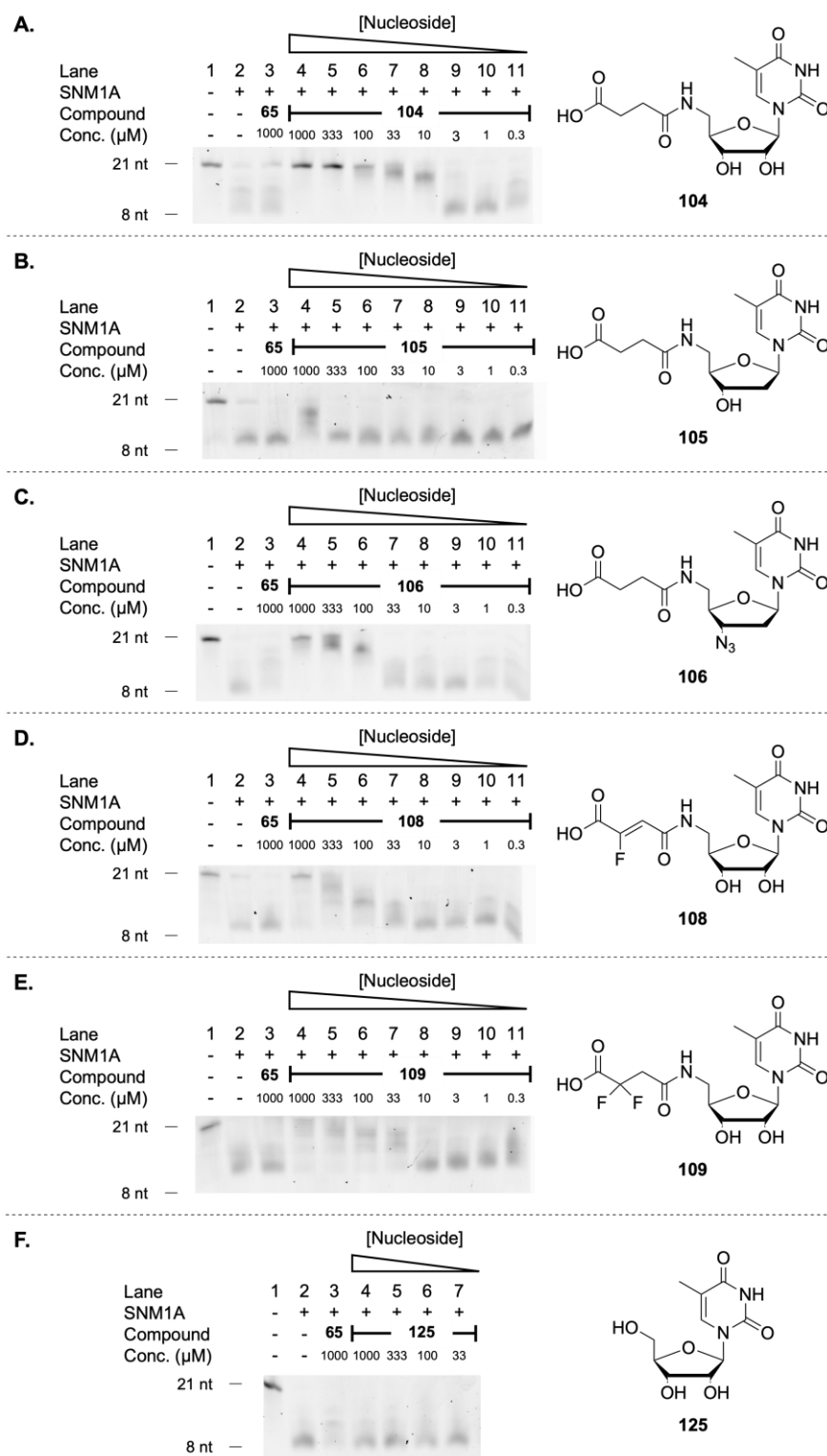


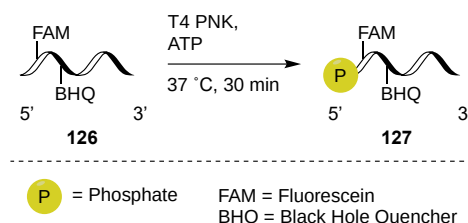
Figure 3.6: Concentration dependence assays of nucleoside analogues **104-106**, **108**, **109** and **125**, containing 5'-succinic acid-derived modifications. Thymidine **65** was incubated with SNM1A at 1 mM to act as a control. Modified nucleosides **104-106**, **108**, **109** and **125** were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min at various concentrations (1 mM – 0.3 μ M). Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

While at 1 mM, all five compounds show effective inhibition of SNM1A (**Figure 3.6A-E**, Lane 4), the difference in their activity is apparent at lower concentrations (**Figure 3.6A-E**). 5-Methyluridine derivative **104**, containing a 5'-succinic acid modification, provided the best results, demonstrating inhibition as low as 10 μ M (**Figure 3.6A**, Lane 8). Interestingly, the closely related thymidine derivative **105**, was not a highly effective inhibitor, with inhibition displayed only at 1 mM (**Figure 3.6B**, Lane 4). Meanwhile the 3'-azido derivative **106** sustained activity with inhibition at 100 μ M (**Figure 3.6C**, Lane 6). The comparison of the activity of **104**, **105** and **106** (**Figure 3.6A-C**) provides interesting insights into the role of the ribose/deoxyribose scaffold in terms of enzyme inhibition. The lower inhibition of the thymidine-containing analogues (**105**, **106**) was unexpected as this scaffold more closely resembles the native substrate of SNM1A, which has a typical preference for DNA substrates over RNA.^{45, 59} Clear preference for the 5-methyluridine derivative **104** in the succinate series (**104-106**), was observed (**Figure 3.6A-C**), suggesting the 2'-OH plays an important role in inhibition. Nucleoside analogue **104** is not the only reported SNM1A inhibitor based on a ribonucleoside scaffold. Previous work in the McGouran Group described potent inhibitors of SNM1A derived from uridine (**Section 1.5.2**).²¹⁷ The fluorinated compounds **108** and **109** demonstrated inhibition of SNM1A as low as 100 μ M and 33 μ M respectively (**Figure 3.6D**, Lane 6 and **Figure 3.6E**, Lane 7). Of the compounds evaluated with concentration dependence assays, 5-methyluridine derivative **104**, containing a 5'-succinic acid modification, was the most effective inhibitor and was selected for further investigation. To determine whether the 5-methyluridine scaffold had any effect on the inhibitory activity of **104**, 5-methyluridine, **125**, was evaluated for inhibition of SNM1A using a similar concentration dependence assay (**Figure 3.6F**). 5-Methyluridine (**125**), did not demonstrate inhibition of SNM1A at any of the concentrations tested (**Figure 3.6F**, Lanes 4-7) and hence, the inhibitory activity of **104** is due to its 5'-succinic acid modification.

3.3. IC₅₀ Determination

Given that **104** exhibited the most promise as an inhibitor of SNM1A, demonstrating moderate inhibition as low as 10 μ M, the extent to which **104** could inhibit the enzyme was further investigated through IC₅₀ determination. A modified procedure of a reported real-time fluorescence assay was carried out.^{209, 216, 217, 220} Oligonucleotide **126**, featuring

a fluorescein (FAM) fluorophore and a black hole quencher (BHQ) moiety was obtained commercially (sequence of **126**: 5'-AXA ATT TGA YCA TCT ATT AT-3', X = FAM thymidine, Y = BHQ thymidine). Oligonucleotide **126** was phosphorylated at the 5'-end using T4 PNK to furnish oligonucleotide **127** (**Scheme 3.1**)



Scheme 3.1: Enzymatic phosphorylation of oligonucleotide **126** to afford fluorescent substrate oligonucleotide **127**. Sequence of oligonucleotide **126**: 5'-AXA ATT TGA YCA TCT ATT AT-3', X = FAM thymidine, Y = BHQ thymidine

Oligonucleotide **127** was then used as a substrate for SNM1A in a real-time fluorescence assay (**Figure 3.7**). Digestion of substrate oligonucleotide **127** by SNM1A led to separation of the FAM and BHQ moieties and a subsequent increase in fluorescence (**Figure 3.7A**). Measurement of this increasing fluorescence signal over time allowed the rate of the nuclease reaction to be quantified. This experiment was carried out in the presence of **104** at a range of concentrations which enabled IC₅₀ calculation. Compound **104** proved to be the most potent nucleoside-derived inhibitor of SNM1A reported to date with an IC₅₀ of 12.3 μM (**Figure 3.7B**).

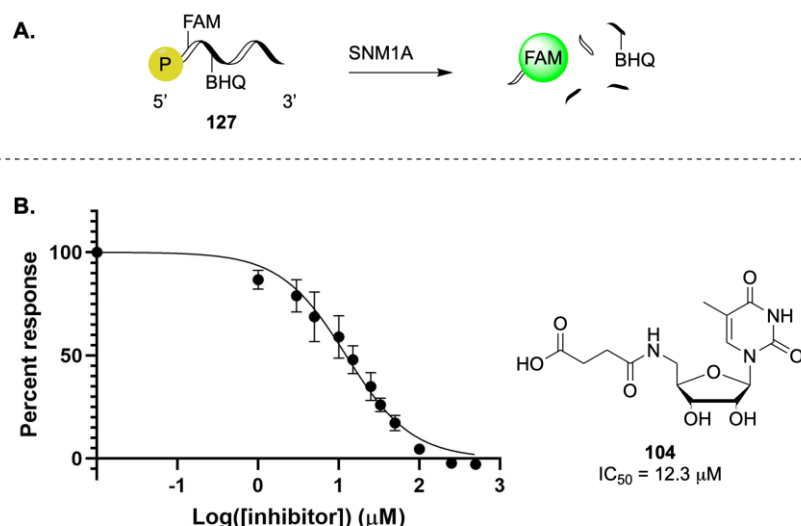


Figure 3.7: IC_{50} determination of **104** in a real-time fluorescence assay. SNM1A (5 nM) was pre-incubated with the modified nucleoside **104** at a range of concentrations (0 – 500 μM) for 6.5 min at rt prior to addition of the 20mer oligonucleotide substrate **127** (125 nM). Fluorescent spectra were measured at 37 °C, with readings taken every 46 seconds for 35 min. For each concentration of inhibitor, fluorescence intensity was plotted against time, and the slope of the linear range of this graph was taken as the rate of enzyme activity. The rate of enzyme activity observed for the zero-inhibitor control was set as 100, and the other rates were normalised to this to obtain percent response values. Percent response was plotted against log([inhibitor]), and a nonlinear regression was performed to calculate the IC_{50} . Error bars generated from 5 independent replicates.

3.4. Circular Dichroism Titrations

The ability of **104** to bind to ssDNA was investigated using CD spectroscopy. Ligand binding to DNA oligonucleotides has previously been reported to disrupt nuclease activity.³⁷⁸⁻³⁸⁰ Hence, the aim of the CD-based assay was to validate the mechanism of action of **104**. Ideally, **104** should bind to the metal ion(s) in the SNM1A active site and show no binding to substrate oligonucleotides. CD spectroscopy is an optical technique that measures the difference in the absorption of left and right circularly polarised light and is widely used to examine conformational changes of DNA upon ligand binding.³⁸¹⁻³⁸³ Change of the CD spectrum of an oligonucleotide upon addition of a ligand indicates conformational changes due to ligand binding.³⁸¹⁻³⁸³ A CD-based assay, utilising an unmodified 21mer ssDNA oligonucleotide (**128**, sequence: 5'-TTAG CAG TCA GTC AGT CAT CG-3'), was developed based on a previously reported assay.³⁸⁴ In order to confirm that the assay was suitable for demonstrating ligand binding to ssDNA, the assay was initially conducted with established ssDNA-binder methylene blue (**129**, **Figure 3.8**).³⁸⁵ The CD spectrum of oligonucleotide **128** was measured in the presence of varying concentrations of **129** (0 – 100 μM) (**Figure 3.8**). CD spectra of **129** alone, without the presence of oligonucleotide **128**, were also recorded at the same concentrations used in

the titrations. These were subtracted from the corresponding spectra measured during the titrations, to allow only changes in the CD spectrum of the oligonucleotide to be visualised. The CD spectrum of oligonucleotide **128** changed considerably upon addition of **129**, particularly at high concentration ($\geq 25 \mu\text{M}$), indicating conformational changes due to binding of **129** to the ssDNA oligonucleotide.

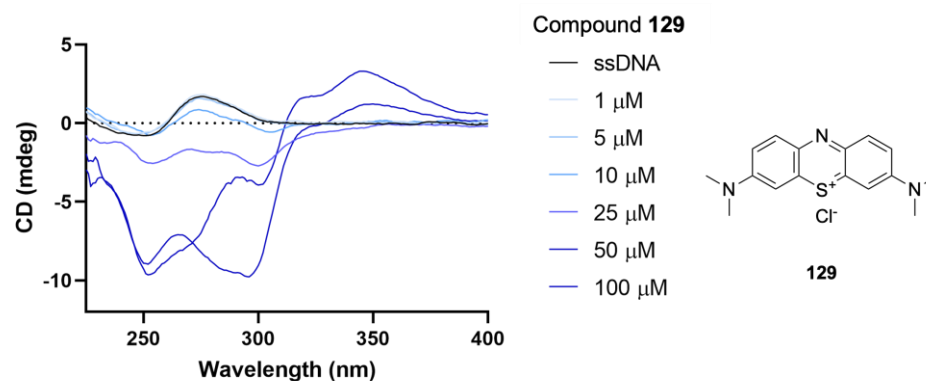


Figure 3.8: CD spectrum of 21mer oligonucleotide **128** ($1.22 \mu\text{M}$ in H_2O) in the presence of varying concentrations of **129** ($0 - 100 \mu\text{M}$), recorded at 25°C . The change in spectrum indicated conformational changes due to binding of **129** to oligonucleotide **128**. Sequence of **128**: 5'-TTAG CAG TCA GTC AGT CAT CG-3'.

The CD-based assay was then carried out with **104**. The CD spectrum of oligonucleotide **128** was measured in the presence of varying concentrations of **104** ($0 - 100 \mu\text{M}$) (**Figure 3.9**). As before, CD spectra of **104** at these concentrations were also measured and subtracted from the corresponding spectra measured during the titrations to enable only changes in the CD spectrum of oligonucleotide **128** to be visualised. No change in the CD spectrum of oligonucleotide **128** upon addition of **104** was observed. Hence, **104** does not bind to ssDNA and therefore, its inhibitory activity is likely due to binding of **104** to the SNM1A active site.

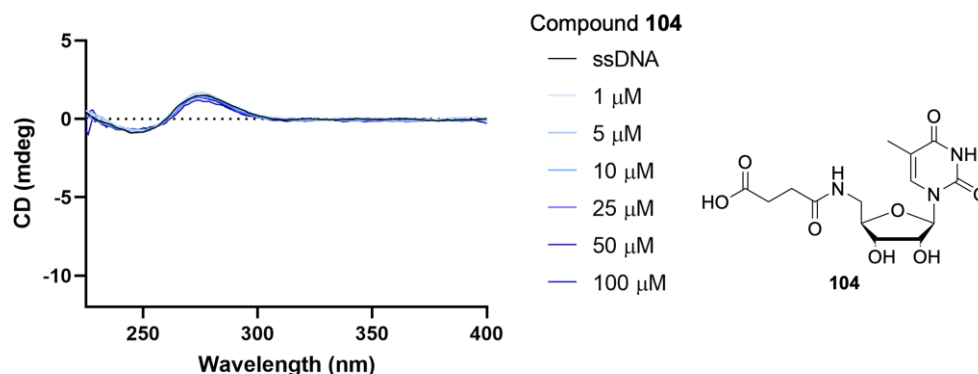


Figure 3.9: CD spectrum of 21mer oligonucleotide **128** (1.22 μM in H_2O) in the presence of varying concentrations of **104** (0 – 100 μM), recorded at 25 $^\circ\text{C}$. No change in spectrum indicated that **104** was not binding to oligonucleotide **128**. Sequence of **128**: 5'-TTAG CAG TCA GTC AGT CAT CG-3'

3.5. Conclusions

To summarise, nucleoside analogues featuring succinic, aspartic, glutamic and citric acid-derived modifications in the 5'-position were evaluated as inhibitors of SNM1A. Integration of MBGs at the 5'-position of 5-methyluridine, thymidine and 3'-azidothymidine scaffolds rendered the 2'- and/or 3'-positions available for further functionalisation and potential probe development. The nucleoside analogues were screened for inhibition of SNM1A using the previously described gel electrophoresis-based assay. Gratifyingly, 18 of the 21 compounds tested inhibited SNM1A during the initial screening at 1 mM and were brought forward for further testing. The succinic-acid derivatives showed particular promise with four compounds (**104**, **106**, **108** and **109**) demonstrating inhibition as low as 100 μM . Concentration-dependence assays illustrated that **106** and **108** did not inhibit SNM1A at concentrations below 100 μM , while **109** displayed moderate inhibition as low as 33 μM . 5-Methyluridine derivative **104** was identified as the most effective inhibitor, displaying moderate inhibition as low as 10 μM . A real-time fluorescence assay determined an IC_{50} of 12.3 μM for SNM1A inhibition by **104**. A CD-based assay was employed to evaluate the ability of **104** to bind ssDNA, in order to validate its mechanism of action as binding to the active site of SNM1A. The assay was conducted using the known ssDNA binder, methylene blue (**129**),³⁸⁵ in order to confirm the suitability of the assay for the evaluation of ligand binding to ssDNA. The assay was then carried out with nucleoside analogue **104** and confirmed that **104** was not binding to ssDNA. It is, therefore, likely that inhibition of SNM1A by **104** is due to the binding of the MBG to the active site metal

ion(s). This work marks a significant advancement in the study of SNM1A, as **104** stands as the most potent nucleoside-based inhibitor of SNM1A documented to date.

Chapter 4

Two-Step Validation Approach for Tools to Study SNM1A

4. Two-Step Validation Approach for Tools to Study SNM1A

4.1. Introduction

Although a number of inhibitors of SNM1A have been reported to date,^{209, 215-220} there remains a need to develop chemical tools to facilitate the study of this poorly characterised enzyme. The incorporation of MBGs into nucleosides and oligonucleotides is a particularly effective method of generating inhibitors of SNM1A (**Section 1.5.2**).²¹⁶⁻²²⁰ In fact, the inclusion of an oligonucleotide scaffold has been proven to enhance substrate recognition and increase binding affinity.²¹⁹ Incorporation of MBGs into the 5'-position of oligonucleotide strands enables the simultaneous targeting of the metal-containing active site of SNM1A and the distal DNA-binding groove,²¹⁹ both of which are featured in the crystal structure of SNM1A and are key for enzymatic activity (**Figure 4.1A**).⁵⁸ However, the synthesis of native nucleosides and oligonucleotides to evaluate the recognition of novel substrates is challenging, time-consuming and expensive.³⁸⁶ Synthesis of modified nucleoside phosphoramidites suitable for solid-phase oligonucleotide synthesis typically includes upwards of six synthetic steps prior to generation of the phosphoramidites which are susceptible to degradation due to their instability towards water and oxygen.²¹⁹ New, facile synthetic and screening methodologies are therefore required to rapidly evaluate the efficiency of substrate binding and to access non-native SNM1A substrates. Although screens of bioactive molecules have previously succeeded in identifying several inhibitors of SNM1A,^{209, 215} there is currently no screening method focused solely on the identification of MBGs, which have been demonstrated as key for targeting SNM1A.²¹⁶⁻²²⁰

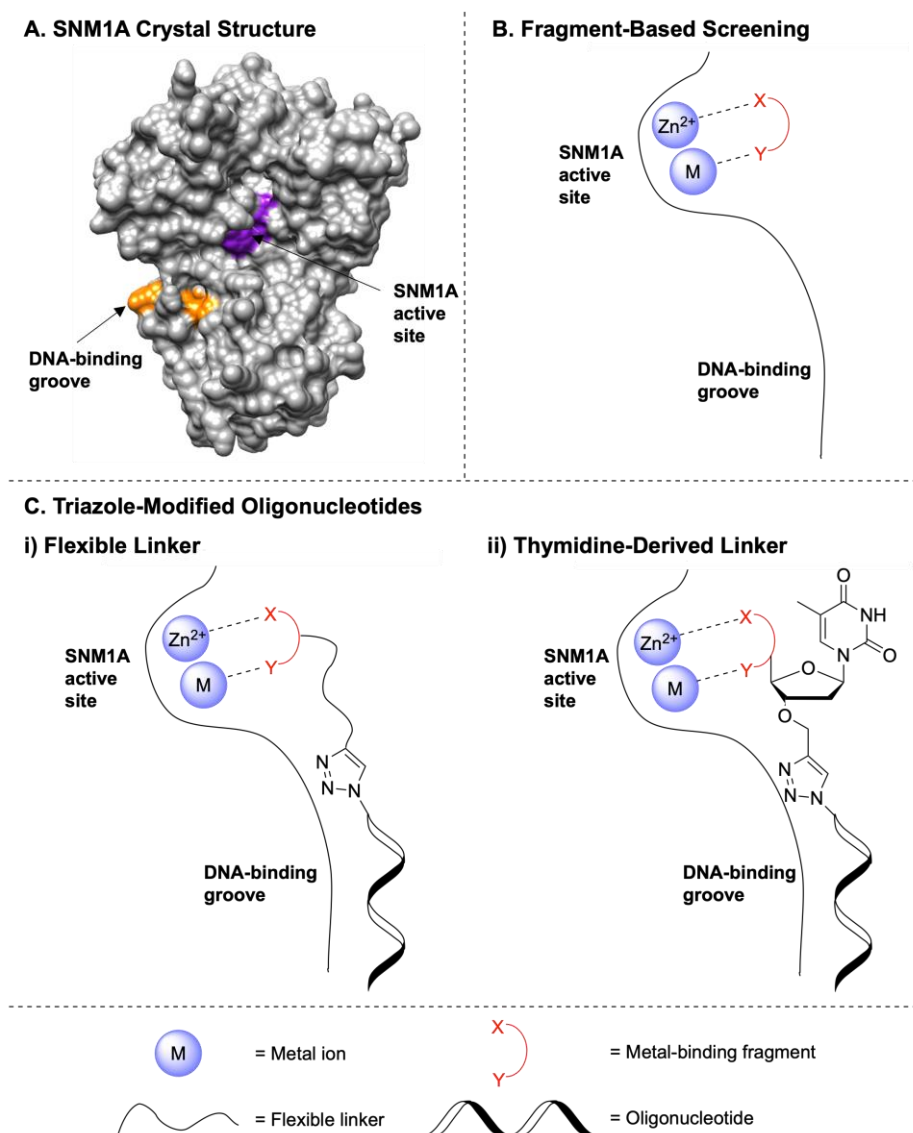


Figure 4.1: Two-step validation of MBGs suitable for targeting SNM1A. A. Crystal structure of SNM1A (PDB: 5AHR) showing the active site (purple) and distal DNA-binding groove (orange).⁵⁸ Graphics created using UCSF Chimera.⁶¹ B. Fragment-based screening for identification of MBGs suitable for targeting the SNM1A active site. C. Triazole-modified oligonucleotides for targeting SNM1A. Both a flexible linker and a thymidine-derived linker were investigated.

This chapter describes a two-step validation approach to evaluate the suitability of MBGs for targeting SNM1A. First, a fragment-based screening approach was used to identify metal-binding fragments suitable for targeting the enzyme (**Figure 4.1B**). Following this, the activity of these metal-binding fragments was confirmed through incorporation of the fragments into oligonucleotides *via* the CuAAC reaction (**Figure 4.1C**). Initially a flexible linker was investigated for incorporating the fragments into oligonucleotide strands (**Figure 4.1C(i)**). While metal-binding fragments alone targeted SNM1A at a concentration of 5 mM, these triazole-modified oligonucleotides were found to successfully target SNM1A at the >1000-fold lower concentration of 2.8 μ M. Although

the unnatural triazole linkage was recognised by SNM1A at low concentrations, the triazole-modified oligonucleotides, containing a flexible linker, displayed a lower affinity for SNM1A compared to naturally-linked oligonucleotides.²¹⁹ It was hypothesised that replacement of the flexible linker with a thymidine-derived linker, may enhance recognition of the oligonucleotides by SNM1A. To investigate this, a series of thymidine derivatives featuring a 3'-alkyne handle were designed and synthesised and incorporated into oligonucleotide strands *via* the CuAAC reaction (**Figure 4.1C(ii)**). These modified oligonucleotides, containing a triazole internucleotide linkage, targeted SNM1A at 0.27 μ M. This novel approach of incorporating functional fragments into oligonucleotides *via* click chemistry is broadly applicable in the generation of modified oligonucleotide structures with high affinity for DNA damage repair enzymes.

4.2. Fragment-Based Screening for Targeting SNM1A

Fragment-based screening approaches evaluate the ability of low molecular weight molecules to exert a desired biological action.³⁸⁷ Although the fragments themselves may only have low binding affinity for the desired target, they can be combined with other scaffolds or functional groups to create a final drug molecule or biochemical tool with high binding affinity. Since fragments often have low affinity for the biological target, they are usually screened at high concentrations.^{387, 388} Several drugs derived from fragment-based screening have been approved by the FDA, including vemurafenib, used in the treatment of late stage melanoma.³⁸⁹ Fragment-based screening has been extensively used as an initial screening method and has been successful in the generation of new hits for inhibitors of both DNA processing enzymes, such as the DNA damage repair enzyme meiotic recombination 11 homolog 1 (MRE11),³⁹⁰ and metalloenzymes, including the metallo- β -lactamase NDM-1.³⁹¹ However, fragment-based screening has not yet been used to identify inhibitors of SNM1A. This section describes the exploration of a fragment-based screening approach for the identification of MBGs suitable for targeting SNM1A. Successful implementation of this method may allow a wide array of metal binding fragments to be rapidly screened for inhibition of DNA damage repair enzymes prior to their incorporation into nucleoside and oligonucleotide scaffolds to create biochemical tools. Such a screening method may also increase the efficiency of the development of SNM1A inhibitors and lead to new insights into its activity.

4.2.1. Synthesis of Metal-Binding Fragments

Fragments were selected for inclusion in this proof-of-principle assay based on their known metal binding abilities³³⁵ and their previous success in targeting the active site of SNM1A when incorporated into a nucleoside or oligonucleotide scaffold. Fragments chosen for screening included squaric acid and squaramide derivatives,^{216, 217, 219} benzoic acid and hydroxamic acid derivatives,^{218, 220} as well as mesylate and sulfonamide derivatives (**Figure 2.4**).²¹⁷ *o*-Phenanthroline (**130**) was included in the screening as a positive control for inhibition of SNM1A, as it is a known zinc chelator and inhibitor of SNM1A.⁵⁸

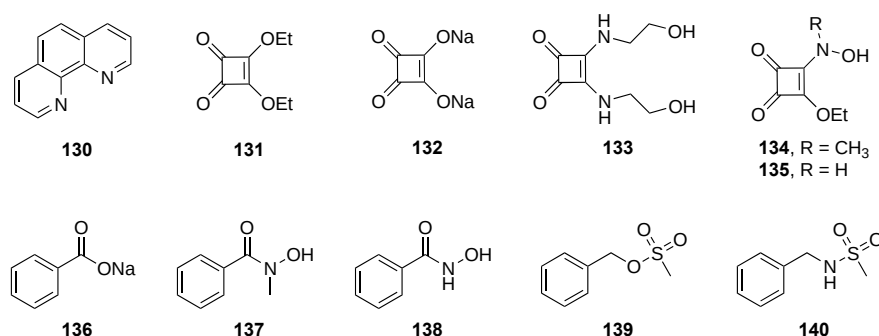
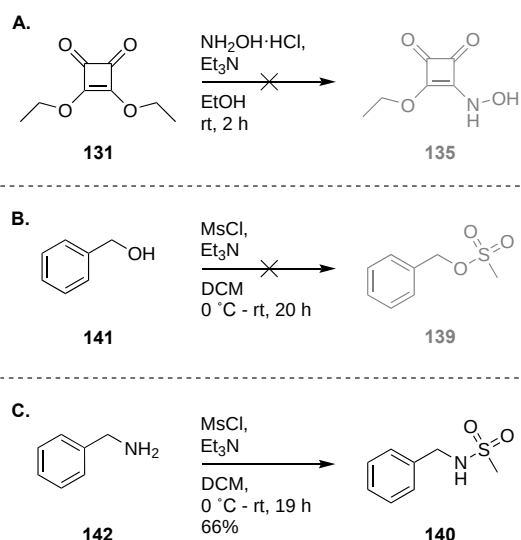


Figure 4.2: Fragments selected for the proof-of-principle fragment-based screening approach. Fragments were selected based on their reported metal-binding abilities.

All fragments were obtained commercially or in moderate to high yields in one step from commercially available starting materials. Diethyl squarate (**131**) is commercially available. Disodium squarate **132** and sodium benzoate **136** were generated by ion exchange from their corresponding commercially available acids. Squaramides **133** and **134** were synthesised from diethyl squarate (**131**) in the McGouran Group by Dr Werner Jauslin.³⁹² Hydroxamic acid derivatives **137** and **138** were synthesised from benzoyl chloride in the McGouran Group by Ailish Newton.³⁹³ Synthetic efforts towards the generation of **135**, **139** and **140** are illustrated in **Scheme 4.1**.



Scheme 4.1: Synthetic efforts towards the generation of **135**, **139** and **140**.

Attempted synthesis of **135** through reaction of diethyl squarate (**131**) with $\text{NH}_2\text{OH}\cdot\text{HCl}$ yielded a complex mixture and **135** could not be isolated. Mesylation of benzyl alcohol (**141**) with methanesulfonyl chloride (MsCl) and Et_3N resulted in little to no reaction progress seen by TLC analysis after 20 h and none of the desired product **139** was isolated. Reaction of benzylamine (**142**) with MsCl in the presence of Et_3N successfully furnished **140** in 66% yield. The ^1H NMR spectrum of the purified product displayed a singlet at 2.86 ppm, corresponding to the CH_3 of the sulfonamide group. Further characterisation using ^{13}C , HSQC and HMBC NMR experiments as well as IR spectroscopy and HRMS confirmed the structure of **140**.

4.2.2. Biochemical Evaluation of Metal-Binding Fragments

Fragments **130-134**, **136-138** and **140**, were examined for binding of SNM1A using the gel electrophoresis-based inhibition assay discussed previously (Section 2.2.1).^{209, 219} In the absence of an inhibitor, SNM1A digests the fluorescent oligonucleotide substrate **64** to generate shorter oligonucleotides (Figure 4.3A(i)). In cases where hydrolysis of oligonucleotide **64** is less efficient, it is possible that the fragment is binding to the metal ion(s) in the active site of the enzyme, leading to inhibition of enzymatic activity (Figure 4.3A(ii)). During fragment-based screening, fragments are often evaluated at high concentrations.^{387, 388} 5 mM was chosen in this instance as this was the concentration at which *o*-phenanthroline (**130**) previously demonstrated inhibition of SNM1A.⁵⁸ The fragments were incubated at a concentration of 5 mM in solution with SNM1A at $37\text{ }^\circ\text{C}$ for 5 min. Fluorescent substrate oligonucleotide **64** was then added. Digestion of the

oligonucleotide substrate **64** was examined by PAGE (**Figure 4.3B**). As enzymatic activity is sensitive to pH, fragments **132** and **136** were evaluated as sodium salts, rather than as their respective acidic parent compounds, in order to minimise perturbations in pH during the enzymatic assay.

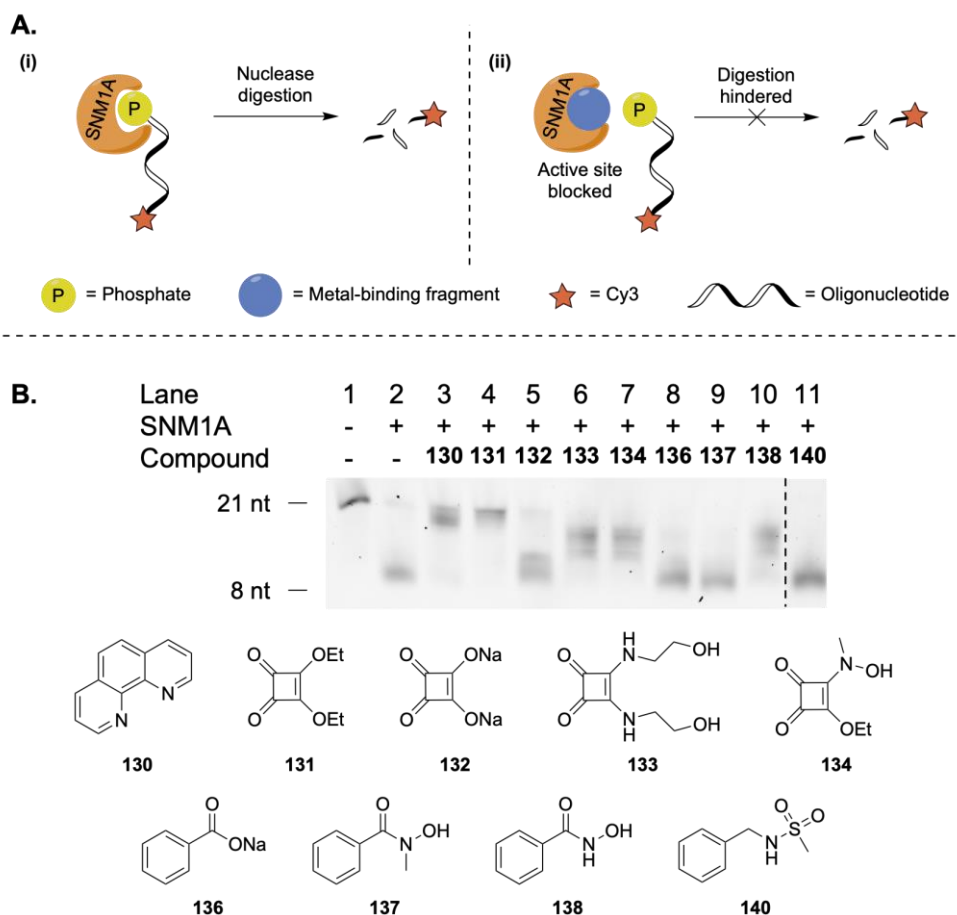


Figure 4.3: Evaluation of fragments **130-134**, **136-138** and **140** for inhibition of SNM1A. A. (i) In the absence of an inhibitor, SNM1A digests substrate oligonucleotide **64** with 5'-3' directionality. (ii) If fragments are binding to the SNM1A active site, enzymatic digestion of oligonucleotide **64** is hindered. B. Fragments **130-134**, **136-138** and **140** (5 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

As previously, a control without SNM1A (**Figure 4.3**, Lane 1) and a control with SNM1A (**Figure 4.3**, Lane 2) were used as the negative and positive controls for digestion of oligonucleotide **64**, respectively. As expected, known inhibitor of SNM1A o-phenanthroline **130** hindered hydrolysis of oligonucleotide **64** by SNM1A (**Figure 4.3**, Lane 3). Interestingly, diethyl squarate **131** hindered SNM1A activity (**Figure 4.3**, Lane 4) but the disodium salt **132** did not (**Figure 4.3**, Lane 5). This result is conflicting with results from a previous study which found that when incorporated into a nucleoside scaffold, the diethyl squarate derivative was not an effective inhibitor of

SNM1A whereas the sodium salt derivative displayed effective inhibition.²¹⁶ Squaric ester derivatives have been shown to react with lysine under physiological conditions.³⁹⁴ It is, therefore, possible that the electrophilic nature of **131** caused it to act as a pan-assay interference compound in this case and it was non-specifically disrupting the enzyme, rather than binding to the metal ion(s) in the SNM1A active site. Squaramides **133** and **134** demonstrated moderate inhibition of SNM1A (**Figure 4.3**, Lanes 6 and 7). This corresponds to a previous report in which such squaramide-derived MBGs successfully targeted the SNM1A active site when incorporated into a nucleoside scaffold.²¹⁶ Of the benzoic acid derivatives, sodium benzoate (**136**) and methylated hydroxamic acid derivative **137** did not affect SNM1A activity, as digestion of substrate oligonucleotide **64** is evident (**Figure 4.3**, Lanes 8 and 9). However, hydroxamic acid **138** successfully reduced SNM1A activity, hindering hydrolysis of oligonucleotide **64** (**Figure 4.3**, Lane 10). Once again, this is in agreement with results from a previous study in which a nucleoside analogue containing a 5'-hydroxamic acid moiety was shown to inhibit SNM1A.²²⁰ Sulfonamide **140** did not affect SNM1A activity (**Figure 4.3**, Lane 11). Fragments identified during this proof-of-concept assay correspond to MBGs that have previously been recognised by SNM1A,²¹⁶⁻²²⁰ demonstrating that fragment-based screening is a suitable approach for the identification of metal-binding groups capable of targeting the enzyme.

4.3. Triazole-Modified Oligonucleotides for Targeting SNM1A

Considering oligonucleotides containing an MBG at the 5'-end have been shown to target SNM1A at low concentrations,²¹⁹ it was hypothesised that combining metal-binding fragments with an oligonucleotide strand may enhance binding to SNM1A. While the metal-binding fragments target the metal ion(s) in the SNM1A active site, inclusion of the oligonucleotide strand may enable the targeting of the distal DNA-binding region. Oligonucleotides modified with a metal binding fragment in the 5'-position may display increased affinity for SNM1A compared to the fragments alone. DNA modification *via* click chemistry provided an elegant method of rapidly installing metal-binding fragments into the 5'-position of oligonucleotides. The use of click chemistry and the CuAAC reaction to modify DNA has been extensively studied and reviewed.^{312, 395, 396} Modifications installed using click chemistry include fluorophores,³⁹⁷ affinity handles³⁹⁸ and metal complexes.³⁹⁹ Over the last number of years, there has been significant interest

in the generation of biocompatible oligonucleotides with a triazole linkage replacing the phosphodiester bond (**Section 1.6.2.1**).²⁹⁰⁻³⁰⁰ In fact, triazole-modified oligonucleotides can be replicated and transcribed in cells.³⁰¹⁻³⁰³

Although the incorporation of fragments into peptides *via* click chemistry to investigate protein-protein interactions has been reported,⁴⁰⁰ integration of fragments into DNA to examine DNA-protein interactions has not yet been explored. DNA-encoded fragment libraries have been widely employed for identification of small molecule ligands of protein targets.^{401, 402} Whereas in this area of research, the DNA strand is important for the construction and identification of the fragment library,^{401, 402} in the work described in this section, the oligonucleotide strand is key for recognition by SNM1A.

This section details the synthesis of compounds containing an alkyne handle, a metal-binding fragment, and a linker. Initially, a flexible linker was investigated for the incorporation of metal-binding fragments into oligonucleotide strands (**Figure 4.4A**). Subsequently, a thymidine-derived linker was explored with the aim of enhancing recognition by SNM1A (**Figure 4.4B**). These alkyne-containing compounds were incorporated into oligonucleotides *via* the CuAAC reaction and the resulting modified oligonucleotides were evaluated for recognition by SNM1A.

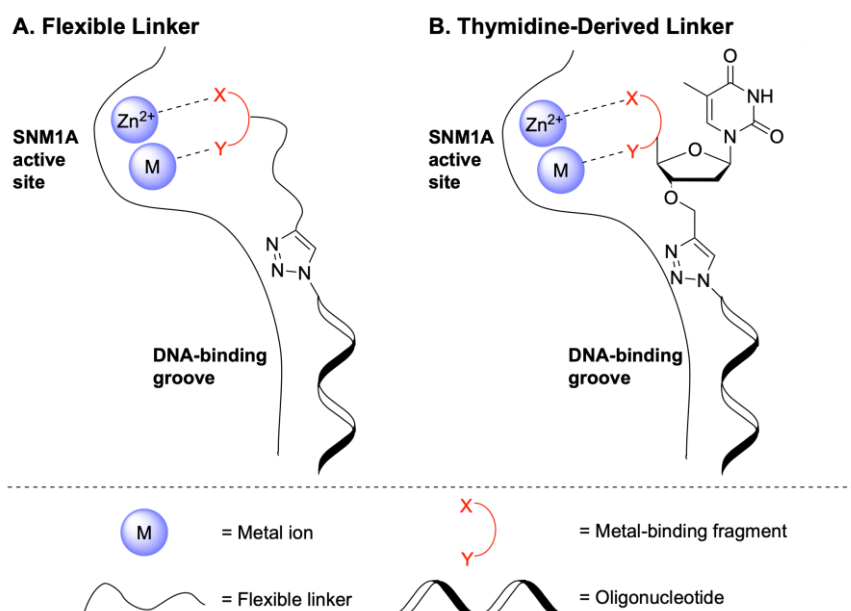


Figure 4.4: Triazole-modified oligonucleotides for targeting SNM1A. Incorporation of MBGs into the 5'-position of oligonucleotides enables the simultaneous targeting of both the SNM1A active site and the DNA-binding groove. MBGs were integrated into oligonucleotides *via* the CuAAC reaction. Both a flexible linker (A) and a thymidine-based linker (B) were investigated.

4.3.1. Triazole-Modified Oligonucleotides: Flexible Linker Derivatives

A series of compounds containing an alkyne handle, a metal-binding fragment and a flexible linker were designed (**Figure 4.5**). The metal-binding fragments included the hydroxamic acid and squaramide motifs identified by fragment-based screening, as well as sulfonamide groups which have had previous success in the inhibition of SNM1A when incorporated into a nucleoside scaffold.²¹⁷ Mesylate groups were also investigated. Alcohol, amine and phosphate groups were used in this method as negative and positive binding controls. The selected 1,4-triazole modification was derived from a 5'-azido-containing oligonucleotide and an alkyne-containing flexible linker. This modification was selected based on the facile installation of a 5'-azido group during solid-phase oligonucleotide synthesis. DNA with triazole modifications of this type have displayed high compatibility with polymerase enzymes. They can be replicated and transcribed by polymerases in cells while retaining high fidelity readthrough.^{296, 301-303} However, this triazole modification displays a reduction in binding affinity to a complementary DNA or RNA target^{295, 304-306} and induces a slowdown in polymerase chain reaction (PCR) replications compared to other triazole modifications,³⁰³ suggesting that this triazole modification introduces a distortion into the DNA strand when incorporated between two furanose rings. Therefore, the flexible linker was selected to allow facile coordination of the MBG to the active site of SNM1A by minimising perturbances caused by the triazole linkage. The linker length was also varied with a one atom extension to investigate whether the distance between the metal-binding fragment and the oligonucleotide strand affects enzyme recognition of the modified oligonucleotide.

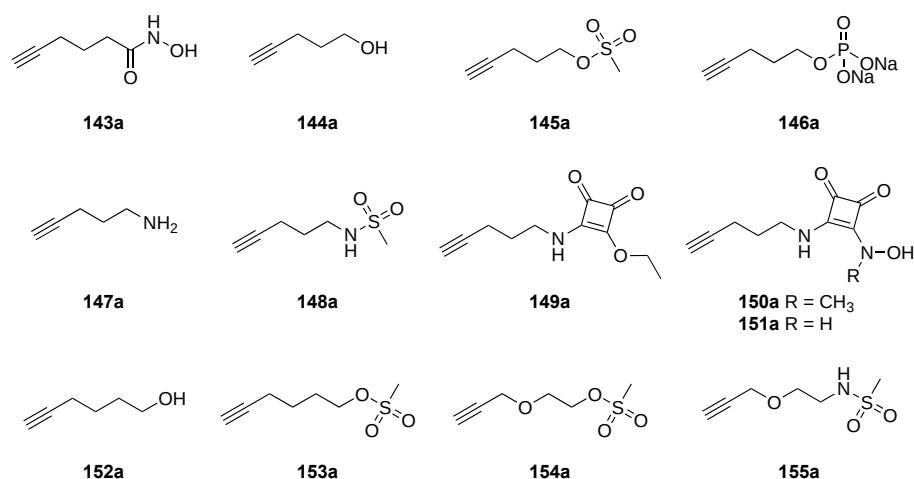
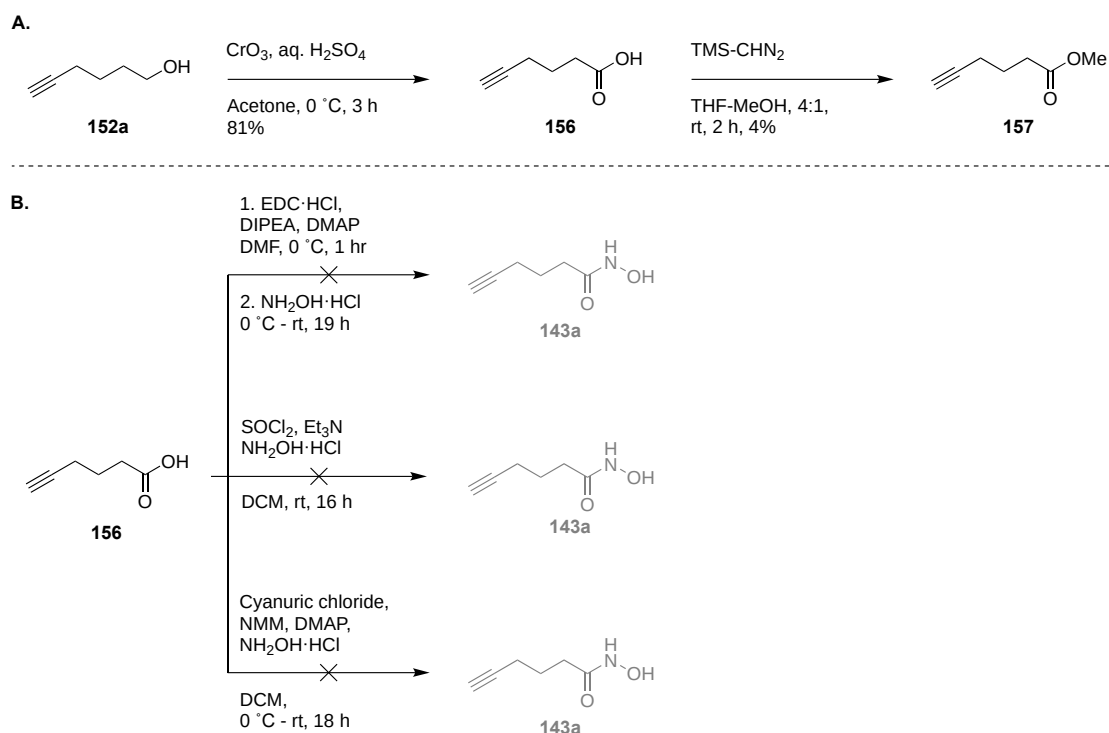


Figure 4.5: Target compounds containing an alkyne handle, a metal-binding fragment and a flexible linker were designed. These compounds are suitable for incorporation into oligonucleotides *via* the CuAAC reaction with a 5'-azido oligonucleotide.

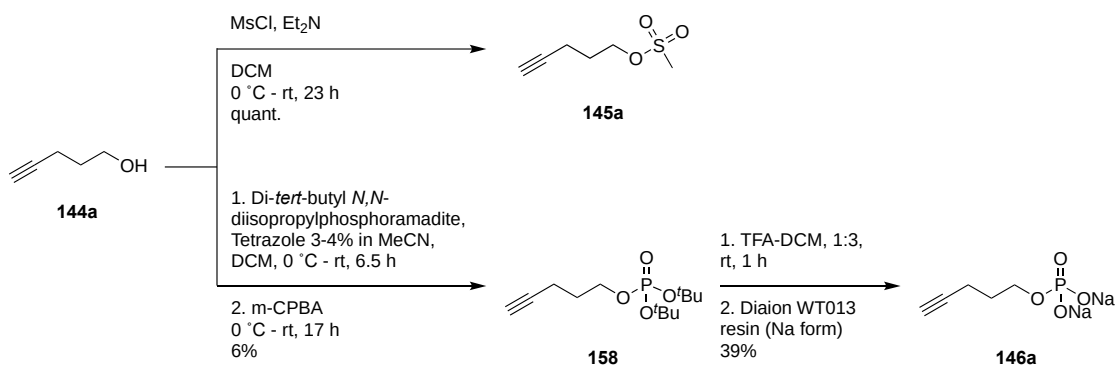
4.3.1.1. Synthesis and Biochemical Evaluation of Metal-Binding Fragments Containing an Alkyne Handle

Initially, the generation of hydroxamic acid **143a** was of particular interest due to the success of this MBG during the fragment-based screening (**Figure 4.3, Section 4.2.2**), as well as its previous efficiency in targeting SNM1A when incorporated into a nucleoside scaffold.²²⁰ Synthetic efforts towards the generation of **143a** are illustrated in **Scheme 4.2**.



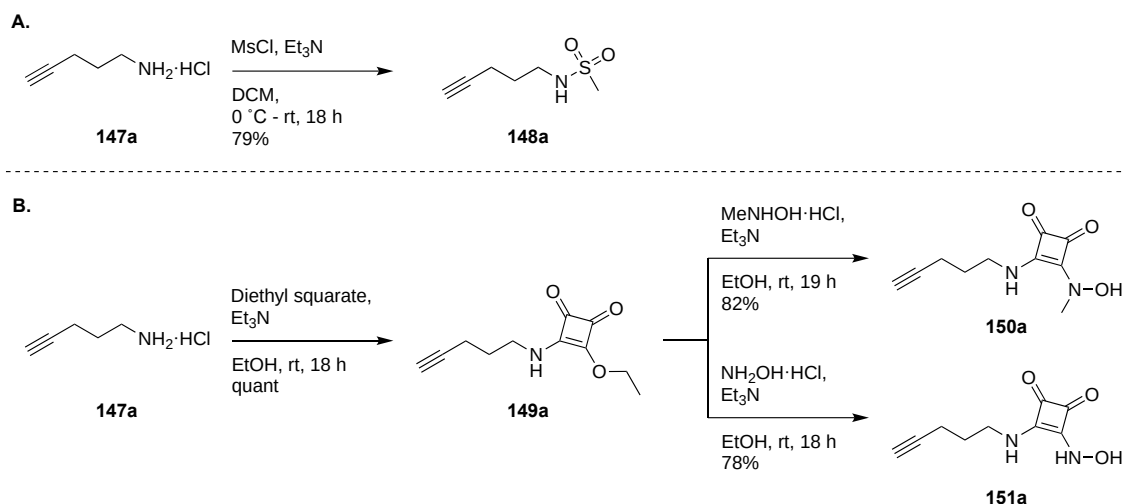
Scheme 4.2: Synthetic efforts towards the generation of hydroxamic acid **143a**.

Oxidation of 5-hexyn-1-ol, **152a**, with Jones' reagent, furnished carboxylic acid **156** in 81% yield. Methylation of carboxylic acid **156** was then carried out in order to generate methyl ester **157**, with the aim of subsequently generating hydroxamic acid **143a** *via* aminolysis with NH_2OH . However, after methylation with TMS-CHN_2 , only 4% of **157** was isolated due to its volatility. As an alternative, synthesis of hydroxamic acid **143a** was attempted *via* EDC-mediated coupling of carboxylic acid **156** with $\text{NH}_2\text{OH}\cdot\text{HCl}$.⁴⁰³ However, TLC analysis after 19 h indicated no reaction progress. Synthesis of hydroxamic acid **143a** was then attempted with the acid chloride derivative of **156**. Initially SOCl_2 was used as a chlorinating agent,⁴⁰⁴ but this resulted in a complex mixture of products after 16 h and **143a** was not isolated. Cyanuric chloride, was employed as a mild chlorinating agent,³⁵⁵ but again a complex mixture was formed after 18 h and none of the desired product **143a** was isolated. Due to the difficulties encountered during the attempted synthesis of **143a**, alternative MBGs were explored. The synthesis of target compounds derived from 4-pentyn-1-ol, **144a**, is illustrated in **Scheme 4.3**.



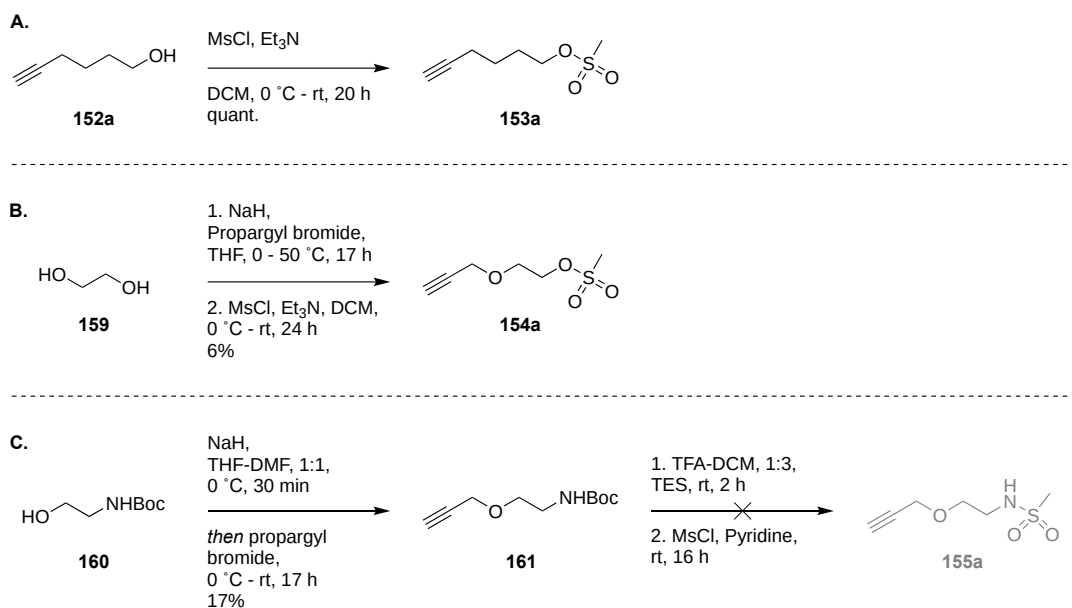
Scheme 4.3: Synthesis of target compounds **145a** and **146a** from 4-pentyn-1-ol **144a**.

Mesylation of 4-pentyn-1-ol, **144a**, using a literature procedure furnished mesylate **145a** in quantitative yield.⁴⁰⁵ The ^1H NMR spectrum featured a singlet at 3.03 ppm, indicating successful formation of the mesylate. Further characterisation using ^{13}C , HSQC and HMBC NMR experiments as well as IR spectroscopy confirmed the structure of **145a**. Treatment of 4-pentyn-1-ol, **144a**, with di-*tert*-butyl *N,N*-diisopropylphosphoramidite followed by oxidation with *meta*-chloroperoxybenzoic acid (*m*-CPBA) afforded protected phosphate **158** in 6% yield. This low yield may be due to the relatively mild conditions used,⁴⁰⁶ and an increased yield may have been achieved using a phosphorylating reagent with higher reactivity, such as phosphoryl chloride.⁴⁰⁷⁻⁴⁰⁹ However, since only a small quantity of **146a** was required, the amount of **158** obtained was sufficient and the reaction conditions were not optimised further. The phosphate group was deprotected followed by ion exchange to furnish disodium phosphate **146a** in 39% yield. The presence of a signal at 3.06 ppm in the ^{31}P NMR spectrum of the product indicated the presence of the phosphate. Further characterisation using ^{13}C , HSQC and HMBC NMR experiments as well as IR spectroscopy and HRMS confirmed the structure of **146a**. The synthesis of target compounds **148a-151a**, derived from 4-pentyn-1-amine, **147a**, was then carried out as shown in **Scheme 4.4**.



Scheme 4.4: Synthesis of target compounds derived from 4-pentyn-1-amine, **147a**.

4-Pentyn-1-amine hydrochloride, **147a**, was mesylated to afford sulfonamide **148a** in 79% yield. A singlet at 2.98 ppm in the ^1H NMR spectrum of the purified product indicated the presence of the sulfonamide. Further characterisation using ^{13}C , HSQC and HMBC NMR experiments as well as IR spectroscopy and HRMS confirmed the structure of **148a**. Monosquaramide **149a** was generated in quantitative yield, *via* reaction of 4-pentyn-1-amine hydrochloride **147a** with diethyl squarate. Monosquaramide **149a** was treated with *N*-methylhydroxylamine hydrochloride or hydroxylamine hydrochloride to furnish squaramides **150a** and **151a** in 82% and 78% yield respectively. The ^{13}C NMR spectra of the purified squaramides **149a-151a** featured signals in the characteristic region of 160 – 190 ppm, corresponding to the squaramide functional groups. Full characterisation using ^1H , ^{13}C , HSQC and HMBC NMR experiments, IR spectroscopy and HRMS confirmed the structures of **149a-151a**. Synthesis of target compounds **153a-155a**, in which the linker length was extended by one atom, was then attempted (Scheme 4.5).



Scheme 4.5: Synthesis of target compounds with an extended flexible linker.

5-Hexyn-1-ol, **152a**, was mesylated following a literature procedure to give mesylate **153a** in quantitative yield (**Scheme 4.5A**).⁴¹⁰ A singlet at 3.01 ppm in the ^1H NMR spectrum indicated formation of the mesylate and characterisation by ^1H , ^{13}C NMR and IR spectroscopy confirmed the structure of **153a**. A one-pot propargylation-mesylation was carried out to furnish **154a** in 6% yield (**Scheme 4.5B**). A singlet at 3.07 ppm, corresponding to the sulfonamide group, and peaks at 2.47 ppm (triplet) and 4.22 ppm (doublet), corresponding to the propargyl group, were observed in the ^1H NMR spectrum. Characterisation using ^{13}C , HSQC and HMBC NMR experiments, IR spectroscopy and HRMS confirmed the structure of **154a**. Despite the low yield, a sufficient quantity of **154a** was isolated and, therefore, the reaction conditions were not optimised further. Synthesis of the sulfonamide analogue **155a** was then attempted (**Scheme 4.5C**). Propargylation of *N*-Boc-ethanolamine (**160**) using a literature procedure afforded **161** in 17% yield.⁴¹¹ Generation of **155a** was attempted *via* Boc deprotection with TFA-DCM, 1:3, followed by mesylation with MsCl in pyridine. However, NMR analysis of the crude product revealed only the unprotected amine derivative and none of the desired product **155a** was isolated. As **145a-146a**, **148a-151a**, **153a** and **154a** had already been successfully synthesised, it was determined that a sufficient number of compounds had been generated in order to assess the feasibility of utilising the CuAAC reaction for incorporating MBGs into oligonucleotides as a strategy for targeting SNM1A. Therefore, the synthesis of **155a** was not pursued further.

Prior to their incorporation into oligonucleotides, compounds **144a-154a**, containing an alkyne handle and an MBG, were tested for inhibition of SNM1A. This was to enable comparison between **144a-154a** and their oligonucleotide counterparts. Like in the fragment-based screening (Section 4.2.2), **144a-154a** were tested for inhibition of SNM1A at 5 mM using the gel electrophoresis-based assay (Figure 4.6). As previous, **146a** was evaluated as the disodium salt, rather than as the acidic phosphate group, in order to minimise perturbations in pH during the enzymatic assay.

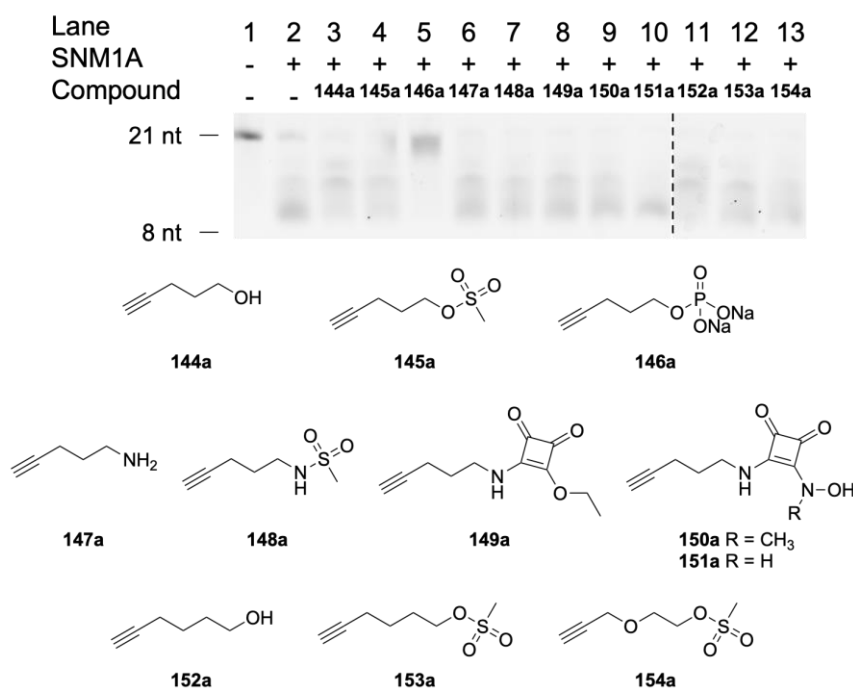
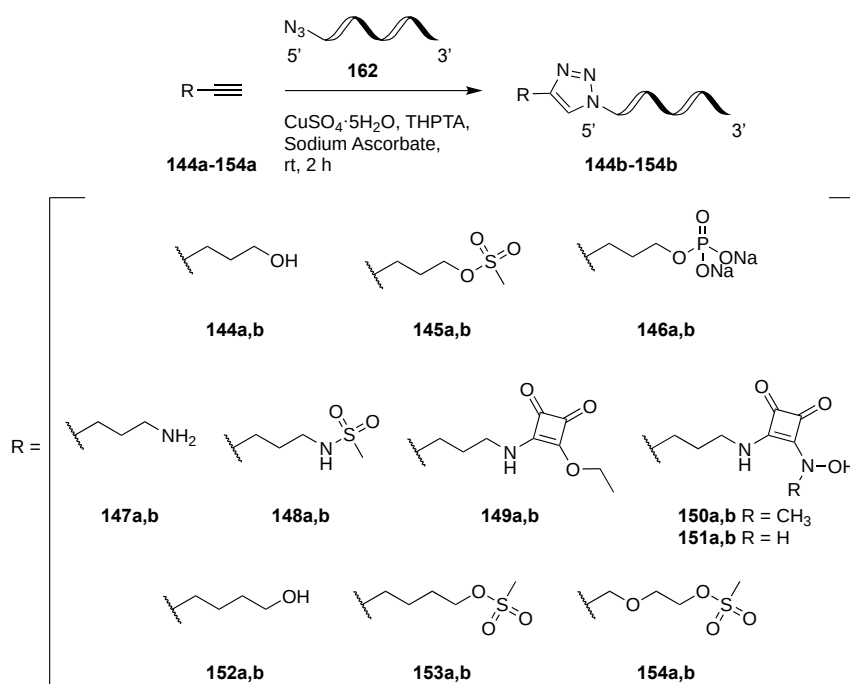


Figure 4.6: Evaluation of **144a-154a** as inhibitors of SNM1A. Fragments **144a-154a** (5 mM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

A control without SNM1A (Figure 4.6, Lane 1) and a control with SNM1A (Figure 4.6, Lane 2) were used as the negative and positive controls for digestion of substrate oligonucleotide **64**, respectively. Phosphate **146a** was the only compound to display any notable inhibition of SNM1A (Figure 4.6, Lane 5). Digestion of the substrate oligonucleotide, **64**, was seen in the case of all the other compounds (Figure 4.6, Lanes 3-4, 6-13). Comparison of these results with those obtained with the corresponding oligonucleotides may provide useful insights into the effect of the inclusion of the oligonucleotide strand on SNM1A binding.

4.3.1.2. Synthesis and Biochemical Evaluation of Modified Oligonucleotides

5'-Azido oligonucleotide **162** (sequence of **162**: 5'-(azidoT)AG CAG TCA GTC AGT CAT GC-3') was provided by the Brown Group, University of Oxford. A CuAAC reaction of compounds **144a-154a** with 5'-azido oligonucleotide **162** afforded modified oligonucleotides **144b-154b** (Scheme 4.6).



Scheme 4.6: Late-stage modification of oligonucleotides. Alkyne-containing compounds **144a-154a** reacted with 5'-azido oligonucleotide **162** in a CuAAC reaction to give modified oligonucleotides **144b-154b**. Sequence of 5'-azido oligonucleotide **162**: 5'-(azidoT)AG CAG TCA GTC AGT CAT GC-3'

The CuAAC reaction was carried out using a modified literature procedure to furnish modified oligonucleotides **144b-154b**, featuring a 5'-MBG linked to the oligonucleotide strand *via* a triazole moiety.²⁸ Until now, the incorporation of metal-binding fragments into oligonucleotide strands *via* click chemistry had not yet been reported. All Cu(I) catalysed triazole formations attempted proceeded smoothly with high conversions demonstrating the wide applicability of this novel method.

Triazole-modified oligonucleotides **144b-154b** were evaluated for binding to SNM1A using a similar inhibition assay to that described previously (Section 2.2.1). In the absence of an inhibitor, SNM1A digests fluorescent substrate oligonucleotide **64** with 5'-3' directionality to produce shorter fluorescent products (Figure 4.7A). However, if a modified oligonucleotide binds to SNM1A, the active site is blocked, digestion of

substrate oligonucleotide **64** is hindered and the full length 21mer **64** is visible in the PAGE readout (**Figure 4.7B**).

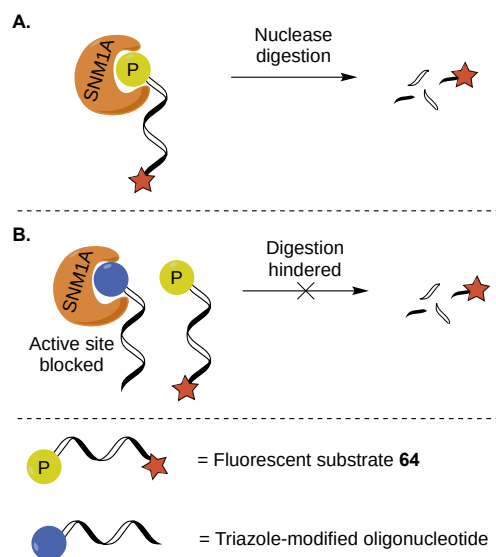


Figure 4.7: Evaluation of the ability of triazole-modified oligonucleotides **144b-154b** to bind to SNM1A. A. In the absence of an inhibitor, SNM1A digests substrate oligonucleotide **64** to produce shorter fluorescent products. B. If a modified oligonucleotide binds to SNM1A, the active site is blocked, digestion of **64** is hindered.

The DNA strand is highly important for substrate recognition by SNM1A.⁵⁸ Therefore, whereas fragments **144a-154a** were evaluated for inhibition of SNM1A at 5 mM, modified oligonucleotides **144b-154b** were assessed at the >1000 fold lower concentration of 2.8 μ M. The results were analysed by PAGE (**Figure 4.8**).

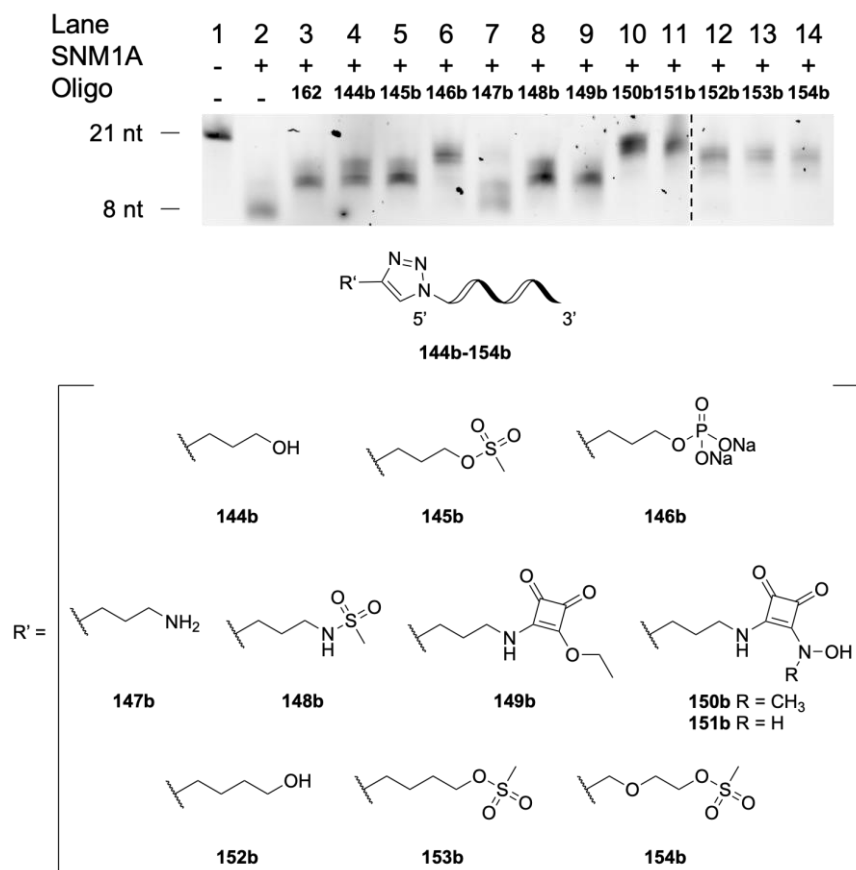


Figure 4.8: Evaluation of binding of **144b-54b** to SNM1A using the gel electrophoresis-based inhibition assay. Modified oligonucleotides **162**, **144b-154b** (2.8 μM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

A control without SNM1A (**Figure 4.8**, Lane 1) and a control with SNM1A (**Figure 4.8**, Lane 2) were used as the negative and positive controls for digestion of oligonucleotide **64**, respectively. To investigate the extent to which binding of the modified oligonucleotides to the enzyme was due to the oligonucleotide strand or whether the MBG had some influence on binding, 5'-azido oligonucleotide **162** was included in the assay. Despite the fact that **162** demonstrated moderate inhibition of SNM1A (**Figure 4.8**, Lane 3), it was clear that varying the MBG did influence substrate binding and enzyme inhibition. Alcohol **144b** (**Figure 4.8**, Lane 4) and mesylate **145b** (**Figure 4.8**, Lane 5) had little effect on SNM1A activity as substrate oligonucleotide **64** was digested. Phosphate **146b** (**Figure 4.8**, Lane 6) demonstrated effective inhibition of enzymatic activity at this concentration, therefore suggesting that the flexible linker enabled the phosphate group to effectively bind to the active site. As anticipated, amine **147b** had no effect on enzymatic activity (**Figure 4.8**, Lane 7) while sulfonamide **148b** and monosquaramide **149b** displayed moderate inhibition of SNM1A

(**Figure 4.8**, Lanes 8 and 9). Similar to the results obtained during the fragment-based screening (**Section 4.2.2**), *N*-hydroxysquaramides **150b** and **151b** considerably reduced SNM1A activity (**Figure 4.8**, Lanes 10 and 11). The one carbon extension of the flexible linker did not notably affect binding of the modified oligonucleotides to SNM1A (**Figure 4.8**, Lanes 12-14). Although alcohol **152b** (**Figure 4.8**, Lane 12) and mesylates **153b** and **154b** (**Figure 4.8**, Lanes 13 and 14) displayed slightly increased levels of inhibition compared to **144b** and **145b** (**Figure 4.8**, Lanes 4 and 5), modified oligonucleotides **152b-154b** did not substantially reduce enzyme activity. In order to further investigate the binding ability of the modified oligonucleotides, **144b-154b** were also evaluated for inhibition of SNM1A at the lower concentration of 1.6 μ M (**Figure 4.9**).

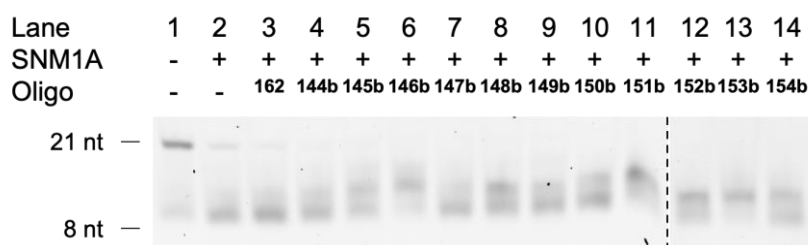


Figure 4.9: Evaluation of recognition of **144b-154b** by SNM1A at 1.6 μ M. Modified oligonucleotides **144b-154b** (1.6 μ M) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. nt = nucleotides

As presented in **Figure 4.9**, **144b-154b** did not demonstrate any inhibition of SNM1A activity at 1.6 μ M. Digestion of fluorescent substrate oligonucleotide **64** was observed in all cases, indicating that the modified oligonucleotides were not binding to SNM1A.

Inclusion of the oligonucleotide strand greatly enhanced substrate binding, from 5 mM of the metal-binding fragments alone to 2.8 μ M when incorporated into an oligonucleotide strand. When the click fragments **144a-154a** were tested against SNM1A at 5 mM, phosphate **146a** reduced SNM1A activity whereas squaramide moieties **150a** and **151a** did not. However, incorporation of the squaramide moieties into an oligonucleotide strand enhanced their binding as **150b** and **151b** reduced enzyme activity at 2.8 μ M. The large discrepancy in inhibitory activity can be rationalised by the oligonucleotide anchoring the MBGs within the active site of the protein. This anchoring effect increases the avidity of the molecule, blocking the active site at much lower concentrations. Incorporation of MBGs into the 5'-position of an oligonucleotide strand

via the CuAAC reaction is, therefore, an effective method of generating non-natural oligonucleotides capable of binding to SNM1A.

4.3.2. Triazole-Modified Oligonucleotides: Thymidine-Derived Linker

While the un-natural triazole linkage was recognised by SNM1A at low concentrations, these modified oligonucleotides (**144b-154b**, **Figure 4.10A**) displayed a lower affinity for SNM1A compared to naturally linked oligonucleotides.²¹⁹ Naturally-linked oligonucleotides featuring squaramide-derived MBGs in the 5'-position have previously demonstrated good inhibition of SNM1A at 0.27 μ M and moderate inhibition as low as 80 nM (**55**, **56**, **Figure 4.10A**, previously discussed in **Section 1.5.2**).²¹⁹ This disparity in activity could potentially be due to perturbances introduced by the triazole linkage, or may be due to a lack of recognition of the flexible linker by the enzyme. It was hypothesised that replacement of the flexible linker with a recognition motif, such as a thymidine-derived linker, may enhance nuclease binding (**Figure 4.10B**). To investigate this, a series of thymidine derivatives featuring a 3'-alkyne handle were designed and synthesised (**163a-165a**, **Figure 4.10C**). These thymidine derivatives were incorporated into the 5'-position of oligonucleotide strands *via* the CuAAC reaction, as previously. The ability of the modified oligonucleotides to bind to SNM1A was evaluated and compared to an unmodified oligonucleotide. 5'-Hydroxyl and 5'-phosphate derivatives were explored as well as a 5'-hydroxamic acid derivative in order to evaluate whether any variation in oligonucleotide binding of SNM1A was due to the 5'-modification and/or the triazole linkage.

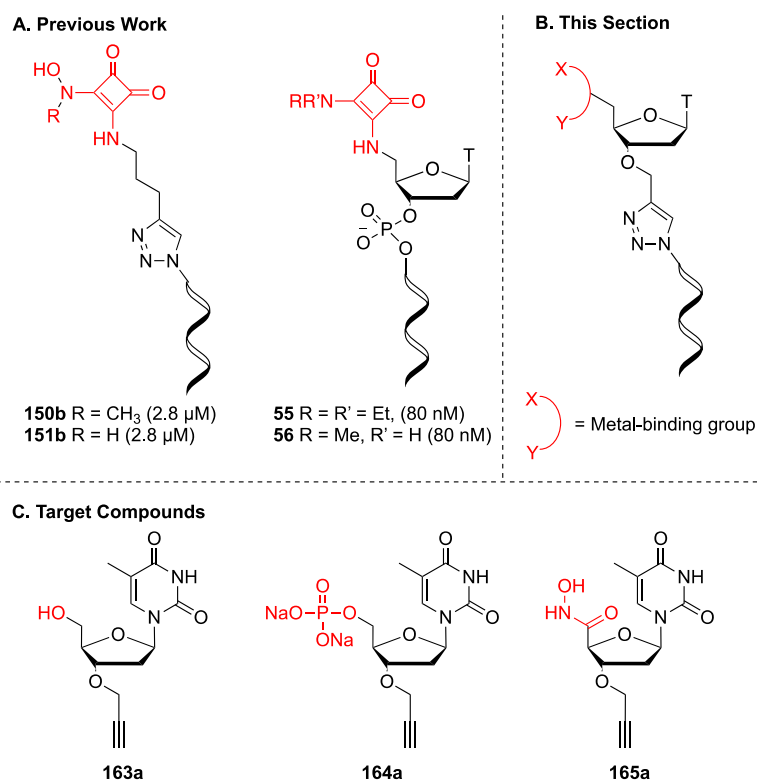
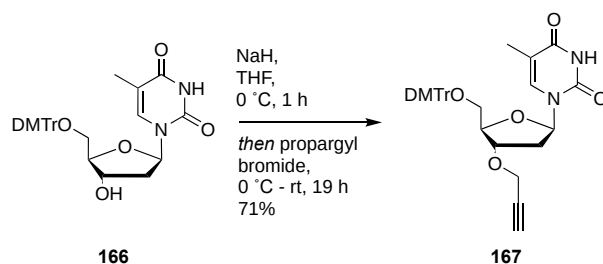


Figure 4.10: Oligonucleotide modification for targeting SNM1A. **A.** Oligonucleotides identified as inhibitors of SNM1A in **Section 4.3.1** and in previous studies in the McGouran Group.²¹⁹ **B.** Oligonucleotide design for this section. **C.** Thymidine derivatives designed for incorporation into oligonucleotides *via* the CuAAC reaction. Numbers in brackets indicate lowest inhibitory concentration for inhibition of SNM1A. Metal-binding groups are highlighted in red.

4.3.2.1. Synthesis of Thymidine Derivatives Containing an Alkyne Handle

The initial step in the synthesis of target compounds **163a-165a**, involved the propargylation of 5'-DMTr protected thymidine **166** to give **167** (**Scheme 4.7**).

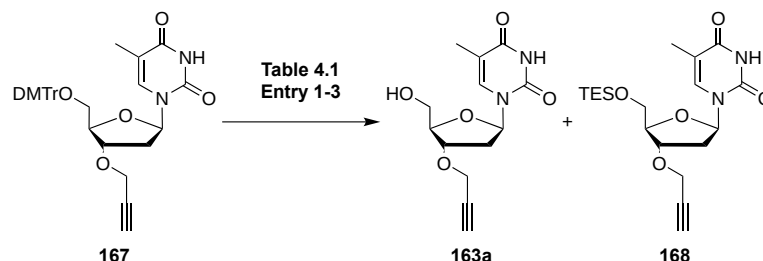


Scheme 4.7: Propargylation of DMTr-protected thymidine **166**.

5'-DMTr protected thymidine **166** was propargylated in the 3'-position using a modified literature procedure.⁴¹² Use of a toluene-acetone eluent during column chromatography to separate **167** from the *N*³-propargylated derivative, enabled isolation of **167** in 71%

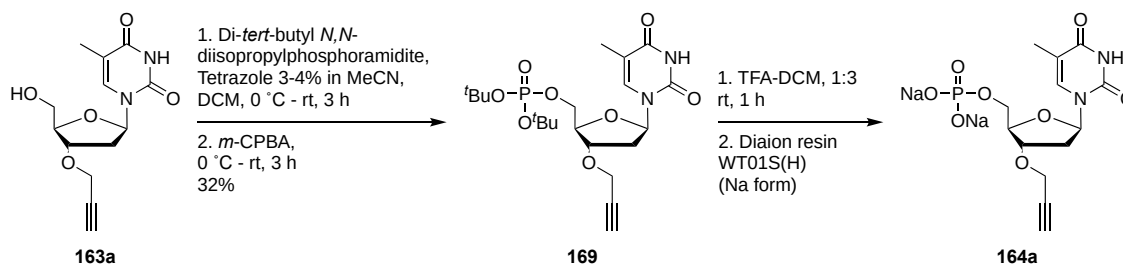
yield. Presence of the N-H in the N^3 -position was confirmed by ^1H - ^{15}N HSQC NMR experiments. Removal of the DMTr group was optimised as illustrated in **Table 4.1**.

Table 4.1: Optimisation of detritylation of **167**.



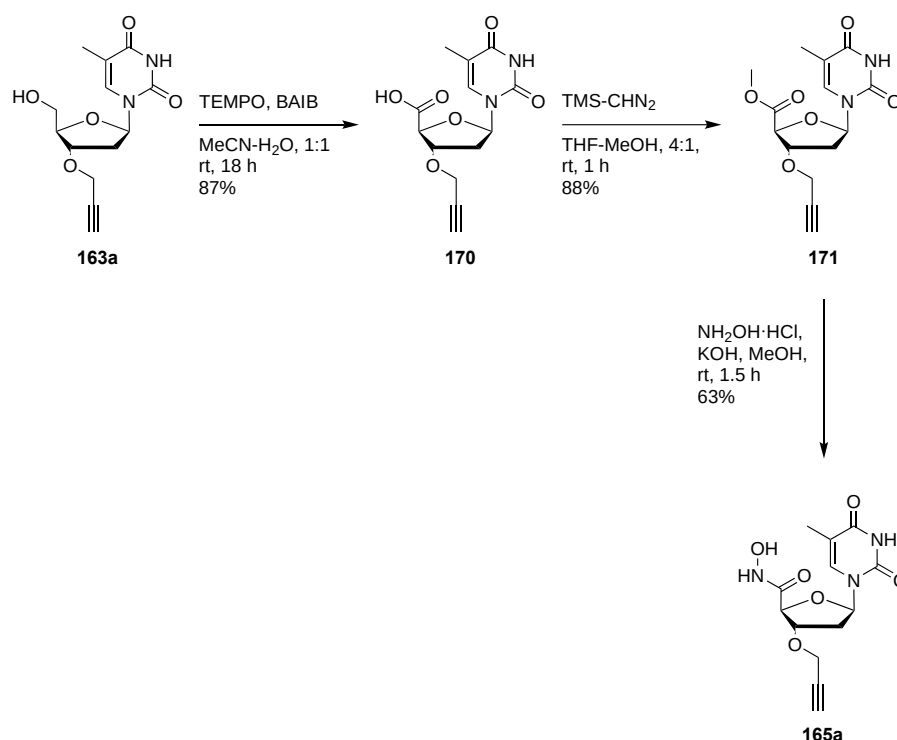
Entry	Deprotection reagents	Temp.	Time (min)	Result
1	3% TFA in DCM, TES (5 eq.)	rt	10	163a : 59% 168 : 24%
2	3% TFA in DCM	rt	17	163a : 56%
3	80% AcOH in H_2O	rt	50	163a : 73%

DMTr removal was initially attempted utilising the optimised reaction conditions from **Section 2.3.1**: 3% TFA in DCM with TES. This, however, afforded **163a** in only a moderate yield of 59% as well as **168**, featuring a TES group in the 5'-position, in 24% yield (**Table 4.1**, Entry 1). The reaction was repeated using 3% TFA in DCM without TES to avoid the generation of the unwanted side-product **168**. This resulted in a lower yield, however, with **163a** furnished in 56% yield (**Table 4.1**, Entry 2). Finally, use of 80% AcOH in H_2O afforded **163a** in 73% yield (**Table 4.1**, Entry 3). The signals corresponding to the DMTr aromatic groups (multiplets in the region of 6.8 – 7.4 ppm) and methoxy groups (singlet at 3.74 ppm) were no longer present in the ^1H NMR spectrum of the purified product and full characterisation confirmed the structure of **163a**. **163a** was then used to generate phosphate **164a** (**Scheme 4.8**).



Scheme 4.8: Synthesis of phosphate **164a** from alcohol **163a**.

Alcohol **163a** was first treated with di-*tert*-butyl *N,N*-diisopropylphosphoramidite followed by oxidation with *m*-CPBA to afford protected phosphate **169** in 32% yield. Removal of the *tert*-butyl (*t*Bu) groups was carried out using TFA-DCM, 1:3 and diaion resin WT01S(H) (Na form) was used to generate the disodium phosphate **164a**. A signal at 2.73 ppm in the ^{31}P NMR spectrum of the purified product indicated the presence of the 5'-phosphate group. Full characterisation by ^1H , ^{13}C NMR, IR spectroscopy and HRMS confirmed the structure of **164a**. Alcohol **163a** was also used to generate **165a** bearing a 5'-hydroxamic acid group (**Scheme 4.9**).

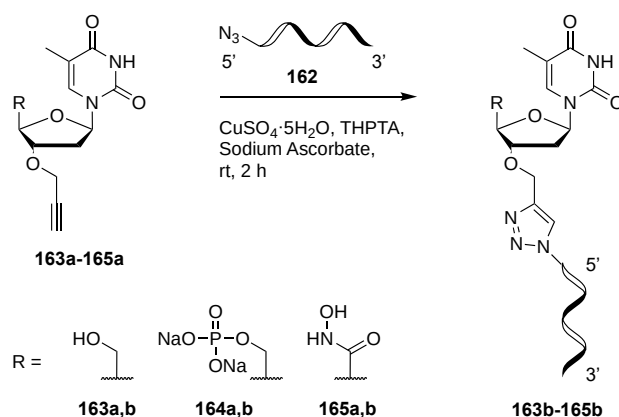


Scheme 4.9: Synthesis of target compound **165a** from **163a**.

Oxidation of the 5'-position was carried out using TEMPO/BAIB-mediated oxidation to furnish carboxylic acid **170** in 87% yield. Methylation of the carboxylic acid using TMS-CHN₂ afforded **171** in 88% yield. Finally, aminolysis was carried out with NH₂OH·HCl to produce **165a** in 63% yield. The ^1H NMR spectrum of the purified product no longer featured a peak corresponding to the methyl ester (singlet at 3.83 ppm) and instead, peaks corresponding to the -OH (broad singlet at 9.22 ppm) and -NH (singlet at 11.07 ppm) groups of the hydroxamic acid was observed. Characterisation by ^1H , ^{13}C NMR, IR spectroscopy and HRMS confirmed the structure of **165a**.

4.3.2.2. Synthesis and Biochemical Evaluation of Modified Oligonucleotides Containing a Triazole Internucleotide Linkage

With **163a**, **164a** and **165a** in hand, modified oligonucleotides with a triazole internucleotide linkage could be generated. As before (Section 4.3.1.2), **163a-165a** underwent a CuAAC reaction with 5'-azido oligonucleotide **162** (Scheme 4.10).²⁸



Scheme 4.10: Late-stage modification of oligonucleotides. Thymidine derivatives containing a 3'-propargyl group, **163a-165a**, reacted with 5'-azido oligonucleotide **162** in a CuAAC reaction to give modified oligonucleotides **163b-165b**. Sequence of 5'-azido oligonucleotide **162**: 5'-(azidoT)AG CAG TCA GTC AGT CAT GC-3'

The CuAAC reaction was carried out to furnish modified oligonucleotides **163b-165b**, featuring a triazole internucleotide linkage and a 5'-OH, 5'-phosphate or 5'-hydroxamic acid moiety. Compared to oligonucleotides **144b-154b**, which utilised a flexible linker, **163b-165b** feature a thymidine-derived linker for enhanced recognition by SNM1A. Triazole-modified oligonucleotides **163b-165b** were evaluated for binding to SNM1A using a similar inhibition assay to that described previously (Section 2.2.1). The results are displayed in Figure 4.11.

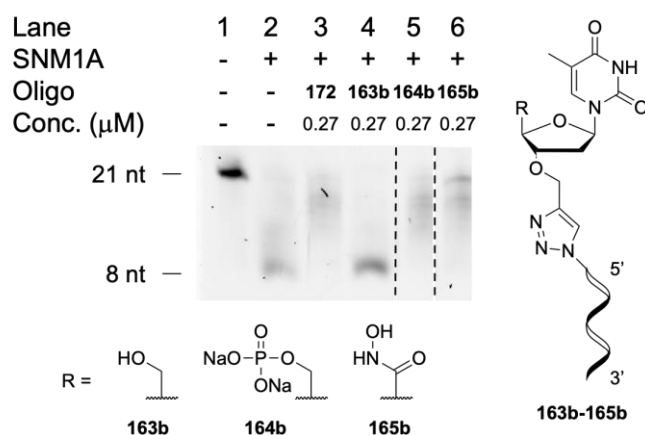


Figure 4.11: Evaluation of binding of **163b-165b** to SNM1A using the gel electrophoresis-based inhibition assay. Modified oligonucleotides **163b-165b** (0.27 μ M) were pre-incubated with SNM1A (25 fmol, 2.5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. Sequence of naturally-linked oligonucleotide 21mer **172**: 5'-TTAG CAG TCA GTC AGT CAT CG-3'; nt = nucleotides

Compared to triazole-linked oligonucleotides **144b-154b**, utilising a flexible linker, oligonucleotides **163b-165b**, employing a thymidine-derived linker, were expected to more closely resemble a natural oligonucleotide strand. Therefore, **163b-165b** were evaluated for inhibition of SNM1A at the lower concentration of 0.27 μ M. This corresponds to the concentration at which naturally-linked oligonucleotides featuring squaramide-derived MBGs in the 5'-position have previously demonstrated notable inhibition of SNM1A (Section 1.5.2).²¹⁹ **Figure 4.11**, Lanes 1 and 2 display the negative and positive controls for digestion of substrate oligonucleotide **64**, respectively. A control using a 21mer oligonucleotide (**172**, sequence: 5'-TTAG CAG TCA GTC AGT CAT CG-3'), containing a 5'-OH was used in place of a modified oligonucleotide to investigate any effect of the triazole and/or 5'-MBG on enzyme activity. At this concentration, oligonucleotide **172** hinders enzyme activity (**Figure 4.11**, Lane 3). Interestingly, triazole-modified oligonucleotide **163b**, featuring a 5'-OH group did not reduce activity of SNM1A, as full digestion of substrate oligonucleotide **64** was observed (**Figure 4.11**, Lane 4). Comparison of the results obtained with naturally-linked oligonucleotide **172** (**Figure 4.11**, Lane 3) and modified oligonucleotide **163b** (**Figure 4.11**, Lane 4), both bearing a 5'-OH, indicates that the triazole modification affects enzyme recognition. The triazole modification may induce a distortion of the oligonucleotide strand, disturbing the binding of **163b** to SNM1A. Oligonucleotide **164b**, bearing a 5'-phosphate group, hindered enzyme activity (**Figure 4.11**, Lane 5), therefore suggesting that any perturbation introduced by the triazole modification was mitigated by the additional binding of the 5'-phosphate to the metal ion(s) in the SNM1A active site. Similarly,

oligonucleotide **165b** also reduced enzymatic activity (**Figure 4.11**, Lane 6), indicating that the 5'-hydroxamic acid effectively targeted the active site of SNM1A. Although oligonucleotides **164b** and **165b**, featuring a 5'-phosphate and a 5'-hydroxamic acid group respectively, reduced SNM1A activity at the low concentration of 0.27 μ M, these triazole-linked oligonucleotides did not show improved binding compared to the naturally-linked oligonucleotide **172**.

4.4. Conclusions

To conclude, this chapter describes a two-step validation process for the identification of metal-binding fragments suitable for targeting SNM1A. This consisted of a fragment-based screening approach followed by incorporation of fragments into oligonucleotides *via* click chemistry (**Figure 4.12**).

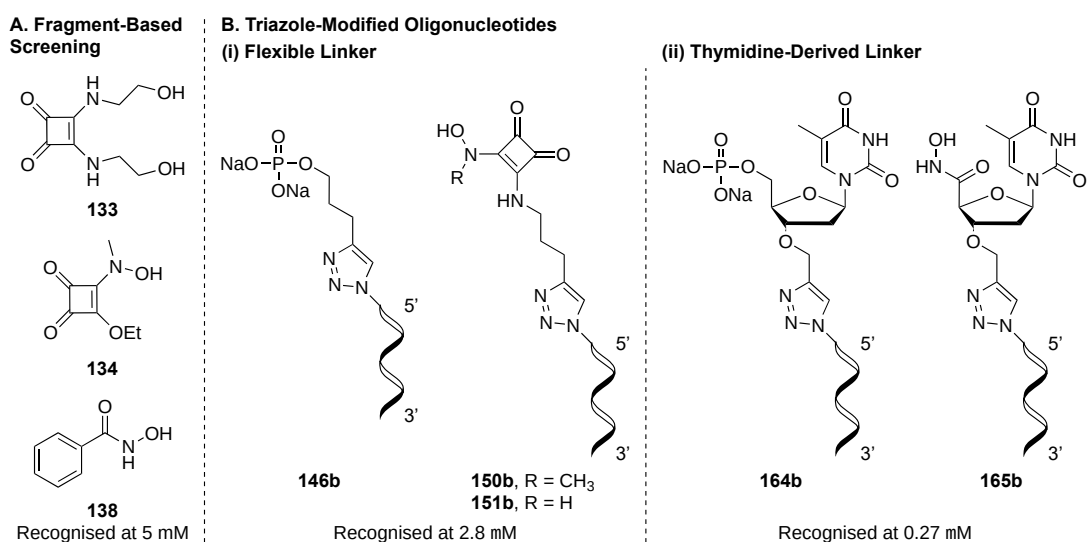


Figure 4.12: Two-step validation approach for the identification of MBGs suitable for targeting SNM1A. A. Fragment-based screening identified metal-binding fragments **133**, **134** and **138** which obstructed enzyme activity at 5 mM. B. Metal-binding fragments were incorporated into oligonucleotides *via* click chemistry to generate triazole-modified oligonucleotides. (i) Modified oligonucleotides **146b**, **150b** and **151b**, utilising a flexible linker, targeted SNM1A at 2.8 μ M. (ii) Modified oligonucleotides **164b** and **165b**, employing a thymidine-derived linker, successfully targeted SNM1A at 0.27 μ M.

The fragment-based screening approach successfully identified squaramide (**133** and **134**) and hydroxamic acid (**138**) metal-binding fragments suitable for targeting SNM1A (**Figure 4.12A**). This is the first time fragment-based screening has been used to identify fragments for targeting SNM1A and demonstrates the extensive utility of a fragment-based screening approach.

This work also demonstrates that incorporation of metal-binding fragments into an oligonucleotide strand *via* the CuAAC reaction is an effective method of rapidly generating non-natural oligonucleotides recognised by SNM1A and screening metal-binding fragments suitable for targeting the enzyme. Previously, the generation of modified nucleosides/oligonucleotides targeting SNM1A required extensive synthesis of modified nucleosides or phosphoramidites, which is both time-consuming and expensive.^{217, 219} Here, a reliable method of rapidly installing 5'-modifications into oligonucleotides using the CuAAC reaction was demonstrated.

Inclusion of the oligonucleotide strand greatly increased substrate recognition by SNM1A, from 5 mM of the metal-binding fragments alone to 2.8 μ M when incorporated into an oligonucleotide strand *via* a flexible linker. Triazole-modified oligonucleotides **146b**, **150b** and **151b** utilising a flexible linker and featuring 5'-phosphate and 5'-squaramide functionalities demonstrated notable inhibition of SNM1A activity at 2.8 μ M, suggesting that these oligonucleotides were targeting both the SNM1A active site and the DNA-binding groove (**Figure 4.12B(i)**). When oligonucleotides employing a thymidine-derived linker were evaluated, modified oligonucleotides **164b** and **165b** containing a 5'-phosphate and 5'-hydroxamic acid group respectively, were recognised by SNM1A at concentrations as low as 0.27 μ M (**Figure 5.12B(ii)**). However, these modified oligonucleotides did not show enhanced SNM1A binding compared to a naturally-linked oligonucleotide (**172**). Despite this, the generation of oligonucleotide **165b**, *via* the CuAAC reaction is of particular significance as previous efforts by the McGouran Group to incorporate a thymidine derivative featuring a 5'-hydroxamic acid group into an oligonucleotide strand *via* traditional solid-phase oligonucleotide synthesis methods were unsuccessful.²¹⁹

The well-established strategy of peptide tethering involves the incorporation of fragments into peptides to enhance their binding to targeted protein pockets.^{400, 413} The novel approach detailed here, involving the incorporation of fragments into oligonucleotides, may be similarly applied to generate modified oligonucleotides or DNA mimics with high affinity for DNA damage repair enzymes. The linker between the fragment and the oligonucleotide strand may be optimised in terms of linker length and type to further enhance binding of target enzymes. The two-step validation process described in this chapter may be applied in the future for the rapid identification of fragments capable of

selectively targeting DNA damage repair enzymes. These fragments may then be incorporated into nucleoside/oligonucleotide scaffolds or combined to generate probes to study DNA damage repair. These results demonstrate a novel synthetic methodology for rapidly installing metal-binding fragments on the 5'-position of oligonucleotides. This is a facile method of generating oligonucleotides with metal-binding capabilities.

Chapter 5

Oligonucleotides with Alternative Backbones for Targeting SNM1A

5. Oligonucleotides with Alternative Backbones for Targeting SNM1A

5.1. Introduction

With the surge in oligonucleotide-based therapeutics available on the market,¹⁵⁸ there have been extensive research efforts dedicated to the modification of the DNA backbone, involving the replacement of phosphodiester bonds with alternative groups. Introduction of alternative backbone moieties is carried out for a number of purposes, including to enhance binding affinity to complementary strands, to bolster resistance to degradation by nucleases and to improve membrane permeability – all crucial factors in the development of effective oligonucleotide therapeutics.²⁵⁶ This chapter describes the investigation of oligonucleotides featuring various alternative backbones for targeting SNM1A. Oligonucleotides in which one phosphodiester linkage was replaced with a non-natural internucleotide linkage were designed (**Figure 5.1**). The bridging groups included: (thio)urea, oxyurea, *N*-oxyamide, (thio)squaramide, and thio/sulfinyl/sulfonyl acetamide groups. If successful in targeting SNM1A, such modified oligonucleotides may serve as suitable scaffolds from which to build probes to study the enzyme.

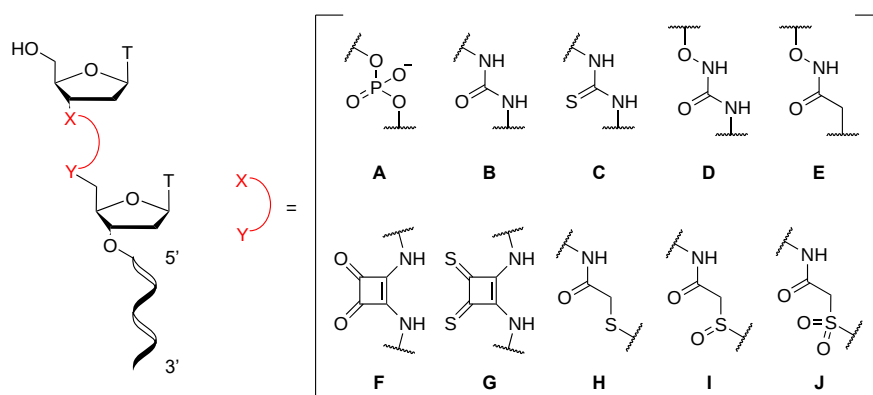


Figure 5.1: Oligonucleotides with modified backbones designed to target SNM1A. A. Natural phosphate, B. Urea, C. Thiourea, D. *N*-Oxyurea, E. *N*-Oxyamide (hydroxamate), F. Squaramide, G. Thiosquaramide, H. Thioacetamide, I. Sulfinyl acetamide, J. Sulfonyl acetamide

The bridging groups were designed based on their reported metal-binding capabilities and/or their previous use as phosphate replacements in oligonucleotide strands. The metal binding ability of (thio)urea, oxyurea, *N*-oxyamide (hydroxamate), (thio)squaramide and sulfoxide groups is well documented (**Figure 5.1B-G, J**).^{72, 73, 77, 216, 414-425} Moreover, a number of the selected bridging groups have previously been investigated as phosphate replacements in nucleosides/oligonucleotides and have been shown to be stable to solid phase oligonucleotide synthesis. Investigations into the use of urea groups (**Figure 5.1B**)

as phosphate replacements have demonstrated that oligonucleotides featuring such modifications exhibit polymerase compatibility⁴²⁶ and stability to enzymatic hydrolysis.²⁸⁵ The *N*-oxyamide (hydroxamate) (**Figure 5.1E**) linkage is of particular interest as one of the first inhibitors of SNM1A developed in the McGouran Group was a thymidine derivative featuring a 5'-hydroxamic acid group (**31**, **Section 1.5.2**).²²⁰ Furthermore, *N*-oxyamide internucleotide linkages have been shown to withstand solid phase synthesis.⁴²⁷ The exploration of squaramide moieties (**Figure 5.1F**) as alternative backbones holds promise in the targeting of SNM1A due to their strong polarisation and interaction with metal ions.²⁸⁷⁻²⁸⁹ Nucleoside and oligonucleotide derivatives featuring (thio)squaramide (**Figure 5.1F, G**) modifications, generated in the McGouran Group, have been shown to inhibit SNM1A.^{216, 219} Squaramide moieties have also been utilised in oligonucleotide strands both as standard 3'-5' linkages as well as unnatural 2'-5' linkages.^{288, 289, 426} Thioacetamide linkages (**Figure 5.1H**) have been investigated as phosphate replacements. Oligonucleotide strands containing such modifications displayed higher RNA binding selectivity compared to complementary DNA.⁴²⁸ Moreover, thioacetamides have been identified as effective MBL inhibitors.⁴²⁹⁻⁴³⁴

This chapter details the synthesis of a number of dinucleoside phosphoramidites featuring unnatural linkages. The phosphoramidites were incorporated into oligonucleotide strands *via* manual SPOS. The ability of the modified oligonucleotides to target SNM1A was evaluated using both a gel electrophoresis-based inhibition assay and a real-time fluorescence assay.

5.2. Synthesis of Dinucleoside Phosphoramidites

Artificial backbones are typically integrated into oligonucleotide strands through modified on-resin coupling of a monomer to form the unnatural linkage, or by coupling of a dinucleoside phosphoramidite featuring the artificial linkage.³²⁰ In this case, it was deemed that the latter was the most suitable approach. A series of dinucleosides with artificial linkages, a 5'-DMTr group and a 3'-OH group were synthesised in the McGouran Group by Dr William Doherty (**173-181**, **Figure 5.2**).

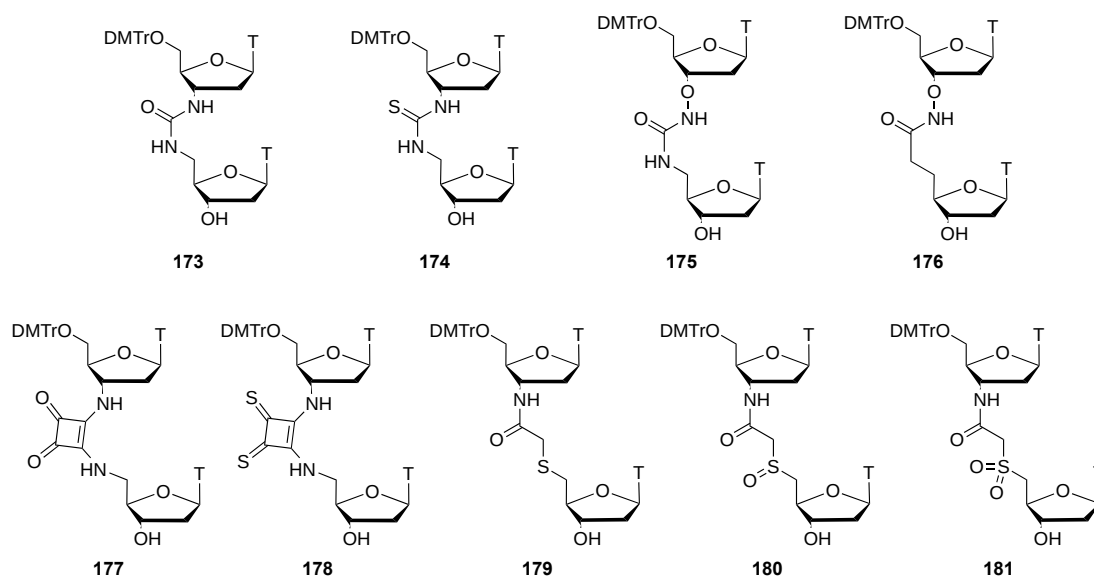
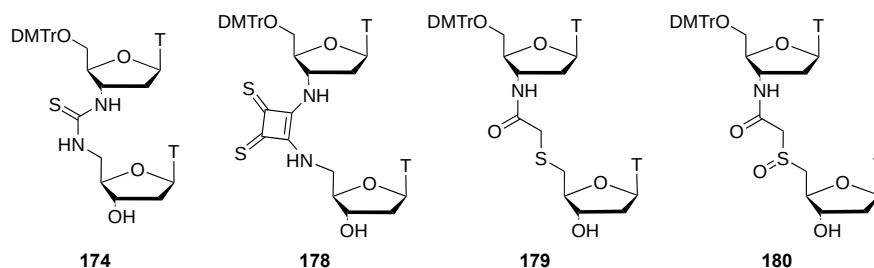


Figure 5.2: Dinucleosides with unnatural linkages synthesised by Dr William Doherty in the McGouran Group. T = Thymine

Dinucleosides **173-181** are suitable precursors to the phosphoramidites required for incorporation into oligonucleotides *via* SPOS. Prior to the generation of phosphoramidites, evaluation of the stability of a number of the dinucleosides towards oxidation conditions used during standard SPOS was carried out. As thiourea and thiosquaramide groups have been shown to be prone to oxidation,^{435, 436} it was investigated whether dinucleosides **174** and **178** would be stable to I_2 oxidation, the standard oxidative conditions utilised during SPOS.³¹⁰ Similarly, it is possible that dinucleosides **179** and **180**, featuring thioacetamide and sulfinyl acetamide groups respectively, may be prone to oxidation.⁴³⁷⁻⁴³⁹ As urea and squaramide linkages have previously been introduced into oligonucleotide backbones *via* SPOS,^{285, 288} evaluation of the stability of dinucleosides featuring such linkages towards oxidative conditions was not required. The stability of dinucleosides **174**, **178-180** towards the standard I_2 oxidation conditions utilised in SPOS was investigated (**Table 5.1**).³¹⁰

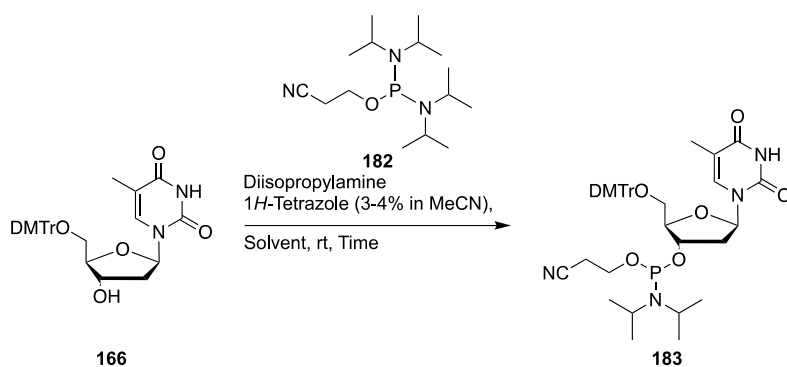
Table 5.1: Stability testing of **174**, **178-180** towards oxidation, results as observed by TLC analysis. **174**, **178-180** were stirred in 0.02 M I₂ in 0.4% pyridine in THF at rt for 24 h. TLC analysis was conducted after 1 min, 10 min, 1 h and 24 h.



Entry	Compound	1 min	10 min	1 h	24 h
1	174	Multiple products	-	-	-
2	178	Multiple products	-	-	-
3	179	Intact 179	Intact 179	Intact 179	Intact 179
4	180	Intact 180	Intact 180	Intact 180	Intact 180

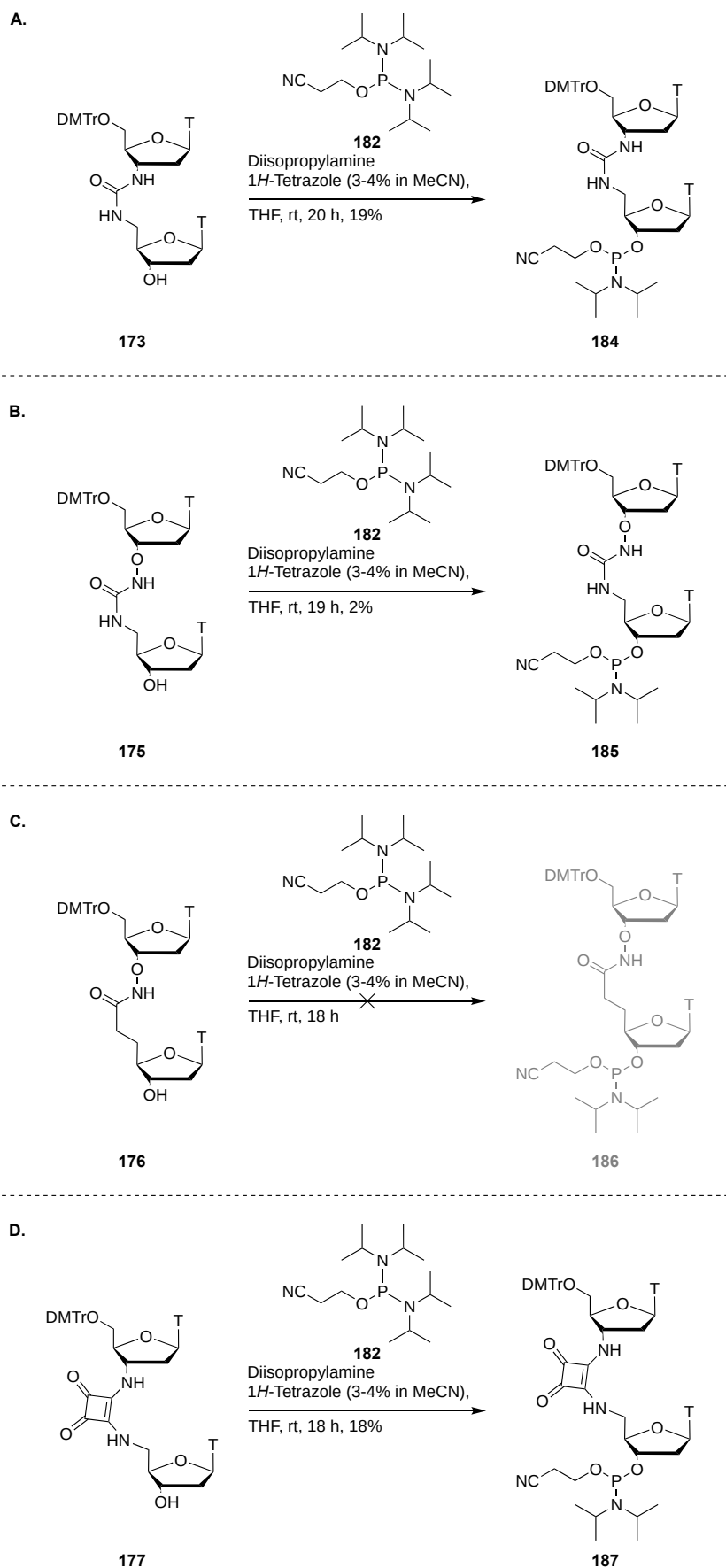
The dinucleosides were each stirred in a solution of 0.02 M I₂ in 0.4% pyridine in THF at rt. The reactions were monitored by TLC analysis over 24 h (**Table 5.1**). Thiourea **174** and thiosquaramide **178** both degraded after 1 min, as a complex reaction mixture was observed by TLC analysis in both cases (**Table 5.1**, Entries 1 and 2). These linkages are therefore not suitable for incorporation into an oligonucleotide strand *via* standard SPOS methods. On the other hand, thioacetamide **179** and sulfinyl acetamide **180** displayed stability to the oxidative conditions, as intact **179** and **180** were observed by TLC analysis after 24 h (**Table 5.1**, Entries 3 and 4). These dinucleosides, **179** and **180**, as well as **173**, **175-177** and **181** were deemed suitable for the generation of phosphoramidites for use in SPOS.

The corresponding phosphoramidites of **173**, **175-177**, **179-181** may be generated through phosphitylation at the 3'-OH. Due to limited quantities of 3'-OH dinucleosides **173**, **175-177**, **179-181**, it was not possible to optimise reaction conditions for each dinucleoside. Therefore, the phosphitylation reaction conditions were optimised using 5'-DMTr-protected thymidine **166** to generate thymidine phosphoramidite **183** (**Table 5.2**).

Table 5.2: Optimisation of phosphitylation conditions.

Entry ^{Ref}	Eq. 182	Eq. DIPA	Eq. 1 <i>H</i> -Tetrazole	Solvent	Time (h)	Yield 183 (%)
1 ⁴⁴⁰	1.5	0.8	0.4	MeCN-DMF (6:1)	2	7
2	3	1.6	0.8	MeCN-DMF (6:1)	3	17
3	4.5	4.5	4.5	THF	6	18
4	4.5	4.5	4.5	THF	17.5	65

Phosphitylation of 5'-DMTr-protected thymidine **166** was initially attempted using modified literature conditions, however, this furnished a disappointing 7% of thymidine phosphoramidite **183** (Table 5.2, Entry 1).⁴⁴⁰ A number of reports suggested that increased equivalents (eq.) of 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite (**182**), diisopropylamine (DIPA) and 1*H*-tetrazole as well as prolonged reaction times may improve the yield of **183**.^{441, 442} Utilising 3 eq. of **182**, 1.6 eq. of DIPA and 0.8 eq. of 1*H*-tetrazole, with a reaction time of 2 h resulted in an increased yield of **183** (17%) (Table 5.2, Entry 2). Due to the low solubility of **166** in MeCN-DMF, the solvent was changed to THF. Utilising THF along with 4.5 eq. each of **182**, DIPA and 1*H*-tetrazole, added in portions over 6 h, afforded **183** in 18% yield (Table 5.2, Entry 3). Finally, it was found that addition of 4.5 eq. each of **182**, DIPA and 1*H*-tetrazole in portions over 17.5 h afforded **183** in 65% yield (Table 5.2, Entry 4). The optimised phosphitylation conditions were then used to generate the dinucleoside phosphoramidites, starting with the urea, *N*-oxyamide and squaramide derivatives (Scheme 5.1).

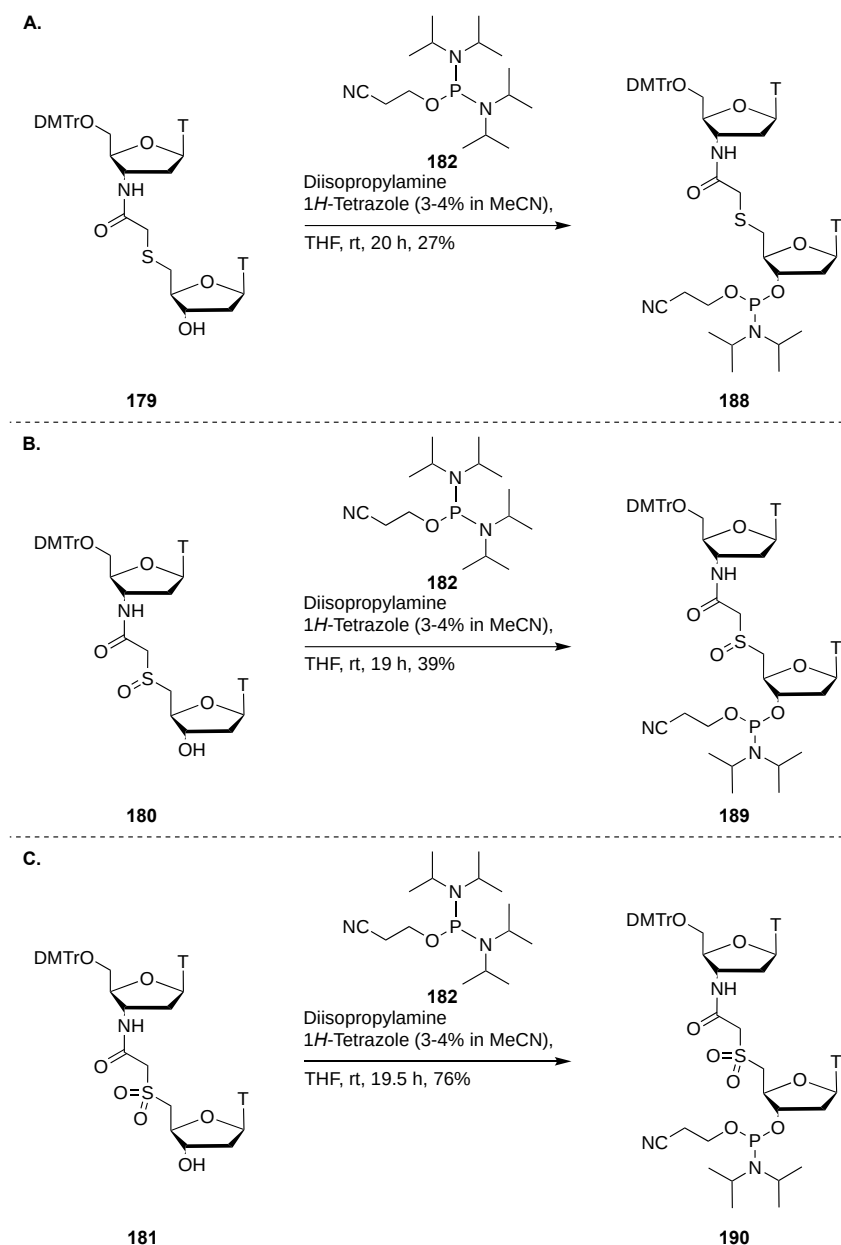


Scheme 5.1: Phosphitylation reactions of dinucleosides **173**, **175-177**. T = Thymine

Phosphitylation of urea-containing dinucleoside **173** was attempted using the optimised conditions (**Scheme 5.1A**). The reaction resulted in a complex mixture of products, as observed by TLC analysis, and some unreacted starting material (**173**) remained. However, the desired product **184** was isolated in 19% yield, along with 34% unreacted starting material **173**. Phosphitylation of *N*-oxyurea dinucleoside **175** also proved troublesome (**Scheme 5.1B**), as very little reaction progress was observed by TLC analysis and 93% of unreacted starting material **175** was recovered. Although dinucleoside phosphoramidite **185** was isolated in 2% yield, this did not provide enough material for use in SPOS. Phosphitylation of *N*-oxyamide derived dinucleoside **176** was unsuccessful under these conditions (**Scheme 5.1C**). Very little reaction progress was observed by TLC analysis and any product generated could not be isolated. Phosphitylation of squaramide-linked dinucleoside **177** resulted in a complex mixture of products (**Scheme 5.1D**). Desired phosphoramidite **187** was isolated in 18% yield and 20% of starting material **177** was also recovered.

Isolation of the dinucleoside phosphoramidites with urea-, *N*-oxyamide and squaramide-derived linkages proved challenging as the phosphitylation reactions generally resulted in a complex mixture of products, including unreacted 3'-OH dinucleosides **173**, **175-177**. The relatively low reactivity of the phosphitylating reagent **182** required prolonged reaction times.³⁸⁶ Therefore, as phosphoramidites are typically unstable to water and oxygen,³⁸⁶ the increased reaction times may have caused degradation of the desired products, resulting in complex mixtures. Yields of the desired phosphoramidites may be increased through use of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite, which has increased reactivity compared to **182**.⁴⁴³ However, due to limited quantities of the dinucleosides available, the reaction conditions could not be optimised further.

Phosphitylation of thioacetamide-, sulfinyl acetamide- and sulfonyl acetamide-linked dinucleosides **179**, **180** and **181** was more successful, with phosphoramidites **188**, **189** and **190** being generated in moderate to high yields (**Scheme 5.2**).



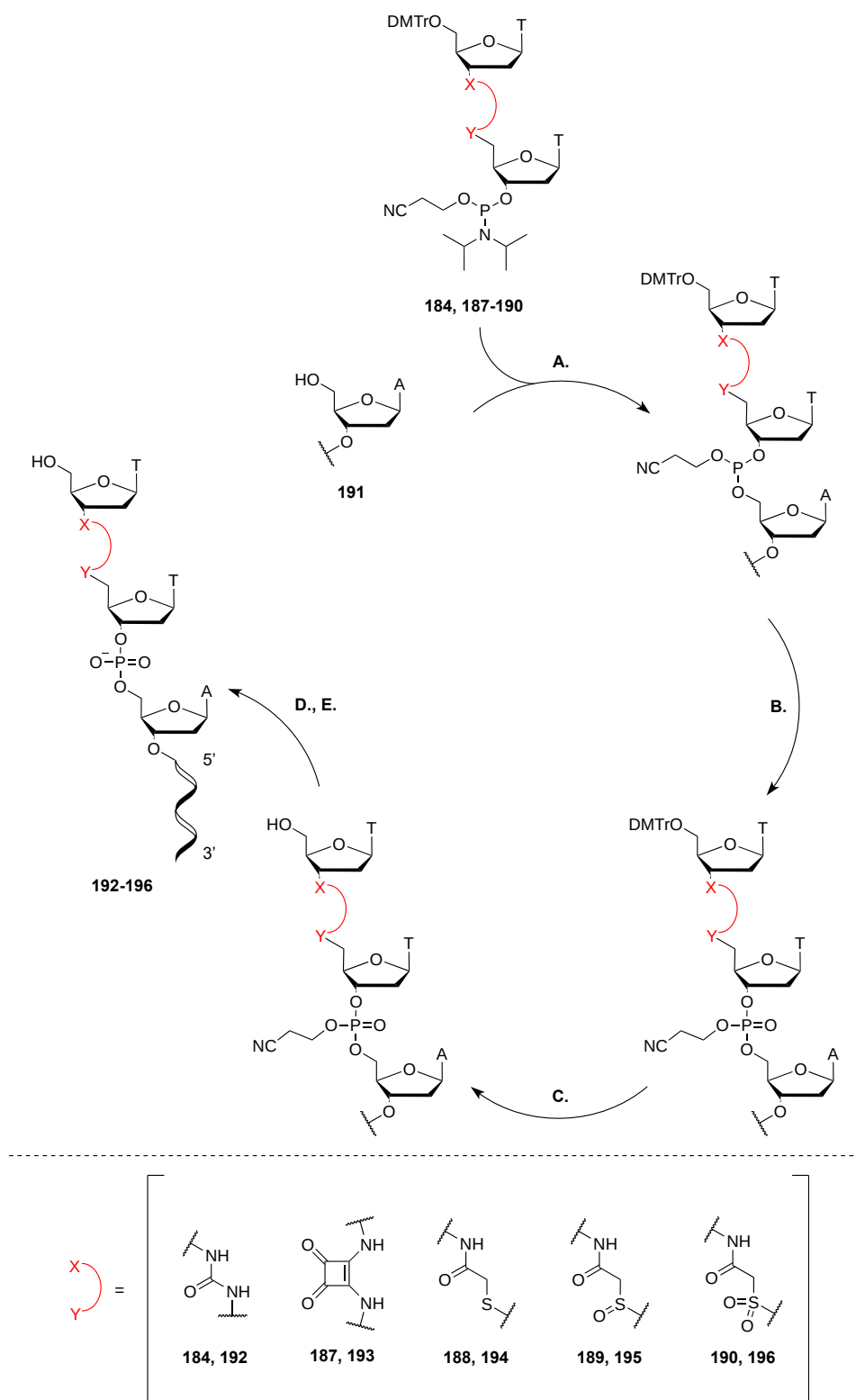
Scheme 5.2: Phosphitylation of dinucleosides **179-181**. T = Thymine

Phosphitylation of thioacetamide-linked dinucleoside **179** was carried out using the same reaction conditions (**Scheme 5.2A**). Although complete consumption of starting material **179** was observed by TLC analysis, a complex mixture was generated and isolation of **188** was challenging. However, phosphoramidite **188** was isolated in 27% yield. Phosphitylation of sulfinyl acetamide-linked dinucleoside **180** afforded phosphoramidite **189** in a moderate yield of 39% (**Scheme 5.2B**). Phosphitylation of sulfonyl acetamide-linked dinucleoside **181** was also successful and phosphoramidite **190** was generated in 76% yield (**Scheme 5.2C**).

For all the dinucleoside phosphoramidites synthesised, the presence of the phosphoramidite group was confirmed by ^{31}P NMR spectroscopy. Two signals at the characteristic value of 147-149 ppm were observed in the case of **184**, **185**, **187**, **188**, and **190**, corresponding to two diastereoisomers. The ^{31}P NMR spectrum of **189** displayed four signals in this characteristic region, confirming the presence of four stereoisomers due to the additional stereocentre at the sulfoxide modification. Dinucleoside phosphoramidites **184**, **187-190** were then incorporated into oligonucleotides *via* SPOS.

5.3. Incorporation of Dinucleosides into Oligonucleotides

A 19mer oligonucleotide, **191** (sequence: 5'-AGC AGT CAG TCA GTC ATC G-3'), was synthesised by automated SPOS by Dr Mark Berney, Ferguson Group, UCD. The support-bound 19mer **191** was used in SPOS with phosphoramidites **184**, **187-190** to generate the corresponding 21mer oligonucleotides **192-196** (sequences: 5'-[TT]AGC AGT CAG TCA GTC ATC G-3', [TT] = thymidine dinucleoside with a modified internucleotide linkage) *via* the manual syringe method (**Scheme 5.3**).



Scheme 5.3: Incorporation of dinucleoside phosphoramidites **184, 187-190** into an oligonucleotide scaffold via manual SPOS to generate 21mer oligonucleotides **192-196**. A. 0.3 M BTT in MeCN, B. 0.02 M I₂ in pyridine-H₂O, 9:1. C. 3% DCA in toluene. D. 20% Diethylamine in MeCN. E. aq. NH₃ (~ 35%), 55 °C. Sequence of support-bound 19mer oligonucleotide **191**: 5'-AGC AGT CAG TCA GTC ATC G-3'. T = Thymine, A = Adenine

A modified literature protocol was followed for solid-phase oligonucleotide synthesis *via* the manual syringe method.⁴⁴⁴ The support-bound 19mer oligonucleotide, **191**, featured a free 5'-OH which was available for coupling with the dinucleoside phosphoramidites. Dinucleoside phosphoramidites **184**, **187-190** were mixed with an activator, 5-(benzylthio)-1*H*-tetrazole (BTT). The support-bound 19mer **191** was treated with this mixture, resulting in the formation of a support-bound phosphite triester (**Scheme 5.3A**).³¹⁴ The phosphite-triester is an acid-sensitive P(III) species and therefore, required oxidation to a stable P(V) species prior to the next detritylation step. Oxidation was carried out using iodine in the presence of H₂O and pyridine (**Scheme 5.3B**).³¹⁰ The next step was removal of the 5'-DMTr group at the 5'-end of the resin-bound oligonucleotide (**Scheme 5.3C**). Detritylation was carried out with 3% dichloroacetic acid (DCA) in toluene. An orange colour, produced by the DMTr cation, was observed in the case of all the oligonucleotides, suggesting manual coupling had been successful. The cyanoethyl groups were then removed using 20% diethylamine in MeCN (**Figure 5.3D**). Although the cyanoethyl groups can be removed during cleavage of the oligonucleotides from the solid support, acrylonitrile, a by-product of the phosphodiester deprotection, is a Michael acceptor that can add to heterocyclic bases, forming 2-cyanoethyl adducts.⁴⁴⁵ Hence, the cyanoethyl groups were selectively removed prior to cleavage and acrylonitrile was removed by washing the solid support with MeCN.⁴⁴⁵ The support-bound oligonucleotides were then treated with aq. NH₃ (~ 35%) at 55 °C to effect simultaneous cleavage from the support and removal of the nucleobase protecting groups (**Scheme 5.3E**).³¹⁰ Following deprotection and cleavage, oligonucleotides **192-196** were purified by semi-preparative reversed-phase HPLC (RP-HPLC) and characterised by analytical RP-HPLC. Both diastereoisomers of **195**, **195A** and **195B**, were successfully separated and isolated. In the case of oligonucleotide **196**, RP-HPLC analysis indicated the presence of multiple products and **196** was not found by MS. It is possible that the sulfonyl acetamide linkage was unstable to the basic conditions used for global deprotection and cleavage. Unmodified 19mer oligonucleotide **191** was also deprotected and cleaved from the solid support to give 19mer oligonucleotide **197** which was isolated by preparative RP-HPLC and characterised by analytical RP-HPLC. MS analysis was carried out by Dr Mark Berney and confirmed the presence of the desired oligonucleotides **192-195**, **197**. Oligonucleotides **192-195**, **197** were then brought forward for biochemical evaluation.

5.4. Biochemical Evaluation of Modified Oligonucleotides

The ability of oligonucleotides **192-195**, **197** to bind to SNM1A was assessed using a similar inhibition assay to that detailed previously (Section 2.2.1). In the absence of an inhibitor, SNM1A digests the fluorescent substrate oligonucleotide (**64**), yielding shorter fluorescent products which are observed in the PAGE readout (Figure 5.3A). Conversely, if a modified oligonucleotide binds to SNM1A, the active site is blocked. This impedes the digestion of substrate oligonucleotide **64**, resulting in the retention of the full length 21mer **64**, as evidenced in the PAGE readout (Figure 5.3B).

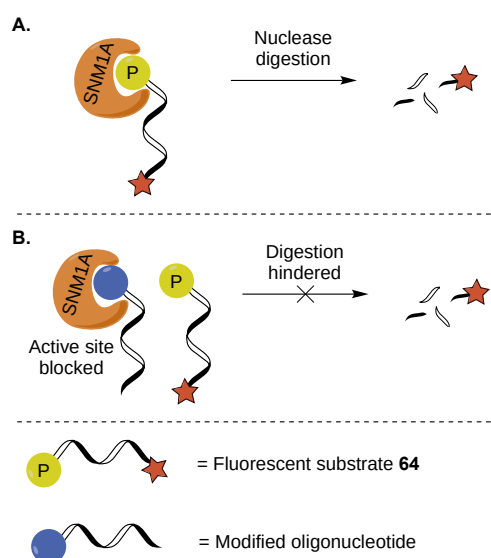


Figure 5.3: Evaluation of the ability of modified oligonucleotides **192-195**, **197** to bind to SNM1A. A. In the absence of an inhibitor, SNM1A digests substrate oligonucleotide **64** to produce shorter fluorescent products. B. If a modified oligonucleotide binds to SNM1A, the active site is blocked and digestion of **64** is hindered.

Modified oligonucleotides **192-195** were designed to target both the SNM1A active site and the DNA-binding groove. Oligonucleotides **192-195** feature an unnatural bridging group in place of one phosphodiester linkage. Consequently, these bridging groups may have the ability to bind to the metal ion(s) in the SNM1A active site. Given the importance of the DNA strand for substrate recognition by SNM1A,⁵⁸ it was hypothesised that inclusion of the oligonucleotide strand would anchor the bridging groups in the active site of the enzyme, thereby enhancing binding ability. The modified oligonucleotides **192-195** were screened for inhibition of SNM1A at a concentration of 450 nM and the results were analysed by PAGE (Figure 5.4). Unmodified 19mer oligonucleotide **197** was also included in the assay to determine whether incorporation of the bridging groups enhanced nuclease binding.

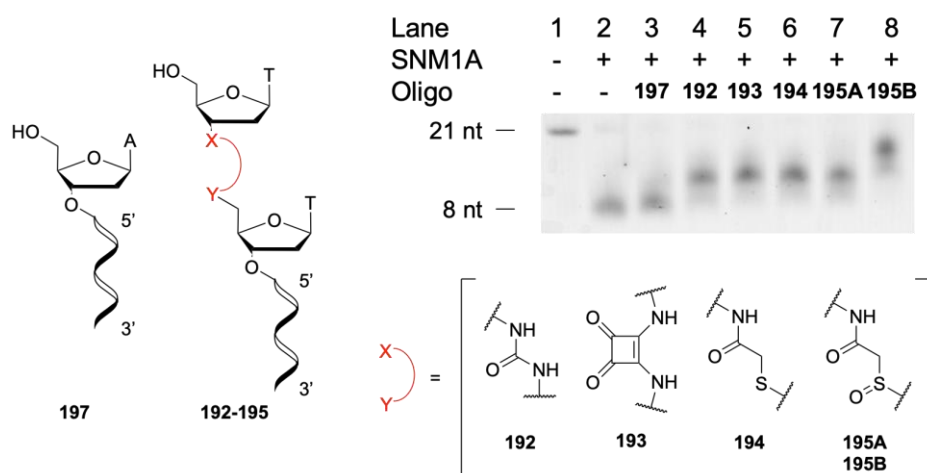


Figure 5.4: Evaluation of binding of **197**, **192-195** to SNM1A using the gel electrophoresis-based inhibition assay. Oligonucleotides **197**, **192-195** (450 nM) were pre-incubated with SNM1A (50 fmol, 5 nM in reaction) at 37 °C for 5 min. Fluorescent substrate oligonucleotide **64** (0.8 pmol, 80 nM in reaction) was then added and incubation at 37 °C was continued for 1 h. 25% of the reaction mixture was used for PAGE. A = Adenine, T = Thymine, nt = nucleotides

A control without SNM1A (**Figure 5.4**, Lane 1) and a control with SNM1A (**Figure 5.4**, Lane 2) were used as the negative and positive controls for digestion of oligonucleotide **64**, respectively. Unmodified 19mer oligonucleotide **197** did not exhibit any inhibition of SNM1A, suggesting it does not bind to the enzyme at this concentration (**Figure 5.4**, Lane 3). Gratifyingly, all of the modified oligonucleotides **192-195** displayed some level of SNM1A inhibition at this concentration (**Figure 5.4**, Lanes 4-8), indicating that the incorporation of the bridging groups may have improved the affinity of the oligonucleotides for SNM1A. Oligonucleotides containing urea (**192**), squaramide (**193**) and thioacetamide (**194**) bridging groups displayed moderate levels of inhibition of SNM1A at this concentration (**Figure 5.4**, Lanes 4-6). In the case of the sulfinyl acetamide modification, while one diastereoisomer, **195A**, only moderately hindered SNM1A activity (**Figure 5.4**, Lane 7), the other diastereoisomer, **195B**, displayed effective inhibition of SNM1A (**Figure 5.4**, Lane 8). The stereochemical configuration of the sulfinyl acetamide group in **195B** may have enabled improved coordination to the metal ion(s) in the SNM1A active site, compared to **195A**.

In order to gain further insight into the ability of modified oligonucleotides **192-195** to target SNM1A, IC_{50} values were determined using a real-time fluorescence assay similar to that described in **Section 3.3** (**Figure 5.5**).

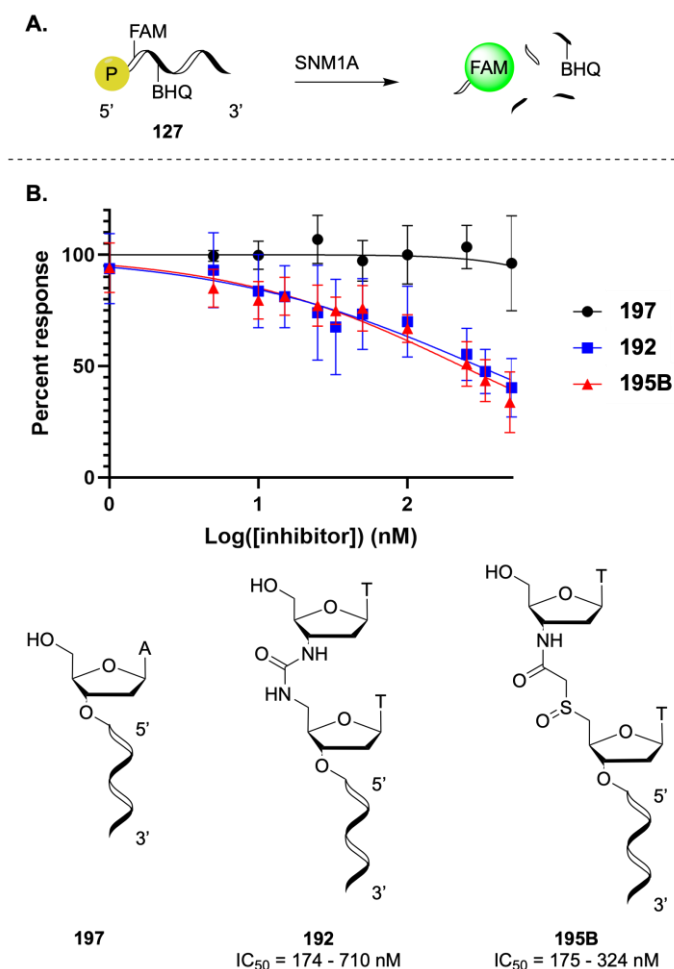


Figure 5.5: IC₅₀ determination of oligonucleotides **192**, **195B**, **197** in a real-time fluorescence assay. SNM1A (5 nM) was pre-incubated with the oligonucleotides **192**, **195B**, **197** at a range of concentrations for 6.5 min at rt prior to addition of the 20mer oligonucleotide substrate **127** (125 nM). Fluorescent spectra were measured at 37 °C, with readings taken every 50 seconds for 35 min. For each concentration of oligonucleotide, fluorescence intensity was plotted against time, and the slope of the linear range of this graph was taken as the rate of enzyme activity. The rate of enzyme activity observed for the zero-inhibitor control was set as 100, and the other rates were normalised to this to obtain percent response values. Percent response was plotted against log([inhibitor]), and a nonlinear regression was performed to calculate the IC₅₀. Error bars were generated from 3-4 independent replicates. A = Adenine, T = Thymine

Digestion of substrate oligonucleotide **127** by SNM1A led to separation of the FAM and BHQ moieties and an increase in fluorescence (**Figure 5.5A**). Measurement of this increasing fluorescence signal over time allowed the rate of the nuclease reaction to be quantified. This experiment was carried out in the presence of modified oligonucleotides **192-195** at a range of concentrations to enable IC₅₀ calculation. The assay was also carried out with unmodified oligonucleotide **197** in order to evaluate the effect of the bridging modifications. Oligonucleotide **197** did not demonstrate binding of SNM1A (**Figure 5.5B**). Urea-modified oligonucleotide **192** was determined to have an IC₅₀ of 174 – 710 nM (**Figure 5.5B**) and **195B** displayed noable binding of SNM1A, with an IC₅₀ value of 175 – 324 nM (**Figure 5.5B**). More precise IC₅₀ values for **192** and **195B** may be determined through increasing the number of replicates.

Unfortunately, in the case of squaramide- (**193**) and thioacetamide- (**194**) modified oligonucleotides and one of the diastereoisomers of the sulfinyl acetamide-modified oligonucleotide (**195A**), although some level of inhibition was observed, IC_{50} values could not be accurately determined as they appeared to be above the range of concentrations tested (**Figure 5.6**).

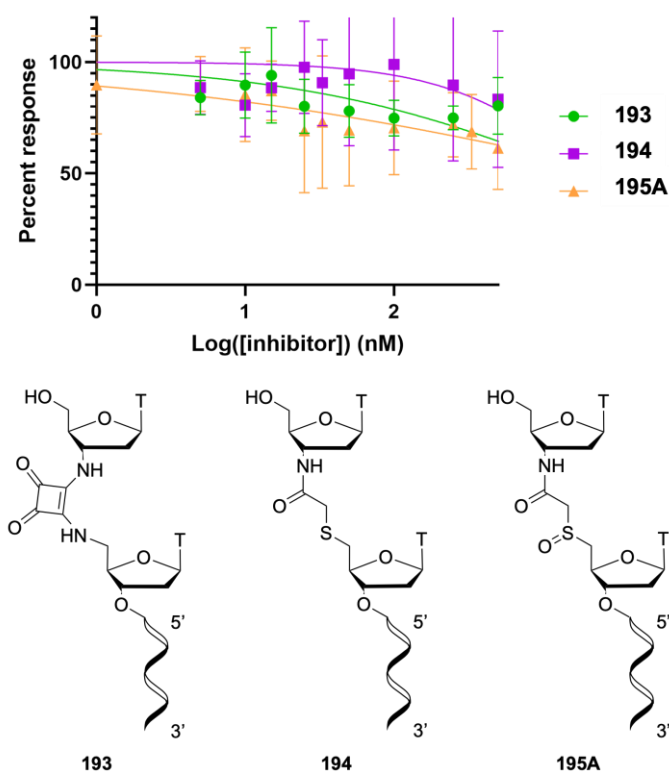


Figure 5.6: IC_{50} determination of oligonucleotides **193**, **194**, **195A** in a real-time fluorescence assay. SNM1A (5 nM) was pre-incubated with the oligonucleotides **193**, **194**, **195A** at a range of concentrations for 6.5 min at rt prior to addition of the 20mer oligonucleotide substrate **127** (125 nM). Fluorescent spectra were measured at 37 °C, with readings taken every 50 seconds for 35 min. For each concentration of oligonucleotide, fluorescence intensity was plotted against time, and the slope of the linear range of this graph was taken as the rate of enzyme activity. The rate of enzyme activity observed for the zero-inhibitor control was set as 100, and the other rates were normalised to this to obtain percent response values. Percent response was plotted against log([inhibitor]), and a nonlinear regression was performed to calculate the IC_{50} . Error bars were generated from 3-4 independent replicates. T = Thymine

The trends observed in the real-time fluorescence assay are in agreement with those ascertained in the gel electrophoresis-based assay. All of the modified oligonucleotides **192-195** exhibited enhanced binding of SNM1A compared to unmodified oligonucleotide **197**, suggesting that the bridging groups successfully targeted the active site of SNM1A. Oligonucleotides featuring urea (**192**), squaramide (**193**) and thioacetamide (**194**) linkages and one of the diastereoisomers of the sulfinyl acetamide-containing oligonucleotide (**195A**), displayed moderate binding affinity for SNM1A. Meanwhile, **195B**, the second diastereoisomer of the sulfinyl acetamide-containing oligonucleotide,

exhibited the most effective binding of SNM1A. This suggests that the stereocentre at the sulfinyl acetamide linkage is important for SNM1A binding.

5.5. Conclusions

This chapter describes the investigation of a number of modified oligonucleotides, featuring alternative backbones, for targeting SNM1A. The groups explored included: (thio)urea, oxyurea, *N*-oxyamide, (thio)squaramide, and thio/sulfinyl/sulfonyl acetamide groups. A series of dinucleosides with artificial linkages, suitable for phosphitylation to generate phosphoramidites for SPOS were synthesised in the McGouran Group by Dr William Doherty. The stability of **174**, **178**, **179** and **180**, containing thiourea, thiosquaramide, thioacetamide and sulfinyl acetamide linkages, towards the standard oxidation conditions utilised in SPOS was investigated. Unfortunately, thiourea **174** and thiosquaramide **178** were found not to be stable in the conditions tested and, therefore, were not used in SPOS. Alternative SPOS conditions may be investigated to enable incorporation of thiourea and thiosquaramide alternative backbones into oligonucleotides. For example, camphorsulfonyloxaziridine has previously been used in the synthesis of oligonucleotides containing disulfide linkages *via* SPOS, in order to prevent premature oxidation of thiols.⁴⁴⁶ Dinucleosides containing thioacetamide (**179**) and sulfinyl acetamide (**180**) linkages were found to be stable to oxidative conditions used in SPOS and so were included in the series of compounds used to generate phosphoramidites. Phosphitylation reaction conditions were optimised using 5'-DMTr-protected thymidine **166** to generate thymidine phosphoramidite **183**. The optimised conditions were successful in the generation of dinucleoside phosphoramidites containing urea (**184**), squaramide (**187**), thioacetamide (**188**), sulfinyl acetamide (**189**) and sulfonyl acetamide (**190**) linkages. Unfortunately, phosphitylation of the *N*-oxyurea dinucleoside (**175**) was too low yielding to generate enough material for use in SPOS. Furthermore, phosphitylation of a dinucleoside containing an *N*-oxyamide linkage (**176**) was not successful under these conditions.

Phosphoramidites **184**, **187-190** were used to generate the corresponding 21mer oligonucleotides **192-196** *via* manual syringe SPOS. Modified oligonucleotides **192-195** were successfully synthesised and purified by semi-preparative RP-HPLC. In the case of oligonucleotide **196**, RP-HPLC analysis indicated the presence of multiple products and **196** was not found by MS. An unmodified 19mer oligonucleotide **197** was also

synthesised. The ability of oligonucleotides **192-195**, **197** to bind to SNM1A was initially assessed using a gel electrophoresis-based inhibition assay. Whereas unmodified 19mer oligonucleotide **197** did not hinder SNM1A activity, all of the modified oligonucleotides **192-195** displayed some level of SNM1A inhibition, indicating that the incorporation of the bridging groups may have improved the affinity of the oligonucleotides for SNM1A. One of the diastereoisomers of the sulfinyl acetamide-modified oligonucleotide, **195B**, demonstrated particular activity. This trend was confirmed using a real-time fluorescence assay to determine IC₅₀ values for SNM1A inhibition by oligonucleotides **192-195**. Once again, unmodified oligonucleotide **197** did not demonstrate binding of SNM1A. Unfortunately, IC₅₀ values could not be accurately determined for squaramide- (**193**) and thioacetamide- (**194**) modified oligonucleotides and one of the diastereoisomers of the sulfinyl acetamide-modified oligonucleotide (**195A**), as they appeared to be above the range of concentrations tested. However, urea-modified oligonucleotide **192** was determined to have an IC₅₀ of 174 – 710 nM and **195B** displayed the most effective binding of SNM1A, with an IC₅₀ value of 175 – 324 nM. This is the first time oligonucleotides containing unnatural internucleotide linkages have been evaluated for targeting SNM1A. This approach has proven successful in generating oligonucleotides with notable affinity for SNM1A, as all of the modified oligonucleotides displayed moderate to high levels of SNM1A binding at a concentration of 450 nM.

It has been reported that a terminal phosphate moiety is required for substrate recognition by SNM1A.⁵⁹ Furthermore, previous work in the McGouran Group has shown that modified nucleosides bearing a 5'-phosphate group display enhanced levels of inhibition compared to that of their un-phosphorylated counterparts.²¹⁷ The binding ability of oligonucleotides **192-195** may, therefore, be enhanced through phosphorylation of the 5'-position, which can be carried out during SPOS.⁴⁴⁷ Such oligonucleotides would target the three main structural features that are important for the activity of SNM1A – the metal-containing active site, the phosphate binding pocket and the distal DNA binding groove – and may be used as scaffolds from which probes to study SNM1A and related enzymes may be built.

Chapter 6

Conclusions and Future Directions

6. Conclusions and Future Directions

This work explored the modification of nucleosides and oligonucleotides as a strategy to target SNM1A. Nucleoside modification (**Figure 6.1A,B**) encompassed both the expansion of established inhibitor scaffolds and the development of novel derivatives. Oligonucleotide modification (**Figure 6.1C, D**) involved the generation of a series of triazole-modified oligonucleotides, incorporating MBGs, for targeted inhibition of SNM1A. The investigation extended to the evaluation of a series of oligonucleotides featuring alternative backbones for their affinity for SNM1A.

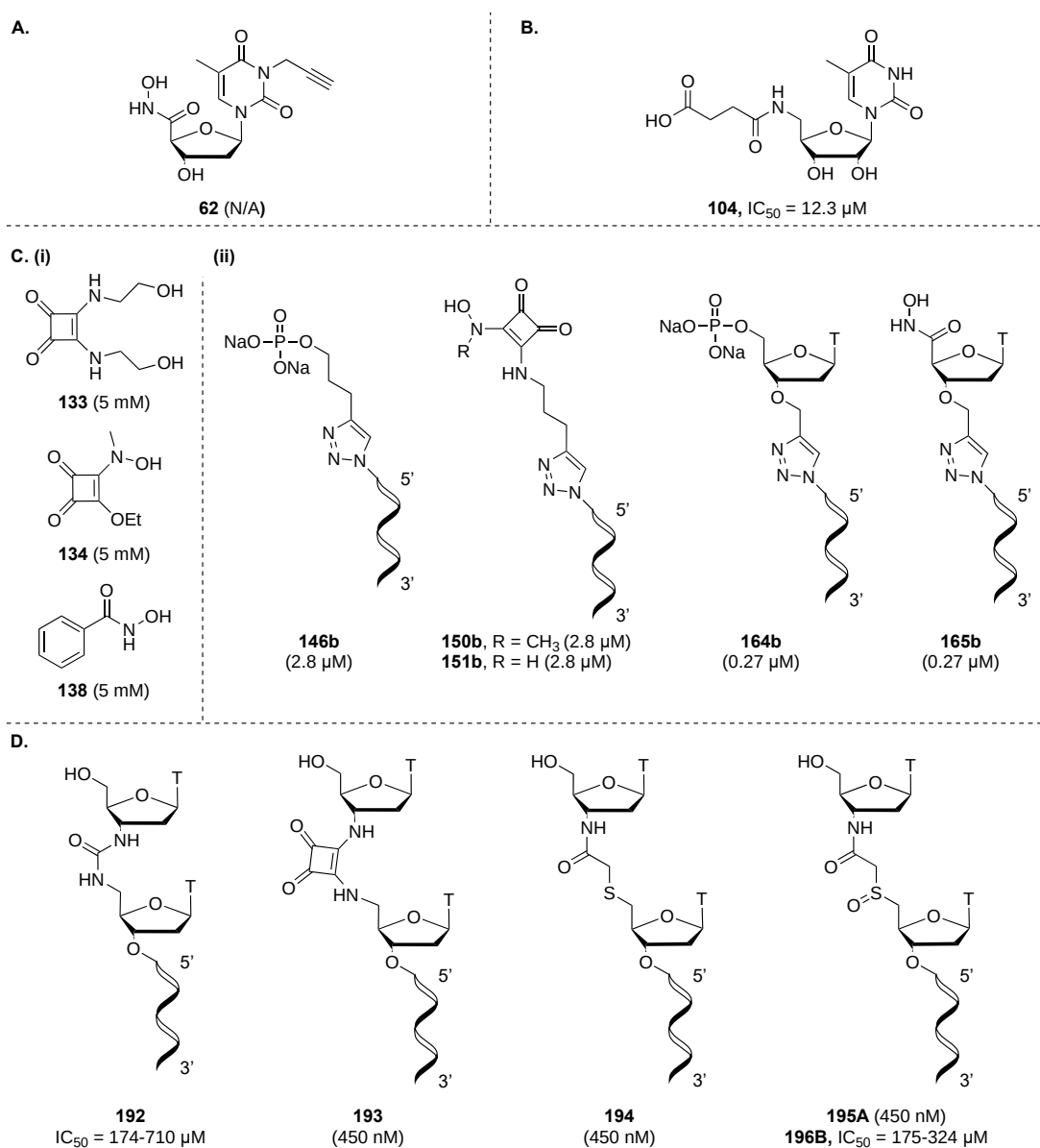


Figure 6.1: Nucleoside and oligonucleotide modification explored in this thesis. Numbers in brackets indicate the lowest inhibitory concentration for inhibition of SNM1A by the respective compound. N/A indicates that the compound did not demonstrate inhibition of SNM1A at 1 mM.

Initially, the generation of chemical tools to study SNM1A through the expansion of previously reported inhibitor scaffolds was explored, as described in Chapter 2 (**Figure 6.1A**). The modification of reported nucleoside-derived inhibitors offers a promising avenue for the development of inhibitors with enhanced potency and membrane permeability and has the potential to enable the investigation of peripheral binding pockets of SNM1A. Thymidine derivative **62**, with a 5'-hydroxamic acid MBG and an *N*³-propargyl group for functionalisation, was designed based on lead inhibitor **31** and was successfully synthesised. The biochemical activity of **62** was evaluated at a range of concentrations (1 mM – 1 μ M) and compared to that of lead inhibitor **31**. Surprisingly, despite its similarity to lead inhibitor **31**, **62** did not demonstrate inhibition of SNM1A. The fact that incorporation of this minimal handle at the *N*³-position prevented enzyme inhibition suggested that this position is important for optimal binding of lead inhibitor **31** to the SNM1A active site. Molecular docking studies supported this hypothesis, as the *N*³-position was shown to stabilise the binding of **31** to the active site *via* a hydrogen bond interaction with the backbone of the protein. Thus, functionalisation at the *N*³-position of thymidine holds less promise for the development of potent and selective inhibitors of SNM1A.

A series of novel carboxylate-rich nucleoside-derived inhibitors of SNM1A were identified in Chapter 3 (**Figure 6.1B**). Succinic, aspartic, glutamic and citric acid-derived modifications were incorporated at the 5'-position of 5-methyluridine, thymidine and 3'-azidothymidine scaffolds, with the 2'- and/or 3'-positions available for further functionalisation. Encouragingly, of the 21 compounds screened, 18 demonstrated SNM1A inhibition at 1 mM. The succinic acid derivatives proved particularly effective with **104**, **106**, **108** and **109** hindering enzyme activity at 100 μ M. The most potent inhibitor, **104**, was further evaluated using a real-time fluorescence assay, which determined an IC₅₀ of 12.3 μ M for SNM1A inhibition by **104**. A CD-based assay confirmed that **104** was not binding to ssDNA, suggesting that inhibition of SNM1A by **104** was due to the binding of the MBG to the active site metal ion(s). The findings presented in this chapter mark crucial progress in the study of SNM1A as **104** is the most potent nucleoside-derived inhibitor of the enzyme reported to date.

A two-step validation process for the identification of metal-binding fragments suitable for targeting the active site of SNM1A was successfully developed (**Figure 6.1C**). This

approach, detailed in Chapter 4, comprised of fragment-based screening followed by integration of fragments into oligonucleotides *via* click chemistry. The proof-of-concept fragment-based screening method identified squaramide (**133**, **134**) and hydroxamic acid (**138**) metal-binding fragments suitable for targeting SNM1A at a concentration of 5 mM (**Figure 6.1C(i)**). Metal-binding fragments were then incorporated into oligonucleotides *via* the CuAAC reaction to generate triazole-modified oligonucleotides featuring 5'-MBGs (**Figure 6.1C(ii)**). Triazole-modified oligonucleotides utilising a flexible linker and featuring 5'-phosphate (**146b**) and 5'-squaramide (**150b**, **151b**) moieties substantially hindered SNM1A activity at 2.8 μ M, indicating their potential binding of both the SNM1A active site and the DNA-binding groove. Inclusion of a thymidine-derived linker further increased substrate recognition as oligonucleotides **164b** and **165b**, featuring a 5'-phosphate group and a 5'-hydroxamic acid group respectively, exhibited SNM1A inhibition at concentrations as low as 0.27 μ M. The work presented in Chapter 4 demonstrates the reliability of incorporating metal-binding fragments into an oligonucleotide strand through the CuAAC reaction, providing a swift and effective means of generating non-natural oligonucleotides recognised by SNM1A. This method is particularly valuable as prior efforts to generate SNM1A inhibitors based on modified nucleosides/oligonucleotides demanded extensive synthesis of modified nucleosides or phosphoramidites, a process that is both time-consuming and expensive.^{217, 219} Furthermore, the described two-step validation process holds promise in future applications for promptly identifying fragments capable of targeting DNA damage repair enzymes.

A number of modified oligonucleotides, featuring alternative backbones, were evaluated for their ability to bind to SNM1A, as described in Chapter 5 (**Figure 6.1D**). Dinucleoside phosphoramidites containing urea, squaramide, thioacetamide, sulfinyl acetamide and sulfonyl acetamide linkages were successfully synthesised *via* phosphitylation of the dinucleoside precursors. The dinucleoside phosphoramidites were used to generate 21mer oligonucleotides containing urea (**192**), squaramide (**193**), thioacetamide (**194**) and sulfinyl acetamide (**195**) bridging groups *via* manual syringe SPOS. Generation of an oligonucleotide featuring a sulfonyl acetamide linkage was unsuccessful. An unmodified 19mer oligonucleotide (**197**) was also successfully synthesised. Initial biochemical screening using a gel electrophoresis-based assay demonstrated that all of the modified oligonucleotides **192-195** displayed some level of SNM1A inhibition at

450 nM, while the unmodified 19mer **197** did not hinder enzyme activity at this concentration. The binding affinity of the oligonucleotides for SNM1A may have been enhanced through incorporation of the artificial backbones. Of the oligonucleotides investigated, **195B**, one of the diastereoisomers of the oligonucleotide featuring a sulfinyl acetamide modification, exhibited particularly effective inhibition of SNM1A. The observed trend was corroborated through a real-time fluorescence assay which was used to calculate IC₅₀ values for SNM1A inhibition by oligonucleotides **192-195**. Notably, an IC₅₀ of 174 – 710 nM was determined for urea-modified oligonucleotide **192**, while **195B** exhibited the most potent binding of SNM1A, with an IC₅₀ value of 175 – 324 nM. Unfortunately, the range of concentrations tested was not high enough to accurately determine IC₅₀ values for squaramide- (**193**) and thioacetamide- (**194**) modified oligonucleotides and one of the diastereoisomers of the sulfinyl acetamide-modified oligonucleotide (**195A**). Overall, this strategy has successfully generated oligonucleotides with notable affinity for SNM1A, as evidenced by the moderate to high levels of SNM1A binding observed for all modified oligonucleotides when evaluated at a concentration of 450 nM. This work holds significant importance as this is the first time SNM1A has been successfully targeted with oligonucleotides containing unnatural internucleotide linkages.

In addition to the generation of a number of modified nucleosides and oligonucleotides that effectively targeted SNM1A, this work has yielded valuable insights that may shape future investigations of this enzyme. The 5-methyluridine derivative **104**, described in Chapter 3, stands as the most potent nucleoside-based inhibitor of SNM1A documented to date. Nucleoside **104** features 2'-OH and 3'-OH groups, offering avenues for additional functionalisation of the inhibitor scaffold, which presents opportunities to overcome challenges such as membrane permeability, or to generate probes that facilitate detailed study of SNM1A. Nucleoside **104** also holds the potential to be incorporated into oligonucleotides *via* SPOS or using the CuAAC method detailed in Chapter 4. The two-step validation approach developed in Chapter 4 offers a prospective avenue for exploring MBGs that have not yet been evaluated for their capacity to target SNM1A. For example, sulfonylhydrazide^{448, 449} and carbamoyl phosphonate⁴⁵⁰⁻⁴⁵² MBGs hold promise for investigating SNM1A, given their previous success in targeting zinc ions in MMPs (**Figure 6.2Ai-ii**). Furthermore, the method of incorporation of MBGs into oligonucleotides *via* the CuAAC reaction described in Chapter 4 offers a viable approach

for the introduction of thiourea and thiosquaramide MBGs into oligonucleotides as these groups demonstrated instability towards traditional SPOS conditions in Chapter 5 (**Figure 6.2Aiii-iv**). The inclusion of a phosphate group at the 5'-position of oligonucleotides **192-195** may augment their binding ability (**Figure 6.2B**). Such oligonucleotides would strategically target the metal ion(s) in the active site, the phosphate binding pocket and the DNA-binding region – the three structural elements crucial for SNM1A activity. Consequently, they may serve as scaffolds for the development of probes to investigate SNM1A and its related enzymes.

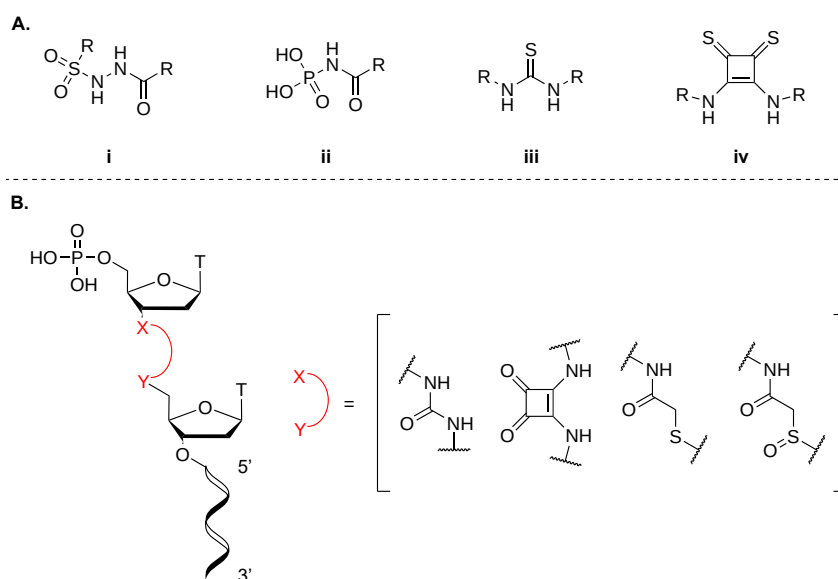


Figure 6.2: Possible future avenues. A. MBGs with the potential to be evaluated for their ability to target SNM1A via the two-step approach developed in Chapter 4. B. Structures of oligonucleotides featuring a 5'-phosphate group and an alternative backbone modification. These oligonucleotides may have the ability to target the SNM1A active site, phosphate-binding pocket, and DNA-binding groove.

Chapter 7

Experimental

7. Experimental

7.1. General Methods

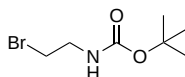
Chemicals were purchased from Acros Organics, Aldrich, Alfa Aesar, Carbosynth, Fisher Scientific, Fluorochem, Sigma-Aldrich, Merck and TCI Chemicals and were used as purchased without further purification. THF and DCM were dried using a PureSolv MD solvent purification system. Anhydrous MeOH was purchased from Acros Organics. Anhydrous DCM and THF used in phosphitylation reactions were purchased from Thermo Scientific and Sigma-Aldrich respectively and used without purification. Tetrazole (3-4% in MeCN) and DIPA were purchased from Thermo Scientific and Sigma-Aldrich respectively and dried over 3 Å molecular sieves prior to use in phosphitylation reactions. Glassware was oven dried prior to use in anhydrous reactions. Oxygen-free anhydrous argon was obtained from BOC gases. Petroleum ether (pet. ether) refers to the fraction of petroleum ether that boils at 40-60 °C. Room temperature (rt) refers to 19-21 °C. Flash column chromatography was carried out using silica gel, particle size 0.04-0.063 mm, purchased from Sigma Aldrich or VWR. TLC analysis was performed on TLC Silica gel 60 F₂₅₄ plates purchased from Merck and was visualised by UV irradiation (254 nm), potassium permanganate stain (3 g KMnO₄, 20 g K₂CO₃, 300 mL H₂O), ninhydrin stain (1.5 g ninhydrin, 5 mL AcOH, 500 mL 95% EtOH), anisaldehyde stain (9.2 mL *p*-methoxybenzaldehyde, 3.75 mL AcOH, 338 mL 95% EtOH, 12.5 mL conc. H₂SO₄) or iodine. ¹H, ¹³C and ³¹P NMR spectra were recorded using Bruker DPX 400 (400.13 MHz for ¹H NMR and 100.61 MHz for ¹³C NMR), Bruker AV 600 (600.13 MHz for ¹H NMR and 150.90 MHz for ¹³C NMR), Bruker AV 400 (400.13 MHz for ¹H NMR and 100.61 MHz for ¹³C NMR) or Agilent MR400 (400.13 MHz for ¹H NMR and 100.61 MHz for ¹³C NMR) instruments. NMR spectra are recorded in CDCl₃, DMSO-*d*₆, acetone-*d*₆, methanol-*d*₄ or D₂O and referenced to residual CDCl₃ (δ_H = 7.26 ppm, δ_C = 77.16 ppm), DMSO-*d*₆ (δ_H = 2.50 ppm, δ_C = 39.52), acetone-*d*₆ (δ_H = 2.05 ppm, δ_C = 29.84 ppm), methanol-*d*₄ (δ_H = 3.31 ppm, δ_C = 49.00 ppm) or D₂O (δ_H = 4.79 ppm).⁴⁵³ Chemical shifts (δ) are reported in ppm and coupling constants are reported in Hertz (Hz) accurate to 0.2 Hz. ¹³C NMR spectra are proton-decoupled. NMR spectra were assigned using HSQC, HMBC, DEPT and TOCSY experiments. Infrared (IR) spectra were obtained on a Perkin Elmer spectrophotometer. Melting points were measured using a Griffin melting point apparatus and are

uncorrected. ESI mass spectra were acquired using a Bruker microTOF-Q III spectrometer interfaced to a Dionex UltiMate 3000 LC in positive and negative modes as required. The instrument was calibrated using a tune mix solution (Agilent Technologies ESI-I Low concentration tuning mix) which was also used as an internal lock mass. Operating conditions were as follows: end-plate offset 500 V, capillary 4500 V, nebulizer 2.0 Bar, dry gas 8.0 L/min, and dry temperature 180 °C. MicroTof control 3.2 and HyStar 3.2 software were used to carry out the analysis. APCI experiments were carried out on the same machines. Agilent tuning mix APCI-TOF was used to calibrate the system. Operating conditions were as follows: Capillary voltage 4000 V, corona 4000 nA, nebulizer gas 2.0 Bar, dry gas 3.0 L/min, dry gas temperature 100-200 °C, vapour temperature 100-400 °C. MicroTof control 3.2 and HyStar 3.2 software were used to carry out the analysis.

7.2. Synthetic Methods

7.2.1. Synthetic Methods for Chapter 2

tert-butyl-(2-Bromoethyl)carbamate (**72**)³⁴³



72

72 was prepared according to a literature procedure.³⁴³ Under argon, 2-bromoethylamine hydrobromide (2.00 g, 9.76 mmol) and DIPEA (1.87 mL, 10.74 mmol) were dissolved in anhydrous DCM (12 mL) and cooled on ice. Boc₂O (2.38 g, 10.85 mmol) in anhydrous DCM (8 mL) was added dropwise over 10 min. The mixture was stirred at 0 °C – rt over 17.5 h. After this time TLC analysis (DCM-MeOH, 9:1) showed consumption of the starting material (R_f = 0.2) and the formation of a new product (R_f = 0.9). The orange solution was diluted with DCM (30 mL) and H₂O (20 mL). The organic layer was washed with sat. aq. NaHCO₃ (30 mL) and brine (30 mL), dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The orange oil was purified *via* flash column chromatography (DCM) to give **72** as an orange oil (2.18 g, 99%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3328 (N-H), 2978 (C-H), 2934 (C-H), 1683 (C=O), 1530 (C=O), 1449, 1428 (C-H), 1390, 1364 (C-H), 1274, 1254, 1217, 1160, 1143, 1040, 1005, 954, 911, 866, 838, 779, 757, 738 (C-H), 656 (C-Br).

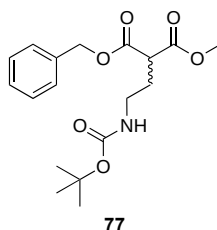
^1H NMR (400 MHz, CDCl_3): δ = 1.45 (s, 9H, 3 x CH_3^{Boc}), 3.46 (t, $J_{\text{CH}_2, \text{CH}_2}$ = 5.4 Hz, 2H, CH_2), 3.52-3.56 (m, 2H, CH_2), 4.93 (br s, 1H, NH) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 28.5 (CH_3^{Boc}), 33.0 (CH_2), 42.5 (CH_2), 80.0 (qC^{Boc}), 155.7 (CO^{Boc}) ppm.

HRMS (ESI^+): m/z calc. for $\text{C}_7\text{H}_{14}^{79}\text{BrNO}_2\text{Na}$ = 246.0100 $[\text{M}+\text{Na}]^+$, found = 246.0099

Spectroscopic data are consistent with that reported in the literature.³⁴³

1-Benzyl 3-methyl 2-(2-((*tert*-butoxycarbonyl)amino)ethyl)malonate (**77**)



Under argon, **72** (479 mg, 2.14 mmol) was dissolved in anhydrous DMF (11 mL) and K_2CO_3 (652 mg, 4.72 mmol) was added. Benzyl methyl malonate (0.39 mL, 2.14 mmol) was added dropwise, and the white suspension was stirred at rt for 23.5 h. After this time, TLC analysis (hexane-EtOAc, 7:3) showed consumption of the malonate starting material (R_f = 0.6) and the formation of a new product (R_f = 0.4). The reaction mixture was diluted with EtOAc (40 mL) and 1 M aq. HCl (40 mL). The organic layer was washed with sat. aq. NH_4Cl (40 mL), H_2O (40 mL) and brine (2 x 30 mL), dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The product was purified *via* flash column chromatography (hexane-EtOAc, 9:1 $\rightarrow$ 8:2) to give **77** as a colourless oil (391 mg, 52%).

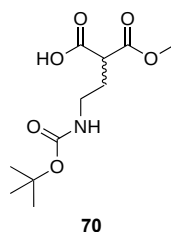
IR (ATR, DCM) $\nu_{\text{max}}/\text{cm}^{-1}$: 3397 (N-H), 2977 (C-H), 1731 (C=O), 1710 (C=O), 1514, 1454, 1437 (C-H), 1391, 1366, 1335 (C-H), 1248, 1158, 1040, 1004, 909, 864 781, 738 (C-H), 697.

^1H NMR (600 MHz, CDCl_3): δ = 1.42 (s, 9H, 3 x CH_3^{Boc}), 2.11 (app. q, J = 6.9 Hz, 2H, β - CH_2), 3.17-3.20 (m, 2H, γ - CH_2), 3.48 (t, $J_{\alpha,\beta}$ = 7.3 Hz, 1H, α -CH), 3.71 (s, 3H, OCH_3), 4.59 (br s, 1H, NH), 5.18 (app. dd, J = 4.5, 12.4 Hz, 2H, CH_2^{Bn}), 7.32-7.34 (m, 5H, 5 x CH^{Bn}) ppm.

^{13}C NMR (150 MHz, CDCl_3): δ = 28.5 (CH_3^{Boc}), 29.2 (β - CH_2), 38.5 (γ - CH_2), 49.5 (α -C), 52.7 (OCH_3), 67.4 (CH_2^{Bn}), 79.5 (qC^{Boc}), 128.3 (CH^{Bn}), 128.5 (CH^{Bn}), 128.7 (CH^{Bn}), 135.5 (qC^{Bn}), 155.9 (CO^{Boc}), 169.1 (BnOCO), 169.6 (CH_3CCO) ppm.

HRMS (APCI): m/z calc. for $\text{C}_{18}\text{H}_{24}\text{NO}_6$ = 350.1622 $[\text{M}-\text{H}]^-$, found 350.1623

4-((*tert*-Butoxycarbonyl)amino)-2-(methoxycarbonyl)butanoic acid **70**⁴⁵⁴



77 (96 mg, 0.27 mmol) was dissolved in MeOH (13 mL) and added to a flask containing Pd/C (10%, 19 mg) and the suspension was flushed with argon. The suspension was sparged with H_2 while stirring for 1 h. After this time, TLC analysis (EtOAc) showed complete consumption of starting material (R_f = 0.9) and the formation of a new product (R_f = 0.1). The reaction mixture was flushed with argon and filtered through celite and the filtrate was concentrated to afford **70** as a colourless film (68 mg, 96%).

IR (ATR, DCM) $\nu_{\text{max}}/\text{cm}^{-1}$: 3384 (N-H, O-H), 2979 (C-H), 1693 (C=O), 1518 (C=O), 1438 (C-H), 1393, 1367 (C-H), 1248, 1162, 1040, 1006, 861, 780, 735 (C-H), 702.

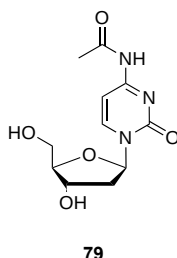
^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 1.36 (s, 9H, 3 x CH_3^{Boc}), 1.81-1.84 (m, 2H, β - CH_2), 2.91 (app. dd, J = 6.9, 13.0 Hz, γ - CH_2), 3.26 (t, $J_{\alpha,\beta}$ = 7.2 Hz, 1H, α -CH), 3.61 (s, 3H, OCH_3), 6.47 (br s, 1H, OH), 6.84-6.86 (m, 1H, NH) ppm.

^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): δ = 28.3 (CH_3^{Boc}), 28.9 (β - CH_2), 38.1 (γ - CH_2), 49.8 (α -CH), 51.8 (OCH_3), 77.5 (qC^{Boc}), 155.6 (CO^{Boc}), 170.2 (CO), 170.4 (CO) ppm.

HRMS (ESI⁺): m/z calc. $\text{C}_{11}\text{H}_{19}\text{NO}_6\text{Na}$ = 284.1105 $[\text{M}+\text{Na}]^+$, found = 284.1110

Spectroscopic data are consistent with that reported in the literature.⁴⁵⁴

***N*⁴-Acetyl-2'-deoxycytidine (79)**⁴⁵⁵



79 was synthesised according to a literature procedure.³⁵⁰ 2'-Deoxycytidine **78** (500 mg, 2.20 mmol) was suspended in anhydrous DMF (11 mL) under argon. Acetic anhydride (0.23 mL, 2.42 mmol) was added and the colourless solution was stirred at rt for 18 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed complete consumption of the starting material (R_f = 0.1) to and the formation of a new product (R_f = 0.7). The solvent was removed *in vacuo* and residual DMF was removed *via* coevaporation with toluene. Et₂O was added to the residue, resulting in the precipitation of a white solid. The supernatant was removed and the solid was washed with excess Et₂O. The white solid product **79** was recrystallised from ethanol and dried under reduced pressure (502 mg, 85%); mp (EtOH) 122-124 °C (lit.⁴⁵⁶ 150-170 °C).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3229 (N-H), 3127 (N-H), 2973 (O-H), 2925 (C-H), 2861 (C-H), 1667 (C=O), 1633 (N-H), 1608 (C=C), 1564 (N-H), 1491 (C-N, C=N), 1432 (C-N), 1386 (C=CH), 1304 (C-N), 1251 (C-N), 1174 (C-O), 1115 (C-O), 1070 (N-C-H), 1007 (C-C-H), 936 (C-C), 695 (N-H).

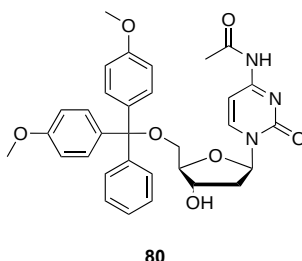
¹H NMR (600 MHz, DMSO-*d*₆): δ = 1.99-2.03 (m, 1H, H-2'a), 2.09 (s, 3H, CH₃^{Ac}), 2.26-2.30 (m, 1H, H-2'b), 3.54-3.63 (m, 2H, H-5'), 3.84-3.86 (m, 1H, H-4'), 4.20-4.22 (m, 1H, H-3'), 5.03 (t, $J_{5',OH}$ = 5.1 Hz, 1H, 5'-OH), 5.26 (d, $J_{3',OH}$ = 9.2 Hz, 1H, 3'-OH), 6.10 (app. t, J = 6.4 Hz, 1H, H-1'), 7.19 (d, $J_{5,6}$ = 7.5 Hz, 1H, H-5), 8.31 (d, $J_{5,6}$ = 7.5 Hz, 1H, H-6), 10.84 (br s, 1H, NH-4) ppm.

¹³C NMR (150 MHz, DMSO-*d*₆): δ = 24.4 (CH₃^{Ac}), 40.9 (C-2'), 61.0 (C-5'), 70.0 (C-3'), 86.2 (C-1'), 87.9 (C-4'), 95.2 (C-5), 145.0 (C-6), 154.5 (C-2), 162.3 (C-4), 171.0 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for $C_{11}H_{15}N_3O_5Na = 292.0904$ $[M+Na]^+$, found = 292.0903

Spectroscopic data are consistent with that reported in the literature.⁴⁵⁵

***N*⁴-Acetyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-cytidine (**80**)⁴⁵⁶**



Method 1:

*N*⁴-Acetyl-2'-deoxycytidine **79** (490 mg, 1.82 mmol) was dissolved in pyridine (4.6 mL) and DMTr-Cl (678 mg, 2.00 mmol) was added in three portions over 15 min. The orange solution was stirred at rt and another two portions of DMTr-Cl (123 mg, 0.40 mmol) were added after 18 h and 20 h. After 22 h TLC analysis (DCM-MeOH, 9:1) showed consumption of starting material ($R_f = 0.2$) and the formation of a new product ($R_f = 0.5$). The reaction was quenched with sat. aq. $NaHCO_3$ (10 mL) and diluted with DCM (20 mL) and H_2O (20 mL). The product was extracted with DCM (3 x 10 mL) and the combined organic layers were washed with H_2O (2 x 20 mL) and brine (20 mL), dried over $MgSO_4$ and filtered. The solvent was removed *in vacuo* and the white foam was purified by flash column chromatography (DCM-MeOH, 39:1 $\rightarrow$ 19:1) to give the product **80** as a white foam (345 mg, 33%).

Method 2:

*N*⁴-Acetyl-2'-deoxycytidine **79** (1.99 g, 7.4 mmol) was suspended in DCM (25 mL). DIPEA (3.87 mL, 22.2 mmol) was added to the suspension followed by DMTr-Cl (2.76 g, 8.1 mmol) in portions. Further portions of DMTr-Cl were added (0.50 g, 1.5 mmol and 1.25 g, 3.7 mmol) after 4 h and 6 h respectively. The orange suspension was stirred at rt and after 71 h TLC analysis (DCM-MeOH, 9:1) showed complete consumption of the starting material ($R_f = 0.2$) and the formation of a new product ($R_f = 0.5$). The solvent was removed *in vacuo* and the resulting brown oil was purified by flash column chromatography (DCM-MeOH, 9:1) to afford **80** as an off-white foam (3.03 g, 72%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3245 (N-H), 2931 (C=C-H, C-H^{Ar}), 2840 (C-H), 1644 (C=O), 1609 (C=C, C=N), 1556 (N-H, C-N), 1386 (C=C-H), 1306 (C-N), 1242 (C-N), 1176 (C-O-C), 1083 (N-C-H), 964 (C-C), 824 (N-H), 758 (N-H), 701 (C-C-N).

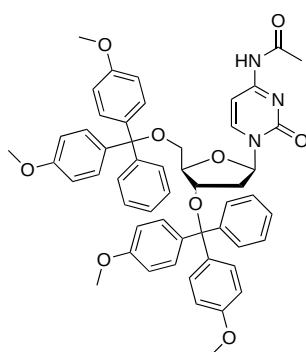
¹H NMR (600 MHz, DMSO-*d*₆): δ = 2.09 (s, 3H, CH₃^{Ac}), 2.09-2.14 (m, 1H, H-2'a), 2.31-2.35 (m, 1H, H-2'b), 3.23-3.28 (m, 2H, H-5'), 3.74 (s, 6H, 2 x OCH₃^{DMTr}), 3.94-3.96 (m, 1H, H-4'), 4.26-4.29 (m, 1H, H-3'), 5.36 (d, $J_{3',OH}$ = 3.4 Hz, 1H, 3'-OH), 6.11 (app. t, J = 5.8 Hz, 1H, H-1'), 6.89 (app. d, J = 7.6 Hz, 4H, 4 x CH^{DMTr}), 7.04 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-5), 7.22-7.32 (m, 7H, 7 x CH^{DMTr}), 7.36-7.38 (m, 2H, 2 x CH^{DMTr}), 8.13 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-6), 10.86 (br s, 1H, NH-4) ppm.

¹³C NMR (150 MHz, DMSO-*d*₆): δ = 24.3 (CH₃^{Ac}), 40.8 (C-2'), 55.0 (OCH₃^{DMTr}), 62.9 (C-5'), 69.3 (C-3'), 85.7 (C-1'), 85.9 (qC^{DMTr} + C-4'), 95.2 (C-5), 113.2 (CH^{DMTr}), 126.8 (CH^{DMTr}), 127.7 (CH^{DMTr}), 127.9 (CH^{DMTr}), 129.7 (CH^{DMTr}), 135.3 (qC^{DMTr}), 144.5 (qC^{DMTr}), 144.6 (C-6), 154.3 (C-2), 158.1 (qC^{DMTr}), 162.3 (C-4), 171.0 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for C₃₂H₃₃N₃O₇Na = 594.2211 [M+Na]⁺, found = 594.2217

Spectroscopic data are consistent with that reported in the literature.⁴⁵⁶

Doubly DMTr-protected nucleoside **81** (R_f = 0.5, DCM-MeOH; 19:1) was also isolated as a yellow foam (0.93 g, 15%).



81

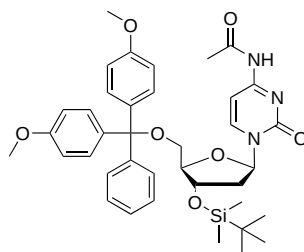
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 2933 (C-H), 1665 (C=O), 1607 (C=C), 1556 (N-H), 1507 (C=N, C-N), 1443 (C-N), 1391 (C=CH), 1302, 1246 (C-N), 1174 (C-O), 1029 (N-C-H), 700 (N-H), 583 (C-O-C).

^1H NMR (600 MHz, $\text{DMSO-}d_6$): δ = 1.65-1.70 (m, 1H, H-2'a), 2.03-2.10 (m, 4H, CH_3^{Ac} + H-2'b), 2.96 (dd, $J_{4',5'a}$ = 4.3 Hz, $J_{5'a,5'b}$ = 10.8 Hz, 1H, H-5'a), 3.14 (dd, $J_{4',5'b}$ = 2.1 Hz, $J_{5'a,5'b}$ = 10.8 Hz, 1H, H-5'b), 3.71-3.73 (m, 12H, 4 x $\text{OCH}_3^{\text{DMTr}}$), 3.90-3.91 (m, 1H, H-4'), 4.03-4.05 (m, 1H, H-3'), 6.08 (app. t, J = 6.6 Hz, 1H, H-1'), 6.77-6.85 (m, 8H, 8 x CH^{DMTr}), 7.02 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-5), 7.05-7.32 (m, 18H, 18 x CH^{DMTr}), 7.96 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-6), 10.88 (br s, 1H, NH-4) ppm.

^{13}C NMR (150 MHz, $\text{DMSO-}d_6$): δ = 24.3 (CH_3^{Ac}), 40.1 (C-2'), 55.0 ($\text{CH}_3^{\text{DMTr}}$), 63.0 (C-5'), 74.1 (C-3'), 85.0 (C-4'), 86.0 (qC^{DMTr}), 86.8 (C-1'), 95.2 (qC^{DMTr}), 113.1 (CH^{DMTr}), 113.3 (CH^{DMTr}), 126.8 (qC^{DMTr}), 126.9 (qC^{DMTr}), 127.6 (CH^{DMTr}), 127.8 (CH^{DMTr}), 127.9 (CH^{DMTr}), 129.6 (CH^{DMTr}), 129.8 (CH^{DMTr}), 129.9 (CH^{DMTr}), 135.1 (qC^{DMTr}), 135.7 (qC^{DMTr}), 135.8 (qC^{DMTr}), 144.4 (qC^{DMTr}), 145.1 (C-6 + qC^{DMTr}), 154.3 (C-2), 158.1 (qC^{DMTr}), 158.2 (qC^{DMTr}), 158.3 (qC^{DMTr}), 162.3 (C-3) and 171.0 (CO^{Ac}) ppm.

HRMS (ESI⁺): m/z calc. for $\text{C}_{53}\text{H}_{51}\text{N}_3\text{O}_9\text{Na}$ = 896.3518 $[\text{M}+\text{Na}]^+$, found = 896.3513

***N*⁴-Acetyl-2'-deoxy-3'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-cytidine (82)**



82

*N*⁴-Acetyl-2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-cytidine **80** (3.03 g, 5.3 mmol) and imidazole (1.44 g, 21.1 mmol) were dissolved in DMF (17 mL) under argon. TBDMS-Cl (1.60 g, 10.6 mmol) in DMF (10 mL) was added dropwise. The solution was stirred at rt and after 18 h, TLC analysis (DCM-MeOH, 19:1) showed complete consumption of the starting material (R_f = 0.2) and the formation of a new product (R_f = 0.6). H_2O (30 mL) was added and the product was extracted with Et_2O (3 x 30 mL). The combined organic layers were washed with H_2O (50 mL) and brine (50 mL), dried over MgSO_4 and filtered. The solvent was removed *in vacuo* to give an off-white foam. The product was purified

by flash column chromatography (pet. ether-EtOAc, 1:9 → EtOAc) to give **82** as a white foam (2.88 g, 79%).

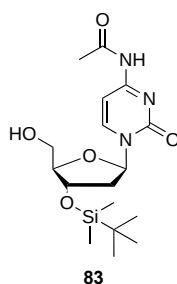
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 2941 (C-H^{Ar}), 2850 (C-H), 1665 (C=O), 1611 (C=C, N-H), 1550 (C=N, C-N), 1494 (C-N), 1444 (C-N), 1392 (C=CH), 1309 (C-N), 1245 (C-N), 1176 (C-O), 1031 (N-C-H), 1002 (C-C-H), 966 (C-C), 828 (Si-C), 725 (N-H).

¹H NMR (600 MHz, DMSO-*d*₆): δ = -0.09 (s, 3H, CH₃^{TBDMS}), -0.02 (s, 3H, CH₃^{TBDMS}), 0.75 (s, 9H, 3 x CH₃^{TBDMS}), 2.09 (s, 1H, CH₃^{Ac}), 2.18-2.22 (m, 1H, H-2'a), 2.27-2.31 (m, 1H, H-2'b), 3.19-3.21 (m, 2H, H-5'), 3.73 (s, 6H, 2 x OCH₃^{DMTr}), 3.86-3.88 (m, 1H, H-4'), 4.37-4.40 (m, 1H, H-3'), 6.08-6.10 (m, 1H, H-1'), 6.87-6.89 (m, 4H, 4 x CH^{DMTr}), 7.06 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-5), 7.23-7.32 (m, 7H, 7 x CH^{DMTr}), 7.35-7.37 (m, 2H, 2 x CH^{DMTr}) 8.25 (d, $J_{5,6}$ = 7.4 Hz, 1H, H-6), 10.86 (br s, 1H, NH-4) ppm.

¹³C NMR (150 MHz, DMSO-*d*₆): δ = -5.2 (CH₃^{TBDMS}), -4.7 (CH₃^{TBDMS}), 17.6 (qC^{TBDMS}), 24.4 (CH₃^{Ac}), 25.6 (CH₃^{TBDMS}), 40.8 (C-2'), 55.1 (CH₃^{DMTr}), 62.0 (C-5'), 70.3 (C-3'), 85.4 (C-4'), 86.9 (C-1'), 86.1 (qC^{DMTr}), 95.3 (C-5), 113.3 (CH^{DMTr}), 126.9 (CH^{DMTr}), 127.8 (CH^{DMTr}), 127.9 (CH^{DMTr}), 129.8 (CH^{DMTr}), 135.2 (qC^{DMTr}), 144.4 (qC^{DMTr}), 144.7 (C-6), 154.4 (C-2), 158.3 (qC^{DMTr}), 162.4 (C-3), 171.1 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for C₃₈H₄₈N₃O₇Si = 686.3256 [M+H]⁺, found = 686.3235

N⁴-Acetyl-2'-deoxy-3'-O-(*tert*-butyldimethylsilyl)-cytidine (**83**)



A solution of TES (0.58 mL, 3.64 mmol) and 3% TFA in DCM (6.70 mL) was added to DMTr-protected cytidine **82** (500 mg, 0.73 mmol). The bright red solution was stirred at rt and a gradual colour change to pale orange was observed. After 10 min, TLC analysis (DCM-MeOH, 19:1) showed consumption of the starting material (R_f = 0.4) and the formation of a new product (R_f = 0.2). Sat. aq. NaHCO₃ was added until effervescence ceased (~ 40 mL) and the reaction mixture was diluted with DCM (10 mL). The layers

were separated and the organic layer was washed with sat. aq. NaHCO_3 (25 mL). The aqueous layers were combined and the product was extracted with DCM (3 x 20 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL), dried over MgSO_4 and filtered. The solvent was removed *in vacuo* to give an off-white solid residue. The product was purified by flash column chromatography (DCM-MeOH, 99:1 $\rightarrow$ 19:1) to give the white solid product **83** (247 mg, 88%); mp (DCM-MeOH) 200-204 °C (decomp.).

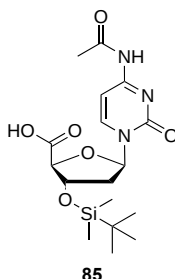
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3518 (O-H), 3298 (N-H), 3102 (N-H), 2930 (C-H), 2857 (C-H), 1673 (C=O), 1655 (C=O, N-H, C-N), 1614 (C=C), 1499 (C=N, C-N), 1465 (C-N), 1434 (C-N), 1358 (C=CH), 1287 (C-N), 1250 (C-N), 1197 (C-O), 1024 (N-C-H), 947 (C-C), 829 (Si-C), 709 (N-H), 669 (O-H), 591 (C=O).

^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 0.07 (s, 6H, 2 x $\text{CH}_3^{\text{TBDMS}}$), 0.87 (s, 9H, 3 x $\text{CH}_3^{\text{TBDMS}}$), 2.06-2.12 (m, 4H, H-2'a + CH_3^{Ac}), 2.23-2.39 (m, 1H, H-2'b), 3.52-3.64 (m, 2H, H-5'), 3.85 (app. q, J = 7.7 Hz, 1H, H-4'), 4.38-4.41 (m, 1H, H-3'), 5.11 (t, $J_{5',\text{OH}}$ = 5.1 Hz, 1H, 5'-OH), 6.09 (app. t, J = 6.3 Hz, 1H, H-1'), 7.20 (d, $J_{5,6}$ = 7.5 Hz, 1H, H-5), 8.28 (d, $J_{5,6}$ = 7.5 Hz, 1H, H-6), 10.85 (br s, 1H, NH-4) ppm.

^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): δ = -4.9 ($\text{CH}_3^{\text{TBDMS}}$), 17.7 (qC^{TBDMS}), 24.4 (CH_3^{Ac}), 25.7 ($\text{CH}_3^{\text{TBDMS}}$), 41.0 (C-2'), 60.0 (C-5'), 71.6 (C-3'), 86.0 (C-1'), 88.0 (C-4'), 95.4 (C-5), 145.0 (C-6), 154.5 (C-2), 162.3 (C-3), 171.1 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for $\text{C}_{17}\text{H}_{30}\text{N}_3\text{O}_5\text{Si}$ = 384.1949 $[\text{M}+\text{H}]^+$, found = 384.1949

***N*⁴-Acetyl-2',5'-deoxy-3'-*O*-(*tert*-butyldimethylsilyl)-5'-oxo-cytidine (**85**)**



Alcohol **83** (807 mg, 2.10 mmol) was suspended in MeCN- H_2O , 1:1 (10.5 mL). BAIB (1.62 g, 5.0 mmol) and TEMPO (99 mg, 0.63 mmol) were added sequentially and the reaction mixture was stirred at rt for 4.5 h. After this time, TLC analysis

(EtOAc-MeOH, 4:1) showed consumption of the starting material ($R_f = 0.6$) and the formation of a new product ($R_f = 0.3$). Sat. aq. NaHCO_3 (~ 60 mL) was added to the reaction mixture which was then stirred at rt for 20 min. The reaction mixture was washed with DCM (3 x 30 mL). The aqueous layers were cooled on ice and carefully acidified with 2 M aq. HCl until pH ~ 2. The white precipitate was collected *via* vacuum filtration and washed with ice cold H_2O (20 mL). The product was further dried under high vacuum to yield pure carboxylic acid **85** as a white powder (659 mg, 77%); mp (H_2O) 190 °C (decomp.).

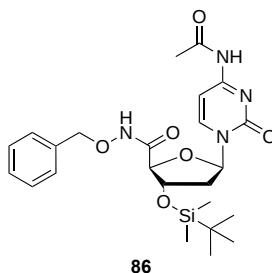
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3136 (N-H, O-H), 2952 (C-H), 2932 (C-H), 2891 (C-H), 2857 (C-H), 1730 (C=O), 1674 (C=O), 1618 (C=C), 1547 (N-H), 1529 (N-H, C-N), 1498 (C=N, C-N), 1433 (C-N), 1335 (C=CH), 1289 (C-N), 1251 (C-N), 1236 (C-N), 1184 (C-O), 1114 (C-O), 1069 (N-C-H), 997 (C-CH), 951 (C-C), 829 (Si-C), 698 (N-H), 628 (C=O).

^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 0.12 (s, 6H, 2 x $\text{CH}_3^{\text{TBDMS}}$), 0.89 (s, 9H, 3 x $\text{CH}_3^{\text{TBDMS}}$), 1.94-1.98 (m, 1H, H-2'a), 2.10 (s, 3H, CH_3^{Ac}), 2.33-2.36 (m, 1H, H-2'b), 4.38-4.39 (m, 1H, H-4'), 4.64-4.65 (m, 1H, H-3'), 6.25 (dd, $J_{1',2'a} = 5.5$ Hz, $J_{1',2'b} = 8.5$ Hz, 1H, H-1'), 7.24 (d, $J_{5,6} = 7.5$ Hz, 1H, H-5), 8.69 (d, $J_{5,6} = 7.5$ Hz, 1H, H-6), 10.89 (br s, 1H, NH-4) ppm.

^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): δ = -5.0 ($\text{CH}_3^{\text{TBDMS}}$), 17.7 (qC^{TBDMS}), 24.4 (CH_3^{Ac}), 25.6 ($\text{CH}_3^{\text{TBDMS}}$), 40.4 (C-2'), 75.6 (C-3'), 85.3 (C-4'), 87.7 (C-1'), 95.5 (C-5), 145.3 (C-6), 154.5 (C-2), 162.5 (C-3), 171.1 (CO^{Ac}), 172.1 (C-5') ppm.

HRMS (ESI^+): m/z calc. for $\text{C}_{17}\text{H}_{27}\text{N}_3\text{O}_6\text{SiNa} = 420.1561$ $[\text{M}+\text{Na}]^+$, found = 420.1562

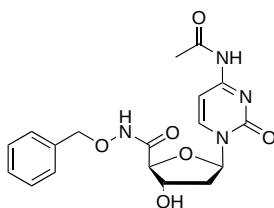
***N*⁴-Acetyl-2',5'-deoxy-3'-*O*-(*tert*-butyldimethylsilyl)-5'-benzyloxyamino-5'-oxo-cytidine (**86**)**



Carboxylic acid **85** (300 mg, 0.75 mmol), DMAP (128 mg, 1.04 mmol), DIPEA (0.36 mL, 2.06 mmol) and EDC·HCl (196 mg, 1.03 mmol) were added to anhydrous DMF (3 mL) under argon and the reaction mixture was stirred at 0 °C for 1 h. After this time *O*-benzylhydroxylamine hydrochloride (164 mg, 1.03 mmol) in DMF (1 mL) was added. The reaction mixture was stirred at 0 °C – rt for 22.5 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed consumption of the starting material ($R_f = 0.2$) and the formation of a new product ($R_f = 0.9$). The reaction mixture was diluted with EtOAc (20 mL) and washed with sat. aq. NaHCO₃ (10 mL) and brine (10 mL). The organic layer was dried over MgSO₄ and filtered. The solvent was removed *in vacuo* to give a colourless oil. Purification was attempted by flash column chromatography in EtOAc. However, separation from *O*-benzylhydroxylamine proved difficult and the product was carried through as a mixture (99 mg, 5:1, **86**: *O*-benzylhydroxylamine).

¹H NMR (400 MHz, acetone-*d*₆): δ = 0.14 (s, 6H, 2 x CH₃^{TBDMS}), 0.91 (s, 9H, 3 x CH₃^{TBDMS}), 2.23 (s, 3H, CH₃^{Ac}), 2.29-2.34 (m, 1H, H-2'a), 2.46-2.52 (m, 1H, H-2'b), 4.28-4.29 (m, 1H, H-4'), 4.63-4.64 (m, 1H, H-3'), 4.95 (s, 2H, CH₂^{Bn}), 6.23 (dd, $J_{1',2'a} = 5.7$ Hz, $J_{1',2'b} = 8.0$ Hz, 1H, H-1'), 7.33-7.47 (m, 6H, 5 x CH^{Bn} + H-5), 8.44 (d, $J_{5,6} = 7.4$ Hz, 1H, H-6), 9.70 (br s, 1H, O-NH), 10.99 (br s, 1H, NH-4) ppm.

HRMS (ESI⁺): m/z calc. for C₂₄H₃₅N₄O₆Si = 503.2320 [M+H]⁺, found = 503.2313

***N*⁴-Acetyl-2',5'-deoxy-5'-benzyloxyamino-5'-oxo-cytidine (**88**)****88**

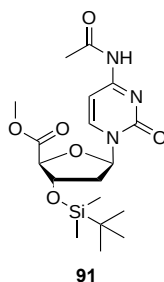
Silyl ether **86** (86 mg, 0.17 mmol) was dissolved in THF (1.8 mL). TBAF·3H₂O (84 mg, 0.27 mmol) was added and the colourless solution was stirred at rt for 19 h. After this time, TLC analysis (EtOAc-MeOH, 9:1) showed complete consumption of the starting material (R_f = 0.7) and the formation of a new product (R_f = 0.2). The solvent was removed *in vacuo* and the resulting residue was purified by flash column chromatography (EtOAc-MeOH, 9:1) to give **88** as a white solid (44 mg, 67%); mp (EtOAc-MeOH) 182-185 °C.

IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3231 (O-H, N-H), 3130 (C-H^{Ar}), 2935 (C-H), 2858 (C-H), 1650 (C=O), 1567 (C=C, N-H), 1488 (C-N), 1366 (C=CH), 1318 (C-N), 1283 (C-N), 1231 (C-N), 1188 (C-O), 1031 (N-C-H), 997 (C-C-H), 956 (C-C), 695 (N-H), 617 (C=O).

¹H NMR (400 MHz, methanol-*d*₄): δ = 2.19 (s, 3H, CH₃^{Ac}), 2.22-2.30 (m, 1H, H-2'a) 2.43-2.49 (m, 1H, H-2'b), 4.25 (d, $J_{3',4'} = 2.3$, 1H, H-4'), 4.41-4.43 (m, 1H, H-3'), 4.59 (br s, 1H, 3'-OH), 4.91 (s, 2H, CH₂^{Bn}), 6.31-6.35 (m, 1H, H-1'), 7.34-7.46 (m, 6H, 5 x CH^{Bn} + H-5), 8.51 (d, $J_{5,6} = 7.5$ Hz, 1H, H-6) ppm.

¹³C NMR (100 MHz, methanol-*d*₄): δ = 24.5 (CH₃^{Ac}), 41.2 (C-2'), 75.3 (C-3'), 79.1 (CH₂^{Bn}), 85.9 (C-4'), 90.3 (C-1'), 98.2 (C-5), 129.5 (CH^{Bn}), 129.8 (CH^{Bn}), 130.5 (CH^{Bn}), 136.7 (qC^{Bn}), 147.1 (C-6), 157.9 (C-2), 164.6 (C-4), 169.4 (C-5'), 173.0 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for C₁₈H₂₁N₄O₆ = 389.1456 [M+H]⁺, found = 389.1457

***N*⁴-Acetyl-2',5'-deoxy-3'-*O*-(*tert*-butyldimethylsilyl)-5'-methoxycarbonyl-5'-oxo-cytidine (91)**

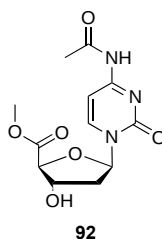
Carboxylic acid **85** (100 mg, 0.25 mmol) was suspended in anhydrous THF-MeOH, 4:1 (2.36 mL) under argon. TMS-diazomethane (2 M in hexane, 0.14 mL, 0.28 mmol) was added dropwise to give a yellow suspension which was stirred at rt for 1 h. After this time, TLC analysis (EtOAc-MeOH, 4:1) showed complete consumption of the starting material ($R_f = 0.3$) and the formation of a new product ($R_f = 0.7$). The reaction was quenched with a few drops of AcOH until the yellow colour disappeared and the reaction mixture was then stirred for 30 min. After this time, the solvent was removed *in vacuo* and the residue was purified by flash column chromatography (DCM-MeOH, 19:1→9:1) to give **91** as a white foam (74 mg, 72%).

IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3420 (N-H), 3152 (N-H), 2953 (C-H), 2933 (C-H), 2893 (C-H), 2858 (C-H), 1737 (C=O), 1644 (C=C), 1503 (C=N, C-N), 1466 (C-N), 1437 (C-H), 1367 (C=CH), 1289 (C-N), 1256 (C-N), 1219 (C-N), 1116 (C-O), 1079 (N-C-H), 998 (C-C-H), 934 (C-C), 831 (Si-C), 704 (N-H), 630 (C=O).

¹H NMR (600 MHz, methanol-*d*₄): δ = 0.16 (s, 3H, CH₃^{TBDMS}), 0.16 (s, 3H, CH₃^{TBDMS}), 0.94 (s, 9H, 3 x CH₃^{TBDMS}), 1.98-2.02 (m, 1H, H-2'a), 2.19 (s, 3H, CH₃^{Ac}), 2.55 (ddd, $J_{2'b,3'} = 1.8$ Hz, $J_{1',2'b} = 5.4$ Hz, $J_{2'a,2'b} = 13.6$ Hz, 1H, H-2'b) 3.81 (s, 3H, COOCH₃), 4.57 (d, $J_{3',4'} = 1.3$ Hz, 1H, H-4'), 4.70-4.71 (m, 1H, H-3'), 6.38 (dd, $J_{1',2'b} = 5.4$ Hz, $J_{1',2'a} = 8.4$ Hz, 1H, H-1'), 7.47 (d, $J_{5,6} = 7.5$, 1H, H-5), 8.81 (d, $J_{5,6} = 7.5$, 1H, H-6) ppm.

¹³C NMR (150 MHz, methanol-*d*₄): δ = -4.91 (CH₃^{TBDMS}), -4.86 (CH₃^{TBDMS}), 18.8 (qC^{TBDMS}), 24.5 (CH₃^{Ac}), 26.1 (CH₃^{TBDMS}), 41.9 (C-2'), 53.1 (COOCH₃), 77.2 (C-3'), 87.2 (C-4'), 90.4 (C-1'), 98.1 (C-5), 146.7 (C-6), 157.9 (C-2), 164.4 (C-4), 172.8 (C-5'), 173.1 (CO^{Ac}) ppm

HRMS (APCI⁺): m/z calc. for C₁₈H₃₀N₃O₆Si = 412.1898 [M+H]⁺, found = 412.1898

***N*⁴-Acetyl-2',5'-deoxy-5'-methoxycarbonyl-5'-oxo-cytidine (92)**

Silyl ether **91** (170 mg, 0.41 mmol) was dissolved in THF (4.1 mL). TBAF·3H₂O (197 mg, 0.62 mmol) was added and the colourless solution was stirred at rt for 18 h. After this time, TLC analysis (EtOAc) showed complete consumption of the starting material (R_f = 0.6) and the formation of a new product (R_f = 0.3). The solvent was removed *in vacuo* and the resulting residue was purified by flash column chromatography (DCM-MeOH, 39:1 → 19:1) to give **92** as a white solid (55 mg, 45%); mp (DCM-MeOH) 196-204 °C (decomp.).

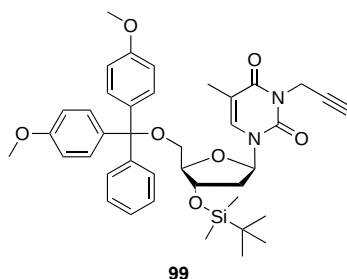
IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3368 (N-H), 3269 (O-H), 3228 (N-H), 3081 (C-H), 3045 (C-H), 2938 (C-H), 2854 (C-H), 1746 (C=O), 1690 (C=O), 1637 (C=O), 1609 (C=C), 1569 (N-H), 1525 (C=N, C-N), 1490 (C-H), 1442 (C-N), 1367 (C=CH), 1299 (C-N), 1216 (C-N), 1173 (C-O), 1081 (N-C-H), 1001 (C-C-H), 953 (C-C), 645 (N-H), 588 (C=O).

¹H NMR (400 MHz, D₂O): δ = 2.20 (s, 3H, CH₃^{Ac}), 2.24-2.31 (m, 1H, H-2'a), 2.55-2.61 (m, 1H, H-2'b), 3.78 (s, 3H, COOCH₃), 4.66 (d, $J_{3',4'} = 2.7$ Hz, 1H, H-4'), 4.72-4.75 (m, 1H, H-3'), 6.31 (app. t, $J = 6.6$ Hz, 1H, H-1'), 7.34 (d, $J_{5,6} = 7.5$ Hz, 1H, H-5), 8.52 (d, $J_{5,6} = 7.5$ Hz, 1H, H-6) ppm.

¹³C NMR (100 MHz, D₂O): δ = 24.1 (CH₃^{Ac}), 39.1 (C-2'), 53.1 (COOCH₃), 73.7 (C-3'), 85.1 (C-4'), 89.4 (C-1'), 98.1 (C-5), 146.4 (C-6), 157.1 (C-2), 162.9 (C-4), 172.4 (C-5'), 174.2 (CO^{Ac}) ppm.

HRMS (APCI⁺): m/z calc. for C₁₂H₁₆N₃O₆ = 298.1034 [M+H]⁺, found = 298.1041

***N*³-Propargyl-3'-*O*-(*tert*-butyldimethylsilyl)-5'-*O*-(4,4'-dimethoxytrityl)-thymidine (99)**



Protected thymidine **98** (300 mg, 0.45 mmol) and K₂CO₃ (190 mg, 1.37 mmol) were suspended in acetone-DMF, 1:1 (3 mL). Propargyl bromide (80% wt in toluene, 76 μ L, 0.68 mmol) was added dropwise. The reaction mixture was stirred at 50 °C for 18 h. After this time a white precipitate had formed and TLC analysis (pet. ether-EtOAc, 2:1) showed complete consumption of starting material (R_f = 0.4) and the formation of a new product (R_f = 0.7). The reaction mixture was filtered and the solid washed with a minimum amount of acetone. The filtrate was concentrated under reduced pressure. H₂O (10 mL) was added to the residue and the product was extracted with DCM (2 x 10 mL). The combined organic layers were washed with brine (20 mL), dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The product was purified *via* flash column chromatography (pet. ether-EtOAc, 4:1) to yield **99** as a white foam (277 mg, 87%).

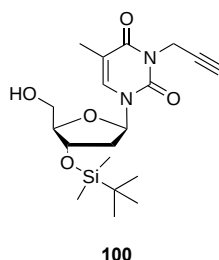
IR (ATR, DCM) $\nu_{\max}/\text{cm}^{-1}$: 3290 (C $\equiv$ C-H), 2930 (C-H), 2857 (C-H), 1707 (C=O), 1671 (C=C), 1651 (C=C), 1608 (C=C), 1464 (CH₃), 1412 (CH₃), 1300 (C-H), 1252 (C-N, C=CH), 1151 (CH₃), 1068 (N-C-H), 1033 (C-C $\equiv$ C), 832 (C-C $\equiv$ C), 770 (C-CH₃), 727 (C-H), 702, 585 (N-C-C, C-O-C).

¹H NMR (400 MHz, acetone-*d*₆): δ = 0.03 (s, 3H, CH₃^{TBDMS}), 0.08 (s, 3H, CH₃^{TBDMS}), 0.86 (9H, 3 x CH₃^{TBDMS}), 1.55 (d, $J_{\text{CH}_3,6}$ = 1.1 Hz, 3H, CH₃^T), 2.30-2.36 (m, 1H, H-2'a), 2.40-2.46 (m, 1H, H-2'b), 2.62 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.5 Hz, 1H, HC $\equiv$ C), 3.34 (dd, $J_{4',5'a}$ = 3.6 Hz, $J_{5'a,5'b}$ = 10.6 Hz, 1H, H-5'a), 3.44 (dd, $J_{4',5'b}$ = 3.6 Hz, $J_{5'a,5'b}$ = 10.6 Hz, 1H, H-5'b), 3.79 (s, 6H, 2 x OCH₃^{DMTr}), 4.00 (app. q, J = 3.4 Hz, 1H, H-4'), 4.64 (d, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.5 Hz, 2H, H₂C-C $\equiv$ CH), 4.66-4.69 (m, 1H, H-3'), 6.37 (app. t, J = 6.6 Hz, 1H, H-1'), 6.89-6.92 (m, 4H, 4 x CH^{DMTr}), 7.23-7.39 (m, 7H, 7 x CH^{DMTr}), 7.49-7.51 (m, 2H, 2 x CH^{DMTr}), 7.72 (app. d, J = 1.1 Hz, 1H, H-6) ppm.

^{13}C NMR (100 MHz, acetone- d_6): δ = -4.7 ($\text{CH}_3^{\text{TBDMS}}$), -4.5 ($\text{CH}_3^{\text{TBDMS}}$), 12.9 (CH_3^{T}), 18.5 (qC^{TBDMS}), 26.1 ($\text{CH}_3^{\text{TBDMS}}$), 30.7 ($\text{H}_2\text{C}-\text{C}\equiv\text{C}$), 41.7 (C-2'), 55.5 ($\text{OCH}_3^{\text{DMTr}}$), 64.0 (C-5'), 71.7 ($\text{HC}\equiv\text{C}$), 73.0 (C-3'), 79.8 ($\text{HC}\equiv\text{C}$), 86.2 (C-1'), 87.3 (C-4'), 87.5 (qC^{DMTr}), 110.2 (C-5), 114.0 (CH^{DMTr}), 127.8 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.0 (CH^{DMTr}), 131.0 (CH^{DMTr}), 135.5 (C-6), 136.5 (CH^{DMTr}), 136.6 (CH^{DMTr}), 145.8 (CH^{DMTr}), 150.9 (C-2), 159.8 (CH^{DMTr}), 162.8 (C-4) ppm.

HRMS (APCI $^+$): m/z calc. for $\text{C}_{40}\text{H}_{47}\text{N}_2\text{O}_7\text{Si}$ = 697.3304 $[\text{M}+\text{H}]^+$, found = 697.3303

***N*³-Propargyl-3'-*O*-(*tert*-butyldimethylsilyl) (**100**)**



A solution of TES (2.84 mL, 17.79 mmol) and 3% TFA in DCM (18 mL) was added to fully protected thymidine **99** (1.24 g, 1.78 mmol). The bright red solution was stirred at rt and a gradual colour change to colourless was observed. After 10 min, TLC analysis (DCM-MeOH, 19:1) showed complete consumption of starting material (R_f = 0.9) and the formation of a new product (R_f = 0.5). After this time, sat. aq. NaHCO_3 was added until effervescence ceased (~ 30 mL) and the reaction mixture was diluted with DCM (10 mL). The organic layer was washed with sat. aq. NaHCO_3 (20 mL). The product was extracted from the combined aq. layers with DCM (3 x 20 mL). The combined organic layers were washed with H_2O (40 mL) and brine (40 mL), dried over MgSO_4 and filtered. The solvent was removed *in vacuo* and the crude yellow oil was purified *via* flash column chromatography (DCM $\rightarrow$ DCM-MeOH, 49:1) to yield **100** as an off-white hygroscopic foam (592 mg, 84%).

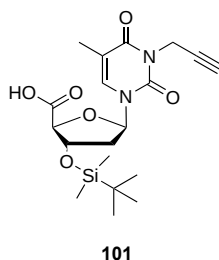
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3468 (O-H), 3265 ($\text{C}\equiv\text{C}-\text{H}$), 2930 (C-H), 2857 (C-H), 2161 ($\text{C}\equiv\text{CH}$), 1703 (C=O), 1640 (C=C), 1465 (CH_3), 1412 (CH_3), 1362 (CH_3), 1251 (C-N, C=C-H), 1193 (CH_3), 1100 (C-O), 1063 (N-C-H), 1026 (C-O), 1006 (C-N-C), 939 (C-C-H), 919 (C-O), 873 (Si-C), 832 (C-C), 769 (C- CH_3), 621 (N-C-C).

^1H NMR (400 MHz, methanol- d_4): δ = 0.13 (s, 6H, 2 x $\text{CH}_3^{\text{TBDMS}}$), 0.93 (s, 9H, 3 x $\text{CH}_3^{\text{TBDMS}}$), 1.93 (d, $J_{\text{CH}_3,6}$ = 0.9 Hz, 3H, CH_3^{T}), 2.16-2.28 (m, 2H, H-2'), 2.53 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 1H, $\text{HC}\equiv\text{C}$), 3.71 (dd, $J_{4',5'a}$ = 3.5 Hz, $J_{5'a,5'b}$ = 12.0 Hz, 1H, H-5'a), 3.79 (dd, $J_{4',5'b}$ = 3.5 Hz, $J_{5'a,5'b}$ = 12.0 Hz, 1H, H-5'b), 3.91 (app. q, J = 3.3 Hz, 1H, H-4'), 4.50-4.53 (m, 1H, H-3'), 4.66 (d, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 2H, $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 6.29 (app. t, J = 6.7 Hz, 1H, H-1'), 7.84 (app. d, J = 0.9 Hz, 1H, H-6) ppm.

^{13}C NMR (100 MHz, methanol- d_4): δ = -4.8 ($\text{CH}_3^{\text{TBDMS}}$), -4.6 ($\text{CH}_3^{\text{TBDMS}}$), 13.1 (CH_3^{T}), 18.8 (qC^{TBDMS}), 26.2 ($\text{CH}_3^{\text{TBDMS}}$), 31.2 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 41.8 (C-2'), 62.4 (C-5'), 71.8 ($\text{HC}\equiv\text{C}$), 73.4 (C-3'), 79.2 ($\text{HC}\equiv\text{C}$), 87.3 (C-1'), 89.4 (C-4'), 110.8 (C-5), 136.8 (C-6), 151.7 (C-2), 164.3 (C-4) ppm.

HRMS (APCI $^+$): m/z calc. for $\text{C}_{19}\text{H}_{30}\text{N}_2\text{O}_5\text{SiNa}$ = 417.1816 $[\text{M}+\text{Na}]^+$, found = 417.1817

***N*³-Propargyl-3'-*O*-(*tert*-butyldimethylsilyl)-5'-deoxy-5'-oxo-thymidine (101)**



Alcohol **100** (575 mg, 1.46 mmol) was suspended MeCN- H_2O , 1:1 (8 mL). BAIB (1.13 g, 3.50 mmol) and TEMPO (70 mg, 0.45 mmol) were added sequentially. The orange suspension was stirred at rt for 2 h. After this time TLC analysis (DCM-MeOH, 19:1) showed complete consumption of the starting material (R_f = 0.6) and the formation of a new product (R_f = 0.1). The reaction was quenched with sat. aq. NaHCO_3 (30 mL) and stirred at rt for 20 min. The product was extracted with DCM (3 x 20 mL). The combined organic layers were washed with H_2O (20 mL), dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography (DCM-MeOH, 19:1) to give **101** as a white foam (290 mg, 49%).

IR (ATR, IPA) $\nu_{\text{max}}/\text{cm}^{-1}$: 3313 (O-H, $\text{C}\equiv\text{C}-\text{H}$), 2958 (C-H), 2930 (C-H), 2858 (C-H), 1705 (C=O), 1667 (C=C), 1640 (C=C), 1466 (CH_3), 1413 (CH_3), 1380 (CH_3), 1274 (C-N), 1253 (C=C-H), 1231 (C-N), 1184 (CH_3), 1150 (CH_3), 1074 (N-C-H), 1005

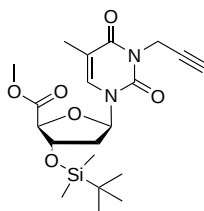
(C-C≡C), 980 (C-N-C), 950 (C-C-H), 917 (C-C≡C), 833 (Si-C), 815 (C-C), 772 (C-CH₃), 662, 621 (N-C-C), 564 (C-O-C).

¹H NMR (400 MHz, methanol-*d*₄): δ = 0.17 (s, 3H, CH₃^{TBDMS}), 0.18 (s, 3H, CH₃^{TBDMS}), 0.95 (s, 9H, 3 x CH₃^{TBDMS}), 1.95 (s, 3H, CH₃^T), 2.01-2.08 (m, 1H, H-2'a), 2.29 (app. dd, *J* = 5.2 Hz, 13.3 Hz, 1H, H-2'b), 2.53 (t, *J*_{HC≡C-CH₂} = 2.4 Hz, 1H, HC≡C), 4.42 (br s, 1H, H-4'), 4.66 (d, *J*_{HC≡C-CH₂} = 2.4 Hz, 2H, H₂C-C≡CH), 4.70 (app. d, *J* = 4.2 Hz, 1H, H-3'), 6.47 (dd, *J*_{1',2'a} = 5.2 Hz, *J*_{1',2'b} = 9.1 Hz, 1H, H-1'), 8.41 (s, 1H, H-6) ppm.

¹³C NMR (100 MHz, methanol-*d*₄): δ = -4.8 (CH₃^{TBDMS}), 13.2 (CH₃^T), 18.8 (qC^{TBDMS}), 26.2 (CH₃^{TBDMS}), 31.2 (H₂C-C≡CH), 40.7 (C-2'), 71.8 (HC≡C), 77.5 (C-3'), 79.2 (HC≡C), 87.3 (C-4'), 88.9 (C-1'), 110.8 (C-5), 137.4 (C-6), 151.8 (C-2), 164.3 (C-4), 182.4 (C-5') ppm.

HRMS (ESI⁺): *m/z* calc. for C₁₉H₂₉N₂O₆Si = 409.1789 [M+H]⁺, found = 409.1789

N³-Propargyl-3'-O-(*tert*-butyldimethylsilyl)-5'-deoxy-5'-methoxycarbonyl-5'-oxo-thymidine (102)



102

Carboxylic acid **101** (49 mg, 0.12 mmol) was dissolved in anhydrous THF-MeOH, 4:1 (1.13 mL) under argon. TMS-diazomethane (2 M in hexane, 0.07 mL, 0.13 mmol) was added dropwise. The colourless solution was stirred at rt for 1.5 h. After this time, TLC analysis (DCM-MeOH, 19:1) showed consumption of the starting material (*R*_f = 0.3) and the formation of a new product (*R*_f = 0.9). The reaction was quenched with a few drops of AcOH and the reaction mixture was stirred at rt for 35 min. After this time, the solvent was removed *in vacuo* and the residue was purified by flash column chromatography (pet. ether-EtOAc, 4:1) to give **102** as a colourless oil (24 mg, 47%).

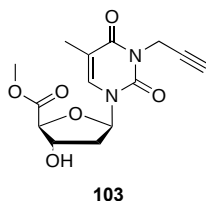
IR (ATR, DCM) $\nu_{\text{max}}/\text{cm}^{-1}$: 3285 (C≡C-H), 2955 (C-H), 2858 (C-H), 1745 (C=O), 1709, 1673 (C=C), 1654, 1465 (CH₃), 1335 (C-H), 1275 (C-N), 1214 (C-N), 1149 (CH₃), 1086 (N-C-H), 1009 (C-C≡C), 834 (Si-C), 770 (C-CH₃).

¹H NMR (400 MHz, CDCl₃): δ = 0.11 (s, 3H, CH₃^{TBDMS}), 0.12 (s, 3H, CH₃^{TBDMS}), 0.90 (s, 9H, 3 x CH₃^{TBDMS}), 1.90-1.96 (m, 1H, H-2'a), 2.00 (d, $J_{\text{CH}_3,6}$ = 1.1 Hz, 3H, CH₃^T), 2.16 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.5 Hz, 1H, HC≡C), 2.34 (ddd, $J_{2'b,3'}$ = 0.9 Hz, $J_{1',2'b}$ = 5.1 Hz, $J_{2'a,2'b}$ = 8.1 Hz, 1H, H-2'b), 3.81 (s, 3H, COOCH₃), 4.45 (br s, 1H, H-4'), 4.51 (app. d, J = 4.5, 1H, H-3'), 4.72 (d, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.5 Hz, 2H, H₂C-C≡CH), 6.57 (dd, $J_{1',2'b}$ = 5.1 Hz, $J_{1',2'a}$ = 9.2 Hz, 1H, H-1'), 8.09 (app. d, J = 1.1 Hz, 1H, H-6) ppm.

¹³C NMR (100 MHz, CDCl₃): δ = -4.8 (CH₃^{TBDMS}), 13.5 (CH₃^T), 18.1 (qC^{TBDMS}), 25.7 (CH₃^{TBDMS}), 30.5 (H₂C-C≡CH), 39.9 (C-2'), 52.7 (COOCH₃), 70.6 (HC≡C), 75.8 (C-3'), 78.4 (H₂C-C≡CH), 85.3 (C-4'), 87.5 (C-1'), 110.6 (C-5), 134.7 (C-6), 150.5 (C-2), 162.6 (C-4), 171.9 (C-5') ppm.

HRMS (ESI⁺): m/z calc. for C₂₀H₃₁N₂O₆Si = 423.1946 [M+H]⁺, found = 423.1946

N³-Propargyl-5'-deoxy-5'-methoxycarbonyl-5'-oxo-thymidine (103)



Silyl ether **102** (96 mg, 0.23 mmol) was dissolved in THF (2.3 mL). TBAF·3H₂O (109 mg, 0.35 mmol) was added and the reaction mixture was stirred at rt for 16 h. After this time, TLC analysis (EtOAc) showed consumption of the starting material (R_f = 0.9) and the formation of a new product (R_f = 0.7). The solvent was removed *in vacuo* and the yellow residue was purified by flash column chromatography (pet. ether-EtOAc, 1:1) to give **103** as a colourless film (20 mg, 29%).

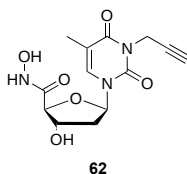
IR (ATR, MeOH) $\nu_{\text{max}}/\text{cm}^{-1}$: 3371 (O-H, C≡C-H), 2923 (C-H), 2849 (C-H), 2357, 1746 (C=O), 1704, 1653 (C=C), 1470 (CH₃), 1331 (CH₃), 1294 (C-H), 1271 (C-H), 1252 (C-N), 1215 (C-N), 1105 (C-O), 1078 (N-C-H), 1004 (C-C≡C), 952 (C-C-H), 773 (C-CH₃).

^1H NMR (600 MHz, methanol- d_4): δ = 1.96 (d, $J_{\text{CH}_3,6}$ = 1.0 Hz, 3H, CH_3^{T}), 2.04-2.11 (m, 1H, H-2'a), 2.37 (ddd, $J_{2'b,3'} = 1.3$ Hz, $J_{1',2'b} = 5.5$ Hz, $J_{2'a,2'b} = 13.6$ Hz, 1H, H-2'b), 2.53 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2} = 2.4$ Hz, 1H, $\text{HC}\equiv\text{C}$), 3.81 (s, 3H, COOCH_3), 4.51 (br s, 1H, H-4'), 4.57 (app. d, $J = 4.8$ Hz, 1H, H-3'), 4.86 (d, $J_{\text{HC}\equiv\text{C}-\text{CH}_2} = 2.4$ Hz, 2H, $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 6.49 (dd, $J_{1',2'b} = 5.5$ Hz, $J_{1',2'a} = 8.9$ Hz, 1H, H-1'), 8.26 (app. d, $J = 1.0$ Hz, 1H, H-6) ppm.

^{13}C NMR (150 MHz, methanol- d_4): δ = 13.3 (H-5), 31.2 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 39.8 (C-2'), 53.0 (COOCH_3), 71.8 ($\text{HC}\equiv\text{C}$), 75.6 (C-3'), 79.2 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 86.4 (C-4'), 88.9 (C-1'), 110.9 (C-5), 137.0 (C-6), 151.7 (C-2), 164.3 (C-4), 173.3 (C-5') ppm.

HRMS (ESI $^+$): m/z calc. for $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_6\text{Na} = 331.0901$ $[\text{M}+\text{Na}]^+$, found = 331.0903

***N*³-Propargyl-5'-deoxy-5'-hydroxyamino-5'-oxo-thymidine (62)**



$\text{NH}_2\text{OH}\cdot\text{HCl}$ (86 mg, 1.23 mmol) and KOH (86 mg, 1.54 mmol) were added to MeOH (1.2 mL) which was gently warmed to 40 °C to dissolve. The reaction mixture was filtered and added to methyl ester **103** (19 mg, 0.06 mmol). The pale orange solution was stirred at rt for 2 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed complete consumption of the starting material ($R_f = 0.7$) and the formation of a new product ($R_f = 0.3$). The reaction mixture was cooled on ice and neutralised with 1 M aq. HCl . The solvent was removed *in vacuo* and the crude product was purified by flash column chromatography (DCM-MeOH, 9:1) to give **62** as a colourless film (13 mg, 68%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3265 (N-H, O-H), 2925 (C-H), 1698 (C=O), 1628 (C=C), 1528 (N-H, C=N), 1468 (CH_3), 1416 (CH_3), 1374 (CH_3), 1334 (C=N), 1270 (C=N), 1151 (CH_3), 1100 (C=O), 1057 (N-C-H), 1028 (C-N-H), 987 (C-N-C), 953 (C-C-H), 770 (C- CH_3), 622 (N-H).

^1H NMR (600 MHz, methanol- d_4): δ = 1.95 (s, 3H, CH_3^{T}), 2.28-2.42 (m, 2H, H-2'), 2.54 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2} = 2.4$ Hz, 1H, $\text{HC}\equiv\text{C}$), 4.23 (br s, 1H, H-4'), 4.50-4.51 (m, 1H, H-3'), 4.66

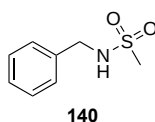
(d, $J_{HC\equiv C-CH_2} = 2.4$ Hz, 2H, $\underline{H_2C-C\equiv CH}$), 6.46 (dd, $J_{1',2'a} = 5.8$ Hz, $J_{1',2'b} = 8.1$ Hz, 1H, H-1'), 8.14 (s, 1H, H-6) ppm.

^{13}C NMR (150 MHz, methanol- d_4): $\delta = 13.1$ (H-5), 31.3 ($\underline{H_2C-C\equiv CH}$), 40.3 (C-2'), 71.8 ($\underline{HC\equiv C}$), 75.2 (C-3'), 79.2 ($\underline{H_2C-C\equiv CH}$), 85.5 (C-4'), 88.6 (C-1'), 111.0 (C-5), 137.4 (C-6), 151.8 (C-2), 164.3 (C-4), 169.4 (C-5') ppm.

HRMS (APCI⁺): m/z calc. for $C_{13}H_{16}N_3O_6 = 310.1034$ $[M+H]^+$, found = 310.1035

7.2.2. Synthetic Methods for Chapter 4

N-Benzylmethanesulfonamide (**140**)⁴⁵⁷



Benzylamine **142** (0.1 mL, 0.92 mmol) and Et_3N (0.32 mL, 2.29 mmol) were dissolved in anhydrous DCM (3 mL) under argon. $MsCl$ (78 μL , 1.01 mmol) was added dropwise on ice. The reaction mixture was brought to rt and stirred for 19 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed consumption of starting material ($R_f = 0.2$) and the formation of a new product ($R_f = 0.8$). The reaction mixture was diluted with DCM (3 mL) and 1 M aq. HCl (5 mL). The layers were separated and the aq. layer was extracted with DCM (3 x 5 mL). The combined organics were washed with brine (20 mL), dried over $MgSO_4$, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-EtOAc, 1:1) to give **140** as a white solid (112 mg, 66%); mp (hexane-EtOAc) 63-65 °C (lit.⁴⁵⁸ 64-65 °C).

IR (ATR, neat) ν_{max}/cm^{-1} : 3224 (N-H), 3016 (C-H), 2933 (C-H), 1605, 1493, 1455, 1435, 1412, 1380, 1337 (C-H), 1294, 1204, 1162, 1131, 1082, 1060, 1015 (S=O), 974, 910, 874, 764, 737, 695.

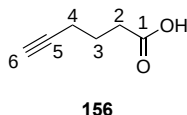
^1H NMR (400 MHz, $CDCl_3$): $\delta = 2.86$ (s, 3H, CH_3), 4.32 (d, $J_{CH_2,CHAr} = 5.6$ Hz, 2H, CH_2), 4.72 (br s, 1H, NH), 7.29-7.41 (m, 5H, CH^Ar) ppm.

^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 41.3$ (CH_3), 47.4 (CH_2), 128.0 (*o*-CH), 128.3 (*p*-CH), 129.1 (*m*-CH), 136.8 (qC) ppm.

HRMS (ESI⁻): m/z calc. for C₈H₁₀NO₂S = 184.0438 [M-H]⁻, found = 184.0442

Spectroscopic data are consistent with that reported in the literature.⁴⁵⁷

Hex-5-ynoic acid (**156**)⁴⁵⁹



5-Hexyn-1-ol **152a** (273 μ L, 2.48 mmol) was dissolved in acetone (80 mL). A fresh solution of Jones reagent (3 mL) was added dropwise at 0 °C over 5 min. The reaction mixture was stirred at 0 °C for 2.5 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed complete consumption of starting material (R_f = 0.73) and the formation of a new product (R_f = 0.67). IPA (20 mL) was added to quench and the reaction mixture was stirred at rt 1 h. During this time, H₂O (~ 80 mL) was added gradually to dissolve precipitated solids. Volatile organics were removed *in vacuo*. The product was extracted with EtOAc (3 x 50 mL) and the combined organics were washed with brine (5 x 50 mL), dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH, 19:1) to give **156** as a colourless oil (200 mg, 72%).

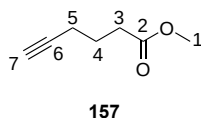
IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3298 (OH), 2926 (C-H), 1708 (C=O), 1413 (C=O), 1245, 920 (C-C $\equiv$ C), 750 (C-H).

¹H NMR (400 MHz, CDCl₃): δ = 1.86 (app. quin, J = 7.2 Hz, 2H, H-3), 1.98 (t, $J_{4,6}$ = 2.6 Hz, 1H, H-6), 2.29 (td, $J_{3,4}$ = 6.9 Hz, $J_{4,6}$ = 2.6 Hz, 2H, H-4), 2.52 (t, $J_{2,3}$ = 7.4 Hz, 2H, H-2) ppm.

¹³C NMR (100 MHz, CDCl₃): δ = 17.9 (C-4), 23.4 (C-3), 32.6 (C-2), 69.5 (C-6), 83.2 (C-5), 178.6 (C-1) ppm.

HRMS (ESI⁺): m/z calc. for C₆H₈O₂ = 111.0451 [M-H]⁺, found = 111.0453

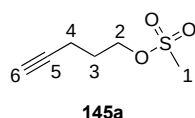
Spectroscopic data are consistent with that reported in the literature.⁴⁵⁹

Methyl hex-5-ynoate (157)⁴⁵⁹

Hex-5-ynoic acid **156** (26 mg, 0.23 mmol) was dissolved in anhydrous THF-MeOH (4:1, 2.3 mL) under argon. TMS-diazomethane (2 M in hexanes, 0.13 mL, 0.26 mmol) was added dropwise and the reaction mixture was stirred at rt for 2 h. After this time TLC analysis (hexane-EtOAc, 4:1) showed consumption of starting material ($R_f = 0.1$) and the formation of a new product ($R_f = 0.7$). AcOH (~ 0.2 mL) was added to quench and the solution changed from pale yellow to colourless. The reaction mixture was stirred for 1 h. The reaction mixture was diluted with H₂O (5 mL) and the product was extracted with DCM (3 x 5 mL). The combined organics were dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography (hexane-EtOAc, 19:1) to give the desired product **157** as a colourless film (2 mg, 7 %). Due to its instability **157** was only characterised by ¹H NMR.

¹H NMR (400 MHz, CDCl₃): δ = 1.85 (app. quin, $J = 7.2$ Hz, 2H, H-4), 1.97 (t, $J_{5,7} = 2.6$ Hz, 1H, H-7), 2.27 (td, $J_{4,5} = 6.9$ Hz, $J_{5,7} = 2.6$ Hz, 2H, H-5), 2.46 (t, $J_{3,4} = 7.4$ Hz, 2H, H-3) 3.68 (s, 3H, H-1) ppm.

Spectroscopic data are consistent with that reported in the literature.⁴⁵⁹

Pent-4-yn-1-yl methanesulfonate (145a)⁴⁰⁵

145a was synthesised according to a literature procedure.⁴⁰⁵ 4-Pentyn-1-ol **144a** (100 μ L, 1.07 mmol) was dissolved in anhydrous DCM (10 mL) under argon and Et₃N (0.18 mL, 1.29 mmol) was added. The reaction mixture was cooled to 0 °C and MsCl (91 μ L, 1.18 mmol) was added dropwise. The reaction mixture was brought to rt and stirred for 3 h. After this time, a further portion of Et₃N (0.20 mL, 1.43 mmol) was added, the reaction mixture was cooled to 0 °C and MsCl (100 μ L, 1.30 mmol) was added dropwise. The reaction mixture was brought to rt and stirred for another 20 h. After this time, TLC

analysis (DCM-MeOH, 19:1) showed complete consumption of starting material ($R_f = 0.6$) and the formation of a new product ($R_f = 0.9$). H₂O (10 mL) was added, and the product was extracted with DCM (2 x 10 mL). The combined organics were dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-DCM, 1:1) to give **145a** as a colourless oil (182 mg, quant.).

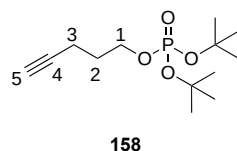
IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3288 (C≡C-H), 2941 (C-H), 1469, 1436 (C-H), 1347, 1332 (C-H), 1169, 1088 (S=O), 1012, 967 (C-C≡C), 923, 826, 770, 723 (C-H).

¹H NMR (400 MHz, CDCl₃): δ = 1.96 (app. quin, $J = 6.5$ Hz, 2H, H-3), 2.01 (t, $J_{4,6} = 2.7$ Hz, 1H, H-6), 2.37 (td, $J_{4,6} = 2.7$ Hz, $J_{3,4} = 6.8$ Hz, 2H, H-4), 3.03 (s, 3H, H-1), 4.36 (t, $J_{2,3} = 6.1$ Hz, 2H, H-2) ppm.

¹³C NMR (100 MHz, CDCl₃): δ = 14.8 (C-4), 27.9 (C-3), 37.4 (C-1), 68.4 (C-2), 69.9 (C-6), 82.2 (C-5) ppm.

Spectroscopic data are consistent with that reported in the literature.⁴⁰⁵

Di-*tert*-butyl pent-4-yn-1-yl phosphate (158)



4-Pentyn-1-ol **144a** (100 μ L, 1.07 mmol) was dissolved in anhydrous DCM (8 mL) under argon. Tetrazole (3-4% in MeCN, 9.41 mL) was added and the reaction mixture was stirred on ice. Di-*tert*-butyl diisopropylphosphoramidite (0.54 mL, 1.71 mmol) was added dropwise on ice. The reaction mixture was warmed to rt and stirred for 3 h. After this time, another portion of di-*tert*-butyl diisopropylphosphoramidite (0.43 mL, 1.08 mmol) was added dropwise on ice. The reaction mixture was warmed to rt and stirred for another 3.5 h. After this time TLC analysis (hexane-EtOAc, 1:1) showed consumption of the starting material ($R_f = 0.7$) and the formation of a new intermediate ($R_f = 0.4$). The mixture was cooled on ice and *m*-CPBA (70-75% purity, 689 mg) was added. The reaction mixture was warmed to rt and stirred for 17 h. After this time TLC analysis (hexane-EtOAc, 1:1) showed the appearance of a new product ($R_f = 0.5$). The

reaction was quenched by addition of 10% (w/v) aq. sodium thiosulfate (20 mL) and stirred at rt for 1.5 h. The layers were separated and the aq. layer was extracted with DCM (3 x 30 mL). The combined organics were washed with sat. aq. NaHCO₃ (50 mL), dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-EtOAc, 4:1 → 1:1) to give the product **158** as a colourless oil (25 mg, 8%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3299 (C≡C-H), 3222 (C≡C-H), 2980 (C-H), 1476 (P=O), 1395, 1370 (C≡C-H), 1260 (C≡C-H), 1174, 1091, 1037 (P-O), 980 (C-C≡C), 919, 860, 833, 807, 742 (P-C).

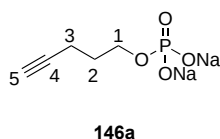
¹H NMR (400 MHz, CDCl₃): δ = 1.49 (s, 18H, 6 x CH₃^{tBu}), 1.84-1.91 (m, 2H, H-2), 1.96 (t, $J_{3,5}$ = 2.6 Hz, 1H, H-5), 2.33 (td, $J_{3,5}$ = 2.6 Hz, $J_{2,3}$ = 7.1 Hz, 2H, H-3), 4.05 (app. q, J = 6.3 Hz, 2H, H-1) ppm.

¹³C NMR (100 MHz, CDCl₃): δ = 15.0 (C-3), 29.3 (d, $J_{C,P}$ = 7.4 Hz, C-2), 30.0 (d, $J_{C,P}$ = 4.3 Hz, CH₃^{tBu}), 65.3 (d, $J_{C,P}$ = 6.3 Hz, C-1), 69.0 (C-5), 82.3 (d, $J_{C,P}$ = 7.0 Hz, qC^{tBu}), 83.4 (C-4) ppm.

³¹P NMR (162 MHz, CDCl₃): δ = -9.74 ppm

HRMS (ESI⁺): m/z calc. for C₁₃H₂₅O₄PNa = 299.1383 [M+Na]⁺, found = 299.1383

Disodium pent-4-yn-1-yl phosphate (146a)



A solution of TFA-DCM, 1:3 (0.65 mL) was added to **158** (18 mg, 65 μ mol) and the pink solution was stirred at rt for 1 h. After this time, TLC analysis (hexane-EtOAc, 1:4) showed consumption of the starting material (R_f = 0.7) and the formation of a new product (R_f = 0.04). Toluene (~ 5 mL) was added and the solvent was removed *in vacuo*. Excess TFA was removed by coevaporation with toluene (3 x 3 mL). The pink residue was redissolved in DCM-MeOH, 9:1 and eluted through Diaion resin WT01S(H) (Na form) in MeOH. The eluent was dried under reduced pressure to afford **146a** as a white film (5.5 mg, 39%).

IR (ATR, MeOH) $\nu_{\max}/\text{cm}^{-1}$: 3284 (C $\equiv$ C-H), 2957 (C-H), 1649 (P=O), 1440 (C-H), 1192, 1085, 1052 (P-O), 983 (C-C $\equiv$ C), 896, 817, 782

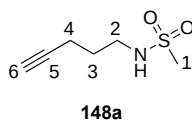
^1H NMR (400 MHz, methanol- d_4): δ = 1.81 (app. quin, J = 6.8 Hz, 2H, H-2), 2.15 (t, $J_{3,5}$ = 2.7 Hz, 1H, H-5), 2.29 (td, $J_{3,5}$ = 2.7 Hz, $J_{2,3}$ = 7.3 Hz, 2H, H-3), 3.90 (app. q, J = 6.2 Hz, 2H, H-1) ppm.

^{13}C NMR (100 MHz, methanol- d_4): δ = 15.8 (C-3), 31.5 (d, $J_{C,P}$ = 7.8 Hz, C-2), 64.3 (d, $J_{C,P}$ = 5.0 Hz, C-1), 69.3 (C-4), 84.9 (C-5) ppm.

^{31}P NMR (162 MHz, methanol- d_4): δ = 3.06 ppm

HRMS (ESI $^+$): m/z calc. for $\text{C}_5\text{H}_8\text{Na}_2\text{O}_4\text{P}$ = 208.9950 $[\text{M}+\text{H}]^+$, found = 208.9949

***N*-(Pent-4-yn-1-yl)methanesulfonamide (148a)**



4-Pentyn-1-amine hydrochloride **147a** (50 mg, 0.42 mmol) was suspended in anhydrous DCM (4.2 mL) under argon. Et_3N (0.15 mL, 1.08 mmol) was added to give a colourless solution and the mixture was stirred on ice. MsCl (49 μL , 0.63 mmol) was added dropwise on ice and the reaction mixture was stirred at 0 °C – rt over 18 h. After this time TLC analysis (DCM-MeOH, 19:1) showed consumption of starting material (R_f = 0.1) and the formation of a new product (R_f = 0.7). The reaction was quenched on addition of MeOH (1 mL) and the solvent was removed *in vacuo*. The product was purified *via* flash column chromatography (hexane-EtOAc, 1:1) to give the product **148a** as a colourless oil (53 mg, 79%).

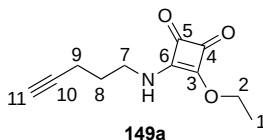
IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3282 (N-H), 2929 (C-H), 1434 (C-H), 1411, 1308 (C-H), 1142, 1079 (S=O), 976 (C-C $\equiv$ C), 850, 820, 762 (C-H).

^1H NMR (400 MHz, CDCl_3): δ = 1.80 (app. quin, J = 6.8 Hz, 2H, H-3), 2.01 (t, $J_{4,6}$ = 2.6 Hz, 1H, H-6), 2.32 (td, $J_{4,6}$ = 2.6 Hz, $J_{3,4}$ = 6.8 Hz, 2H, H-4), 2.98 (s, 3H, H-1), 3.29 (app. q, J = 6.2 Hz, 2H, H-2), 4.38 (br s, 1H, NH) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 15.8 (C-4), 28.6 (C-3), 40.6 (C-1), 42.2 (C-2), 69.9 (C-6), 82.8 (C-5) ppm.

HRMS (ESI $^+$): m/z calc. for $\text{C}_6\text{H}_{11}\text{NO}_2\text{SNa}$ = 184.0403 $[\text{M}+\text{Na}]^+$, found = 184.0410

3-Ethoxy-4-(pent-4-yn-1-ylamino)cyclobut-3-ene-1,2-dione (**149a**)



4-Pentyn-1-amine hydrochloride **147a** (50 mg, 0.42 mmol) and Et_3N (0.12 mL, 0.86 mmol) were dissolved in EtOH (4 mL). Diethyl squarate (87 μL , 0.59 mmol) was added. The reaction mixture was stirred at rt for 18 h. After this time, TLC analysis (DCM-MeOH 9:1) showed the complete consumption of starting material (R_f = 0.1) and the formation of a new product (R_f = 0.7). The solvent was removed *in vacuo* and the crude product was purified *via* flash column chromatography (hexane-EtOAc, 1:1) to give the desired product **149a** as a white solid (87 mg, quant.); mp (hexane-EtOAc) 57-58 $^\circ\text{C}$.

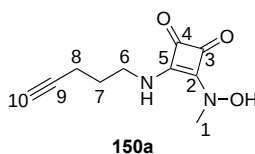
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3296 (N-H), 3198 (C-H), 2935 (C-H), 2117, 1698 (C=O), 1577 (C=O), 1490, 1464, 1433, 1376 (C-H), 1357, 1338, 1317, 1257, 1198, 1153, 1117, 1050, 1004, 989, 874, 852, 808, 788.

^1H NMR (400 MHz, CDCl_3): δ = 1.46 (t, $J_{1,2}$ = 7.1 Hz, 3H, H-1), 1.86 (app. quin, J = 6.8 Hz, 2H, H-8), 2.01 (t, $J_{9,11}$ = 2.6 Hz, 1H, H-11), 2.31 (td, $J_{9,11}$ = 2.6 Hz, $J_{8,9}$ = 6.9 Hz, 2H, H-9), 3.57-3.61(m, 1.5H, H-7), 3.79 (br s, 0.5H, H-7), 4.76-4.79 (m, 2H, H-2), 5.53 (br s, 0.3H, NH), 6.54 (br s, 0.7H, NH) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 15.7 (C-9), 16.0 (C-1), 29.1 (C-8), 43.9 (C-7), 69.9 (C-2, C-11), 82.5 (C-10), 172.6 (C-6), 177.7 (C-3), 183.0 (C-5), 189.5 (C-4) ppm.

Note: 149a (CDCl₃, rt) exhibits rotamers in NMR spectroscopy

HRMS (APCI): m/z calc. for $\text{C}_{11}\text{H}_{12}\text{NO}_3$ = 206.0823 $[\text{M}-\text{H}]^-$, found = 206.0825

3-(hydroxy(methyl)amino)-4-(pent-4-yn-1-ylamino)cyclobut-3-ene-1,2-dione (150a)

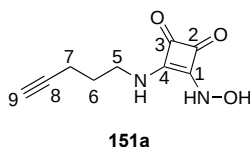
149a (49 mg, 0.24 mmol) was dissolved in EtOH (2.4 mL). *N*-Methylhydroxylamine hydrochloride (32 mg, 0.38 mmol) and Et₃N (0.1 mL, 0.72 mmol) were added and the reaction mixture was stirred at rt for 19 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed complete consumption of the starting material ($R_f = 0.7$) and the formation of a new product ($R_f = 0.3$). The solvent was removed *in vacuo* and the product was purified *via* flash column chromatography (DCM-MeOH, 9:1) to give **150a** as a white solid (40 mg, 82 %); mp (DCM-MeOH) 130-132 °C.

IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3274 (N-H), 3066 (O-H), 2772 (C-H), 1799, 1642 (C=O), 1527 (C=O), 1447, 1421, 1397, 1351 (C-H), 1275, 1234, 1140, 1120, 1012 (C-C $\equiv$ C), 885, 866, 821, 761 (C-H).

¹H NMR (400 MHz, CDCl₃): δ = 1.73 (app. quin, $J = 7.1$ Hz, 2H, H-7), 2.18 (td, $J_{8,10} = 2.7$ Hz, $J_{7,8} = 7.3$ Hz, 2H, H-8), 2.78 (t, $J_{8,10} = 2.7$ Hz, 1H, H-10), 3.34 (s, 3H, H-1), 3.52 (app. q, $J = 6.7$ Hz, 2H, H-6), 7.69 (br s, 1H, NH), 10.57 (br s, 1H, OH) ppm.

¹³C NMR (100 MHz, CDCl₃): δ = 15.0 (C-8), 29.7 (C-7), 40.8 (C-1), 42.8 (C-6), 71.5 (C-10), 83.8 (C-9), 165.9 (C-2), 167.1 (C-5), 178.3 (C-4), 180.1 (C-3) ppm.

HRMS (ESI⁺): m/z calc. for C₁₀H₁₁N₂O₃ = 207.0775 [M-H]⁺, found = 207.0776

3-(Hydroxyamino)-4-(pent-4-yn-1-ylamino)cyclobut-3-ene-1,2-dione (151a)

149a (54 mg, 0.26 mmol), hydroxylamine hydrochloride (29 mg, 0.42 mmol) and Et₃N (0.11 mL, 0.79 mmol) were dissolved in EtOH (2.6 mL) and stirred at rt for 18 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the complete consumption of starting material ($R_f = 0.7$) and the formation of a new product ($R_f = 0.2$). The solvent was

removed *in vacuo* and the crude product was purified *via* flash column chromatography (DCM-MeOH, 17:3) to give the product **151a** as a pale red solid (40 mg, 78%); mp (DCM-MeOH) 140 °C (decomp.).

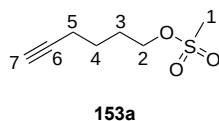
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3292 (N-H), 3176 (O-H), 2835 (C-H), 1802, 1647 (C=O), 1615, 1543 (C=O), 1475, 1447, 1420, 1350, 1309 (C-H), 1254, 1125, 1103, 1062, 1031, 994 (C-C $\equiv$ C), 866, 838, 767, 724 (C-H), 664, 639, 612.

^1H NMR (600 MHz, DMSO- d_6): δ = 1.72 (app. quin, J = 7.2 Hz, 2H, H-6), 2.18 (td, $J_{7,9}$ = 2.6 Hz, $J_{6,7}$ = 7.3 Hz, 2H, H-7), 2.78 (t, $J_{7,9}$ = 2.6 Hz, 1H, H-9), 3.51 (br s, 2H, H-5), 7.57 (br s, 1H, NH), 9.68 (s, 1H, NHOH), 10.59 (br s, 1H, NHOH) ppm.

^{13}C NMR (150 MHz, DMSO- d_6): δ = 15.0 (C-7), 29.7 (C-6), 42.9 (C-5), 71.5 (C-9), 83.8 (C-8), 166.6 (C-1), 166.9 (C-4), 178.9 (C-3), 180.7 (C-2) ppm.

HRMS (ESI): m/z calc. for $\text{C}_9\text{H}_9\text{N}_2\text{O}_3$ = 193.0619 $[\text{M-H}]^-$, found = 193.0622

Hex-5-yn-1-yl methanesulfonate (**153a**)⁴⁶⁰



153a was synthesised according to a modified literature procedure.⁴¹⁰ 5-Hexyn-1-ol **152a** (110 μL , 1.02 mmol) and Et_3N (170 μL , 1.22 mmol) were dissolved in anhydrous DCM (10 mL) under argon. MsCl (87 μL , 1.10 mmol) was added dropwise at 0 °C. The reaction mixture was stirred from 0 °C – rt over 20 h. After this time TLC analysis (DCM-MeOH, 9:1) showed consumption of starting material (R_f = 0.7) and the formation of a new product (R_f = 0.9). The reaction mixture was washed with 0.05 M aq. HCl (20 mL) and sat. aq. NaHCO_3 (20 mL). The combined aq. layers were extracted with DCM (2 x 20 mL) and EtOAc (2 x 20 mL). The combined organic layers were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH, 19:1) to give the product **153a** as a colourless oil (147 mg, 82%).

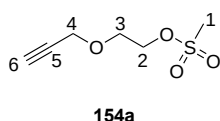
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3288 (C $\equiv$ C-H), 2944 (C-H), 1435, 1347 (C-H), 1332, 1168, 1013 (S=O), 973 (C-C $\equiv$ C), 928, 870, 819, 749 (C-H).

^1H NMR (400 MHz, CDCl_3): δ = 1.63-1.70 (m, 2H, H-4), 1.86-1.93 (m, 2H, H-3), 1.97 (t, $J_{5,7}$ = 2.6 Hz, 1H, H-7), 2.26 (td, $J_{5,7}$ = 2.6 Hz, $J_{4,5}$ = 7.0 Hz, 2H, H-5), 3.01 (s, 3H, H-1), 4.27 (t, $J_{2,3}$ = 6.4 Hz, 2H, H-2) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 18.0 (C-5), 24.4 (C-4), 28.2 (C-3), 37.6 (C-1), 69.3 (C-7), 69.5 (C-2), 83.5 (C-6) ppm.

Spectroscopic data are consistent with that reported in the literature.⁴⁶⁰

2-(Prop-2-yn-1-yloxy)ethyl methanesulfonate (**154a**)⁴⁶¹



Ethylene glycol **159** (0.18 mL, 3.22 mmol) was dissolved in anhydrous THF (16 mL) under argon. NaH (60% in mineral oil, 258 mg, 6.44 mmol) was added on ice and the reaction mixture was stirred on ice for 40 min. Propargyl bromide (80% in toluene, 0.52 mL, 4.83 mmol) was added dropwise on ice and the reaction mixture was stirred at 50 °C for 17 h. After this time TLC analysis (hexane-EtOAc, 1:1) showed consumption of starting material (R_f = 0.1) to give an intermediate (R_f = 0.3). The reaction was cooled to rt and quenched with addition of TFA (0.5 mL). The reaction mixture was placed on ice and Et_3N (2.3 mL, 16.5 mmol) was added. MsCl (0.37 mL, 4.78 mmol) was added dropwise on ice. The reaction mixture was stirred at 0 °C – rt over 24 h. After this time, TLC analysis (hexane-EtOAc, 1:1) showed consumption of the intermediate (R_f = 0.3) and the formation of a new product (R_f = 0.5). The reaction was quenched by addition of H_2O (10 mL) and the product was extracted with DCM (2 x 10 mL). The combined organics were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-EtOAc, 1:1) to give desired product **154a** as a pale yellow oil (37 mg, 6%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3281($\text{C}\equiv\text{C-H}$), 2929 (C-H), 1455 (C-H), 1341 (C-H), 1170, 1105 (S=O), 1018, 995, 971 (C-C $\equiv$ C), 915, 836, 800.

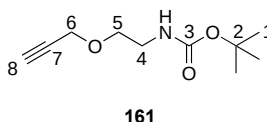
^1H NMR (400 MHz, CDCl_3): δ = 2.47 (t, $J_{4,6}$ = 2.4 Hz, 1H, H-6), 3.07 (s, 3H, H-1), 3.80-3.83 (m, 2H, H-3), 4.22 (d, $J_{4,6}$ = 2.4 Hz, 2H, H-4), 4.39-4.41 (m, 2H, H-2) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 37.9 (C-1), 58.6 (C-4), 67.6 (C-3), 68.8 (C-2), 75.4 (C-6), 78.9 (C-5) ppm.

HRMS (ESI $^+$): m/z calc. for $\text{C}_6\text{H}_{10}\text{O}_4\text{SNa}$ = 201.0192 $[\text{M}+\text{Na}]^+$, found = 201.0187

Spectroscopic data are consistent with that reported in the literature.⁴⁶¹

***tert*-Butyl (2-(prop-2-yn-1-yloxy)ethyl)carbamate (**161**)**



161 was synthesised according to a literature procedure.⁴¹¹ *N*-(*tert*-Butoxycarbonyl)ethanolamine **160** (0.48 mL, 3.10 mmol) was dissolved in anhydrous THF-DMF, 1:1 (16 mL) and stirred on ice. NaH (60% in mineral oil, 311 mg, 7.78 mmol) was added and the reaction mixture was stirred on ice for 30 min. Propargyl bromide (80% in toluene, 0.5 mL, 4.64 mmol) was added dropwise over 5 min and the reaction mixture was brought to rt and stirred for 17 h. After this time TLC analysis showed consumption of starting material (R_f = 0.1, hexane-EtOAc, 4:1) and the formation of a new product (R_f = 0.1, hexane-EtOAc, 9:1). H_2O (3 mL) was added to quench and the volatile organics were removed *in vacuo*. The reaction mixture was diluted with EtOAc (15 mL) and the organics were washed with brine (2 x 15 mL). The combined aq. layers were extracted with EtOAc (2 x 20 mL). The organics were combined and dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-EtOAc, 19:1 $\rightarrow$ 9:1) to give the product **161** as a yellow oil (105 mg, 17%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3296 (N-H), 2977 ($\text{C}\equiv\text{C-H}$), 2933 (C-H), 2177, 1695 (C=O), 1511 (C=O), 1455, 1392, 1366 (C-H), 1270, 1250, 1168, 1098, 1036, 925 (C-C $\equiv$ C), 865, 780, 757 (C-H).

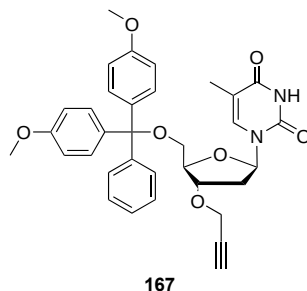
^1H NMR (400 MHz, CDCl_3): δ = 1.44 (s, 9H, H-1), 2.44 (t, $J_{6,8}$ = 2.4 Hz, 1H, H-8), 3.34 (app. q, J = 5.1 Hz, 2H, H-4), 3.59 (t, $J_{4,5}$ = 5.1 Hz, 2H, H-5), 4.16 (d, $J_{6,8}$ = 2.4 Hz, 2H, H-6), 4.89 (br s, 1H, NH) ppm.

^{13}C NMR (100 MHz, CDCl_3): δ = 28.5 (C-1), 40.5 (C-4), 58.4 (C-6), 69.3 (C-5), 74.8 (C-8), 79.5 (C-2), 79.6 (C-7), 156.1 (C-3) ppm.

HRMS (ESI^+): m/z calc. for $\text{C}_{10}\text{H}_{17}\text{NO}_3\text{Na}$ = 222.1101 $[\text{M}+\text{Na}]^+$, found = 222.1101

Spectroscopic data are consistent with that reported in the literature.⁴⁶²

3'-*O*-propargyl-5'-*O*-(4,4'-dimethoxytrityl)-thymidine (**167**)⁴¹²



167 was synthesised according to a modified literature procedure.⁴¹² Under argon, **166** (501 mg, 0.92 mmol) was suspended in anhydrous THF (9 mL) and placed on ice. NaH (60% in mineral oil, 92 mg, 2.3 mmol) was added and the reaction mixture was stirred on ice for 1 h. After this time, propargyl bromide (80% in toluene, 119 μL , 1.10 mmol) was added dropwise on ice. The reaction mixture was stirred at 0°C – rt for 17 h. After this time, TLC analysis (hexane-EtOAc, 4:1) showed complete consumption of starting material (R_f = 0.3) and formation of a new product (R_f = 0.7). H_2O (1 mL) was added to quench and the organics were removed *in vacuo*. The residue was redissolved in H_2O (20 mL) and EtOAc (20 mL). The product was extracted with EtOAc (2 x 20 mL) and the combined organics were washed with brine (40 mL), dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (toluene-acetone, 8:2) to give **167** as a white foam (379 mg, 71%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3248 (N-H), 2932 (C-H), 1682 (C=O), 1606 (C=C), 1507, 1464 (CH_3), 1248 (C-N, C=CH), 1175, 1091 (N-C-H), 1031 (C-C $\equiv$ C), 913, 827, 790 (C-CH $_3$), 755, 726 (C-H), 700.

^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 1.43 (d, $J_{\text{CH}_3,6}$ = 0.7 Hz, 3H, CH_3^T), 2.27-2.37 (m, 2H, H-2'), 3.17 (dd, $J_{4',5'a}$ = 3.5 Hz, $J_{5'a,5'b}$ = 10.5 Hz, 1H, H-5'a), 3.25 (dd, $J_{4',5'b}$ = 4.0 Hz, $J_{5'a,5'b}$ = 10.5 Hz, H-5'b), 3.51 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 1H, $\text{HC}\equiv\text{C}$), 3.74 (s, 6H, 2 x $\text{OCH}_3^{\text{DMTr}}$), 4.03 (app. dd J = 3.5, 6.5 Hz, 1H, H-4'), 4.22 (app. dd, J = 2.4, 3.9 Hz,

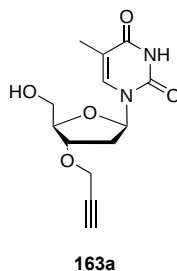
^1H , $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 4.45-4.47 (m, 1H, H-3'), 6.12 (dd, $J_{1',2'a} = 6.1$ Hz, $J_{1',2'b} = 8.0$ Hz, 1H, H-1'), 6.89-6.92 (m, 4H, 4 x CH^{DMTr}), 7.23-7.27 (m, 7H, 7 x CH^{DMTr}), 7.39-7.40 (m, 2H, 2 x CH^{DMTr}), 7.50 (app. d, $J = 0.7$ Hz, 1H, H-6), 11.35 (br s, 1H, NH-3) ppm.

^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): $\delta = 11.7$ (CH_3^{T}), 36.2 (C-2'), 55.1 ($\text{OCH}_3^{\text{DMTr}}$), 56.0 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 63.7 (C-5'), 77.5 ($\text{C}\equiv\text{CH}$), 78.1 (C-3'), 80.0 ($\text{C}\equiv\text{CH}$), 82.6 (C-4'), 83.8 (C-1'), 86.1 (qC^{DMTr}), 109.8 (C-5), 113.3 (CH^{DMTr}), 126.9 (CH^{DMTr}), 127.6 (CH^{DMTr}), 128.0 (CH^{DMTr}), 129.7 (CH^{DMTr}), 135.2 (qC^{DMTr}), 135.4 (qC^{DMTr}), 135.5 (C-6), 144.6 (qC^{DMTr}), 150.4 (C-2), 158.2 (qC^{DMTr}), 163.6 (C-4) ppm.

HRMS (ESI⁺): m/z calc. for $\text{C}_{34}\text{H}_{34}\text{N}_2\text{O}_7\text{Na} = 605.2258$ $[\text{M}+\text{Na}]^+$, found = 605.2269

Spectroscopic data are consistent with that reported in the literature.⁴¹²

3'-O-propargyl-thymidine (163a)⁴⁶³



Method 1:

A solution of TES (0.23 mL, 1.44 mmol) and 3% TFA in DCM (2.77 mL) was added to **167** (168 mg, 0.29 mmol) and a bright red colour was immediately observed. The reaction mixture was stirred rt and a gradual colour change from red to colourless was observed. After 10 min, TLC analysis (DCM-MeOH, 19:1) showed complete consumption of the starting material ($R_f = 0.6$) and the formation of a new product ($R_f = 0.3$). The reaction was quenched with sat. aq. NaHCO_3 until effervescence ceased (~ 10 mL) and the reaction mixture was diluted with DCM (10 mL). The product was extracted with DCM (3 x 20 mL). The combined organic layers were washed with H_2O (20 mL) and brine (20 mL). The combined aqueous layers were extracted with EtOAc (3 x 20 mL). The combined organics were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH,

99:1 → 19:1) to give the product **163a** as a white solid (48 mg, 59%); mp (DCM-MeOH) 142-143 °C (lit.⁴⁶⁴ 144.7 °C).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3467 (N-H), 3302 (O-H), 3273 (C≡C-H), 3192 (C-H), 3056 (C-H), 2936 (C-H), 1703 (C=O), 1662 (C=C), 1475 (CH₃), 1406 (CH₃), 1368 (CH₃), 1332, 1300, 1276, 1262 (C-N), 1240 (C=C-H), 1204 (CH₃), 1130 (C-O), 1097, 1087, 1061 (N-C-H), 1049, 975, 958, 937 (C-C-H), 919 (C-O), 878, 835 (C-C), 788 (C-CH₃), 704.

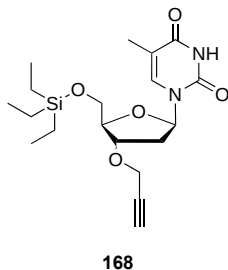
¹H NMR (600 MHz, acetone-*d*₆): δ = 1.81 (d, $J_{\text{CH}_3,6}$ = 1.2 Hz, 3H, CH₃^T), 2.25-2.29 (m, 1H, H-2'a), 2.38 (ddd, $J_{2'b,3'}$ = 2.1 Hz, $J_{1',2'b}$ = 5.9 Hz, $J_{2'a,2'b}$ = 13.8 Hz, 1H, H-2'b), 2.98 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 1H, HC≡C), 3.77-3.38 (m, 2H, H-5'), 4.07 (app. dd, J = 3.2, 5.7 Hz, 1H, H-4'), 4.26 (app. t, J = 2.4 Hz, 2H, H₂C-C≡CH), 4.37 (t, $J_{5',\text{OH}}$ = 5.1 Hz, 1H, 5'-OH), 4.41-4.43 (m, 1H, H-3'), 6.25 (dd, $J_{1',2'b}$ = 5.9 Hz, $J_{1',2'a}$ = 8.5 Hz, 1H, H-1'), 7.78 (app. d, J = 1.2 Hz, 1H, H-6), 9.98 (br s, 1H, NH-3) ppm.

¹³C NMR (150 MHz, acetone-*d*₆): δ = 12.6 (CH₃^T), 37.7 (C-2'), 56.9 (H₂C-C≡CH), 63.1 (C-5'), 76.1 (HC≡C), 80.0 (C-3'), 80.7 (H₂C-C≡CH), 85.7 (C-1'), 85.9 (C-4'), 110.8 (C-5), 137.0 (C-6), 151.4 (C-2), 164.3 (C-4) ppm.

HRMS (ESI⁺): m/z calc. for C₁₃H₁₆N₂O₅ = 303.0951 [M+Na]⁺, found = 303.0951

Spectroscopic data are consistent with that reported in the literature.⁴⁶³

3'-O-propargyl-5'-O-(triethylsilyl)-thymidine (168) was also recovered as a by-product of the DMTr deprotection as a colourless film (28 mg, 24%).



IR (ATR, DCM) $\nu_{\text{max}}/\text{cm}^{-1}$: 3186 (N-H), 3056, 2954 (C≡C-H), 2913 (C-H), 2876 (C-H), 1683 (C=O), 1465 (CH₃), 1414 (CH₃), 1360 (CH₃), 1323, 1272 (C-N), 1246 (C=C-H),

1200 (CH₃), 1125 (C-O), 1092 (N-C-H), 1061 (C-O), 1003 (C-N-C), 959 (C-C-H), 930 (C-O), 883 (Si-C), 850 (C-C), 807, 781 (C-CH₃), 728, 666 (N-C-C).

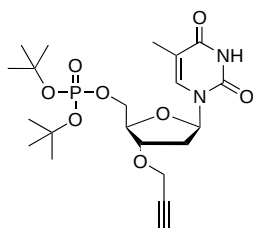
¹H NMR (400 MHz, acetone-*d*₆): δ = 0.70 (q, J_{CH_2,CH_3} = 8.0 Hz, 6H, 3 x CH₂^{TES}), 1.01 (t, J_{CH_2,CH_3} = 8.0 Hz, 9H, 3 x CH₃^{TES}), 1.84 (d, $J_{CH_3,6}$ = 1.2 Hz, 3H, CH₃^T), 2.16-2.21 (m, 1H, H-2'a), 2.40 (ddd, $J_{2'b,3'}$ = 1.9 Hz, $J_{1',2'b}$ = 5.7 Hz, $J_{2'a,2'b}$ = 13.7 Hz, 1H, H-2'b), 3.00 (t, $J_{HC\equiv C-CH_2}$ = 2.4 Hz, 1H, HC $\equiv$ C), 3.87-3.92 (m, 2H, H-5'), 4.09-4.11 (m, 1H, H-4'), 4.27 (app. t, J = 2.4 Hz, 2H, H₂C-C $\equiv$ CH), 4.41-4.43 (m, 1H, H-3'), 6.26 (dd, $J_{1',2'b}$ = 5.7 Hz, $J_{1',2'a}$ = 8.7 Hz, 1H, H-1'), 7.60 (app. d, J = 1.2 Hz, 1H, H-6), 10.00 (br s, 1H, NH-3) ppm.

¹³C NMR (100 MHz, acetone-*d*₆): δ = 4.9 (CH₂^{TES}), 7.1 (CH₃^{TES}), 12.6 (CH₃^T), 37.9 (C-2'), 56.8 (H₂C-C $\equiv$ CH), 64.1 (C-5'), 76.2 (HC $\equiv$ C), 79.9 (C-3'), 80.6 (H₂C-C $\equiv$ CH), 85.4 (C-1'), 85.6 (C-4'), 110.8 (C-4), 136.2 (C-6), 151.3 (C-2), 164.2 (C-3) ppm.

HRMS (ESI⁺): m/z calc. for C₁₉H₃₀N₂O₅SiNa = 417.1816 [M+Na]⁺, found = 417.1822

Method 2:

AcOH (80% v/v in H₂O, 3 mL) was added to **167** (168 mg, 0.29 mmol) and a bright red colour was immediately observed. The reaction mixture was stirred at rt for 50 min. After this time TLC analysis (DCM-MeOH, 19:1) showed complete consumption of the starting material (R_f = 0.6) and the formation of a new product (R_f = 0.3). The reaction mixture was neutralised with sat. aq. K₂CO₃ until pH ~ 7. The product was extracted with EtOAc (3 x 10 mL) and the combined organic layers were dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH, 99:1 $\rightarrow$ 19:1) to give the product **163a** as a white solid (59 mg, 73%).

3'-O-propargyl- thymidine-5'-O-(di-tert-butyl)phosphate (169)**169**

Alcohol **163a** was suspended in anhydrous DCM (1.1 mL). Tetrazole (3-4% in MeCN, 1.2 mL) was added and the suspension was stirred on ice. Di-*tert*-butyl *N,N*-diisopropylphosphoramidite (0.12 mL, 0.36 mmol) was added dropwise and the reaction mixture was stirred on ice for 20 min and then at rt for 3 h. After this time a white precipitate was seen and TLC analysis (DCM-MeOH, 19:1) showed complete consumption of the starting material ($R_f = 0.27$) to give an intermediate ($R_f = 0.37$). The mixture was cooled on ice and *m*-CPBA (70-75% purity, 89 mg) was added resulting in the disappearance of the white precipitate. After stirring on ice for 5 min the mixture was warmed to rt and stirred for 1 h. After this time TLC analysis (DCM-MeOH, 19:1) showed the formation of a new product ($R_f = 0.42$). The reaction was quenched by addition of 10% (w/v) aq. sodium thiosulfate (10 mL) and stirred at rt for 1 h. The reaction mixture was diluted with DCM (20 mL). The aq. layer was extracted with DCM (20 mL). The combined organics were washed with sat. aq. NaHCO_3 (20 mL), dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH, 99:1 $\rightarrow$ 19:1) to give the product **169** as a colourless oil (21 mg, 32%).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3214 (N-H), 2981 (C-H), 1683 (C=O), 1470 (CH_3), 1397, 1371 (CH_3), 1268 (C-N), 1200, 1173 (CH_3), 1088 (C-O), 1039 (C-N-H), 984 (C-N-C), 920 (C-C-H), 885, 838, 808, 782 (C- CH_3), 733 (P-C), 693 (N-H).

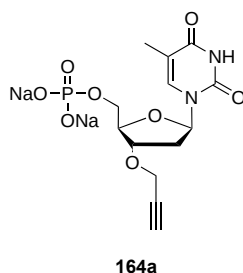
^1H NMR (400 MHz, acetone- d_6): $\delta = 1.50$ (d, $J_{\text{H-P}} = 1.64$ Hz, 18 H, 6 x CH_3^{tBu}), 1.87 (d, $J_{\text{CH}_3,6} = 1.0$ Hz, 3H, CH_3^{T}), 2.22-2.29 (m, 1H, H-2'a), 2.44 (ddd, $J_{2'b,3'} = 2.2$ Hz, $J_{1',2'b} = 5.9$ Hz, $J_{2'a,2'b} = 13.9$ Hz, 1H, H-2'b), 3.02 (t, $J_{\text{HC}\equiv\text{C-CH}_2} = 2.4$ Hz, 1H, $\text{HC}\equiv\text{C}$), 4.13-4.19 (m, 2H, H-5'), 4.21-4.24 (m, 1H, H-4'), 4.30 (app. t, $J = 2.2$ Hz, 2H, $\text{H}_2\text{C-C}\equiv\text{CH}$), 6.31 (dd, $J_{1',2'b} = 5.9$ Hz, $J_{1',2'a} = 8.4$ Hz, 1H, H-1'), 7.65 (s, 1H, H-6), 10.07 (s, 1H, NH-3) ppm.

^{13}C NMR (100 MHz, acetone- d_6): δ = 12.6 (CH_3^{T}), 30.1 (d, $J_{\text{C},\text{P}}$ = 4.4 Hz, CH_3^{tBu}), 37.4 (C-2'), 57.1 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 67.4 (d, $J_{5',\text{P}}$ = 6.4 Hz, C-5'), 76.4 ($\text{HC}\equiv\text{C}$), 80.5 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 82.8 (d, $J_{\text{C},\text{P}}$ = 7.2 Hz, qC^{tBu}), 83.0 (d, $J_{\text{C},\text{P}}$ = 6.7 Hz, qC^{tBu}), 83.6 (d, $J_{\text{C},\text{P}}$ = 8.5 Hz, C-4'), 85.5 (C-1'), 111.2 (C-5), 151.3 (C-2), 164.3 (C-4) ppm.

^{31}P NMR (162 MHz, acetone- d_6): δ = -9.96 ppm

HRMS (ESI): m/z calc. for $\text{C}_{21}\text{H}_{32}\text{N}_2\text{O}_8\text{P}$ = 471.1902 $[\text{M}-\text{H}]^-$, found = 471.1903

3'-O-propargyl-thymidine-5'-O-sodium phosphate (164a)



A solution of TFA-DCM, 1:3 (0.4 mL) was added to **169** (21 mg, 0.04 mmol) and the pink solution was stirred at rt for 1 h. After this time, TLC analysis (DCM-MeOH, 4:1) showed consumption of the starting material (R_f = 0.9) and the formation of a new product (R_f = 0.3). Toluene (2 mL) was added and the solvent was removed *in vacuo*. Excess TFA was removed by coevaporation with toluene (2 x 2 mL). The pink residue was redissolved in MeOH and eluted through Diaion resin WT01S(H) (Na form) in MeOH to afford **164a** as a white solid. Sodium trifluoroacetate (~10%) remained in the sample but **164a** was used without further purification.

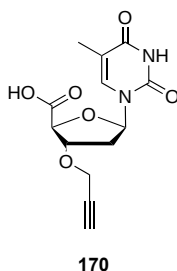
^1H NMR (400 MHz, methanol- d_4): δ = 1.95 (s, 3H, CH_3^{T}), 2.27-2.39 (m, 2H, H-2'), 2.84 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.3 Hz, 1H, $\text{HC}\equiv\text{C}$), 4.03 (app. t, J = 3.6 Hz, 2H, H-5'), 4.21-4.22 (m, 1H, H-4'), 4.24 (d, J = 2.3 Hz, 2H, $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 4.47 (app. d, J = 5.0 Hz, 1H, H-3'), 6.29 (dd, $J_{1',2'a}$ = 5.8 Hz, $J_{1',2'b}$ = 8.8 Hz, 1H, H-1'), 7.93 (s, 1H, H-6) ppm.

^{13}C NMR (100 MHz, methanol- d_4): δ = 12.6 (CH_3^{T}), 36.0 (C-2'), 57.4 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 66.0 (d, $J_{5',\text{P}}$ = 5.5 Hz, C-5'), 75.9 ($\text{HC}\equiv\text{C}$), 80.7 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 81.6 (C-3'), 85.7 (d, $J_{\text{C},\text{P}}$ = 9.5 Hz, C-4'), 86.2 (C-1'), 112.2 (C-5), 138.4 (C-6), 152.6 (C-2), 166.6 (C-3) ppm.

^{31}P NMR (162 MHz, methanol- d_4): δ = 2.73 ppm

HRMS (ESI⁻): m/z calc. $C_{13}H_{16}N_2O_8P = 359.0650$ $[M-2Na+H]^-$, found = 359.0641

3'-*O*-propargyl-5'-deoxy-5'-oxo-thymidine (**170**)



Alcohol **163a** (134 mg, 0.48 mmol) was dissolved in MeCN-H₂O, 1:1 (2.4 mL). BAIB (371 mg, 1.15 mmol) and TEMPO (23 mg, 0.15 mmol) were added sequentially and the reaction mixture was stirred at rt for 17.5 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed complete consumption of the starting material ($R_f = 0.7$) and the formation of a new product ($R_f = 0.1$). Sat. aq. NaHCO₃ (8 mL) was added and the reaction mixture was stirred at rt for 30 min. The mixture was diluted with DCM (10 mL). The aq. layer was washed with DCM (5 x 10 mL). The combined organic layers were extracted with H₂O (3 x 15 mL). The aq. layers were combined, acidified with 2 M aq. HCl until pH ~ 2 and extracted with EtOAc (3 x 20 mL). The combined EtOAc layers were dried over MgSO₄, filtered and the solvent was removed *in vacuo* to afford **170** as a pure white solid (124 mg, 87%); mp (EtOAc) 140-143 °C (decomp.).

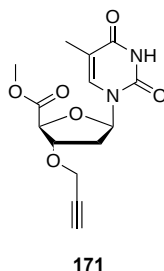
IR (ATR, neat) $\nu_{\max}/\text{cm}^{-1}$: 3186 (N-H), 3073 (O-H, C≡C-H), 2492 (C-H), 1703 (C=O), 1648 (C=O), 1621 (C=C), 1482 (CH₃), 1439, 1409 (CH₃), 1374 (CH₃), 1356, 1332, 1271 (C-N), 1247 (C=C-H), 1224 (C-N), 1182 (CH₃), 1132 (CH₃), 1106, 1079 (N-C-H), 1036 (C-C≡C), 975 (C-N-C), 940 (C-C-H), 838, 801 (C-C), 781 (C-CH₃), 751, 720, 698, 671.

¹H NMR (600 MHz, DMSO-*d*₆): δ = 1.77 (s, 3H, CH₃^T), 1.95-1.99 (m, 1H, H-2'a), 2.29 (app. dd, $J = 5.5, 13.8$ Hz, 1H, H-2'b), 3.48 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2} = 2.3$ Hz, 1H, HC≡C), 4.25 (dd, $J_{\text{HC}\equiv\text{C}-\text{CH}_2} = 2.3$ Hz, $J_{\text{gem}} = 4.9$ Hz, 2H, H₂C-C≡CH), 4.37 (br s, 1H, H-4'), 4.38 (br s, 1H, H-3'), 6.20 (dd, $J_{1',2'b} = 5.5$ Hz, $J_{1',2'a} = 9.1$ Hz, 1H, H-1'), 8.59 (br s, 1H, H-6), 11.27 (br s, 1H, NH-3) ppm.

^{13}C NMR (150 MHz, DMSO- d_6): δ = 12.4 (CH_3^T), 35.7 (C-2'), 55.6 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 77.4 ($\text{HC}\equiv\text{C}$), 80.0 (C-4'), 81.5 (C-3'), 85.4 (C-1'), 109.1 (C-5), 137.5 (C-6), 150.5 (C-2), 163.8 (C-4) ppm.

HRMS (ESI): m/z calc. for $\text{C}_{13}\text{H}_{13}\text{N}_2\text{O}_6$ = 293.0777 $[\text{M}-\text{H}]^-$, found = 293.0778

3'-O-propargyl-5'-deoxy-5'-methoxycarbonyl-5'-oxo-thymidine (171)



Carboxylic acid **170** was first dissolved in a minimum amount of MeOH and passed through Diaion WT01S(H) resin and the solvent was removed *in vacuo*. **170** (140 mg, 0.48 mmol) was dissolved in anhydrous THF-MeOH, 4:1 (4.8 mL) under argon. TMS-diazomethane (2M in hexanes, 0.26 mL, 0.53 mmol) was added dropwise and the bright yellow solution was stirred at rt for 1 h. After this time TLC analysis (DCM-MeOH, 9:1) showed complete consumption of starting material (R_f = 0.1) and the formation of a new product (R_f = 0.9). The reaction was quenched with a few drops of AcOH and stirred at rt for 1 h. After this time the solvent was removed *in vacuo* to give a yellow residue which was immediately purified by flash column chromatography (DCM-MeOH, 19:1) to give **171** as a white foam (129 mg, 88%).

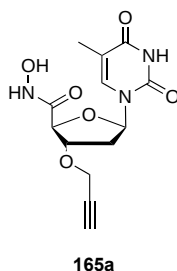
IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3252 ($\text{C}\equiv\text{C}-\text{H}$), 3034 (N-H), 2923 (C-H), 2851 (C-H), 1748 (C=O), 1682 (C=O), 1469 (CH_3), 1435, 1405, 1378, 1360, 1332 (C-H), 1298, 1277 (C-N), 1214 (C-N), 1131 (CH_3), 1102, 1081 (N-C-H), 1040 (C-C $\equiv$ C), 933, 870, 795 (C-C), 753 (C- CH_3), 740, 666.

^1H NMR (600 MHz, CDCl_3): δ = 1.97 (d, $J_{\text{CH}_3,6}$ = 1.2 Hz, 3H, CH_3^T), 1.98-2.01 (m, 1H, H-2'a), 2.50 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 1H, $\text{HC}\equiv\text{C}$), 2.55 (app. dd, J = 5.3, 13.9 Hz, 1H, H-2'b), 3.83 (s, 3H, OCH_3), 4.26 (d, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.4 Hz, 2H, $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 4.46 (app. d, J = 5.0 Hz, 1H, H-3'), 4.66 (br s, 1H, H-4'), 6.46 (dd, $J_{1',2'b}$ = 5.3 Hz, $J_{1',2'a}$ = 9.2 Hz, 1H, H-1'), 8.03, (q, $J_{\text{CH}_3,6}$ = 1.2 Hz, 1H, H-6), 8.34 (br s, 1H, NH-3) ppm.

^{13}C NMR (150 MHz, CDCl_3): δ = 12.8 (CH_3^{T}), 36.6 (C-2'), 52.9 (OCH_3), 56.9 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 75.7 ($\text{HC}\equiv\text{C}$), 78.5 ($\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 81.1 (C-3'), 81.9 (C-4'), 86.8 (C-1'), 111.5 (C-5), 136.2 (C-6), 150.4 (C-2), 163.6 (C-4), 171.5 (C-5') ppm.

HRMS (ESI^+): m/z calc. for $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_6\text{Na}$ = 331.0901 $[\text{M}+\text{Na}]^+$, found = 331.0903

3'-O-propargyl-5'-deoxy-5'-methoxycarbonyl-5'-oxo-thymidine (165a)



Hydroxylamine hydrochloride (207 mg, 2.98 mmol) was suspended in MeOH (3 mL) and warmed to dissolve. KOH (209 mg, 3.73 mmol) was added, the reaction mixture was filtered and the filtrate was added to methyl ester **171** (44 mg, 0.14 mmol). The reaction mixture was stirred at rt for 1.5 h. After this time TLC analysis (DCM-MeOH, 9:1) showed complete consumption of the starting material (R_f = 0.9) and the formation of a new product (R_f = 0.6). The reaction mixture was neutralised by addition of 1 M aq. HCl and the solvent was removed *in vacuo*. The product was purified *via* flash column chromatography (DCM-MeOH, 19:1 $\rightarrow$ 9:1) to afford **165a** as a white solid (28 mg, 63%); mp (DCM-MeOH) 170 °C (decomp.).

IR (ATR, neat) $\nu_{\text{max}}/\text{cm}^{-1}$: 3384 (N-H), 3237 (O-H), 3090 (N-H), 3056, 3000, 2959 (C-H), 2933 (C-H), 2851 (C-H), 2112, 1698 (C=O), 1661 (C=O), 1647 (C=O), 1572 (N-H, C-N), 1468 (CH_3), 1448, 1435, 1406 (CH_3), 1388, 1374 (CH_3), 1354 (C-N), 1312, 1290, 1269 (C-N), 1256, 1236, 1199, 1173 (CH_3), 1136, 1107 (C-O), 1074, 1050 (N-C-H), 1037, 1022 (C-N-H), 990 (C-N-C), 974, 943 (C-C-H), 925, 888, 807, 787, 766 (C- CH_3), 754, 725, 704, 659 (N-H).

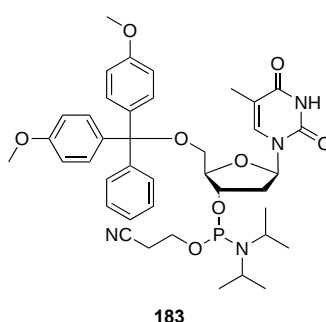
^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ = 1.78 (s, 3H, CH_3^{T}), 2.23-2.27 (m, 1H, H-2'a), 2.36 (app. dd, J = 5.6, 13.7 Hz, 1H, H-2'b), 3.51 (t, $J_{\text{HC}\equiv\text{C}-\text{CH}_2}$ = 2.2 Hz, 1H, $\text{HC}\equiv\text{C}$), 4.27-4.28 (m, 3H, H-3', $\text{H}_2\text{C}-\text{C}\equiv\text{CH}$), 6.24 (dd, $J_{1',2'b}$ = 5.6 Hz, $J_{1',2'a}$ = 8.9 Hz, 1H, H-1'), 8.12 (s, 1H, H-6), 9.22 (br s, 1H, OH), 11.07 (s, 1H, $\text{HN}-\text{OH}$), 11.34 (s, 1H, NH-3) ppm.

^{13}C NMR (150 MHz, $\text{DMSO-}d_6$): δ = 12.4 (CH_3^{T}), 35.6 (C-2'), 55.9 ($\text{H}_2\text{C-C}\equiv\text{CH}$), 77.7 ($\text{HC}\equiv\text{C}$), 79.7 ($\text{H}_2\text{C-C}\equiv\text{CH}$), 80.4 (C-3'), 80.5 (C-4'), 84.8 (C-1'), 109.7 (C-5), 136.4 (C-6), 150.6 (C-2), 163.6 (C-4), 166.1 (C-5') ppm.

HRMS (ESI): m/z calc. for $\text{C}_{13}\text{H}_{14}\text{N}_3\text{O}_6$ = 308.0888 $[\text{M-H}]^-$, found = 308.0881

7.2.3. Synthetic Methods for Chapter 5

5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine-3'-O-[O-(2-cyanoethyl)-N,N'-diisopropylphosphoramidite (183)



Alcohol **166** (100 mg, 0.18 mmol) was coevaporated with anhydrous DCM (3 x 2 mL), dried under high vacuum and dissolved in anhydrous THF (2 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (40 μL , 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.60 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (90 μL , 0.28 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (40 μL , 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.60 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (90 μL , 0.28 mmol) were added $t = 1$ h and $t = 1.5$ h. The reaction mixture was stirred at rt for another 16 h. After this time, TLC analysis (hexane-EtOAc, 1:4) showed the complete consumption of starting material ($R_f = 0.3$) and the formation of a new product ($R_f = 0.8$). The reaction mixture was diluted with DCM (10 mL), washed with sat. aq. NaHCO_3 (10 mL) and brine (10 mL). The combined organic layers were dried over Na_2SO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (hexane-EtOAc-Et₃N, 1:1:0.002) to give the product **183** as a white foam (89 mg, 65%).

^1H NMR (400 MHz, acetone- d_6): δ = 1.10 (d, $J_{\text{CH}_3,\text{CH}} = 7.1$ Hz, 2H, CH_3^{iPr}), 1.18-1.20 (m, 10 H, CH_3^{iPr}), 1.47-1.49 (m, 3H, CH_3^{T}), 2.40-2.54 (m, 2H, H-2'), 2.63 (t,

$J_{CH_2,CH_2} = 6.1$ Hz, 1H, OCH_2CH_2CN), 2.77 (t, $J_{CH_2,CH_2} = 6.0$ Hz, 1H, OCH_2CH_2CN), 3.36 (m, 2H, H-5'), 3.60-3.68 (m, 2H, 2 x CH^{iPr}), 3.70-3.78 (m, 1H, OCH_2CH_2CN), 3.79 (s, 2.4H, OCH_3^{DMTr}), 3.80 (s, 3.6H, OCH_3^{DMTr}), 3.81-3.93 (m, 1H, OCH_2CH_2CN), 4.14 (app. dd, $J = 3.0, 6.4$ Hz, 0.4H, H-4'), 4.20 (app. dd, $J = 2.9, 5.8$ Hz, 0.6H, H-4'), 4.71-4.78 (m, 1H, H-3'), 6.36 (m, 1H, H-1'), 6.90-6.93 (m, 4H, 4 x CH^{DMTr}), 7.23-7.40 (m, 7H, 7 x CH^{DMTr}), 7.49-7.52 (m, 2H, 2 x CH^{DMTr}), 7.60 (q, $J_{CH_3,6} = 1.2$ Hz, 0.4H, H-6), 7.63 (q, $J_{CH_3,6} = 1.2$ Hz, 0.6H, H-6), 9.96 (NH-3) ppm.

^{13}C NMR (100 MHz, acetone- d_6): $\delta = 12.2$ (CH_3^T), 20.7 (app. t, $J = 6.7$ Hz, OCH_2CH_2CN), 24.8-24.9 (m, CH_3^{iPr}), 40.1 (app. t, $J = 5.3$ Hz, C-2'), 43.9 (d, $J = 12.5$ Hz, CH^{iPr}), 55.5 (OCH_3^{DMTr}), 59.4 (d, $J_{C,P} = 4.1$ Hz), 59.6 (d, $J_{C,P} = 4.4$ Hz), 64.3 (C-5'), 64.3 (C-5'), 74.4 (d, $J_{C,P} = 16.7$ Hz, C-3'), 74.7 (d, $J_{C,P} = 17.2$ Hz, C-3'), 85.2 (C-1'), 85.2 (C-1'), 85.8 (d, $J_{C,P} = 5.9$ Hz, C-4'), 86.2 (d, $J_{C,P} = 3.4$ Hz, C-4'), 87.5 (qC^{DMTr}), 87.5 (qC^{DMTr}), 111.0 (C-6), 111.1 (C-6), 114.0 (CH^{DMTr}), 118.8 (CN), 119.0 (CN), 127.8 (CH^{DMTr}), 127.8 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.1 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.0 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.3 (C-6), 136.4 (qC^{DMTr}), 136.5 (qC^{DMTr}), 136.6 (qC^{DMTr}), 145.8 (qC^{DMTr}), 151.2 (C-2), 159.8 (qC^{DMTr}), 164.3 (C-4) ppm.

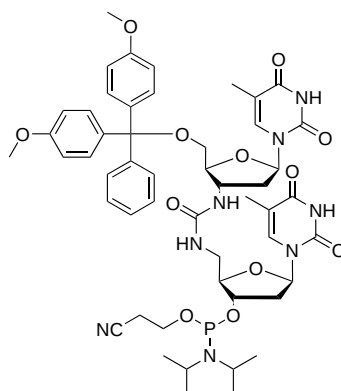
^{31}P NMR (162 MHz, acetone- d_6): $\delta = 148.1$ and 148.2 ppm

Note: NMR spectra are complicated due to the presence of diastereoisomers.

HRMS (ESI⁺): m/z calc. for $C_{40}H_{50}N_4O_8P = 745.3361$ $[M+H]^+$, found = 745.3368

Spectroscopic data are in good agreement with that reported in the literature.⁴⁶⁵

***N*-[3'-*O*-((2-Cyanoethoxy)(diisopropylamino)phosphino)-5'-deoxythymidin-5'-yl]-*N'*-[5'-*O*-(4,4'-dimethoxytrityl)]deoxythymidin-3'-yl-urea (**184**)**



184

Alcohol **173** (300 mg, 0.37 mmol) was coevaporated with anhydrous DCM (3 x 3 mL), dried under high vacuum and dissolved in anhydrous THF (4 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (80 µL, 0.57 mmol) and tetrazole (3-4 % in MeCN, 1.21 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (180 µL, 0.57 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (80 µL, 0.57 mmol), tetrazole (3-4 % in MeCN, 1.21 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (180 µL, 0.57 mmol) were added at $t = 1.5$ and $t = 2.5$ h. The reaction mixture was stirred at rt for another 17.5 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the consumption of starting material ($R_f = 0.4$) and the formation of a new product ($R_f = 0.5$). The reaction mixture was diluted with DCM (20 mL), washed with sat. aq. NaHCO_3 (20 mL) and brine (20 mL). The combined aq. layers were extracted with EtOAc (2 x 40 mL) and the combined organics were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (toluene-acetone- Et_3N , 7:3:0.01 $\rightarrow$ 3:7:0.01) to give **184** as a white solid (71 mg, 19%). **173** (103 mg, 34%) was also recovered from the reaction.

^1H NMR (600 MHz, acetone- d_6): $\delta = 1.20$ (d, $J_{\text{CH}_3,\text{CH}} = 6.5$ Hz, 12H, 4 x CH_3^{iPr}), 1.46 (d, $J_{\text{CH}_3,6} = 1.2$ Hz, 3H, $^5\text{TCH}_3^{\text{T}}$), 1.83 (d, $J_{\text{CH}_3,6} = 1.2$ Hz, 3H, $^3\text{TCH}_3^{\text{T}}$), 2.29-2.33 (m, 1H, $^5\text{T}\text{H}-2'\text{a}$), 2.34-2.37 (m, 2H, $^3\text{T}\text{H}-2'\text{a} + ^3\text{T}\text{H}-2'\text{b}$), 2.42-2.50 (m, 1H, $^5\text{T}\text{H}-2'\text{b}$), 2.77 (t, $J_{\text{CH}_2,\text{CH}_2} = 6.5$ Hz, 2H, $\text{OCH}_2\text{CH}_2\text{CN}$), 3.38-3.48 (m, 3H, $^3\text{T}\text{H}-5'\text{a} + ^3\text{T}\text{H}-5'\text{b} + ^5\text{T}\text{H}-5'\text{a}$),

3.50-3.55 (m, 1H, $^5\text{T}\text{H-5'b}$), 3.63-3.70 (m, 2H, 2 x $\text{CH}^{i\text{Pr}}$), 3.79 (s, 6H, 2 x OCH_3), 3.80-3.83 (m, 1H, $\text{OCH}_2\text{CH}_2\text{CN}$), 3.86-3.92 (m, 1H, $\text{OCH}_2\text{CH}_2\text{CN}$), 4.03-4.05 (m, 1H, $^5\text{T}\text{H-4'}$), 4.07-4.10 (m, 1H, $^3\text{T}\text{H-4'}$), 4.56-4.59 (m, 1H, $^3\text{T}\text{H-3'}$), 4.62-4.67 (m, 1H, $^5\text{T}\text{C-3'}$), 5.78-5.82 (m, 1H, $^3\text{T}\text{NH-5'}$), 6.02-6.06 (m, 1H, $^5\text{T}\text{NH-3'}$), 6.25-6.28 (m, 2H, $^3\text{T}\text{H-1'}$ + $^5\text{T}\text{H-1'}$), 6.89-6.90 (m, 4H, 4 x CH^{DMTr}), 7.22-7.40 (m, 7H, 7 x CH^{DMTr}), 7.51-7.53 (m, 2H, 2 x CH^{DMTr}), 7.56 (q, $J_{\text{CH}_3,6} = 1.2$ Hz, 0.2 H, $^3\text{T}\text{H-6}$), 7.58 (q, $J_{\text{CH}_3,6} = 1.2$ Hz, 0.8 H, $^3\text{T}\text{H-6}$), 7.64-7.65 (m, 1H, $^5\text{T}\text{H-6}$), 9.85 (br s, 2H, $^3\text{T}\text{NH-3}$ + $^5\text{T}\text{NH-3}$) ppm.

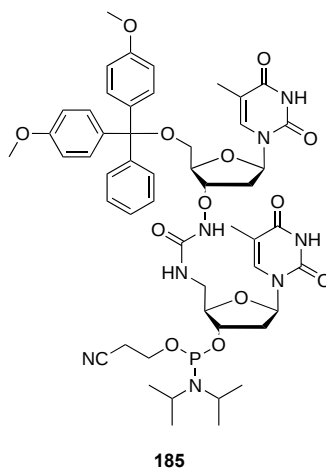
^{13}C NMR (150 MHz, acetone- d_6): $\delta = 12.2$ ($^5\text{T}\text{CH}_3^{\text{T}}$), 12.4 ($^3\text{T}\text{CH}_3^{\text{T}}$), 20.7 (d, $J_{\text{C,P}} = 7.2$ Hz, $\text{OCH}_2\text{CH}_2\text{CN}$), 24.8 (d, $J_{\text{C,P}} = 7.5$ Hz, $\text{CH}_3^{i\text{Pr}}$), 24.9 (d, $J_{\text{C,P}} = 7.5$ Hz, $\text{CH}_3^{i\text{Pr}}$), 39.0 (d, $J_{\text{C,P}} = 4.9$ Hz, $^3\text{T}\text{C-2'}$), 39.3 ($^5\text{T}\text{C-2'}$), 42.7 ($^3\text{T}\text{C-5'}$), 44.0 (d, $J_{\text{C,P}} = 12.5$ Hz, $\text{CH}^{i\text{Pr}}$), 51.6 ($^5\text{T}\text{C-3'}$), 55.6 ($\text{OCH}_3^{\text{DMTr}}$), 64.4 ($^5\text{T}\text{C-5'}$), 75.1 (d, $J_{\text{C,P}} = 17.5$ Hz, $^3\text{T}\text{C-3'}$), 84.9 ($^5\text{T}\text{C-1'}$), 85.1 ($^5\text{T}\text{C-4'}$), 85.9 ($^3\text{T}\text{C-1'}$), 86.2 ($^3\text{T}\text{C-4'}$), 87.3 (qC^{DMTr}), 110.9 ($^5\text{T}\text{C-5}$), 111.1 ($^3\text{T}\text{C-5}$), 114.0 (CH^{DMTr}), 119.3 (CN), 127.7 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.4 ($^5\text{T}\text{C-6}$), 136.6 (qC^{DMTr}), 136.8 (qC^{DMTr}), 137.1 ($^3\text{T}\text{C-6}$), 146.0 (qC^{DMTr}), 151.3 ($^5\text{T}\text{C-2}$), 151.3 ($^3\text{T}\text{C-2}$), 158.6 (NH-CO-NH), 159.7 (qC^{DMTr}), 159.7 (qC^{DMTr}), 164.2 ($^3\text{T}\text{C-4}$ + $^5\text{T}\text{C-4}$) ppm.

^{31}P NMR (162 MHz, acetone- d_6): $\delta = 147.9$ and 148.6 ppm.

Note: NMR spectra are complicated due to the presence of diastereoisomers.

HRMS (ESI $^+$): m/z calc. for $\text{C}_{51}\text{H}_{63}\text{N}_8\text{O}_{12}\text{PNa} = 1033.4195$ [$\text{M}+\text{Na}$] $^+$, found = 1033.4177

***N*-[3'-*O*-((2-Cyanoethoxy)(diisopropylamino)phosphino)-5'-deoxythymidin-5'-yl]-*N'*-[5'-*O*-(4,4'-dimethoxytrityl)]thymidin-3'-*O*-yl-urea (**185**)**



Alcohol **175** (130 mg, 0.16 mmol) was coevaporated with anhydrous DCM (3 x 3 mL), dried under high vacuum and dissolved in anhydrous THF (1.7 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.52 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.52 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) were added at *t* = 1 h and *t* = 2 h. The reaction mixture was stirred at rt for another 17 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the consumption of starting material (*R*_f = 0.5) and the formation of a new product (*R*_f = 0.7). The solvent was removed *in vacuo* and solids were redissolved in DCM (20 mL), washed with sat. aq. NaHCO₃ (2 x 20 mL) and brine (20 mL). The combined organics were dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH-Et₃N, 98:2:0.1 → 9:1:0.01) to give **185** as a white film (4 mg, 2%). **175** (121 mg, 93%) was also recovered from the reaction.

¹H NMR (600 MHz, acetone-*d*₆): δ = 1.18-1.21 (m, 12 H, 4 x CH₃^{iPr}), 1.37 (d, *J*_{CH3-6} = 1.0 Hz, 3H, ⁵TCH₃^T), 1.82 (d, *J*_{CH3-6} = 1.1 Hz, 3H, ³TCH₃^T), 2.32-2.37 (m, 2H, ³TH-2'), 2.39-2.46 (m, 1H, ⁵TH-2'a), 2.64-2.67 (m, 1H, ⁵TH-2'b), 2.76-2.80 (m, 2H, OCH₂CH₂CN), 3.39-3.46 (m, 2H, ⁵TH-5'), 3.48-3.57 (m, 2H, ³TH-5'), 3.64-3.70 (m, 2H, 2 x CH^{iPr}), 3.79 (s, 6H, 2 x OCH₃^{DMTr}), 3.81-3.83 (m, 1H, OCH₂CH₂CN), 3.87-3.92 (m,

^1H , $\text{OCH}_2\text{CH}_2\text{CN}$), 4.12-4.13 (m, 0.3 H, $^3\text{T}\text{H-4'}$), 4.18-4.21 (m, 0.7 H, $^3\text{T}\text{H-4'}$), 4.37-4.38 (m, 1H, $^5\text{T}\text{H-3'}$), 4.59-4.61 (m, $^3\text{T}\text{H-3'}$), 4.74-4.76 (m, 1H, $^5\text{T}\text{H-4'}$), 6.17-6.20 (m, 1H, $^3\text{T}\text{H-1'}$), 6.38-6.41 (m, 1H, $^5\text{T}\text{H-1'}$), 6.90-6.91 (m, 4H, 4 x CH^{DMTr}), 7.25 (app. t, $J=7.3$ Hz, 1H, $^3\text{T}\text{NH-5'}$), 7.32-7.35 (m, 7H, 7 x CH^{DMTr}), 7.46-7.48 (m, 2H, 2 x CH^{DMTr}), 7.55-7.59 (m, 1H, $^3\text{T}\text{H-6}$), 7.64 (app. d, $J=1.1$ Hz, $^5\text{T}\text{H-6}$), 8.67 (app. t, $J=10.5$ Hz, O-NH), 9.93 (br s, 2H, $^3\text{T}\text{NH-3} + ^5\text{T}\text{NH-3}$) ppm. *Note: Signal for $\text{OCH}_2\text{CH}_2\text{CN}$ is obscured by H_2O peak.*

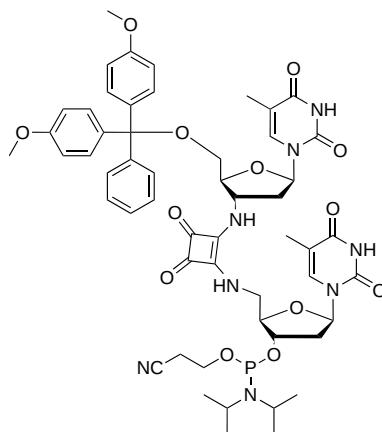
^{13}C NMR (150 MHz, acetone- d_6): $\delta = 12.0$ ($^5\text{T}\text{CH}_3^{\text{T}}$), 12.4 ($^3\text{T}\text{CH}_3^{\text{T}}$), 20.7 (d, $J_{\text{C,P}} = 7.8$ Hz, $\text{OCH}_2\text{CH}_2\text{CN}$), 24.8 (d, $J_{\text{C,P}} = 14.7$ Hz, CH_3^{iPr}), 24.9 (d, $J_{\text{C,P}} = 14.0$ Hz, CH_3^{iPr}), 37.3 ($^5\text{T}\text{C-2'}$), 38.7 (d, $J_{\text{C,P}} = 2.7$ Hz, $^3\text{T}\text{C-2'}$), 38.8 (d, $J_{\text{C,P}} = 2.3$ Hz, $^3\text{T}\text{C-2'}$), 39.0 (d, $J_{\text{C,P}} = 4.8$ Hz, $^3\text{T}\text{C-2'}$), 42.2 ($^3\text{T}\text{C-5'}$), 44.0 (d, $J_{\text{C,P}} = 12.3$ Hz, CH^{iPr}), 55.6 ($\text{OCH}_3^{\text{DMTr}}$), 59.5 (d, $J_{\text{C,P}} = 18.4$ Hz, $\text{OCH}_2\text{CH}_2\text{CN}$), 65.4 ($^5\text{T}\text{C-5'}$), 75.2 (d, $J_{\text{C,P}} = 17.7$ Hz, $^3\text{T}\text{C-3'}$), 75.5 (d, $J_{\text{C,P}} = 16.1$ Hz, $^3\text{T}\text{C-3'}$), 83.2 ($^5\text{T}\text{C-3'}$), 85.3 ($^5\text{T}\text{C-1'}$), 85.8 (d, $J_{\text{C,P}} = 6.0$ Hz, $^3\text{T}\text{C-4'}$), 86.0 (d, $J_{\text{C,P}} = 3.4$ Hz, $^3\text{T}\text{C-4'}$), 86.7 ($^5\text{T}\text{C-4'}$), 86.8 ($^3\text{T}\text{C-1'}$), 87.7 (qC^{DMTr}), 110.1 ($^3\text{T}\text{C-5}$), 111.3 ($^5\text{T}\text{C-5}$), 114.1 (CH^{DMTr}), 119.3 (CN), 127.9 (CH^{DMTr}), 128.8 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.0 (CH^{DMTr}), 136.3 ($^5\text{T}\text{C-6}$), 136.3 (qC^{DMTr}), 136.5 (qC^{DMTr}), 137.3 ($^3\text{T}\text{C-6}$), 145.7 (qC^{DMTr}), 151.2 ($^3\text{T}\text{C-2}$), 151.3 ($^5\text{T}\text{C-2}$), 159.8 (qC^{DMTr}), 159.8 (qC^{DMTr}), 160.9 (CO-NH-O), 164.1 ($^5\text{T}\text{C-4}$), 164.3 ($^3\text{T}\text{C-4}$) ppm.

^{31}P NMR (162 MHz, acetone- d_6): $\delta = 147.8$ and 148.5 ppm.

Note: NMR spectra are complicated due to the presence of diastereoisomers.

HRMS (ESI $^+$): m/z calc. for $\text{C}_{51}\text{H}_{63}\text{N}_8\text{O}_{13}\text{PNa} = 1049.4144$ $[\text{M}+\text{Na}]^+$, found = 1049.4124

***N*-(3'-*O*-((2-Cyanoethoxy)(diisopropylamino)phosphino-5'-deoxythymidin-5'-yl)-*N'*-[5'-*O*-(4,4'-dimethoxytrityl)]deoxythymidin-3'-yl-3,4-dioxocyclobuten-1,2-diamine (**187**)²⁸⁸**



187

Alcohol **177** (100 mg, 0.12 mmol) was coevaporated with anhydrous DCM (3 x 20 mL), dried under high vacuum and dissolved in anhydrous THF (1.3 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.38 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (60 µL, 0.19 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.38 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (60 µL, 0.19 mmol) were added at *t* = 1.5 and *t* = 3.5 h. The reaction mixture was stirred at rt for another 15.5 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the consumption of starting material (*R*_f = 0.4) and the formation of a new product (*R*_f = 0.6). The solvent was removed *in vacuo* and solids were redissolved in DCM (10 mL) and washed with sat. aq. NaHCO₃ (3 x 10 mL) and brine (10 mL). The combined organics were dried over Na₂SO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH-Et₃N, 98:2:0.1 → 19:1:0.02) to give **187** as a white solid (22 mg, 18%). **177** (20 mg, 20%) was also recovered from the reaction.

¹H NMR (600 MHz, acetone-*d*₆): δ = 1.19-1.21 (m, 12H, 4 x CH₃^{iPr}), 1.54 (s, 3H, ⁵TCH₃^T), 1.83 (s, 1H, 3H, ³TCH₃^T), 2.37-2.41 (m, 1H, ³T-H-2'a), 2.44-2.53 (m, 1H, ³T-H-2'b), 2.55-2.58 (m, 1H, ⁵T-H-2'a), 2.64-2.68 (m, 1H, ⁵T-H-2'b), 2.78-2.80 (m, 2H,

OCH₂CH₂CN), 3.38-3.40 (m, 1H, ⁵T_H-5'a), 3.50-3.52 (m, 1H, ⁵T_H-5'b), 3.63-3.71 (m, 2H, 2 x CH^{iPr}), 3.78 (s, 6H, 2 x OCH₃^{DMTr}), 3.80-3.86 (m, 1H, OCH₂CH₂CN), 3.89-3.96 (m, 2H, OCH₂CH₂CN + ³T_H-5'a), 4.02-4.07 (m, 1H, ³T_H-5'b), 4.10-4.12 (m, 1.4H, ³T_H-4' + ⁵T_H-4'), 4.14-4.16 (m, 0.6H, ³T_H-4' + ⁵T_H-4'), 4.62 (br s, 1H, ³T_H-3'), 5.10 (br s, 1H, ⁵T_H-3'), 6.25 (dd, $J_{1',2'a} = 6.6$ Hz, $J_{1',2'b} = 12.7$ Hz, 1H, ⁵T_H-1'), 6.30 (app. dt, $J = 2.6, 6.4$ Hz, 1H, ³T_H-1'), 6.87-6.88 (m, 4H, 4 x CH^{DMTr}), 7.00 (br s, 1H, NH^{Sq}), 7.12 (br s, 1H, NH^{Sq}), 7.21-7.37 (m, 7H, 7 x CH^{DMTr}), 7.28-7.50 (m, 3H, 2 x CH^{DMTr} + ³T_H-6), 7.62-7.63 (m, 1H, ⁵T_H-6), 9.97 (br s, 2H, ³T_{NH}-3 + ⁵T_{NH}-3) ppm. *Note: Signal for OCH₂CH₂CN is obscured by H₂O peak.*

¹³C NMR (150 MHz, acetone-*d*₆): $\delta = 12.3$ (⁵TCH₃^T), 12.4 (³TCH₃^T), 20.7 (d, $J_{C,P} = 6.7$ Hz, OCH₂CH₂CN), 24.8 (d, $J_{C,P} = 12.0$ Hz, CH₃^{iPr}), 24.9 (d, $J_{C,P} = 12.2$ Hz, CH₃^{iPr}), 38.7 (d, $J_{C,P} = 8.2$ Hz, ³T_C-2'), 38.7 (d, $J_{C,P} = 8.3$ Hz, ³T_C-2'), 39.8 Hz (⁵T_C-2'), 43.9 (d, $J_{C,P} = 11.9$ Hz, CH^{iPr}), 44.0 (d, $J_{C,P} = 12.3$ Hz, CH^{iPr}), 46.4 (³T_C-5'), 55.2 (⁵T_C-3'), 55.5 (OCH₃^{DMTr}), 59.4 (d, $J_{C,P} = 19.4$ Hz, OCH₂CH₂CN), 59.5 (d, $J_{C,P} = 18.1$ Hz, OCH₂CH₂CN), 64.1 (⁵T_C-5'), 74.3 (d, $J_{C,P} = 18.3$ Hz, ³T_C-3'), 74.8 (d, $J_{C,P} = 13.4$ Hz, ³T_C-3'), 84.7 (³T_C-1'), 84.9 (³T_C-1'), 85.4 (⁵T_C-4'), 85.6 (³T_C-4'), 86.7 (⁵T_C-1'), 87.5 (qC^{DMTr}), 111.2 (⁵T_C-5), 111.4 (³T_C-5), 111.5 (³T_C-5), 114.0 (qC^{DMTr}), 127.7 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.0 (CH^{DMTr}), 131.1 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.5 (⁵T_C-6), 136.6 (qC^{DMTr}), 137.7 (³T_C-6), 145.9 (qC^{DMTr}), 151.3 (⁵T_C-2), 151.4 (³T_C-2), 159.7 (qC^{DMTr}), 164.1 (⁵T_C-4), 164.2 (³T_C-4), 183.8 (qC^{Sq}), 183.9 (qC^{Sq}), 184.5 (CO^{Sq}), 184.6 (CO^{Sq}) ppm.

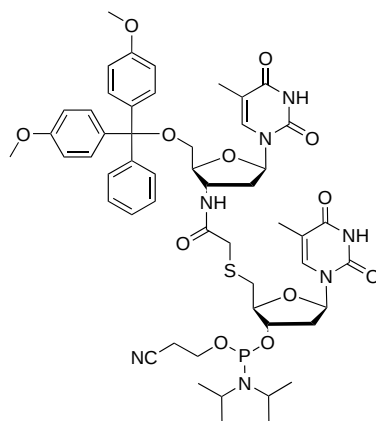
³¹P NMR (162 MHz, acetone-*d*₆): $\delta = 148.1$ and 148.8 ppm

Note: NMR spectra are complicated due to the presence of diastereoisomers.

HRMS (ESI⁺): m/z calc. for C₅₄H₆₃N₈O₁₃PNa = 1085.4144 [M+Na]⁺, found = 1085.4145

Spectroscopic data are in good agreement with that reported in the literature.²⁸⁸

***N*-5'-*O*-(4,4'-Dimethoxytrityl)deoxythymidin-3'-yl-[3'-*O*-((2-cyanoethoxy)(diisopropylamino)phosphino)]-5'-deoxythymidin-yl-mercaptoacetamide (**188**)⁴²⁸**



188

Alcohol **179** (100 mg, 0.12 mmol) was coevaporated with anhydrous DCM (3 x 2 mL), dried under high vacuum and dissolved in anhydrous THF (1.3 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.39 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (60 µL, 0.19 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (30 µL, 0.21 mmol) and tetrazole (3-4 % in MeCN, 0.39 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (60 µL, 0.19 mmol) were added at $t = 1.5$ h and $t = 2.5$ h. The reaction mixture was stirred at rt for another 17.5 h. After this time, TLC analysis (DCM-MeOH, 19:1) showed the complete consumption of starting material ($R_f = 0.3$) and the formation of a new product ($R_f = 0.5$). The solvent was removed *in vacuo* and solids were redissolved in DCM (10 mL), washed with sat. aq. NaHCO_3 (10 mL) and brine (10 mL). The combined organics were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH- Et_3N , 98:2:0.1 $\rightarrow$ 9:1:0.01) to give **188** as a white film (33 mg, 27%).

^1H NMR (600 MHz, acetone- d_6): $\delta = 1.19$ -1.21 (m, 12H, 4 x $\text{CH}_3^{i\text{Pr}}$), 1.47 (d, $J_{\text{CH}_3,6} = 1.2$ Hz, 3H, $^5\text{TCH}_3^{\text{T}}$), 1.82 (d, $J_{\text{CH}_3,6} = 1.2$ Hz, 3H, $^3\text{TCH}_3^{\text{T}}$), 2.35-2.46 (m, 3H, $^3\text{T}\text{H}-2' + ^5\text{T}\text{H}-2'\text{a}$), 2.49-2.54 (m, 1H, $^5\text{T}\text{H}-2'\text{b}$), 2.77-2.78 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CN}$), 2.95-3.04 (m, 2H, $^3\text{T}\text{H}-5'$), 3.22 (d, $J_{a,b} = 14.6$ Hz, 0.4 H, SCH_2a), 3.24 (d, $J_{a,b} = 14.6$ Hz,

0.6 H, SCH₂a), 3.31 (d, $J_{a,b} = 14.6$ Hz, 0.4 H, SCH₂b), 3.32 (d, $J_{a,b} = 14.6$ Hz, 0.6 H, SCH₂b), 3.39-3.41 (m, 1H, $^5\text{T}^{\text{H}}\text{-5'a}$), 3.45-3.48 (m, 1H, $^5\text{T}^{\text{H}}\text{-5'b}$), 3.64-3.71 (m, 2H, 2 x CH^{iPr}), 3.79 (s, 6H, 2 x OCH₃^{DMTr}), 3.81-3.85 (m, 1H, OCH₂CH₂CN), 3.88-3.93 (m, 1H, OCH₂CH₂CN), 4.06-4.09 (m, 1H, $^5\text{T}^{\text{H}}\text{-4'}$), 4.15-4.17 (m, 0.5H, $^3\text{T}^{\text{H}}\text{-4'}$), 4.56-4.60 (m, 1H, $^3\text{T}^{\text{H}}\text{-3'}$), 4.75-4.79 (m, 1H, $^5\text{T}^{\text{H}}\text{-3'}$), 6.27-6.32 (m, 2H, $^3\text{T}^{\text{H}}\text{-1'}$ + $^5\text{T}^{\text{H}}\text{-1'}$), 6.89-6.91 (m, 4H, 4 x CH^{DMTr}), 7.23-7.39 (m, 7H, 7 x CH^{DMTr}), 7.50-7.52 (m, 2H, 2 x CH^{DMTr}), 7.54-7.55 (m, 1H, $^3\text{T}^{\text{CH}}\text{CH}_3^{\text{T}}$), 7.63 (app. t, $J = 1.2$ Hz, 1H, $^5\text{T}^{\text{CH}}\text{CH}_3^{\text{T}}$), 7.76 (app. t, 1H, $^5\text{T}^{\text{NH}}\text{-3'}$), 9.94 (br s, 2H, $^3\text{T}^{\text{NH}}\text{-3}$ + $^5\text{T}^{\text{NH}}\text{-3}$) ppm.

¹³C NMR (150 MHz, acetone-*d*₆): $\delta = 12.2$ ($^5\text{T}^{\text{CH}}\text{CH}_3^{\text{T}}$), 12.5 ($^3\text{T}^{\text{CH}}\text{CH}_3^{\text{T}}$), 20.8 (d, $J_{C,P} = 7.1$ Hz, OCH₂CH₂CN), 24.8-24.9 (m, 4 x CH₃^{iPr}), 35.1 ($^3\text{T}^{\text{C}}\text{-5'}$), 35.2 ($^3\text{T}^{\text{C}}\text{-5'}$), 36.1 (CH₂S), 38.5 ($^5\text{T}^{\text{C}}\text{-2'}$), 38.9 ($^3\text{T}^{\text{C}}\text{-2'}$), 43.9 (d, $J_{C,P} = 12.4$ Hz, CH^{iPr}), 44.0 (d, $J_{C,P} = 12.4$ Hz, CH^{iPr}), 51.0 ($^5\text{T}^{\text{C}}\text{-4'}$), 51.0 ($^5\text{T}^{\text{C}}\text{-4'}$), 55.6 (OCH₃^{DMTr}), 59.4 (d, $J_{C,P} = 19.4$ Hz, OCH₂CH₂CN), 59.5 (d, $J_{C,P} = 18.2$ Hz, OCH₂CH₂CN), 64.5 ($^5\text{T}^{\text{C}}\text{-5'}$), 76.2 (d, $J_{C,P} = 17.6$ Hz, $^3\text{T}^{\text{C}}\text{-3'}$), 76.5 (d, $J_{C,P} = 16.2$ Hz, $^3\text{T}^{\text{C}}\text{-3'}$), 84.7 ($^5\text{T}^{\text{C}}\text{-4'}$), 84.9 ($^5\text{T}^{\text{C}}\text{-1'}$), 85.6 (d, $J_{C,P} = 9.5$ Hz, $^3\text{T}^{\text{C}}\text{-1'}$), 85.7 (d, $J_{C,P} = 6.4$ Hz, $^3\text{T}^{\text{C}}\text{-4'}$), 85.9 (d, $J_{C,P} = 3.9$ Hz, $^3\text{T}^{\text{C}}\text{-4'}$), 87.4 (qC^{DMTr}), 111.1 ($^5\text{T}^{\text{C}}\text{-5}$), 111.2 ($^3\text{T}^{\text{C}}\text{-5}$), 111.3 ($^3\text{T}^{\text{C}}\text{-5}$), 114.0 (CH^{DMTr}), 119.1 (CN), 119.2 (CN), 127.7 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.4 ($^5\text{T}^{\text{C}}\text{-6}$), 136.6 (qC^{DMTr}), 136.7 ($^3\text{T}^{\text{C}}\text{-6}$), 136.8 ($^3\text{T}^{\text{C}}\text{-6}$), 146.0 (qC^{DMTr}), 151.3 ($^3\text{T}^{\text{C}}\text{-2}$ + $^5\text{T}^{\text{C}}\text{-2}$), 159.6 (qC^{DMTr}), 164.2 ($^3\text{T}^{\text{C}}\text{-4}$ + $^5\text{T}^{\text{C}}\text{-4}$), 169.8 (CO^{amide}), 170.0 (CO^{amide}).

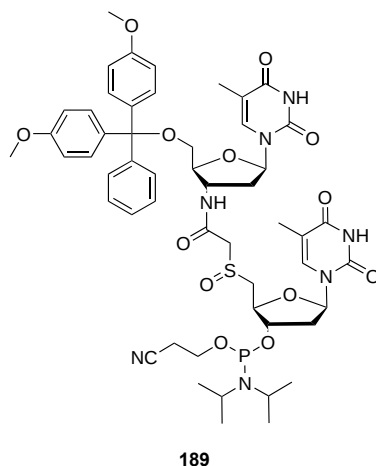
³¹P NMR (162 MHz, acetone-*d*₆): $\delta = 148.2$ and 148.4 ppm.

Note: NMR spectra are complicated due to the presence of diastereoisomers.

HRMS (ESI⁺): m/z calc. for C₅₂H₆₄N₇O₁₂PSNa = 1064.3963 [M+Na]⁺, found = 1064.3977

Spectroscopic data is consistent with that reported in the literature.⁴²⁸

***N*-5'-*O*-(4,4'-Dimethoxytrityl)deoxythymidin-3'-yl-[3'-*O*-((2-cyanoethoxy)(diisopropylamino)phosphino)]-5'-deoxythymidin-yl-sulfinylacetamide (189)**



Alcohol **180** (143 mg, 0.17 mmol) was coevaporated with anhydrous DCM (3 x 3 mL), dried under high vacuum and dissolved in anhydrous THF (1.8 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (40 µL, 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.55 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (40 µL, 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.55 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) were added at t = 1 h and t = 2 h. The reaction mixture was stirred at rt for another 17 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the consumption of starting material (R_f = 0.3) and the formation of a new product (R_f = 0.5). The solvent was removed *in vacuo* and solids were redissolved in DCM (15 mL) and washed with sat. aq. NaHCO₃ (15 mL). The organics were diluted with DCM (60 mL) and washed with brine (60 mL). The combined organics were dried over MgSO₄, filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH-Et₃N, 19:1:0.02) to give **189** as a white film (70 mg, 39%). **180** (58 mg, 41%) was also recovered from the reaction.

¹H NMR (400 MHz, acetone-*d*₆): δ = 1.18-1.26 (m, 12H, 4 x CH₃ⁱPr), 1.45 (d, *J*_{CH₃,6} = 1.0 Hz, 3H, ⁵TCH₃^T), 1.80 (br s, 3H, ³TCH₃^T), 2.35-2.58 (m, 4H, ³T-H-2' + ⁵T-H-2'), 2.77-2.80 (m, 2H, OCH₂CH₂CN), 3.17-3.50 (m, 4.5 H, ³T-H-5' + ⁵T-H-5' +

SOCH₂a), 3.52-3.72 (m, 2.5H, 2 x CH^{iPr} + SOCH₂a), 3.79 (s, 6H, 2 x OCH₃^{DMTr}), 3.81-3.95 (m, 2.65H, OCH₂CH₂CN + SOCH₂b), 4.07-4.13 (m, 1.35H, ⁵T_H-4' + SOCH₂b), 4.37-4.46 (m, 0.6H, ³T_H-4'), 4.48-4.51 (m, 0.2H, ³T_H-4'), 4.54-4.85 (m, 0.2H, ³T_H-4'), 4.64-4.71 (m, 0.5H, ³T_H-3'), 4.75-4.79 (m, 1.5H, ³T_H-3' + ⁵T_H-3'), 6.28-6.36 (m, 2H, ³T_H-1' + ⁵T_H-1'), 6.89-6.91 (m, 4H, 4 x CH^{DMTr}), 7.22-7.39 (m, 7H, 7 x CH^{DMTr}), 7.50-7.52 (m, 2H, 2 x CH^{DMTr}), 7.53-7.59 (m, 1H, ³T_H-6), 7.62-7.64 (m, 1H, ⁵T_H-6), 7.96-7.98 (m, 0.4H, ⁵T_{NH}-3'), 8.06-8.07 (m, 0.6H, ⁵T_{NH}-3'), 9.98 (br s, 2H, ³T_{NH}-3 + ⁵T_{NH}-3) ppm. *Note: Signal for OCH₂CH₂CN is obscured by H₂O peak.*

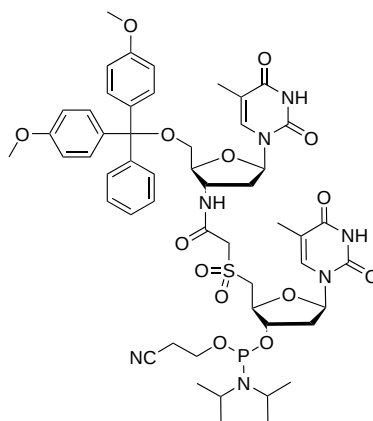
¹³C NMR (100 MHz, acetone-*d*₆): δ = 12.2 (⁵TCH₃^T), 12.4 (³TCH₃^T), 12.5 (³TCH₃^T), 20.7 (d, *J*_{C,P} = 7.2 Hz, OCH₂CH₂CN), 24.9 (app. t, *J* = 6.5 Hz, 4 x CH₃^{iPr}), 38.3 (⁵T_C-2'), 38.6 (³T_C-2'), 44.0 (d, *J*_{C,P} = 12.4 Hz, CH^{iPr}), 44.1 (d, *J*_{C,P} = 12.4 Hz, CH^{iPr}), 51.0 (⁵T_C-3'), 51.4 (⁵T_C-3'), 54.1 (d, *J*_{C,P} = 14.1 Hz), 55.5 (OCH₃^{DMTr}), 56.9 (SOCH₂), 59.0 (SOCH₂), 59.5 (d, *J*_{C,P} = 18.7 Hz, OCH₂CH₂CN), 64.2 (⁵T_C-5'), 64.6 (⁵T_C-5'), 76.8 (d, *J*_{C,P} = 17.1 Hz, ³T_C-3'), 77.1 (d, *J*_{C,P} = 15.3 Hz, ³T_C-3'), 80.1 (d, *J*_{C,P} = 5.5 Hz, ³T_C-4'), 80.3 (d, *J*_{C,P} = 7.6 Hz, ³T_C-4'), 80.6 (d, *J*_{C,P} = 4.1 Hz, ³T_C-4'), 84.6 (⁵T_C-4'), 84.8 (⁵T_C-4'), 85.0 (⁵T_C-1'), 86.1 (d, *J*_{C,P} = 12.0 Hz, ³T_C-1'), 86.5 (d, *J*_{C,P} = 8.3 Hz, ³T_C-1'), 87.4 (qC^{DMTr}), 111.1 (⁵T_C-5), 111.1 (⁵T_C-5), 111.2 (³T_C-5), 111.3 (³T_C-5), 114.0 (CH^{DMTr}), 119.1 (CN), 119.3 (CN), 127.7 (CH^{DMTr}), 128.7 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.3 (⁵T_C-6), 136.5 (⁵T_C-6), 136.7 (qC^{DMTr}), 136.7 (qC^{DMTr}), 137.0 (³T_C-6), 137.2 (³T_C-6), 146.0 (qC^{DMTr}), 151.2 (⁵T_C-2), 151.3 (³T_C-2), 159.7 (qC^{DMTr}), 159.7 (qC^{DMTr}), 159.7 (qC^{DMTr}), 164.2 (³T_C-4 + ⁵T_C-4), 165.0 (CO^{amide}), 165.0 (CO^{amide}), 165.1 (CO^{amide}), 165.2 (CO^{amide}) ppm.

³¹P NMR (162 MHz, acetone-*d*₆): δ = 148.2, 148.2, 148.7 and 148.9 ppm.

Note: NMR spectra are complicated due to the presence of four stereoisomers.

HRMS (ESI⁺): *m/z* calc. for C₅₂H₆₅N₇O₁₃PS = 1058.4093 [M+H]⁺, found 1058.4106

***N*-5'-*O*-(4,4'-Dimethoxytrityl)deoxythymidin-3'-yl-[3'-*O*-((2-cyanoethoxy)(diisopropylamino)phosphino)]-5'-deoxythymidin-yl-sulfonylacetamide (**190**)**



190

Alcohol **181** (151 mg, 0.17 mmol) was coevaporated with anhydrous DCM (3 x 3 mL), dried under high vacuum and dissolved in anhydrous THF (1.8 mL) under argon. Molecular sieves (3 Å) were added followed by the addition of DIPA (40 µL, 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.57 mL). 2-Cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) was added dropwise over 5 min. The reaction mixture was stirred at rt. Further portions of DIPA (40 µL, 0.29 mmol) and tetrazole (3-4 % in MeCN, 0.57 mL) and 2-cyanoethyl *N,N,N',N'*-tetraisopropylphosphorodiamidite **182** (80 µL, 0.25 mmol) were added at $t = 1$ h and $t = 1.5$ h. The reaction mixture was stirred at rt for another 18 h. After this time, TLC analysis (DCM-MeOH, 9:1) showed the complete consumption of starting material ($R_f = 0.5$) and the formation of a new product ($R_f = 0.7$). The solvent was removed *in vacuo* and solids were redissolved in DCM (15 mL) and washed with sat. aq. NaHCO_3 (2 x 15 mL) and brine (15 mL). The combined organics were dried over MgSO_4 , filtered and the solvent was removed *in vacuo*. The crude product was purified *via* flash column chromatography (DCM-MeOH- Et_3N , 98:2:0.1 $\rightarrow$ 19:1:0.02) to give **190** as a white solid (142 mg, 76%).

^1H NMR (600 MHz, acetone- d_6): $\delta = 1.22$ (m, 12H, 4 x CH_3^{iPr}), 1.43-1.44 (m, 3H, $5^{\text{T}}\text{CH}_3^{\text{T}}$), 1.81-1.82 (m, 3H, $3^{\text{T}}\text{CH}_3^{\text{T}}$), 2.37-2.43 (m, 2H, $3^{\text{T}}\text{H}-2^{\text{a}}$ + $5^{\text{T}}\text{H}-2^{\text{a}}$), 2.47-2.57 (m, 2H, $3^{\text{T}}\text{H}-2^{\text{b}}$ + $5^{\text{T}}\text{H}-2^{\text{b}}$), 2.78-2.79 (m, 2H, $\text{OCH}_2\text{CH}_2\text{CN}$), 2.42-3.49 (m, 2.5H, $5^{\text{T}}\text{H}-5^{\text{a}}$ + $3^{\text{T}}\text{H}-5^{\text{a}}$), 3.54-3.60 (m, 0.5H, $3^{\text{T}}\text{H}-5^{\text{a}}$), 3.66-3.73 (m, 2H, 2 x CH^{iPr}), 3.79 (s,

6H, 2 x OCH₃), 3.81-3.87 (m, 1H, OCH₂CH₂CN), 3.90-3.92 (m, 2H, OCH₂CH₂CN + SO₂CH₂a), 4.05-4.12 (m, 2H, ³T_H-5'b + ⁵T_H-4'), 4.21 (d, $J_{a,b}$ = 14.4 Hz, 1H, SO₂CH₂b), 4.49-4.51 (m, 0.5H, ³T_H-4'), 4.54-4.56 (m, 0.5H, ³T_H-4'), 4.70-4.72 (m, 1H, ³T_H-3'), 4.78-4.79 (m, 1H, ⁵T_H-3'), 6.31 (app. dt, J = 1.2, 6.6 Hz, 1H, ⁵T_H-1'), 6.38 (app. q, J = 6.9 Hz, 1H, ³T_H-1'), 6.90-6.92 (m, 4H, 4 x CH^{DMTr}), 7.24-7.39 (m, 7H, 7 x CH^{DMTr}), 7.51-7.52 (m, 2H, 2 x CH^{DMTr}), 7.63 (app. d, J = 1.1 Hz, 1H, ³T_H-6), 7.67 (app. d, J = 1.1 Hz, ⁵T_H-6), 7.96 (br s, 0.2H, ⁵T_{NH}-3'), 8.16 (d, $J_{NH,3'}$ = 7.0 Hz, 0.8H, ⁵T_{NH}-3'), 9.95 (br s, 2H, ³T_{NH}-3 + ⁵T_{NH}-3) ppm. *Note: Signal for OCH₂CH₂CN is obscured by H₂O peak.*

¹³C NMR (150 MHz, acetone-*d*₆): δ = 12.1 (⁵T_{CH₃}^T), 12.5 (³T_{CH₃}^T), 20.7 (d, $J_{C,P}$ = 7.3 Hz, OCH₂CH₂CN), 20.7 (d, $J_{C,P}$ = 7.3 Hz, OCH₂CH₂CN), 24.8 (d, $J_{C,P}$ = 10.6 Hz, CH₃^{iPr}), 24.9 (d, $J_{C,P}$ = 10.1 Hz, CH₃^{iPr}), 38.1 (d, $J_{C,P}$ = 5.3 Hz, ³T_C-3'), 38.4 (⁵T_C-3'), 44.0 (d, $J_{C,P}$ = 12.3 Hz, CH^{iPr}), 44.1 (d, $J_{C,P}$ = 12.3 Hz, CH^{iPr}), 51.4 (⁵T_C-3'), 55.6 (OCH₃^{DMTr}), 56.6 (³T_C-5'), 56.6 (³T_C-5'), 59.5 (d, $J_{C,P}$ = 18.5 Hz, OCH₂CH₂CN), 59.6 (d, $J_{C,P}$ = 19.0 Hz, OCH₂CH₂CN), 60.4 (SO₂CH₂), 60.5 (SO₂CH₂), 64.4 (⁵T_C-5'), 76.8 (d, $J_{C,P}$ = 17.1 Hz, ³T_C-3'), 77.1 (d, $J_{C,P}$ = 16.7 Hz, ³T_C-3'), 81.3 (d, $J_{C,P}$ = 6.4 Hz, ³T_C-4'), 81.4 (d, $J_{C,P}$ = 3.5 Hz, ³T_C-4'), 84.6 (⁵T_C-4'), 84.7 (⁵T_C-4'), 84.9 (⁵T_C-1'), 86.5 (³T_C-1'), 87.5 (qC^{DMTr}), 111.1 (⁵T_C-5), 111.3 (³T_C-5), 111.4 (³T_C-5), 114.0 (CH^{DMTr}), 114.0 (CH^{DMTr}), 119.0 (CN), 119.1 (CN), 127.7 (CH^{DMTr}), 128.8 (CH^{DMTr}), 129.1 (CH^{DMTr}), 131.1 (CH^{DMTr}), 136.3 (⁵T_C-5), 136.5 (qC^{DMTr}), 136.6 (qC^{DMTr}), 137.0 (³T_C-5), 146.0 (qC^{DMTr}), 151.2 (³T_C-2 + ⁵T_C-2), 159.7 (qC^{DMTr}), 159.8 (qC^{DMTr}), 163.2 (⁵T_C-4), 163.3 (³T_C-4), 164.2 (CO^{amide}) ppm.

³¹P NMR (162 MHz, acetone-*d*₆): δ = 148.8 and 149.0 ppm.

HRMS (ESI⁺): m/z calc. for C₅₂H₆₄N₇O₁₄PS = 1096.3862 [M+Na]⁺, found = 1096.3862

Note: NMR spectra are complicated due to the presence of diastereoisomers.

7.3. Synthesis and Characterisation of Modified Oligonucleotides

7.3.1. Sequences of Oligonucleotides

Table 7.1: Sequences of Oligonucleotides in this work. X = Thymidine 5'-phosphate, Y = Cy3, [FamT] = FAM thymidine, [BHQT] = BHQ thymidine, Z = 5'-azido thymidine, R = triazole-linked modification, [TT] = thymidine dinucleoside with modified internucleotide linkage; Extinction coefficients were calculated using the Eurofins Oligo Analysis Tool.⁴⁶⁶

Oligonucleotide	Sequence (5'-3')	Extinction Coefficient (L mol ⁻¹ cm ⁻¹)
63	TTA GCA GTC AGT CAG TCA TCGY	225,800
64	XTA GCA GTC AGT CAG TCA TCGY	225,800
126	A[FamT]A ATT TGA [BHQT]CA TCT AT TAT	N/A
162	ZAG CAG TCA GTC AGT CAT GC	212,150
144b-154b	RTAG CAG TCA GTC AGT CAT GC	212,150
163b-165b	RTAG CAG TCA GTC AGT CAT GC	220,250
172	TTA GCA GTC AGT CAG TCA TCG	225,800
192, 194, 195A, 195B	[TT]AGC AGT CAG TCA GTC ATC G	225,800
193	[TT]AGC AGT CAG TCA GTC ATC G	233,900
197	AGC AGT CAG TCA GTC ATC G	209,700

7.3.2. Synthesis of Fluorescent Substrate Oligonucleotide

Fluorescent oligonucleotide **63** was synthesised by Dr Afaf El-Sagheer, Brown Group, University of Oxford, using standard solid phase oligonucleotide synthesis.²¹⁹ Oligonucleotide **63** was phosphorylated using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's instructions. Oligonucleotide **63** (0.3 nmol), ATP (50 nmol) and T4 PNK (10 units) were incubated in kinase reaction buffer (50 µL; 10 mM MgCl₂, 70 mM Tris-HCl, 5 mM DTT, pH 7.6) at 37 °C for 30 min according to manufacturer instructions. The enzyme was inactivated by heating to 65 °C for 20 min to give the phosphorylated oligonucleotide **64**.

7.3.3. Modified Oligonucleotides from Chapter 4

7.3.3.1. Preparation of Triazole-Linked Oligonucleotides

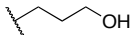
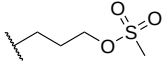
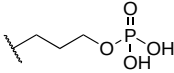
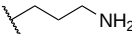
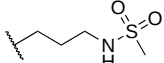
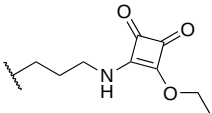
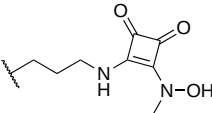
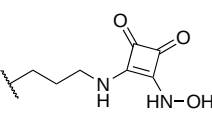
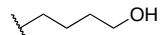
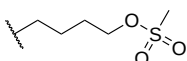
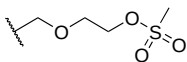
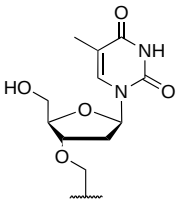
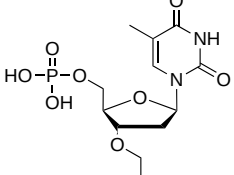
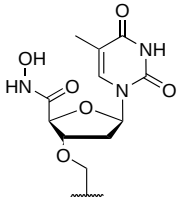
5'-azido oligonucleotide **162** was synthesised by Dr. Afaf El-Sagheer, Brown Group, University of Oxford, using standard solid phase oligonucleotide synthesis.²¹⁹ H₂O was degassed with argon prior to carrying out the reaction. Alkyne-containing compounds **144a-154a**, **163a-165a** were dried under high vacuum over phosphorous pentoxide prior to use in CuAAC reactions and were added to the reaction mixture as a solution in H₂O (0.04 or 0.05 M, 0-40% DMSO). 5'-Azido oligonucleotide **162** (20 uL, 1.5 mM in H₂O, 0.03 μmol) was added to a solution of alkyne-containing **144a-154a**, **163a-165a** (35 μL) and the mixture was degassed with argon. A solution of Cu^I catalyst was prepared by combining tris-hydroxypropyltriazole ligand (THPTA) (1.6 mg, 3.68 μmol) with sodium ascorbate solution (30 uL, 505 mM in H₂O, 15 μmol) followed by a solution of CuSO₄·5H₂O (15 uL, 100 mM in H₂O, 1.5 μmol) and the catalyst solution was degassed with argon. The catalyst was immediately added to the solution of 5'-azido oligonucleotide **25** and alkyne containing compound and degassed with argon. The reaction mixture was incubated at rt for 2 h with shaking at 200 rpm. After this time, the reaction was quenched by addition of a solution of EDTA (2 μL, 0.5 M in H₂O, pH 8) and desalted using NAP-5 column (GE Healthcare) according to manufacturer instructions to give triazole-linked oligonucleotides **144b-154b**, **163b-165b**.

7.3.3.2. Mass Spectrometry of Triazole-Linked Oligonucleotides

Triazole-linked oligonucleotides **144b-154b** were characterised using MALDI-TOF. Oligonucleotides were used as a solution in H₂O (30-60 μM). A solution of 30 mg/mL 3-hydroxypicolinic acid (Sigma-Aldrich) was freshly prepared in H₂O and used as a matrix. Diammonium citrate (30 mg/mL in H₂O) and diammonium tartrate (7.5 mg/mL in H₂O) were used as additives. A mixture of 1:1:1 oligonucleotide:matrix:additive was prepared to give a final oligonucleotide concentration of 10-20 μM. 1 μL of this mixture was spotted on the target plate using the dried droplet method. Positive and negative ion MALDI TOF spectra were obtained in linear mode on a Bruker 'autoflex maX' MALDI TOF/TOF instrument. Electrospray mass spectrometry of oligonucleotides **163b-164b** was performed in H₂O using a Waters ESI HRMS by Dr Afaf El-Sagheer, University

of Oxford. MALDI-TOF of **165b** was carried out by Dr Mark Berney, University College Dublin.

Table 7.2: Mass spectrometry data of the triazole-linked oligonucleotides **144b-154b**, **163b-165b**. ^aMain peak in mass spectra of *N*-hydroxysquaramides corresponds to 5'-amino oligonucleotide **147b** caused by fragmentation of the squaramide moieties. The intact mass is also seen in the mass spectra. ^bMain peak in mass spectrum of mesylate **153b** corresponds to a fragmentation product formed by elimination of the mesylate. The intact ion is also seen in the mass spectrum.

Oligonucleotide	Modification	<i>m/z</i> Calc.	Found
144b		6227 [M+H] ⁺	6227
145b		6305 [M+H] ⁺	6305
146b		6307 [M+H] ⁺	6306
147b		6226 [M+H] ⁺	6225
148b		6304 [M+H] ⁺	6303
149b		6350 [M+H] ⁺	6350
150b		6351 [M+H] ⁺	6350 ^a
151b		6337 [M+H] ⁺	6341 ^a
152b		6241 [M+H] ⁺	6240
153b		6319 [M+H] ⁺	6321 ^b
154b		6321 [M+H] ⁺	6320
163b		6421 [M-H] ⁻	6423
164b		6501 [M-H] ⁻	6502
165b		6450 [M-H] ⁻	6448

7.3.4. Modified Oligonucleotides from Chapter 5

7.3.4.1. Preparation of Oligonucleotides *via* SPOS using the manual syringe method

Dinucleoside phosphoramidites **184**, **187-189** were dried under high vacuum over phosphorous pentoxide prior to use. The solid support used during SPOS was dG(iBu)-CPG 500 Å (49 µmol/g, Eurogentec). Support-bound 19mer oligonucleotide **191** was synthesised by Dr. Mark Berney, Ferguson Group, UCD *via* automated SPOS. Oligonucleotide **191** was used to prepare modified oligonucleotides **192-195** on 0.75 – 1 µM scale *via* the manual syringe method as follows:

1. Wash (3 x 2 mL MeCN)
2. Couple (100 – 200 µL 0.1 M dinucleoside phosphoramidite **184**, **187-189** + 150 – 300 µL BTT, both in MeCN, 10 min at rt then 5 min with gentle heating)
3. Wash (3 x 2 mL MeCN)
4. Oxidise (500 µL, 0.02 M I₂ in pyridine-H₂O, 9:1, 45 sec)
5. Wash (3 x 2 mL MeCN)
6. Wash (1 x 1 mL pyridine-MeCN, 1:9)
7. Wash (3 x 2 mL MeCN)
8. Deblock (3 x 2 mL 3% DCA in toluene)
9. Wash (3 x 2 mL MeCN)
10. Deprotect (1 mL 20% diethylamine in MeCN, 1 min)
11. Wash (3 x 2 mL MeCN)
12. Global deprotection and cleavage (35% aq. NH₃, 55 °C, 16 – 18 h)

The solid support was removed by filtration and the solvent was removed *in vacuo*. The crude oligonucleotides were redissolved in H₂O and desalted using NAP-5 columns

(GE Healthcare). The solvent was removed *in vacuo* and the oligonucleotides were redissolved in 0.1 M TEA-acetic acid, pH = 7 (500 μ L) prior to purification by RP-HPLC.

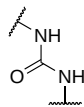
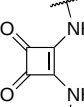
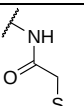
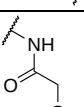
7.3.4.2. Preparation of 19mer Oligonucleotide

19mer oligonucleotide **197** was prepared through global deprotection and cleavage of support-bound 19mer **191**. This was carried out with 35% aq. NH_3 at 55 $^\circ\text{C}$ for 16 h. The solid support was removed by filtration and the solvent was removed *in vacuo*. The crude oligonucleotide was redissolved in H_2O and desalted using NAP-5 columns (GE Healthcare). The solvent was removed *in vacuo* and the oligonucleotide was redissolved in 0.1 M TEA-acetic acid, pH = 7 (500 μ L) prior to purification by RP-HPLC.

7.3.4.3. Purification and characterisation of Oligonucleotides

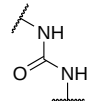
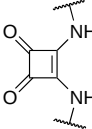
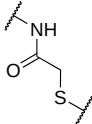
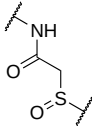
Oligonucleotides were purified by RP-HPLC on a Waters XBridge BEH C18 OBD 130 $\text{\AA}$, 5 μm , 10 mm x 100 mm column, using an Agilent 1260 Infinity instrument. The flow rate was 4.5 mL/min. Solvent A was a 0.1 M aqueous solution of TEA-acetic acid, pH = 7. Solvent B was MeCN. The solvent gradients used for each oligonucleotide are shown in **Table 7.3**.

Table 7.3: Solvent gradients used in semi-preparative RP-HPLC of oligonucleotides **192-195**, **197**.

Oligonucleotide	Modification	Gradient
192		7 – 50% Solvent B over 17 min
193		7 – 50% Solvent B over 16 min
194		7 – 50% Solvent B over 17 min
195		7 – 50% Solvent B over 17 min
197	N/A	7 – 50% Solvent B over 16 min

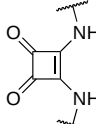
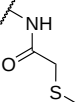
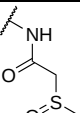
Analytical HPLC of purified fractions was carried out on a Waters XBridge BEH C18 OBD 130 Å, 5 µm, 2.1 mm x 100 mm column using an Agilent 1260 Infinity instrument. The flow rate was 0.22 mL/min. Solvent A was a 0.1 M aqueous solution of TEA-acetic acid, pH = 7. Solvent B was MeCN. The solvent gradients used and retention times for each oligonucleotide are shown in **Table 7.4**.

Table 7.4: Details of analytical RP-HPLC characterisation of modified oligonucleotides **192-195, 197**.

Oligonucleotide	Modification	Gradient	Retention Time (min)
192		7 – 50% Solvent B over 16 min	12.95
193		7 – 50% Solvent B over 16 min	5.03
194		7 – 50% Solvent B over 16 min	11.23
195		7 – 50% Solvent B over 16 min	195A: 13.07 195B: 13.39
197	N/A	7 – 50% Solvent B over 16 min	6.76

Oligonucleotides **192-195, 197** were characterised by MALDI-TOF MS using a Bruker AutoFlex Max instrument in negative mode. MALDI-TOF MS was carried out by Dr Mark Berney, University College Dublin.

Table 7.5: Mass Spectrometry data of modified oligonucleotides **192-195**, **197**. *In the case of squaramide oligonucleotide **193**, the main peak in the mass spectrum corresponds to an adduct formed by fragmentation of the squaramide moiety. Similar fragmentation was observed for oligonucleotides **150b** and **151b**.

Oligonucleotide	Modification	<i>m/z</i> Calc.	Found
192		6379 [M-H] ⁻	6379
193		6431 [M-H] ⁻	6112*
194		6410 [M-H] ⁻	6410
195		6426 [M-H] ⁻	6427
197	N/A	5809 [M-H] ⁻	5809

7.3.5. Determination of Concentration of Oligonucleotide Solutions

Concentrations of oligonucleotides were determined by measuring absorbance in H₂O at 260 nm. UV-Vis absorption spectra were recorded using a Varian Cary 50 spectrophotometer. A spectroscopic window of 600-200 nm was used for all spectra. Baseline correction from blank solvent was used for all spectra. Spectra were measured in a 1 cm quartz cuvette at rt. Concentrations were determined using the Beer-Lambert Law with the extinction coefficients detailed in **Section 7.3.1**.

7.4. Biochemical Methods

7.4.1. Gel Electrophoresis Assay of Modified Nucleosides or Fragments

Gel electrophoresis assays were performed according to a modified literature procedure.²²⁰ Truncated human SNM1A (698-1040) was stored as a 1.0 μM (0.04 mg/mL) solution in reaction buffer (20 mM HEPES-KOH pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.05% (v/v) Triton-X100, 0.1 mg/mL BSA, 5% (v/v) glycerol, 0.5 mM DTT). All incubations were carried out with gentle shaking (80 rpm). Modified nucleosides or fragments were dried under high vacuum over phosphorous pentoxide

prior to use in the assay. Modified nucleosides (1 mM in reaction or as specified) or fragments (5 mM in reaction) were treated with SNM1A (25 fmol or 50 fmol as specified) in reaction buffer (10 μ L) containing 4% DMSO on ice, and then incubated at 37 °C for 5 min. A solution of fluorescent substrate oligonucleotide **64** (1 μ L, 0.8 pmol/ μ L) was added and each reaction was incubated at 37 °C for a further 60 min. The reactions were stopped by addition of 2 μ L of stop solution (95% formamide, 10 mM EDTA) followed by heating at 95 °C for 3 min. Oligonucleotides were separated on 15% acrylamide 6.5 M urea gels (2.9 g urea, 2.7 mL 40% acrylamide-bisacrylamide 25:1, 0.7 mL 10X TBE (0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA pH 8.0), 1.3 mL H₂O) in 1X TBE at 150 V for 75-90 min, alongside bromophenol blue and xylene cyanol as markers for 8 nt and 28 nt respectively. The gels were imaged using a Typhoon FLA 9500. All assays were carried out in at least duplicate.

7.4.2. Gel-Electrophoresis Assay of Modified Oligonucleotides

Gel electrophoresis assays were performed according to a modified literature procedure.²²⁰ Truncated human SNM1A (698-1040) was stored as a 1.0 μ M (0.04 mg/mL) solution in reaction buffer (20 mM HEPES-KOH pH 7.5, 50 mM KCl, 10 mM MgCl₂, 0.05% (v/v) Triton-X100, 0.1 mg/mL BSA, 5% (v/v) glycerol, 0.5 mM DTT). All incubations were carried out with gentle shaking (80 rpm). Oligonucleotides (2.8 μ M in reaction or as specified) were treated with SNM1A (50 fmol) in reaction buffer (10 μ L) on ice, and then incubated at 37 °C for 5 min. A solution of fluorescent substrate oligonucleotide **64** (1 μ L, 0.8 pmol/ μ L) was added and each reaction was incubated at 37 °C for a further 60 min. The reactions were stopped by addition of 2 μ L of stop solution (95% formamide, 10 mM EDTA) followed by heating at 95 °C for 3 min. Oligonucleotides were separated on 15% acrylamide 6.5 M urea gels (2.9 g urea, 2.7 mL 40% acrylamide-bisacrylamide 25:1, 0.7 mL 10X TBE (0.9 M Tris, 0.9 M boric acid, 0.02 M EDTA pH 8.0), 1.3 mL H₂O) in 1X TBE at 150 V for 75-90 min, alongside bromophenol blue and xylene cyanol as markers for 8 nt and 28 nt respectively. The gels were imaged using a Typhoon FLA 9500. All assays were carried out in at least duplicate.

7.4.3. Real-Time Fluorescence Assay of Modified Nucleoside

Real-time fluorescence assays were performed according to a modified literature procedure^{209, 217, 220} utilising a 20-nucleotide ssDNA substrate of the following sequence:

5'-A[FamT]AATTTGA[BHQT]CATCTATTAT-3' (Eurogentec). This oligonucleotide (**126**) contained a fluorescein-conjugated thymine (FamT) as the second residue from the 5'-end, and a black-hole quencher moiety conjugated to a thymine residue (BHQT) eight nucleotides away. The oligonucleotide substrate was phosphorylated at the 5'-end using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocol to give **127** which was made up to 1.25 μM for addition to reactions. Nuclease reactions were carried out in black 384-well microplates in a total volume of 25 μL in reaction buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM MgCl_2 , 0.5 mM DTT, 0.05% (v/v) Triton-X100, 5% (v/v) glycerol) containing 4% DMSO, with 125 nM oligonucleotide substrate (**127**) and 5 nM SNM1A (698-1040). Reactions were carried out in the presence of twelve different concentrations (0-500 μM) of inhibitor **104**. Five replicates of each reaction were performed. SNM1A was incubated with the inhibitor in the above nuclease buffer for 6.5 min at rt, before the reactions were started by the addition of the DNA substrate (**127**). The fluorescence spectra were measured at 37 °C using a BioTek Synergy H1 microplate reader (excitation at 495 nm, emission at 525 nm) with readings taken every 46 s for 35 min. The fluorescence intensity for each reaction was plotted against time, and the rate of increase was determined and normalised to the zero-inhibitor control. This was plotted against inhibitor concentration and the data were fitted using a "log[inhibitor] vs. normalised response" nonlinear regression algorithm on GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) to calculate the IC_{50} values.

7.4.4. Real-Time Fluorescence Assay of Modified Oligonucleotides

Real-time fluorescence assays were performed according to a modified literature procedure^{209, 217, 220} utilising a 20-nucleotide ssDNA substrate of the following sequence: 5'-A[FamT]AATTTGA[BHQT]CATCTATTAT-3' (Eurogentec). This oligonucleotide (**126**) contained a fluorescein-conjugated thymine (FamT) as the second residue from the 5'-end, and a black-hole quencher moiety conjugated to a thymine residue (BHQT) eight nucleotides away. The oligonucleotide substrate was phosphorylated at the 5'-end using T4 polynucleotide kinase (New England Biolabs) according to the manufacturer's protocol to give **127** which was made up to 1.25 μM for addition to reactions. Nuclease reactions were carried out in black 384-well microplates in a total volume of 25 μL in reaction buffer (20 mM HEPES-KOH, pH 7.5, 50 mM KCl, 10 mM MgCl_2 , 0.5 mM

DTT, 0.05% (v/v) Triton-X100, 5% (v/v) glycerol) with 125 nM oligonucleotide substrate **127** and 5 nM SNM1A (698-1040). Reactions were carried out in the presence of twelve different concentrations of modified oligonucleotides **192-195**, **197**. Between three and six replicates of each reaction were performed. SNM1A was incubated with the inhibitor in the above nuclease buffer for 6.5 min at rt, before the reactions were started by the addition of the DNA substrate (**127**). The fluorescence spectra were measured at 37 °C using a BioTek Synergy H1 microplate reader (excitation at 495 nm, emission at 525 nm) with readings taken every 50 s for 35 min. The fluorescence intensity for each reaction was plotted against time, and the rate of increase was determined and normalised to the zero-inhibitor control. This was plotted against inhibitor concentration and the data were fitted using a “log[inhibitor] vs. normalised response” nonlinear regression algorithm on GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) to calculate the IC₅₀ values.

7.4.5. Circular Dichroism Titrations

Circular dichroism measurements were made using a JASCO J-815 spectrometer. A spectroscopic window of 400-200 nm was used, and data points were taken at an interval of 0.5 nm, scanning at 50 nm min⁻¹. Data are presented as an average of 3 accumulations. Baseline correction from blank solvent was used for all spectra. Spectra were measured in a 1 cm quartz cuvette at 25 °C. Titrations were performed through modification of a literature procedure.³⁸⁴ A solution of a 21mer ssDNA oligonucleotide **128** in H₂O was made up and diluted to 1.22 µM in the cuvette. A solution of methylene blue (**129**), or of compound **104**, in 4% DMSO in H₂O was aliquoted into the cuvette such that the volume in the cuvette did not increase by more than 10% during the titration. CD spectra of methylene blue (**129**) and of compound **104** alone, without the presence of the oligonucleotide, were also recorded at the same concentrations used in the titrations, and these were subtracted from the corresponding spectra measured during the titrations, to allow only changes in the CD spectrum of the oligonucleotide to be visualised. Spectra were analysed using the spectral analysis software and smoothed using the ‘adaptive smoothing function’.

Chapter 8

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8. References

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